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Study of gene expression alteration in male androgenetic alopecia: evidence of predominant molecular signaling pathways

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Abbreviations

ACTH : Adrenocorticotropic hormone
AGA : androgenetic alopecia AGRP : agouti-related peptide
α-MSH : α-melanocyte stimulating hormone
AR : androgen receptor
ASIP : agouti signaling protein
BAMBI : BMP and activin membrane-bound inhibitor
BMP : Bone morphogenetic protein
CTNNB1 : β-catenin1
CTSG : cathespin G
CMA1 : chymase
CYP27B1 : Cytochrome p450 family 27 subfamily B, polypeptide 1
DGA : discriminatory genes analyses
DHT : 5-alpha dihydrotestosterone
DKK1 : dickkopf Wnt signaling pathway inhibitor 1
DLX3 : Distal-less homeobox 3
DP : dermal papilla
DSG4 : desmoglein 4
HF: hair follicles
ID3 : Inhibitor of DNA binding 3
ITGB6 : Integrin Beta 6
LEF1 : lymphoid enhancer binding factor 1
LGR5 : Leucin-Rich repeat containing G protein-coupled receptor 5
MC : mast cells
POMC : proopiomelanocortin
PTGDS : prostaglandin D_2 synthase
S100A7 : psoriasin

SC : stem cells
SFRP2 : secreted Frizzled-related protein 2
TH : tyrosine hydroxylase
TPSAB / TPSAD : tryptase alpha / tryptase delta
VGF : Nerve Growth factor inducible
VDR : vitamin D receptor

ABSTRACT

Background: Male androgenetic alopecia (AGA) is the most common form of hair loss in men and is characterized by a distinct pattern of progressive hair loss starting from the frontal area and the vertex of the scalp. Although several genetic risk loci have been identified, relevant genes for AGA remain to be defined.

Objectives: Herein, molecular biomarkers associated with premature AGA were identified through gene expression analysis using cDNA generated from scalp vertex biopsies of hairless/bald men with premature AGA and healthy volunteers.

Results: This monocentric study reveals that genes encoding mast cell granule enzymes, inflammatory and immunoglobulin-associated immune mediators were significantly over-expressed in AGA. In contrast, under-expressed genes appear to be associated with the Wnt/β-catenin and BMP/TGF-β signaling pathways. Although involvement of these pathways in hair follicle regeneration is well-described, functional interpretation of the transcriptomic data highlights different events that account for their inhibition. In particular, one of these events depends on the dysregulated expression of proopiomelanocortin (POMC), as confirmed by RT-qPCR and immunohistochemistry. In addition, lower expression of CYP27B1 in AGA subjects supports the notion that changes in vitamin D metabolism contributes to hair loss.

Conclusion: This study provides compelling evidence for distinct molecular events contributing to alopecia that may pave way for new therapeutic approaches.

INTRODUCTION

Male pattern baldness or male androgenetic alopecia (AGA) is the most common type of baldness, characterized by progressive patterned hair loss from the scalp, starting in the frontal area and the vertex, following a defined pattern[1,2]. It is caused by a shortening of the anagen phase of hair cycling and progressive miniaturization of the hair follicle (HF), which results in the formation of vellus hairs that eventually atrophy[3]. Up to 80% of European men are concerned by AGA during their life. Although the precise etiology and pathogenesis of androgenetic alopecia are still unclear, genetic and androgenic factors play definite roles. . Androgens, especially the metabolite 5-alpha dihydrotestosterone (DHT), affect genetically susceptible cells of the dermal papilla (DP), specialized mesenchymal derived part of the HF, causing progressive HF miniaturization and hair cycle abnormalities leading to male AGA[4].

Polymorphism of the androgen receptor (AR) gene was first identified in association with AGA [5,6]. The paradoxical role of androgens in hair follicle biology remains poorly understood: indeed, androgens trigger hair development at puberty and inversely favor AGA in later life. Kretzchmar *et al.*[7] explored the inhibitory role of androgens by defining a reciprocal relationship between activated Wnt/β-catenin and AR signaling within the HF. They pinpointed AR as a negative regulator of β-catenin signaling. However, identification of various new susceptibility genes, such as those on chromosomes 3q26 and 20p11[8,9], suggests that non-androgen-dependent pathways are also involved, as reviewed by Heilmann-Heimbach et al[10]

HF contains a well-described stem cell niche in the bulge area of the hair, close to the sebaceous glands. After the anagen phase, the catagen phase is followed by migration of stem cells (SC) from the bulge to the bulb to regenerate a new hair. In alopecia, the anagen phase becomes shorter and the number of follicles in the anagen phase decreases. It has been suggested that stem cell pool remain unaffected in AGA patient whereas progenitor cells

arising from stem cells are much more numerous in the follicles of AGA subjects compared to controls[11].

Although genetic predisposition and the role of genes in AGA have been documented in several previous reports [12,13], inheritance of male AGA is not fully understood. Also, the full mechanism of hair growth impairment in this disorder remains to be elucidated. The aim of this study is to focus on biomarkers associated with AGA through transcriptomic analyses of scalp biopsies from AGA subjects

MATERIAL AND METHODS

Selection of individuals with AGA and controls

This monocentric study was approved by at the Ethics Committee of Saint-Louis Hospital and the study described adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants. Individuals with any systemic diseases were excluded from the study, and 28 male young Caucasian volunteers were selected for the study and allocated into the two following groups: - group A: Fourteen hairless/bald participants (alopecia, "A"; age: 29.4±3.4), with premature AGA, with stage V to VII according to the Hamilton's classification as modified by Norwood; - group C: Fourteen control subjects (controls, "C"; age: 26.1±3.6) with <2% white hairs and stage I or II in the Hamilton's classification as modified by Norwood .

Gene analysis from cutaneous biopsies.

Two scalp biopsies (2 mm diameter each) were carried out on the vertex, either at the edges of alopecia area in the group A subjects, or at a similar emplacement for the the control group. Biopsies were immediately frozen and kept for further RNA extraction or immunostaining. RNA was isolated using a standard RNA extraction protocol (Trizol®

Reagent, Ambion by Life Technologies, Courtaboeuf, France). Extracted RNA samples were quality checked usingthe Agilent 2100 Bioanalyzer platform (Agilent Technologies, Les Ulis, France).

Gene expression analysis of human skin biopsies was performed using Agilent Whole Human Genome Oligo Microarrays (*onecolor*, 8x60K, v2). Bioinformatical analysis of the microarray experiments was obtained from the single-color hybridization of human cDNA samples to Agilent microarrays and was structured in four main parts: - data preprocessing that covers all necessary data transformations as well as an assessment of variability between the samples, and the detection of potential outliers by a global correlation analysis; - discriminatory genes analyses (DGAs) of pairwise comparisons between A *vs* C groups in order to characterize the differences between the various sample groups based on their gene expression profiles; - functional annotation analysis (FAA);- annotation enrichment analysis (AEA) of differentially expressed genes in order to provide an overview of the different biological processes and pathways which are regulated in the discriminatory analyses.

Secondary analysis of the main metabolic pathways and their potential dysfunctions was performed using the Java/Perlsoftware Predictsearch® (PS-V5) [14]. The gene expression value cutoff was 60 for all the analyses. This text-mining-based software was used to search and retrieve correlations between modulated genes and biological processes to characterize pathways and functional networks.

Real-Time Quantitative PCR Analysis

Validation of some potent genes depicted by DGA, FAA, and PredictSearch analysis of the identified genes using microarrays was subsequently assessed by analysis of 45 genes by Real Time quantitative PCR (RT-qPCR)[15] using aliquots of the RNA prepared for the microarrays (details in Supplementary Materials and Methods). Furthermore, to improve

our study, the isolated total RNA were also subjected to reverse transcription (Fluidigm™, Les Ulis, France) prior to high throughput RT-qPCR analysis using the same primers [16, 17] (Biomark-HD system, Fluidigm™) (Supplementary Materials and Methods).

Histochemical analysis.

The frozen biopsies from the vertex were cut in 7µm sections using a Leica CM 3050 cryostat. Sections were then mounted on Superfrost® plus silanized glass slides. The sections were blocked for 30 min in 10% goat serum and incubated with anti-POMC (Abcam, ab32893, Paris, France) and anti-ASIP/PARD3 (Abcam, ab64646) before visualized with AlexaFluor 488 (Invitrogen, A11078), and propidium iodide (Sigma, L'Isle d'Abeau, France) for nuclear staining. The microscopic observations were realized using a Leica DMLB or Olympus BX43 microscope (Rungis, France). Pictures were digitized with a numeric DP72 Olympus camera with CellD storing software

Statistical analysis.

One-way analysis of variance (ANOVA) was performed followed by Tukey's honest significant difference (HSD) to perform pairwise comparisons. Statistical significance was assessed using the two-sided Student's t test and the Mann-Whitney statistical test for Fluidigm high throughput qPCR analysis and PredictSearch® analysis. Presented p values are not corrected for multiple testing. Differences were considered significant at $p<0.05$ and tendency to significance for $0.05<p<0.1$.

RESULTS AND DISCUSSION

Patient cohorts and Gene expression analysis.

Fourteen young volunteers with male AGA (alopecia group: A) with appearance of hair loss since 1 to 18 years ago (median, 4.7 years) and 14 young control volunteers (control group: C) were enrolled. Scalp involvement ranged from 10% to 30% (median, 14%) in AGA volunteers. Subjects did not present any other skin involvement or auto-immune disorders. Biopsies were carried out on the scalp as indicated in figure 1.

A single-color hybridization of human cDNA samples to Agilent Whole Human Genome Oligo Microarrays was assessed with RNA extracted from the scalp biopsies derived from the healthy donors or from the AGA subjects. All probes significantly modulated between A and C samples ($|\text{Fold Change}| \geq 1.5$ with a $p<0.05$) were included in a cluster analysis, with hierarchically clustered heat maps (Euclidean distance, complete linkage) of reporters (whole cluster analysis available under request). Genes differentially expressed in the group A relative to the group C (GEO deposit GSE90594) were detected using a bioinformatical analysis of the 28 microarrays (http://193.48.40.18/Diffusions_IUH/L.MICHEL). Briefly, the identification of differentially expressed genes was done by ANOVA and Tukey post-hoc tests, effect size and detectability. This returned 325 genes upregulated in the alopecia samples relative to the healthy controls and 390 genes with the opposite expression trend (Table 1). After Benjamini-Hochberg correction for multiple testing, the analysis evidenced *in fine* a final number of **184** significantly down-regulated and **149** up-regulated candidate gene sequences in “A” comparing to “C”, respectively (Table 1, Fig.2). Hierarchically clustered heat map representation is shown in Fig.3. An overview of the different biological processes and pathways significantly enriched among the differentially expressed genes was provided by the functional annotation analysis (Table 2, Fig.S1a and S1b). Based on

their fold change, a battery of genes was selected and analyzed for mRNA transcript abundancy by high throughput RT-qPCR, confirming for most of them altered gene expression levels in AGA cohort detected by transcriptomic analysis (Fig.4). The DGA gene set was submitted to PredictSearch® analysis for functional interpretation and major over- or under-expressed genes were highlighted and associated by gene families (Tables 3A-D). As detailed thereafter, the present transcriptomic data shows an intense activation of immune and inflammatory responses in AGA cohort whereas an inhibition of the Wnt/β-catenin signaling pathway, of the TGF-β and BMP signaling pathways as well as an alteration of the vitamin D biosynthesis were detected in the AGA subjects.

Activation of immune and inflammatory responses in AGA subjects.

Our data show strong up-regulation of immune and inflammatory genes, as related to B and T lymphocyte recruitment and activation of mast cell (MC), as well as some inflammatory factors related to extracellular matrix as depicted in Fig.4 and Table 3A. Indeed, a significant increase of several chemokines, including *CCL13*, *CCL18*, *CXCL10* and *CXCL12*, was detected in our AGA subjects (Fig.3, Table 3A, $p<0.01$, 0.05, 0.05 and 0.001, respectively). These chemokines play an important role in accumulation of leukocytes during inflammation through potent chemotactic activity, such as CCL13 for recruitment of monocytes, lymphocytes, basophils and eosinophils. Binding of CXCL10 to CXCR3 or CXCL12 to CXCR4 results in pleiotropic effects, including stimulation of monocytes, natural killer and T-cell migration, as well as modulation of adhesion molecule expression. Increased production of CXCL10 from HF has been shown to induce preferential infiltrates of highly chemoattracted Th1 and Tc1 cells during the acute phase of alopecia areata (AA) [18,19]. In AGA subjects, its production, as well as that of CCL8, CCL13, CCL26, and IL-8 (which tended to be increased by RT-PCR), should favor

inflammation maintenance. Regarding immune response, we also noticed significant upregulation of TNFRSF17 in AGA volunteers (p<0.05) Also known as B cell maturation antigen (BCMA), and member of the TNF-receptor superfamily [20], this receptor is preferentially expressed in mature B lymphocytes, and may be important for B cell development and autoimmune responses. Also, CD38, a multifunctional ectoenzyme widely expressed in leukocytes including B and T lymphocytes, is up-regulated in AGA cohort (p<0.02). It has been shown that CD38 might function in cell adhesion, signal transduction and calcium signalling [21].

We observed an over-expression of *HLA-DPB1* as well as *HLA-DQA1*, *HLA-DRB3* and *HLA-DRB4* in AGA volunteers (Fig.4, Table 3A, p<0.005, 0.01, 0.001 and 0.002, respectively). This is in line with previous reports showing that keratinocytes in the bulb of HF from subjects with AA express class II HLA antigens [22]. This suggests that the mononuclear cell activation in AGA might be linked to the expression of class II HLA antigens on HF keratinocytes, thus triggering a putative autoimmune response. The DGA analysis also revealed an up-regulation of genes encoding for B lymphocyte-produced immunoglobulins, including *IGHG1*, *IGHM* and *IGJ*, in some AGA volunteers. Possibly, lymphocytes attack the lower part of the anagen follicle, with ectopic expression of MHC class I and II molecules on the epithelium of affected HF, suggesting that B, T cells and cytokines play an important role in AGA as depicted in AA where chemokines are regarded as key players that mediate autoimmune inflammation [19]. Furthermore, we detected a slight up-regulation of the gene encoding high-affinity IgE-receptor *FcεRI/MAS4A2* (*membrane-spanning 4-domains, subfamily A, member 2*) in AGA subjects (p<0.001), a receptor found on the surface of MCs and basophils and involved in FcεRI-dependent signaling. The relationship between mast cells and male pattern hair loss was supported by previous histological evidences showing that perifollicular fibrosis is

associated with increase in MC numbers [23]. Similarly, we noticed up-regulated expression of several genes including *COL1A1* ($p<0.01$) or *COL1A2* ($p<0.01$) and genes of several metalloproteinases (MMPs), especially *MMP12* ($p<0.02$), as observed during inflammatory wound-healing [24] (Fig.4, Table 3A). Of interest, our data bring evidence of an activation of MCs in AGA subjects as illustrated by the significant over-expression of MC markers, including *prostaglandin D_2 synthase* (*PTGDS*) ($p<0.01$), *KIT/c-kit*, *tryptases* (*TPSAB* ($p<0.001$) and *TPSAD* ($p<0.001$)), *chymase* (*CMA1*) ($p<0.005$) and *cathespin G* (*CTSG*) ($p<0.0005$) (Fig.4, Table 3A). Expression of KIT/c-kit has been shown to be positively controlled by GATA2 in a hair-cycle-dependent manner [25] and its significant up-regulation in the AGA subjects ($p<0.002$) reinforces a functional role of MC and epithelial KIT/c-kit expression in hair growth control. We may suggest that PGD_2 synthesized from prostaglandin H_2 by PTGDS, and previously found highly expressed in AGA [26], could inhibit HF regeneration through binding to the Gpr44 receptor [27]. Altogether, our results reinforce the MC involvement in AGA.

Inhibition of the Wnt signaling in AGA patients.

Among genes eliciting a differential expression pattern in AGA subjects and healthy volunteers, a significant number of genes was related to the Wnt/β-catenin signaling pathway as described in Fig.4 and Table 3B. This pathway plays an important and well-known role in HF development, hair stem cell maintenance, and HF lineage commitment [28] and is required for the induction of hair keratin genes in HF stem cells co-cultured with DP cells [29]. Moreover, several studies have reported the inhibition of this pathway in AGA [7, 30, 31].

SFRP2 (*secreted Frizzled-related protein 2*) and *DKK1* (*dickkopf Wnt signaling pathway inhibitor 1*) are the main known inhibitors of the Wnt signaling [32,33]. DKK1-promoting HF

regression was shown to be up-regulated in follicular keratinocytes of the late anagen in depilation-induced hair cycle progression [34]. While DKK1 expression remained unchanged in our study, expression of *SFRP2* was significantly increased in more than 80% of our AGA subjects (p<0.002). SFRP2, expressed in the epithelial portion of HF, has been shown to be involved in hair fiber differentiation [35] and as well as in the catagen phase by inhibiting keratinocyte differentiation [36].

In accordance with this report, we found that expression of *SFRP2* and Wnt target genes such as *LGR5* (p<0.001), *ID3* (p<0.05), MSX2 (p<0.1), *ALAD* (p<0.05), and *BAMBI* (*BMP and activin membrane-bound inhibitor*) (p<0.05) exhibited an inverse relationship (Fig.3, Table 3B). *LGR5* (*Leucin-Rich repeat containing G protein-coupled receptor 5*) is a major Wnt target gene in hair cell progenitors [37,38], and *ID3* (*Inhibitor of DNA binding 3*) encodes a marker in dermal papilla at the middle anagen and telogen stages [39]. Noteworthy, *BAMBI* [40] encodes the bone morphogenic protein and activin membrane-bound inhibitor, which is known to enhance the Wnt/β-catenin signalling in various cell types [41].

In line with reduced expression of these Wnt/β-catenin target genes, expression of *LEF1* (*lymphoid enhancer binding factor 1*) and *CTNNB1* (*β-catenin1*), although to a lesser extent, is decreased in the AGA patients (Fig.3, Table 3B, p<0.1 and 0.01, respectively). The products of these genes are known to interact and, after nuclear translocation, to bind to promoter regions of Wnt target genes [42,43]. LEF1 is considered as the key transducer of the Wnt signaling pathway, and a potent regulator of hair development [44] while CTNNB1 is the major downstream effector of this pathway [45,46].

Noteworthy, our data suggest that alteration of hair regeneration might result also from a dysregulation of *proopiomelanocortin* (*POMC*) expression, which was reduced in 70% of the AGA cohort compared to controls (Fig.4, Table 3B, p<0.001). POMC is the precursor of two

hormones: Adrenocorticotropic hormone (ACTH) known as an activator of hair growth [47], and α-melanocyte stimulating hormone (α-MSH), which triggers eumelanin synthesis and can protect CTNNB1 from degradation by ubiquitinylation through its inhibitory effect on GSK3β [48].

Moreover, our data showed that one of the highly repressed gene expression in the AGA subjects affected *VGF* ($p<0.02$), an inducible member of the *Nerve Growth Factor* family that might also contribute to POMC downregulation. Indeed, invalidation of this gene has been shown to lead to a strong alteration of *POMC* expression as well as of neuropeptide Y and AGRP (agouti-related peptide), an antagonist of the α-MSH receptor [49].

On the other hand, *ASIP* (*agouti signaling protein*), encoding another antagonist of the α-MSH receptor, was found highly expressed in 86% of the AGA subjects (Fig.4, Table 3B, $p<0.005$). Thus, it can be suggested that a low level of α-MSH together with high levels of ASIP contributes to impaired CTNNB1 stabilization. Of note, our data in AGA did not only evidence the up-regulation of ASIP but also that of its negative regulator *CORIN*, which encodes a transmembrane serine protease expressed specifically in the DP (Fig.4, Table 3B, $p<0.001$). However, it was reported in mice that β-catenin activity in the DP suppresses *ASIP* expression and activates *CORIN* while regulating melanocyte activity by a mechanism independent of both ASIP and CORIN [50]. The opposite regulation of ASIP and CORIN by β-catenin in mice is not in line with their co-expression seen nearly in all AGA subjects and such a discrepancy might illustrate distinct regulations among species. Alternatively, activities of the respective gene products may not follow gene expression.

Besides RT-qPCR analysis of *LEF1*, *POMC*, *ASIP* and *CORIN* gene expression to strengthen bioinformatics analysis of transcriptomic data (Fig.4), a histochemical analysis was performed to evaluate whether some of the gene modulations could be detected at protein levels. While both POMC and ASIP expression levels were strongly impaired in AGA

patients as shown in figure 5, with scoring intensity in Table 4, POMC was barely detectable in AGA subjects, in line with the results from the transcriptomic data. In contrast, ASIP was more expressed in all HF areas of controls (Fig.5 and Table 4). Thus, a decrease of POMC at both mRNA and protein levels should have a more drastic effect on β-catenin stabilization than the increased expression of ASIP. Noteworthy, a lower size of the bulb was observed in AGA subjects as compared to controls.

Furthermore, we found that *SRD5A2* encoding the Steroid 5α-reductase was up-regulated in AGA (Table 3B, $p<0.01$). This enzyme, known to suppress hair growth activity and to be highly active in balding HF [51], is involved in the conversion of testosterone into DHT. Interestingly, DHT was reported to activate the interaction between β-catenin and AR [52]. Such an interaction leads to suppress the β-catenin transcriptional activity, in line with the inhibition of Wnt signaling pathway by androgens in DP cells [30].

TGF-β and BMP signaling pathways in AGA patients.

TGF-β stimulates hair follicle regeneration through Wnt signaling activation and this is achieved via the inhibitory effect of TGF-β on DKK1 [53,54]. Among the different members of the TGF-β family, only the expression of *TGFB2* was found negatively modulated in our AGA patients (Fig.4, Table 3C, $p<0.0001$). Several reports have evidenced the role of TGF-β2 in hair physiology and in particular in HF morphogenesis [55].

TGF-β can be activated through its interaction with Integrin Beta 6 (ITGB6) whose gene, known to be up-regulated in the outer root sheath during early hair regeneration [54], was under-expressed in AGA (Fig.4, Table 3C, $p<0.001$). Thus, the reduced expression of ITGB6 in AGA is in line with an inhibition of the TGF-β_2 signaling, which impacts negatively folliculogenesis and maturation [56]. Moreover, expression of other related ITGB6 or TGF-β_2

genes were found underexpressed. Indeed, as ITGB6, its complex associate, *psoriasin* (*S100A7*) [57], exhibited a decreased expression in our AGA patients together with several other S100A proteins (Fig.4, $p<0.05$). The expression of these S100A proteins closely coincides with epithelial Ca^{++}-regulation, which governs cornified envelope formation in hair follicles [58].

As previously reported in cultured DP cells from AGA [59], an underexpression of bone morphogenetic protein 2 (*BMP2*) was observed in our subjects (Table 3C, $p<0.02$). Together with TGF-β, this reduced expression might decrease the activation of SMAD4 and its target *Distal-less homeobox 3* (*DLX3*) [60] as well as it may explain the under-expression of *desmoglein 4* (*DSG4*) we observed in AGA patients (Fig.4, Table 3C, $p<0.05$ and $p<0.02$, respectively). Indeed, ablation of SMAD4 was reported to inhibit the expression of *DSG4*, involved in hair follicle integrity and hair shaft differentiation [61]. Of interest, Wnt-, BMP- and TGF-β signaling pathways are all potent transcriptional inducers of BAMBI [40]. Conversely, inhibition of BAMBI expression as well as that of DLX3 and KRT16 may be triggered by Mir-31, shown to be markedly increased during the anagen and decreased during the telogen and catagen phases [62]. Accordingly, *KRT16* was also decreased in our AGA patients (Table 3C, $p<0.001$).

Interestingly, DLX3 ablation has been reported to lead to alopecia through a loss of BMP signaling while DLX3 activates hair formation and differentiation, towards its ability to induce the expression of *GATA3* and *homeobox C13* (*HOXC13*) [63]. HOXC13, whose expression was reduced also in our AGA patients (Table 3C, $p<0.01$), plays a role in the expression of keratin genes [64] and in hair follicle differentiation [65]. In addition to *KRT16*, some AGA patients exhibited an underexpression of a subset of genes encoding keratins (*KRT19, KRT26, KRT39, KRT72, KRT81*) and keratin-associated proteins (KRTAPs) that play a crucial role in hair shaft formation [66,67]. We also observed a down-regulation of

mesenchymal stem cell markers (Fig.4) such as *SOX9* ($p<0.01$) and of *Hedgehog-interacting protein* (HHIP) ($p<0.02$), which is a potent morphogen acting in a wide range of developmental processes, involved in the Hedgehog signaling and the epithelial-to-mesenchymal transition [68].

Vitamin D3 signaling in AGA patients.

It has been shown that CTNNB1 and LEF1 can interact with vitamin D receptor (VDR) to form a complex [69], and *VDR* gene was found differentially expressed between AGA and controls (Fig.4, $p<0.05$). Interestingly, VDR–null mice elicit an alopecia phenotype due to a defect in epithelial mesenchymal communication that is required for normal hair cycling [70]. However, this activity was shown to be independent of the binding of its ligand [71]. On the other hand, calcitriol, the active form of vitamin D3, through binding to VDR, was reported to be responsible for the suppressed expression of DKK1 and SFRP2 in murine bone marrow stromal cells [72].

The notion that AGA exhibit an alteration of the vitamin biosynthesis was supported by the decreased expression of *CYP27B1* (Cytochrome p450 family 27 subfamily B, polypeptide 1), in 86% of the AGA subjects (Table 3D, $p<0.02$). Indeed, expression of CYP27B1 is involved in the synthesis of calcitriol, which elicits a protective role in radiation-induced alopecia [73]. Such reduced expression of *CYP27B1* in AGA can be related to the underexpression of *tyrosine hydroxylase* (*TH*) we observed (Table 3D, $p<0.05$). Known as a transcriptional target of vitamin D3 [74], TH is involved in the first step of L-dopa synthesis that activates tyrosinase leading to melanogenesis [75]. Thus, in addition to the role of POMC and ASIP in hair differentiation and melanogenesis towards their respective impacts on CTNNB1, a

decrease of *TH* might explain the progressive replacement of terminal hairs by smaller hairs with less pigmentation observed in AGA.

CONCLUSION

Although RNAs were extracted from whole follicle and not specific follicular compartments, the resulting expression data might reflect the combined amount of distinct RNA populations extracted from different cell types. However, integrating genes within functional networks indicate that the different events observed in our study recapitulate what is known in AGA and therefore ensures consistencies of our results. The present transcriptomic analysis allows us to highlight several specific molecular pathways discriminating AGA individuals from controls as schematized in Fig.6 and to add two new genes POMC and VGF as markers of AGA. It is presently unclear how these events are related to inflammation, mast cell activation, leukocyte recruitment and dysregulation of the immune system. Nevertheless, the activation of these processes in AGA is illustrated by the over-expression of genes encoding chemokines, immunoglobulins, mast cell markers and MHC class II molecules. Our results also bring evidence of the crucial role of the Wnt/β-catenin signaling pathway in hair physiology, indicating that its inhibition in AGA subjects can be triggered by an increased expression of *SFRP2* as well as in some extent by a decreased *CTNNB1* and *LEF1* expression. A reduced β-catenin activity should indeed impair HF integrity and regeneration. Moreover, the alteration of β-catenin stabilization might be linked to the decreased expression of POMC at both RNA and protein levels through the loss of the inhibitory effect of α-MSH on GSK3β. Inhibition of β-catenin activity can be also achieved by an increase production of DHT in the AGA group as a consequence of the higher expression of *SRD5A2*. In addition, we showed that other components might contribute to this inhibition. Indeed, a reduced expression of

BMP2 and *ITGB6* should impact negatively the TGF-β transcriptional activity and TGF-β activation of the Wnt/β-catenin signaling, respectively. Although the upstream mechanisms by which modulation of these genes is achieved in AGA remain to be determined, the increased *SFRP2* expression might result in part from an altered metabolism of vitamin D3. The under-expression of *CYP27B1* leading to a reduced production of calcitriol, known to inhibit *DKK1* and *SFRP2* expression, might contribute to reduce the β-catenin activity.

Altogether, these data bring new evidences that distinct altered mechanisms converging to inhibit Wnt/β-catenin signaling account for AGA in young male subjects. They also evidence B and T cell involvement suggesting an underlying autoimmune-related mechanism that remains to be further highlighted.

CONFLICT OF INTEREST

"The authors state no conflict of interest."

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TABLES

Table 1 - Number of candidate reporters returned by the DGAs (FC: fold-change).

Expression trend	Gene numbers p≤0.05; FC≥1.5x	Adjusted gene numbers p≤0.05; FC≥1.5x
A > C	325	**149**
A < C	390	**184**

Table 2 - Synopsis of up- or down-regulated gene family. Most prominent findings of the Functional Annotation (FA) analysis, highlighted in beige and of the Annotation Enrichment (AEA) analysis, shown with blue background.

A > C	A < C
Innate Immunity	Cytoskeleton
Extracellular matrix (some collagens)	Cell adhesion
Receptor signaling	Keratin-associated proteins
Response to toxins	subset of keratins
B-cell Immunity	connexins/cell communication
Cell differentiation	TGF-beta signaling pathway
Cell proliferation	endopeptidase inhibitor activity
Inflammation	EGF-like protein domain
T-cell Immunity	growth factor activity
Cell migration	Platelet Activation
Angiogenesis	spermatogenesis
Development	
Cell adhesion	
Repressed by IL7 Pathway (only 4 genes)	
Induced by IL1 Pathway (only 5 genes)	
immunoglobulins (antibody chains)	
pos. regulation of leukocyte/T-cell activation	
some MHC II molecules with various associated categories	
some cytokines/chemokines associated with various signaling pathways	
cellular calcium ion homeostasis	
IFNg response	
metalloprotease activity	

Table 3A – Differential gene expression between AGA patients and controls.

Immune and inflammatory genes	Average expression values		P values	Mean of fc in all patients	Nbr. of AGA patients for:		
	AGA	CTRL.			fc ≥ 1.5 and ≤ 2	fc > 2	Total
CCL13	683	351	**	3.1 ± 1.0	1	12	13/14
CXCL10	24	10	*	4.2 ± 2.5	0	12	12/14
CXCL12	15025	9940	***	1.5 ± 0.2	8	1	9/14
CCL8	21	12	**	2.4 ± 0.9	3	8	11/14
CCL18	1842	595	*	20.0 ± 14.7	0	13	13/14
CCL26	50	27	*	3.0 ± 1.5	4	9	13/14
TNFRSF17	17	4	*	8.7 ± 5.4	1	13	14/14
CD38	33	11	*	9.7 ± 4.7	0	14	14/14
IRF4	43	26	***	2.1 ± 0.5	4	7	11/14
HLA-DPB1	3666	2557	**	1.8 ± 0.4	5	6	11/14
HLA-DQA1	1216	310	**	11.4 ± 8.0	1	12	13/14
HLA-DRB3	683	351	***	2.0 ± 0.5	7	5	12/14
HLA-DRB4	3979	2086	**	2.5 ± 0.8	1	10	11/14
MS4A2	527	352	***	1.8 ± 0.2	7	4	11/14

ECM genes	Average expression values		P values	Mean of fc in all patients	Nbr. of AGA patients for:		
	AGA	CTRL.			fc ≥ 1.5 and ≤ 2	fc > 2	Total
COL1A1	24812	11937	**	2.9 ± 0.9	2	11	13/14
COL1A2	26931	15195	**	2.1 ± 0.5	3	8	11/14
MMP12	46	13	*	12.3 ± 8.9	0	13	13/14

MC markers	Average expression values		P values	Mean of fc in all patients	Nbr. of AGA patients for:		
	AGA	CTRL.			fc ≥ 1.5 and ≤ 2	fc > 2	Total
TPSAB1	2627	1630	***	2.1 ± 0.4	3	9	12/14
TPSD1	2154	1308	***	2.3 ± 0.8	4	7	11/14
CMA1	784	511	**	2.2 ± 0.6	4	7	11/14
CTSG	43	26	***	2.3 ± 0.4	1	12	13/14
PTGDS	9301	5162	**	2.7 ± 0.7	3	11	14/14

The average of gene expression values (A.U.) were obtained for selected genes from bioinformatical analysis, with p values established between AGA and control (CTRL) subjects. The mean of fold change (fc) in all subjects and standard deviations were deduced from the mean of the ratios (value of AGA subjects *versus* controls) for each AGA subject. The number of patients eliciting a modulated expression according to the selected threshold is indicated.

p values: *< 0.05; ** <0.01; *** < 0.001.

Table 3B – Wnt/β-catenin signaling related genes

Genes	Average expression values		P values	Mean of fc in all patients	Nbr. of AGA patients for:		
	AGA	CTRL.			fc ≤ 0.7 and >0.5	fc ≤ 0.5	Total
LGR5	70	125	***	0.5 ± 0.2	2	9	11/14
ID3	1176	1445	*	0.7 ± 0.1	7	0	7/14
MSX2	31	48	0.09	0.4 ± 0.2	0	10	10/14
ALAD	544	697	*	0.7 ± 0.1	7	2	9/14
BAMBI	2114	3695	*	0.4 ± 0.2	2	10	12/14
LEF1	863	1221	0.10	0.6 ± 0.2	4	6	10/14
CTNNB1	504	683	**	0.8 ± 0.1	6	0	6/14
POMC	368	628	***	0.5 ± 0.2	3	9	12/14
VGF	124	335	*	0.3 ± 0.2	1	12	13/14

Genes	Average expression values		P values	Mean of fc in all patients	Nbr. of AGA patients for:		
	AGA	CTRL.			fc ≥ 1.5 and ≤ 2	fc > 2	Total
SFRP2	828	407	**	3.3 ± 1.1	2	12	14/14
ASIP	334	202	**	2.3 ± 0.6	5	8	13/14
CORIN	422	229	***	2.3 ± 0.3	3	11	14/14
SRD5A2	96	59	**	2.2 ± 0.7	2	8	10/14

The average of gene expression values (A.U.) and the means of fold change (fc) were calculated as in Table 3A.

Table 3C - TGBβ/BMP-signaling related genes

Genes	Average expression values		P values	Mean of fc in all patients	Nbr. of AGA patients for:		
	AGA	CTRL.			fc ≤ 0.7 and >0.5	fc ≤ 0.5	Total
TGFB2	23	39	***	0.5 ± 0.1	6	6	12/14
ITGB6	24	43	***	0.5 ± 0.1	2	10	12/14
BMP2	31	48	*	0.4 ± 0.1	4	9	13/14
DSG4	180	346	*	0.6 ± 0.3	0	11	11/14
DLX3	401	653	*	0.5 ± 0.2	2	9	11/14
KRT16	27980	46982	***	0.6 ± 0.2	6	6	12/14
HOXC13	569	1066	**	0.4 ± 0.2	3	9	12/14

The average of gene expression values (A.U.) and the means of fold change (fc) were calculated as in Table 3A.

Table 3D – Vitamin D3 – related genes

Genes	Average expression values		P values	Mean of fc in all patients	Nbr. of AGA patients for:		
	AGA	CTRL.			fc ≤ 0.7 and >0.5	fc ≤ 0.5	Total
CYP27B1	107	176	*	0.5 ± 0.2	3	9	12/14
TH	63	98	*	0.6 +/-0.1	4	8	12/14

The average of gene expression values (A.U.) and the means of fold change (fc) were calculated as in Table 3A.

Table 4 – Score of staining intensity of POMC and ASIP/PARD3 according to their distribution along the hair follicle.

Score of staining intensity

0	1	2	3	4	5	6	7	8	9

Volunteer	POMC Infundibulum + Upper root sheath	POMC Bulge	POMC Lower root sheath	POMC Bulb	ASIP/PARD3 Infundibulum + Upper root sheath	ASIP/PARD3 Bulge	ASIP/PARD3 Lower root sheath	ASIP/PARD3 Bulb
Control 1	8	7	5	4	6	5	6	4
Control 2	8	5	5	2	5	6	5	5
Control 3	7	4	6		5	4	5	6
Control 4	**7**	**7**	**3**	**2**	**4**	**3**	**3**	**5**
Control 5	6	2	2	2	3	4	4	
Control 6	7	5	4	4	2	4	4	7
Control 7	7	5	4		3	5	2	
Control 8	7	5	4	3	2		2	7
Control 9	6	5	2	3	2	1	3	7
Control 10	7	5	2	3	2	5	3	5
Control 11	8	7	5	6	2	4	4	2
Control 12	5	2	2		3	4	3	2
Control 13	7	4	2		3	4	4	
Control 14	**7**	**4**	**2**	**2**	**3**	**4**	**3**	**3**
AGA 1	4	2	2	3	3	3	4	
AGA 2	6	2	2	1	2	3	4	3
AGA 3	4	1	1	1	3	1	2	
AGA 4	4	1	2	4	2	3	4	2
AGA 5	4	1	1	2	2	2	3	1
AGA 6	4	1	3	0	2	2	3	2
AGA 7	5	2	2	1	2	3	2	1
AGA 8	3	1	1		3	1	2	3
AGA 9	**4**		**1**		**3**	**3**	**4**	**1**
AGA 10	3	2	2	1	4	4	4	1
AGA 11	6	2	2	1	4	5	5	3
AGA 12	2	2	1	1	4	2	3	2
AGA 13	2	1	1		1	2	4	5
AGA 14	2	1			2	2	3	2

	POMC Infundibulum + Upper root sheath	POMC Bulge	POMC Lower root sheath	POMC Bulb	ASIP/PARD3 Infundibulum + Upper root sheath	ASIP/PARD3 Bulge	ASIP/PARD3 Lower root sheath	ASIP/PARD3 Bulb
Ratio AGA/CT	0,6	0,3	0,5	0,5	0,8	0,6	0,9	0,4
p value	***0,000001***	***0,000001***	***0,0002***	***0,005***	***0,10***	***0,001***	***0,21***	***0,0005***

LEGENDS TO FIGURES

Figure 1. Photographs of two representative AGA subjects (A) and two representative control volunteers (C).

Arrows indicate the site where the scalp biopsies were carried out.

Figure 2. Transcriptomic analysis of scalp biopsies from subjects with AGA (A) and controls (C).

Histogram of differentially expressed genes between alopecia and control volunteers depicts a quite similar number of significantly upregulated and downregulated genes. Blue bar charts and orange bar charts represent Up-regulated and Down-regulated genes in group A *versus* group C, respectively. Legend shows 3 groups of fold changes (fc): fc > 2.5, fc > 2 and < 2.5 and fc >1.5 and <2 for up-regulated genes and fc < -2.5, fc > -2.5 and < -2 and fc < -1.5 and >-2.

Figure 3 – Hierarchically clustered heat maps (Euclidean distance, complete linkage) of reporters with higher expression in the alopecia group (A) relative to the healthy controls (C) (two-dimensional clustering)

Figure 4 - Gene expression analysis of some key factors by qPCR

Selection of differentially expressed genes in volunteers with AGA compared to control ones was obtained after DGA and functional analysis, completed by PredictSearch one. Results are presented as fold changes in mean gene expression (alopecia/control samples), with n=14 samples for each group. † $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

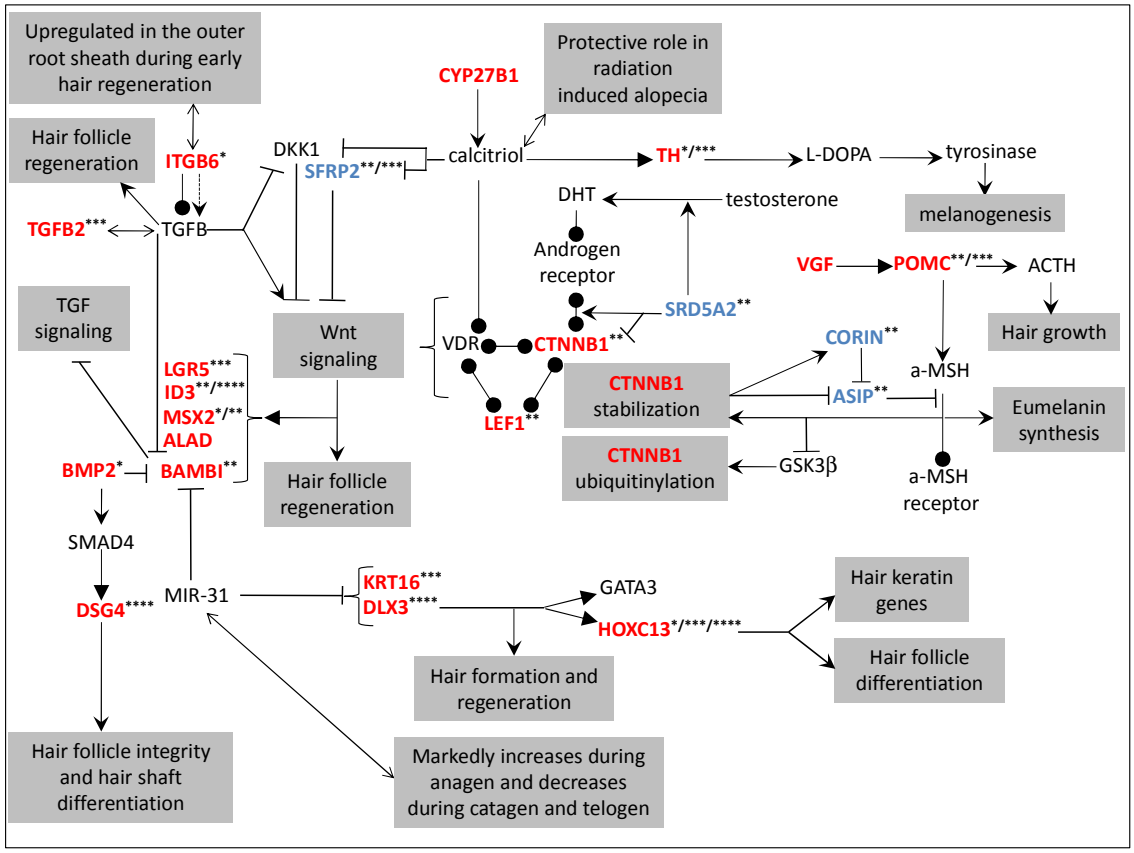
Figure 5 – Immunohistochemistry staining

POMC (4A) and ASIP/PARD3 (4B) immunostaining in the main areas of the hair follicle as depicted in one representative control group volunteer and one representative AGA group volunteer. POMC and ASIP/PARD3 are visualized with AlexaFluor 488 (green) and nuclear staining with propidium iodide (red). Colors and numbers as indicated by the "score of staining intensity" (table 4) represent the intensity of the immunolabeling found for POMC and ASIP according to their distribution along the hair follicle, ranging from 1 (the lowest) to 8 (the highest) staining intensity. Magnification x63.

Figure 6 – Schematic representation of signaling pathways in AGA.

Schematic representation of functional networks. Functional correlations between the modulated genes was designed with the help of the text-mining software PredictSearch (see materials and methods). Modulated genes in AGA are indicated in blue when overexpressed or in red when underexpressed in AGA *versus* volunteers.

*, **, ***, **** indicate in which compartment(s) of the hair follicle (hair matrix, dermal papilla, outer root sheath or inner root sheath, respectively), the gene expression was reported to be detected, with respective references as following: ASIP [76], BAMBI [77], BMP2 [77], CORIN [76], CTNNB1 [46, 50], DLX3 [63], DSG4 [78], HOXC13 [79], ID3 [80], ITGB6 [81], KRT16 [82], LEF1 [83], LGR5 [84], MSX2 [85], POMC [86, 87], SFRP2 [35], SRD5A2 [88], TGFB2 [89, 56], TH [82].



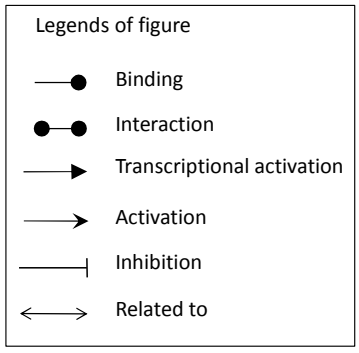


Figure 1

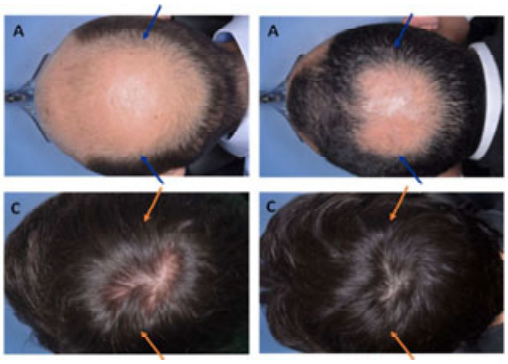


Figure 2

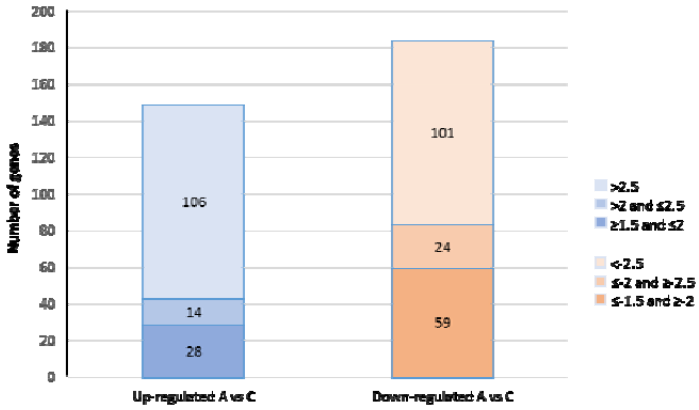


Figure 3

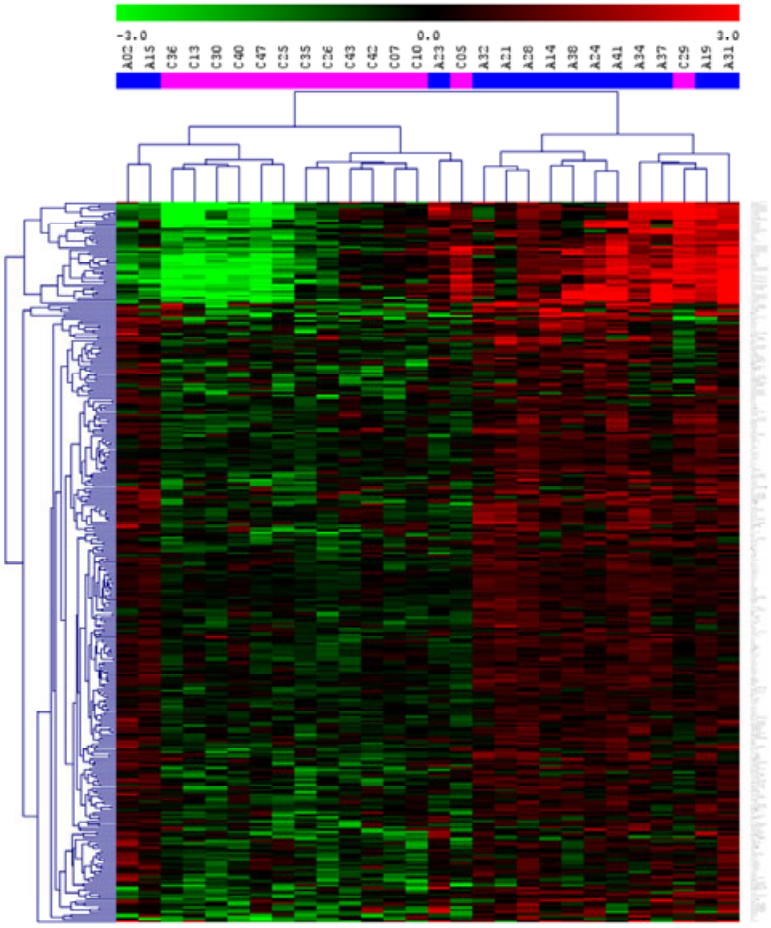


Figure 4

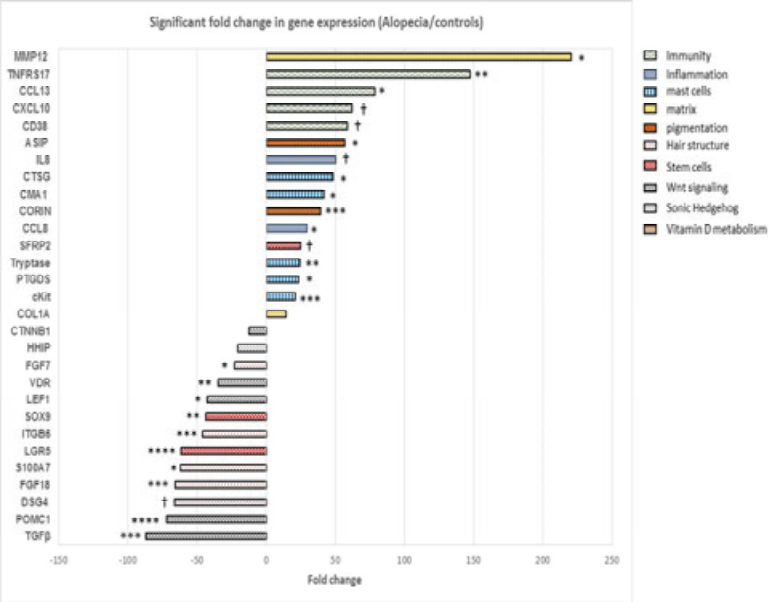


Figure 4