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Changing human hair fibre colour and shape from the follicle



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HIGHLIGHTS

- New pool of genes with different levels of transcription associated with different hair shapes.
- Over-representation analysis points to epigenetic regulation and glutathione metabolism in hair shape setting.

GRAPHICAL ABSTRACT

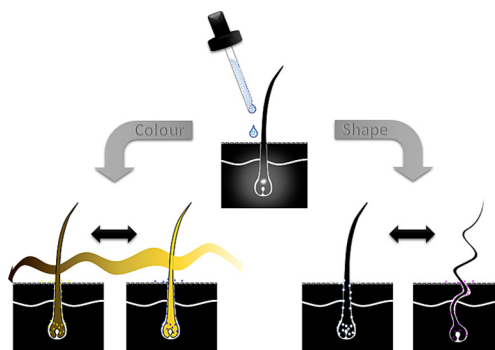
Abbreviations: AChE, Acetylcholinesterase; AMO, Amodiaquin dihydrochloride dihydrate; ANOVA, Analysis of variance; ASIP, Agouti signalling protein coding gene; cAMP, Cyclic adenosine monophosphate; CEP83, Centrosomal protein 83 coding gene; CGA, glycoprotein hormones alpha polypeptide coding gene; cGMP, Cyclic guanosine monophosphate; COX, cyclooxygenase; CREB, cAMP responsive-element-binding protein; DAVID, Database for Annotation, Visualization and Integrated Discovery; DIP, dipyrindamole; DMSO, dimethyl sulfoxide; EDAR, Ectodysplasin A receptor coding gene; EMA, European Medicines Agency; *ERRF11*, ERBB Receptor Feedback Inhibitor 1 coding gene; ETA, Ethacrynic acid; ETP, Entacapone; *FABP7*, Fatty acid binding protein 7 coding gene; FC, Fold change; FDA, Food and Drug Administration; *FGFR2*, Fibroblast Growth Factor Receptor 2 coding gene; *FKBP2*, FK506-binding protein 2 coding gene; *FMO1*, Flavin-containing monooxygenase 1 coding gene; *FMO9P*, Flavin Containing Dimethylalanine Monooxygenase 9, Pseudogene; *FRAS1*, Fraser Extracellular Matrix Complex Subunit 1 coding gene; *GATA3*, GATA binding protein 3 coding gene; GPx, glutathione peroxidase; *Gpx2*, glutathione peroxidase 2 coding gene (mouse); *GPX2*, glutathione peroxidase 2 coding gene; GPX2, human glutathione peroxidase 2; *GPX3*, glutathione peroxidase 3 coding gene; GPX3, human glutathione peroxidase 3; GST, glutathione S-transferase; *GSTM2*, glutathione S-transferase mu 2 coding gene; *GSTM4*, glutathione S-transferase mu 4 coding gene; H3, histone 3; H4, histone 4; *H2AC11*, H2A clustered histone 11 coding gene; *H3C10*, H3 clustered histone 10 coding gene; *H4-16*, H4 histone 16 coding gene; *H4C6*, H4 clustered histone 6; *H4C13*, H4 clustered histone 13 coding gene; HF, Hair follicle; *HOXC13*, Homeobox C13 coding gene; HSP, Heat shock protein; HTS, High throughput screening; IFN γ , Interferon gamma; IGFBP5, IGF-binding protein 5; IRS, inner root sheath; *KRT25*, keratin 25 coding gene; *KRT71*, keratin 71 coding gene; *KRT74*, keratin 71 coding code; *KRTAP*, keratin associated protein coding gene; *LCE3E*, Late cornified envelope 3E coding gene; LGR4, Leucine rich repeat containing G protein-coupled receptor 4 coding gene; LINC00708, Long intergenic non-protein coding RNA 708 gene; *LIPH*, Lipase H coding gene; *LOC100132891*, *Homo sapiens* complex locus LOC100132891, uncharacterized transcript; *LOC101927117*, *Homo sapiens* locus LOC101927117, uncharacterized transcript; *LPAR6*, Lysophosphatidic acid receptor 6 coding gene; MAPK, mitogen-activated protein kinase; *MC1R*, Melanocortin 1 receptor coding gene; MDD, midodrine hydrochloride; *MIR6871*, MicroRNA 6871 gene; MTF, Microphthalmia-associated transcription factor; *MRFAP1*, Morf4 family associated protein 1 coding gene; *MSC*, Musculin coding gene; NADPH, Nicotinamide adenine dinucleotide phosphate; *NBP18P*, NBP member 18, pseudogene; *NDUFV2*, NADH:ubiquinone oxidoreductase core subunit V2 coding gene; NOS, nitric oxide synthase; Nrf2, Nuclear factor E2-related factor 2; *OCA2*, OCA2 melanosomal transmembrane protein coding gene; *OFCC1*, Orofacial cleft 1 candidate 1 coding gene; ORA, Over-representation analysis; *PADI3*, Peptidyl arginine deiminase 3 coding gene; *PEX14*, Peroxisomal biogenesis factor 14 coding gene; PKA, Protein kinase A; *PLCG1*, Phospholipase C gamma 1 coding gene; PPAR- γ , Peroxisome proliferator-activated receptor gamma isoform; *PPDPF*, Pancreatic progenitor cell differentiation and proliferation factor coding gene; *PRSS53*, Serine protease 53 coding gene; *PTK6*, Protein tyrosine kinase 6 coding gene; qPCR, quantitative polymerase chain reaction; RNS, reactive nitrogen species; ROS, reactive oxygen species; RPTN, Repetin coding gene; *S100A10*, S100 calcium binding protein A10 coding gene; *S100A11*, S100 calcium binding protein A11 coding gene; *SEPTIN3*, Septin 3 coding gene; *SLC24A5*, Solute carrier family 24, member 5 coding gene; *SLC45A1*, Solute carrier family 45, member 1 coding gene; *SLC45A2*, Solute carrier family 45, member 2 coding gene; *SRMS*, Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites coding gene; *TCERG1L*, Transcription elongation regulator 1 like coding gene; TCF, T-cell factor; TCHH, Trichohyalin coding gene; *TCHHL1*, Trichohyalin like 1 coding gene; *TGFA*, Transforming growth factor alpha coding gene; TNF α , Tumour necrosis factor alpha; TPM, Topiramate; *TYRP1*, Tyrosinase related protein 1 coding gene; WebGestalt, WEBbased Gene Set Analysis Toolkit; WNT10A, Wnt family member 10A coding gene; ZOM, Zomepirac sodium salt.

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- Most variable genes between very curly and straight hairs are expressed in the inner root sheath.
- A screening methodology to search for molecules able to modulate the hair shape and colour was established.
- Modulation of hair colour and shape in the follicle by topical application of bioactive molecules is feasible.



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ABSTRACT

Introduction: Natural hair curvature and colour are genetically determined human traits, that we intentionally change by applying thermal and chemical treatments to the fibre. Presently, those cosmetic methodologies act externally and their recurrent use is quite detrimental to hair fibre quality and even to our health.

Objectives: This work represents a disruptive concept to modify natural hair colour and curvature. We aim to model the fibre phenotype as it is actively produced in the follicle through the topical delivery of specific bioactive molecules to the scalp.

Methods: Transcriptome differences between curly and straight hairs were identified by microarray. In scalp samples, the most variable transcripts were mapped by *in situ* hybridization. Then, by using appropriate cellular models, we screened a chemical library of 1200 generic drugs, searching for molecules that could lead to changes in either fibre colour or curvature. A pilot-scale, single-centre, investigator-initiated, prospective, blind, bilateral (split-scalp) placebo-controlled clinical study with the intervention of cosmetics was conducted to obtain a proof of concept (RNEC n.92938).

Results: We found 85 genes transcribed significantly different between curly and straight hair, not previously associated with this human trait. Next, we mapped some of the most variable genes to the inner root sheath of follicles, reinforcing the role of this cell layer in fibre shape moulding. From the drug library screening, we selected 3 and 4 hits as modulators of melanin synthesis and gene transcription, respectively, to be further tested in 33 volunteers. The intentional specific hair change occurred: 8 of 14 volunteers exhibited colour changes, and 16 of 19 volunteers presented curvature modifications, by the end of the study.

Conclusion: The promising results obtained are the first step towards future cosmetics, complementary or alternative to current methodologies, taking hair styling to a new level: changing hair from the inside out.

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Introduction

In line with our previous thoughts and hypothesis [1], we propose an unconventional approach to control hair traits through the modulation of gene expression and/or protein activity by the topical delivery of specific bioactive molecules. Hair is one of the most memorable features of our image. Made to protect the scalp from sunburns and the brain from overheating, we have turned this skin appendage into a way of expressing ourselves and manifesting our individuality. Along with the natural diversity of hair phenotypes, we learned how to change its colour and shape by applying thermal and chemical treatments to radically change our look. Currently, all available cosmetic technologies to change colour and shape act on hair fibres, externally.

Hair shafts show striking diversity across and within all human populations, proposing that hair fibre shape and colour have undergone many adaptive changes over the years with a genetic basis far from being completely unveiled [2]. Hair colour is one of the most conspicuous human phenotypes. The natural colouration of hair is a multistep process that involves the synthesis of pigments (melanins) by the melanocytes, their transfer to surrounding pre-cortical keratinocytes, and their incorporation into the forming hair shafts [3,4]. In melanocytes, two chemically

distinct pigments are produced: eumelanin (brown to black) and pheomelanin (yellow to reddish-brown). The diversity in human hair colour arises from the quantity and ratio of eumelanin and pheomelanin, with other physical aspects of the hair shafts intervening as minor modifiers. Several polymorphisms in a wide range of genes affecting melanogenesis (e.g.: *ASIP*, *MC1R*, *TYRP1*, *OCA2*, *SLC24A5*, *SLC45A2*) are known to influence the production of melanins, being responsible for the normal diversity of human hair colour [5–7].

Though less understood than colour, it is known that hair shape is also defined by the follicle: follicle size and curvature define hair curliness. Fibre curvature requires hair bulb axial asymmetry in the processes involving cell proliferation and differentiation [8]. Different genes have been associated with (1) HF cells proliferation and differentiation and, thereby, with hair morphology, (2) with pathological conditions affecting hair morphology, and (3) with human populations' hair morphology traits [9–12].

Natural hair shape and colour are normally fixed traits in adults; thus, on each new restart of the hair cycle, the colour and curvature characteristics of the fibre are restored, as genetically programmed [3,13]. Hair colour always had an enormous social and cosmetic impact as exemplified by the ancient trend of dying the hair to appear more attractive. Currently, the concern with hair

colour as well as length, shape, and density is higher than ever, as society focuses more and more on beauty and youthfulness. Hair colouring, permanent waving, or straightening rely on aggressive chemical and/or physical processes that can result in acute or chronic deleterious traumas, such as hair loss or even cancer development [14–23]. Moreover, these procedures cause split, dry, and dull hair due to cumulative damage to the fibre structure [24,25]. Efforts to develop dye and straightening systems that minimize fibre damage have been made, but progress has been limited and risks remain [26–28]. With this work, we propose a new perspective and approach to control hair shape and change hair colour: the topical delivery of bioactive molecules able to modulate the activity of specific genes and/or proteins in the living cells of the HFs, changing hair from its root. Our approach aims to avoid the negative side effects on hair fibres of current cosmetic treatments.

The fact that there are changes in the appearance of human hair as a side effect of a few clinical treatments and that the reported cases of hair colour and shape changes are reversible and dose-dependent [29] constitute supporting arguments that we can design cosmetic formulations to be applied on the scalp, containing specific molecules, in specific dosages, for the safe control of hair shape and colour as it is produced in the follicle. Case reports on drug-induced changes in hair colour are either as lightening or darkening [30–32]. There are also reports on curly to straight hair transitions and *vice-versa* as a side effect [32–34]. To determine the *in vivo* cosmetic feasibility and relevance of a different approach consisting in the topical use of bioactive ingredients for hair colour and shape modulation at the follicle level, we set in motion a multistep work plan. Due to the reduced available information about the genetic determinants of human hair shape phenotypes, we undertook a high-throughput approach to compare the levels of gene expression between straight hair and very curly hair and we mapped to specific follicle layers some of those protein-coding genes that showed the highest fold changes between the two phenotypes. The collected data on the hair curvature-associated genes was then used to lay in the ground a screening approach for bioactive molecules with the potential to change hair curvature. We already developed a sensitive methodology for melanin quantification and therefore a tool for searching *in vitro* possible colour modulators [35]. We screened Prestwich Chemical Library® (an off-patent collection of bioactive molecules, mostly approved by the FDA, EMA, and other agencies) regarding the effect on melanin production and the ability to change the transcription levels of selected genes. After confirming the modulator activity of the top hit inducers and inhibitors, a clinical pilot study with the intervention of cosmetic products was conducted to validate the suitability of some selected drugs as cosmeceutical ingredients for the modulation (darkening, lightening, waving, or straightening) of hair colour and shape at the follicle.

Experimental

Materials and subjects

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Artur Cavaco-Paulo. The sequence of original primers generated during the study has been fully disclosed in [Appendix A, S1 Table](#). Besides these primers, designed for the synthesis of the riboprobes, and the formulations for scalp topical application of bioactive molecules, this study did not generate any other new unique reagents or material. The formulations are the object of the international provisional patent application WO/2021/260667, published on December 30, 2021. Information on experimental models and participants is provided in [Appendix A, S2–S6 Tables](#).

Microarray analysis of hair follicle transcriptome

Sample collection and RNA isolation

Around 50 pigmented hair follicles (HFs) were plucked from each donor with dark brown hair ([S4 Table](#)), after informed consent, and according to a protocol complying with the rules of ethical conduct and requirements of good practices in experimenting with humans, approved by the competent legal authority, the Ethic Council of the University of Minho (ECUM). The collection procedure consisted in isolating one hair with fingers (protected with gloves) and then pulling it close to the root with tweezers. After visually checking the integrity, the follicle was immersed in a preservation solution (RNAlater). Then, the excess hair shaft was cut off with scissors. The process was repeated until 50 HFs were collected from the entire scalp into the same microtube, which was stored at 4 °C and later transferred to –80 °C.

Samples were thawed on ice and the total RNA was extracted using the SV Total RNA Isolation system according to its protocol. Briefly, the RNAlater® solution was removed, and HFs were lysed with RNA Lysis Buffer. After adding the RNA neutralizing solution, the samples were heated at 70 °C for 3 min. Then, the tubes were centrifuged for 10 min at 12,000 g and the clear solution was transferred to a clean microtube. The remaining hair shafts were discarded at this point. Ethanol was added, and the lysate was transferred to a spin basket assembly, to proceed according to the protocol. The RNA was eluted with 50 µL of nuclease-free water. Concentration and purity were determined by spectrophotometry (NanoDrop ND-1000 UV/VIS, ThermoFisher Scientific, Waltham, Massachusetts, EUA). Integrity was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA).

Target synthesis and hybridization to GeneChips

Total RNA was processed for use on Affymetrix GeneChip HuGene 1.0 ST Arrays, according to the manufacturer's Whole Transcript Sense Target Labeling Assay. Briefly, 100 ng of total RNA containing spiked in Poly-A RNA controls was used in a reverse transcription reaction to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an *in vitro* transcription (IVT) reaction to generate cRNA from which 15 µg were used for a second cycle of first-strand cDNA synthesis. An amount of 5.5 µg of single-stranded cDNA was fragmented and end-labeled. Size distribution of the fragmented and end-labelled cDNA was assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay. Five micrograms of end-labelled, fragmented cDNA were used in a 100 µL hybridization cocktail containing added hybridization controls. A volume of 80 µL of mixture was hybridized on arrays for 17 h at 45 °C. Standard post-hybridization wash and double-stain protocols (Fluidics protocol FS450_0007, Affymetrix) were used on an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an Affymetrix GeneChip scanner 3000 7G.

The data generated was used in the hair shape transcriptome comparison: very curly versus straight hair phenotypes.

GeneChip data and functional enrichment analysis

The data from the eleven arrays, corresponding to the collected pigmented HFs samples from the eleven donors ([S4 Table](#)), were analyzed using Chipster 2.2 [36] using custom cdf file HuGene10stv1_Hs_ENTREZG as available from Brainarray database version 14 [37]. Following RMA normalization and biomaRt annotation, differential expression was determined by empirical Bayes two-group test [38] with Benjamini-Hochberg multiple testing correction and a p-value cut-off of 0.05.

The Over-Representation Analysis (ORA) was performed on the significantly different transcribed genes using two online tools:

WebGestalt [39] and using DAVID [40]. Considering all mapped Entrezgene IDs from the platform affy_hugene_1.0_st.v1 as the reference list, an ORA was performed for each of the following functional categories: Gene Ontology (GO), KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and Wikipathway. The minimum number of genes per category was selected as 3 genes. Only the significant enriched terms with a false discovery rate below 0.05 are represented in the graph (FDR values adjusted by the Benjamini–Hochberg method).

Microarray validation by qPCR

A group of nine genes with the highest fold changes were selected to confirm, by qPCR using TaqMan® probe chemistry, the observed differences in transcription levels in the same samples used in the microarray analysis. The cDNA pre-amplification was performed using the RNA extracted from hair follicles from the same donors (S4 Table) and the iScript™ cDNA Synthesis kit according to the manufacturer's recommendations. The volume of reagents was fixed except for water and the RNA sample; the amount of RNA used per reaction was 200 ng and the water volume was set to complete the final volume of 40 µL. The reaction conditions were 5 min at 25 °C, 60 min at 42 °C and 5 min at 85 °C. The reaction was performed on a thermocycler (Bio-Rad iCycler Thermal Cycler). The cDNA samples were stored at –20 °C.

Quantification of gene transcription was done using a CFX96™ Real Time PCR Detection System. Each reaction mix consisted of 10 µL of TaqMan® Universal master mix, 1 µL of TaqMan® gene assay and 1 µL of the cDNA. RNase-free water was added to complete the final reaction volume of 20 µL. The qPCR cycling conditions were: one cycle at 95 °C for 10 min and 50 cycles of 2 steps at 95 °C for 15 s, and at 60 °C for 1 min. Each sample was normalized to the experimental vehicle control using the $2^{-\Delta\Delta C_q}$ comparative method [41] and the endogenous gene, the (*MTATP6*). Each assay included duplicate reactions for each sample and was repeated once.

Riboprobe design and synthesis

Biological samples and total RNA extraction

Around 50 pigmented HFs were plucked from two donors, using the same procedure described for the microarray analysis sample collection. Total RNA from HFs was quantified by the Nanodrop® ND-1000 spectrophotometer (Nanodrop® Technologies; Wilmington).

Probe design

For each selected gene, the sequence of all possible transcripts known to be expressed in humans was retrieved from the National Center for Biotechnology Information (NCBI) Nucleotide database. For each of the chosen gene transcripts, the coding region (CDS) and untranslated region 3' (3' UTR) were identified. The common sequence in all the different transcripts for a particular gene was identified using the LALIGN alignment tool [42]. The secondary structure of each transcript was obtained by the Mfold Web Server [43]. RNA Folding Form (version 2.3) was used to obtain the predicted secondary structure of all the gene transcripts. The standard program settings were maintained except for the folding temperature which was set at 70 °C.

Primer design

The primers were designed using the free software Primer3Plus [44]. The promoter sequence for T3 RNA Polymerase was added to all the reverse primer sequences (complete primer sequence available in the S1 Table).

Reverse transcription

A mixture of the RNA extracted from both donors was reverse transcribed using the iScript™ cDNA Synthesis kit according to the manufacturer's recommendations. The volume of reagents was fixed except for water and RNA sample; the amount of RNA used per reaction was 1 µg and the water volume was set to complete the final volume of 20 µL. The reaction conditions were 5 min at 25 °C, 60 min at 42 °C and 5 min at 85 °C. The reaction was performed on a thermocycler (Bio-Rad T100™ Thermal Cycler). The cDNA samples were stored at –20 °C.

Target cDNA amplification by PCR

Before performing the synthesis of probes, the DNA template was amplified using the designed primers and the synthesized cDNA obtained as described above. All PCR amplifications used 2x Phusion Flash High-Fidelity Mix following the manufacturer's instructions; the volume fractions of cDNA and each primer were 10 % and 5 % of the final PCR reaction, respectively. The final amplification conditions for each gene were optimized and validated (S7 Table).

PCR product purification

All the PCR products were purified from agarose gel bands. First, they were run at agarose concentrations between 10 and 15 g/L, according to their predicted molecular weights. The gels were previously prepared by dissolving the agarose powder in 0.5x TBE buffer and pre-stained with 0.04 % (v/v) of Midori Green Advance DNA staining. After electrophoresis, the gel was observed on an ultraviolet transilluminator (Transilluminator UV, GenoView with GenoSmart, VWR). The gel band, containing the DNA fragment of interest, was excised using a razor blade, placed in a clean tube and weighed. The PCR product was purified according to the instructions of the PureLink Quick Gel Extraction and PCR Purification Combo Kit.

Probe synthesis

Digoxigenin (DIG) incorporation through the transcription of the cDNA into the cRNA probe was accomplished following Gandrillon's protocol [45].

Briefly, 1 µg of the amplified and purified target cDNA was incubated with the reaction mix containing 7 µL of 5x transcription buffer, 2 µL of T3 RNA polymerase, 2 µL of RNasin®, 2 µL of 10x DIG RNA labelling mixture, 4 µL of 0.1 M DTT (DL-dithiothreitol) and ultrapure water to complete the volume of 35 µL. The reaction was performed at 37 °C for 3 h. The cDNA template was degraded for 30 min at 37 °C after adding 4 µL of RQ1-DNase and 2 µL of RNasin®. The reaction was stopped by the sequential addition of 200 µL of TE buffer, 20 µL of 4 M LiCl and 600 µL of absolute ethanol. The labelled RNA was purified by precipitation at –20 °C overnight (or at –80 °C for 45 min) and centrifugation at 11000 g for 30 min, at 4 °C. The RNA pellet was then washed with 70 % ethanol, centrifuged again for 15 min (11000 g, 4 °C), dried, resuspended in 50 µL of 10 mM EDTA (pH 8.0) and stored at –20 °C. The efficiency of probe synthesis was checked by electrophoresis in an 8 g/L agarose gel as described before.

In situ hybridization (ISH)

Biological samples

The paraffin blocks of *postmortem* scalp skin samples were obtained during clinical autopsies of donors with different hair curliness phenotypes, in Hospital de Santa Maria/Faculdade de Medicina de Lisboa, complying with the rules of ethical conduct and requirements of good practices approved by the competent legal authority, the Comissão de Ética do Centro Académico de Medicina de Lisboa. These paraffin blocks were processed by the

Histology Service from the Life and Health Sciences Research Institute, in Braga, according to their standard procedures. From each paraffin block, many histological sections of 10 µm were cut, with three sections being glued per slide.

Deparaffinization and rehydration of sections

The paraffin was solubilized by immersing the slides in xylene, 3x 5 min, then the skin sections were rehydrated progressively in a series of aqueous ethanol solutions of decreasing ethanol volume fraction: 100 % for 2x 5 min, 90 % for 2 min, 70 % for 2 min and 30 % for 2 min. The last wash was made with 1x Phosphate-buffered saline (PBS, pH 7.4) for 5 min. The procedure was performed in glass jars at room temperature, on a gyratory shaker with gentle agitation.

Protein digestion and probe hybridization

After the rehydration steps, the sections were subjected to proteolytic digestion using 2 µg/mL of proteinase K in PBS, for 14 min at 37 °C, in a water bath with moderate agitation. The enzyme was rinsed off with PBS (2x 2 min, at room temperature). The sections were subsequently fixed with 4 g/L paraformaldehyde in PBS, for 20 min at room temperature. Afterwards, sections were washed with PBS (5 min, at room temperature) to remove the paraformaldehyde and they were equilibrated in 50 mL of 2x Saline Sodium Citrate (SSC, pH 7.5), 2x 10 min at room temperature.

The purified RNA probe solution was diluted a hundred times in a hybridization mix solution with the following composition in RNase-free water: 50 % (v/v) of formamide, 195 mM NaCl, 9 mM Tris-HCl, 1 mM Tris-base, 5 mM NaH₂PO₄·2H₂O, 5 mM Na₂HPO₄, 5 mM EDTA, 100 g/L dextran sulphate, 1 g/L yeast RNA and 1x Denhardt's solution [0.2 g/L Ficoll-type 400, 0.2 g/L polyvinylpyrrolidone, 0.2 g/L bovine serum albumin - fraction V]. A hybridization mix volume of 150–200 µL was applied per section; sections were covered and incubated in a humidified chamber overnight, at 70 °C.

DIG immunological detection

The next day, sections were washed three times at 65 °C in 1x SSC, pH 7.5 containing, in volume fractions, 50 % formamide and 0.1 % Tween 20 (15 min plus 2x 30 min). Then, sections were washed 2x 30 min in freshly prepared maleic acid buffer containing Tween 20 [MABT: 100 mM maleic acid, 149 mM NaCl, 1 % (v/v) Tween 20, pH 7.5]. The sections were then blocked with 400 µL per slide of MABT containing the volume fractions 2 % of Blocking reagent and 20 % of goat serum, for 1.5 h. Anti-DIG antibody was diluted 1:2000 in the blocking solution and a volume of 400 µL was applied to each section. The slides were incubated overnight at room temperature.

Next day, the slides were rinsed four times with MABT for 30 min each washing and equilibrated in NaCl/Tris-HCl/MgCl₂ solution (NTM) (100 mM NaCl, 100 mM Tris-HCl, 50 mM MgCl₂, pH 9.5) for 30 min at room temperature.

Signal development was accomplished using a colour reaction mix containing 6.75 µL NBT (4-nitroblue tetrazolium chloride) and 52.5 µL of BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt) per 15 mL of NTM. The mix volume per slide was 300 µL; the slides were incubated at 37 °C in the dark until the desired intensity was achieved. To stop the alkaline phosphatase colour reaction, the slides were washed twice for 5 min in PBS buffer with 0.1 % Tween 20.

The sections were examined by bright field microscopy using an Olympus IX70 inverted epi-fluorescence microscope and a Leica DM 5000B Microscope.

In vitro screening for hair color modulators

Cell culture conditions

Human skin keratinocytes (NCTC2544 cell line) were purchased from Instituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia, Italy). SK-Mel-1 and SK-Mel-23 cell lines (derived from pigmented human skin melanomas) were kindly provided by Doctor Sofia Magina (Centro de Investigação Médica, Faculdade Medicina do Porto, Portugal) and Doctor Francisco X. Real (Epithelial Carcinogenesis Group, Centro Nacional de Investigaciones Oncológicas, Spain), respectively. SK-Mel-1, SK-Mel-23 and NCTC2544 cell lines were grown in Roswell Park Memorial Institute (RPMI-1640) medium, supplemented with 10 % fetal bovine serum (FBS) and 1 % of antibiotic/antimycotic solution ([S2 Table, Appendix A](#)). The cells were kept at 37 °C in a humidified atmosphere containing 5 % CO₂. Every 2–3 days, the cell culture medium was refreshed.

Molecular library dilution

The Prestwick Chemical Library® (Harvard Institute of Chemistry and Cell Biology) was used in these experiments as the source of molecules with the potential to alter hair colour and shape. This library contained 1200 pharmacological compounds dissolved in DMSO at 10 mM. Most of them are approved by the FDA, EMA and other agencies - selected for their high chemical and pharmacological diversity. All drugs are off-patent, their bioavailability is known and safety data in humans is available.

The 10x dilution of the chemical library was prepared in polystyrene 96-well plates, sterile with round bottom in DMSO and kept at –20 °C. The final concentration of the bulk of the library compounds was 10 µM and the final DMSO volume fraction was 1 %. The more cytotoxic drugs were further diluted according to their toxicity levels, maintaining the DMSO final concentration.

Screening of melanogenesis modulators

SK-MEL-23 was used as a model of human primary melanocytes due to greater ease of manipulation and reproducibility of *in vitro* melanin production, which are very important factors on large-scale tests such as the one necessary to screen the 1200 drugs in cells. SK-Mel-23 cells were treated with the test compounds for three days. Then, melanin was quantified by fluorescence and the amount of pigment produced was normalized to the protein content of each sample before the analysis of the 1200 compounds, several parameters were optimized to adapt the fluorescence-based melanin quantification to a plate format. The optimization was performed using previously known chemical modulators of melanogenesis: forskolin (Fsk, 20 µM) as an inducer of melanogenesis and kojic acid (KA, 2 mM) as an inhibitor of melanin production. A robust large-scale system for *in vitro* screening of melanogenesis modulators was established, based on the fluorescence method for melanin quantification [[35](#)], with the set-up being described below.

SK-Mel-23 cells were seeded on 24-well plates at a density of 9.0x10⁴ cells/well. The next day, the medium was changed, and cells were treated with 500 µL of medium containing 10 µM of the pharmacological drugs for 72 h. Each plate had four controls: 1 % DMSO (solvent control), untreated cells (negative control), 20 µM Fsk (positive control for melanogenesis induction) 2 mM KA (positive control for melanogenesis inhibition). At the end of the treatment, the culture medium was removed, wells were washed twice with PBS, pH 7.4 and cells were lysed with 1 M NaOH containing 10 % (v/v) DMSO. Samples of each lysate were collected to determine protein content by the DC Protein Assay, using bovine serum albumin as the protein standard. Protein content served as an indirect measure of the cytotoxicity of each library compound, and it was used to normalize the cellular level of melanin in different samples. Aqueous

hydrogen peroxide solution was added to each well at the final volume fraction of 30 % and plates were incubated in the dark at 25 °C for 4 h under mild agitation. The fluorescence of oxidized lysates was measured ($\lambda_{\text{ex}} = 470 \text{ nm}$; $\lambda_{\text{em}} = 550 \text{ nm}$), and melanin content was calculated by interpolating the results with a standard curve generated by fluorescence of oxidized *Sepia officinalis* melanin standards. SynergyMx multiwall plate reader spectrophotometer was used for fluorescence measurements. Each compound was tested in triplicate in three independent plates.

The drugs that induced cytotoxicity at 10 μM were reassessed using a decreasing set of concentrations [5, 1, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 (μM)] until the protein content was above 70 % (compared to solvent control).

Dose-response assay of selected hits

SK-Mel-23 and SK-Mel-1 cells were seeded on 24-well plates at a density of 9.0×10^4 cells/well. The cells were treated with different concentrations [1, 5, 10, 50 and 100 (μM)] of dipyrindamole (DIP), phenazopyridine hydrochloride (PHE), amodiaquin dichloride dihydrate (AMO), rivastigmine hydrogen tartrate (RIV), zomepirac sodium salt (ZOM) and paroxetine hydrochloride (PAR) for 24 h, 48 h and 72 h. Several controls were performed simultaneously: negative control (untreated cells), solvent control, melanogenesis inducer (20 μM Fsk) and inhibitor (2 mM KA) positive controls. NCTC2544 cells, treated under the same set of conditions as the melanotic lines, were used to control the fluorescence background. At the end of treatments, cells were processed as described for the primary screening to quantify melanin and total protein.

In vitro cytotoxicity

The cytotoxicity of selected hits was tested in SK-Mel-23 and SK-Mel-1. Cells were seeded on 24-well tissue culture plates at the initial density of 9.0×10^4 cells/well. The next day, fresh medium containing different concentrations of DIP [1, 5, 10, 50 and 100 (μM)], PHE, AMO, RIV, ZOM, and PAR were added to the respective wells. The cell death control with 10 % (v/v) DMSO, the untreated control with just fresh medium and the solvent control were performed in parallel. Cell viabilities at 24 h, 48 h and 72 h of exposure were determined using the MTT reduction assay [46]. At the end of the exposure times, cells were incubated with MTT (0.5 g/L) for 1 h at 37 °C. The crystals were dissolved in 500 μL of a mixture of 1:1 ethanol and DMSO. Absorbance values at 570 and 690 nm were measured in duplicate, using a SynergyMx multiwall plate reader spectrophotometer. Percentages of cell viability were calculated considering the solvent dilution control as the reference for 100 % viability.

In vitro screening for hair shape modulators

Cell culture conditions

The human hair primary IRS cells from ScienCell Research Laboratories (Innoprot) were selected as the cell model to study the capacity of chemical compounds to change the expression levels of the selected genes.

Primary cells were cultured, following the manufacturer's instructions, in poly-L-lysine-coated 75 cm^2 culture vessels containing mesenchymal stem cell medium supplemented with 5 % FBS, 1 % mesenchymal stem cell growth supplement and 1 % penicillin/streptomycin solution. The cells were grown and maintained under a humidified atmosphere containing 5 % CO_2 at 37 °C. IRS primary cells were sub-cultured over 10 population doublings maximum, avoiding confluence; the culture medium was refreshed every 2 to 3 days.

Molecular library dilution

The dilution of the Prestwick Chemical Library® was made to obtain cell viabilities over 80 % regarding solvent control. The dilution was freshly prepared in polystyrene 96-well plates, sterile with a round bottom, the day before cell treatment, and diluted compounds were kept at 4 °C. The final concentration of the bulk of the library compounds was 25 μM and the DMSO final volume percentage was 0.5 %. The other remaining drugs were further diluted according to their toxicity levels tested before the screening, maintaining the solvent final concentration.

In vitro cytotoxicity

Cells were seeded at 2.0×10^4 cells/well on poly-L-lysine-coated 96-well culture plates the day before the experiments. The medium was changed and 1 μL of each prediluted compound was added to each well containing 200 μL of complete medium. The controls performed simultaneously were the cell death control with 10 % (v/v) DMSO, the untreated control without any treatment and the solvent control with DMSO at the same final concentration as in samples treated with the library compounds (0.5 %).

After 24 h of exposure, cell viability was determined using the MTT reduction assay. The medium was replaced by a new medium containing 0.5 mg/mL of MTT and maintained for 2 h at 37 °C under a humidified atmosphere containing 5 % CO_2 . The crystals were dissolved in 150 μL of a mixture of 1:1 ethanol and DMSO. Absorbance values at 570 nm and 690 nm were measured in a Spectramax 340PC microplate reader. Percentages of cell viability were calculated considering the solvent control as the reference for 100 % viability.

Screening of gene transcription modulators

IRS cells were plated at an initial density of 3.0×10^4 cells/well on poly-L-lysine-coated culture 96-well plates. On the next day, the medium (200 μL /well) was changed and cells were treated with 1 μL of each previously diluted library drug, according to the pre-established non-toxic concentration. Two controls were performed in each cell culture plate: the solvent (0.5 % DMSO) and untreated controls. After 24 h of incubation, the RNA isolation and reverse transcription were performed following the protocol of the TaqMan® Gene Expression Cells-to-CT™ kit. The cell lysates were kept at -80°C in the original cell culture plates and cDNA samples were stored at -80°C in low-bind PCR plates.

The cDNA pre-amplification was performed using the TaqMan® PreAmp Master Mix kit, according to its protocol. Briefly, a preamp reaction final volume of 25 μL contained 6.25 μL of cDNA and 6.25 μL of a pool of the TaqMan® assays for the six selected transcripts plus the endogenous gene (S1 Table). The pre-amplification reaction consisted of 14 cycles. The cycling conditions were: one cycle at 95 °C for 10 min and 14 cycles of 2 steps at 95 °C for 15 s, and at 60 °C for 4 min. The pre-amplification product was diluted ten times by adding 225 μL of TE buffer before qPCR and stored at -20°C .

Quantification of gene transcription was done using a CFX96™ Real Time PCR Detection System and a StepOnePlus™ Real-Time PCR System. Each reaction mix consisted of 10 μL of TaqMan® gene expression master mix, 1 μL of TaqMan® gene assay and 9 μL of the diluted preamplified cDNA. The qPCR cycling conditions were: one cycle at 95 °C for 10 min and 50 cycles of 2 steps at 95 °C for 15 s, and at 60 °C for 1 min. Control reactions included the pre-amplified cDNA of non-treated cells, a no-reverse transcription reaction control, and the no-template control. Each sample was normalized to the experimental vehicle control using the $2^{-\Delta\Delta\text{Ct}}$ comparative method [41] and two endogenous genes, the Cyclin Dependent Kinase Inhibitor 1A (CDKN1A) and the 18S ribosomal RNA (18S).

Screening validation

The best 24 selected hit compounds were validated using the same protocol described for the library screening, using the chemicals from a different commercial source.

Pilot cosmetic study

Ethics statement

The clinical study with intervention of cosmetics “Avaliação da eficácia de novos produtos cosméticos no controlo da cor e forma do cabelo” was conducted according to the deliberation of CEICVS - Ethics Committee for Research in Life and Health Sciences, University of Minho (CE.CVS 118/2018), registered on RNEC (National Registry for Clinical Studies) at 13/02/2019, with the number 92938, according to the Portuguese Law n. 21/2014, published in Diário da República n.º 75/2014, Série I, on April 16, 2014. Written informed consent was obtained from all subjects before any study-related procedures were initiated.

Subjects

Thirty-three adult volunteers from both sexes with ages between 18 and 55 years old (**S6 Table**) were considered eligible to participate. The exclusion criteria were as follows: pregnancy or intention to become pregnant during the study, breastfeeding, any known hair or scalp medical condition, known allergy/hypersensitivity to drugs or cosmetics, a significant systemic or chronic disease, use of daily medication (except for birth control), current participation in another clinical trial, pharmacologic or cosmetic treatment within the previous 30 days that could influence the outcomes of the study.

Hair cosmetic formulations under study

The cosmetic formulations used in this study (**S8 Table**) were prepared by dissolving one of the bioactive ingredients under test in a vehicle solution developed by Solfarcos in association with Inovapoteq. This vehicle solution, without any active ingredient, was also used as the placebo (Formulation O).

Study design

The study was designed as a single-centre, investigator-initiated, prospective, blind, bilateral (split-scalp) placebo-controlled, multiarm parallel study whose purpose was to demonstrate the feasibility (proof of concept) of hair colour and shape modulation by scalp topical treatment. The study took place at the Center of Biological Engineering, at the University of Minho, in 2019, and participants were recruited from the local academic community. The allocation of volunteers followed a covariate-adaptive randomization. Each participant was sequentially assigned to one of the arms of the study (Formulations A-G) by considering the hair characteristics and previous assignments (**Table 1**). On day 1, two distinct areas (approx. 1 cm²) were shaved on the back of the subject's scalp. Then, 10 µL of the placebo (left side) and test formulation (right side) were massaged lightly into the areas under treatment; the procedure was performed by one of the investigators. To maximize the absorption of the active ingredient, subjects were encouraged to avoid washing their hair during the next 24 h. The use of a mild shampoo was also recommended to avoid any confounding effects of other topical bioactive agents. Harsh hair treatments (e.g., chemical straightening) were heavily discouraged. Compliance with the instructions was assessed in all subsequent visits until the end of the study. The formulations were applied three times a week, over 5 weeks (15 applications total).

Adverse events assessment

The selected compounds are repurposed drugs that passed all phases of clinical trials and for which the prescribed concentration ranges are known. Since the chosen working concentrations are well below the maximum limit of those ranges, the safety of the topical treatment was mainly assessed by the subjects' self-reporting of adverse events. At the last application, and before the collection of samples, the hair of areas treated with the test formulation and placebo was also measured to infer the influence on hair growth rate.

Efficacy assessment

Before the first application, a photograph capturing simultaneously the two scalp zones under test was taken to set a baseline for hair colour and shape. Then, with each subsequent application, the growing hair was visually inspected for changes in its phenotype. To register the differences between the areas treated with the test formulation and the areas treated with the vehicle formulation, new photographs were taken every week (3rd, 6th, 9th, 12th and 15th application). After the last application, hair samples were also collected for melanin quantification or straightening/curling degree evaluation as described below.

Melanin quantification [35]: hair samples were weighted and digested in 2.5 M NaOH at 25 °C for 24 h. The obtained solutions were diluted to a final hair concentration of 1 mg/mL in 1 M NaOH containing 10 % DMSO. After incubation at 80 °C for 1 h, the complete oxidation of hair lysates (4 h, 25 °C) was performed with aqueous hydrogen peroxide solution at the final volume fraction of 30 %. The melanin content of hair samples, determined as an average of three quantifications, was calculated based on a standard curve generated by the fluorescence (after oxidation) of *Sepia* melanin standards.

Evaluation of straightening/curling degree: the straightening and curling degrees were calculated by dividing the ratio of control hairs (placebo formulation treatment) by the ratio of treated hairs (test formulation). Each ratio was obtained by dividing the length mean of relaxed hairs by the length mean of hairs under stress (Equation (1)). Each hair was individually measured over a white paper (relaxed hair, RH); afterwards, both ends were held to straighten the hair fibre and the length was again measured (hair under stress, SH). Each ratio, for both control and treated samples, was the result of the measurement of at least 5 hair fibres.

Straightening or Curling Degree

$$= \frac{\text{RH length} / \text{SH length (control hair)}}{\text{RH length} / \text{SH length (treated hair)}} \quad (1)$$

Evaluation of the formulations for possible physicochemical modifications of hair shafts.

Blond hair samples were treated with Formulation O and Formulation A. Dark brown hair samples were treated with Formulation O, Formulation B and Formulation C. Very curly hair samples were treated with Formulation O, Formulation D and Formulation E. Straight hair samples were treated with Formulation O, Formulation F and Formulation G. All formulations were prepared as described before (**S8 Table, Appendix A**). Briefly, hair samples were cut by approximately 2 cm and weighted. The samples were incubated at room temperature for 24 h with the formulations in a volume/mass ratio of 200. After the treatment, samples were washed 2 times with PBS containing 10 % ethanol, 2 times with PBS with 0.1 % Tween 20, 3 times with PBS and one time in distilled water. Each washing step was performed at room temperature for 5 min, with agitation. The samples were dried before further analysis. Melanin quantification and straightening/curling degree

Table 1
Allocated and analysed volunteers in the groups of the study.

Group	Treatment aim	Volunteers' hair type	N. of Volunteers	Formulation
Hair color modulation	Hair Darkening	Blond/ light brown	3	A (N = 3)
		Dark brown	3	B (N = 6)
	Hair Lightening	Dark blond	3	
		Dark brown	3	C (N = 5)
		Dark blond/ light brown	2	
Hair shape modulation	Hair Straightening	Wavy/Curly	4	D (N = 4)
		Wavy/Curly	4	E (N = 4)
	Hair curling	Straight	3	F (N = 6)
		Wavy/Curly	3	
		Straight	2	
		Straight	2	G (N = 5)
		Wavy/Curly	3	

determination were performed as described in the previous section.

Quantification and statistical analysis

All results are expressed as mean values more or less standard deviation of at least two independent experiments. HTS-Corrector software version 2.0 was used to analyze the screening data and for hit selection [47]. The cut-off for hit melanogenesis inducers was set at $\mu + 3\sigma$, a commonly used threshold in high throughput screening, corresponding to a false positive error rate of 0.00135. The cut-off for melanogenesis inhibitors was adjusted to $\mu - 2\sigma$ due to a very low incidence of hits when using a more conservative approach. For each gene qPCR data, both the cut-offs were set at $\mu \pm 2\sigma$; compounds were identified as hits if inducing or inhibiting more than two times the standard deviation of the mean level of gene expression of the population, with a probability of being a true hit of 95.7 % [48]. GraphPad Prism (version 6.01) and IBM SPSS Statistics (version 26) were used for the statistical analysis of other experimental data; paired and unpaired Student t-tests, one sample t-test and two-way ANOVA were used in different contexts. Non-parametric tests were used whenever the data did not fulfil the criteria for parametric analysis: Mann-Whitney U test and Wilcoxon signed-rank test. The differences were considered significant at p -values < 0.05.

Data and code availability

The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [49] and are accessible through GEO Series accession number GSE193983 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE193983>).

Results and discussion

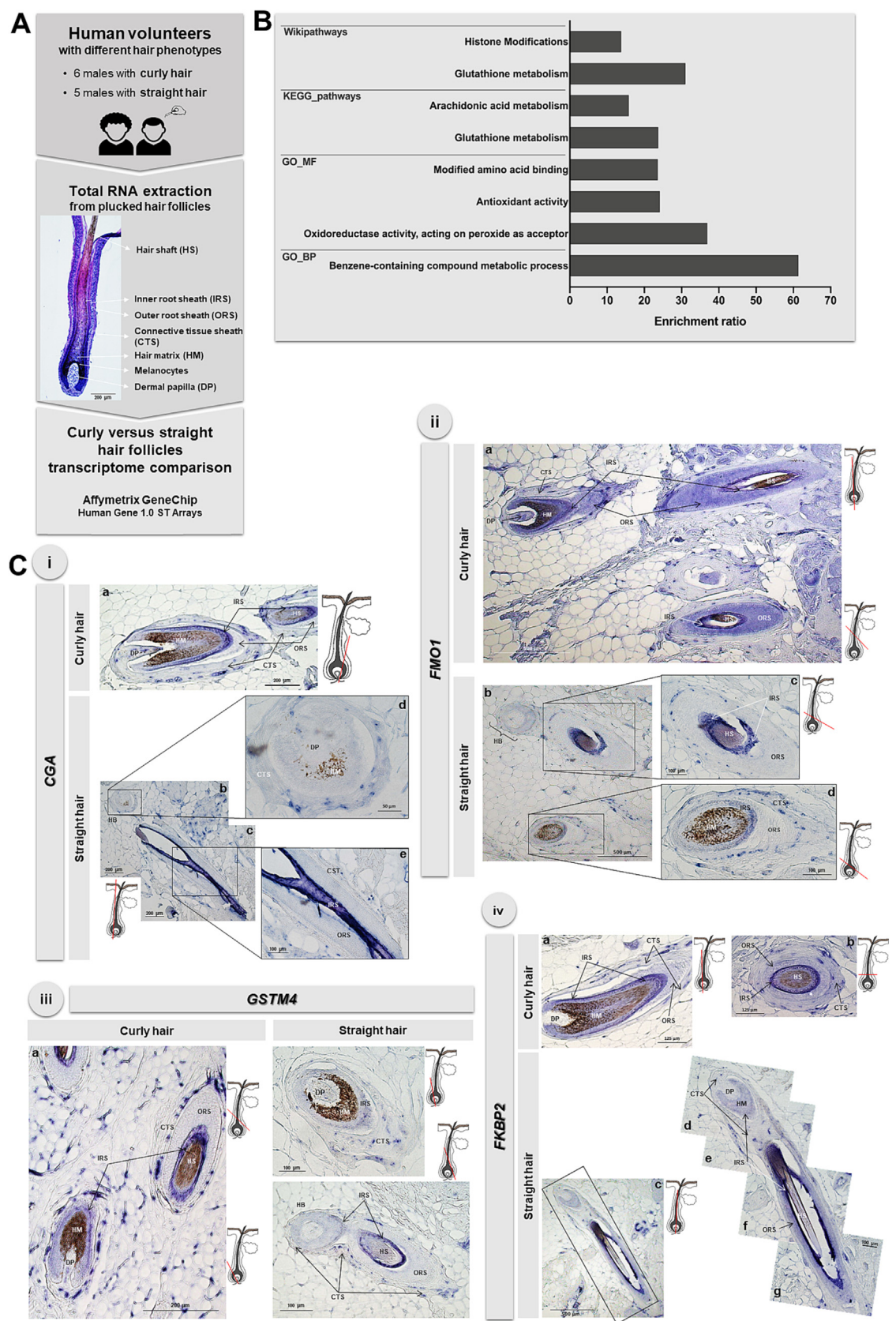
Part I: The search for new gene players in human hair shape

The production of a curved hair fibre may be under epigenetic regulation and may require additional antioxidant and proliferation supports provided by the glutathione metabolism.

In recent years, a continuous effort has been made to elucidate the genetic basis of natural human hair shape diversity. A fast-growing list of genes and their polymorphisms is being associated with different scalp hair shape phenotypes [50]. We sought differences at the transcript levels associated with different hair curvatures, which may reflect different intensities of gene activity, instead of a search more centred on the upstream genetic causes. Such transcriptome analysis can give valuable information which is still missing in the context of human hair shape.

Using microarray technology, we compared the gene transcription profile of very curly to the gene transcription profile of straight HF, plucked from a total of 11 donors (Fig. 1A). We verified by histology that all significant cell layers, including the matrix, were represented in naked-eye visible bulbs of plucked HF (Fig. 1A). However, we cannot exclude that this study leaves out other genes, located in other bulb regions that could be less efficiently plucked and therefore less represented in the extracted RNA pool. Knowing that about 80 % to 90 % of scalp hairs are anagen hairs, others found that plucked scalp HF, obtained less invasively than skin biopsy samples, are useful RNA sources for the genetic analysis of the top 200 genes associated with genodermatoses by gene expression level [51]. Our comparative analysis using plucked HF revealed a set of new genes that were not previously associated with the hair curvature phenotypes. We have found 85 genes that were transcribed with a statistically significant difference between very curly and straight HF - absolute fold change above 1.7 ($|FC| > 1.7$,

Fig. 1. The search for “new” gene players in human hair shape. (A) Methodology used to search for genes involved in hair shape determination. The total RNA was extracted from hair follicles plucked from a total of 11 male donors, six with very curly hair and five with straight hair. Bright-field photo of a haematoxylin-eosin stained longitudinal cryosection of a plucked straight hair follicle (donor 1, S4 Table), where all the major cell layers of an anagen follicle are identified (total magnification: 40x). The levels of gene transcripts were compared between the very curly and straight hair follicles using the microarray technology. (B) The production of a curved hair fibre may be under epigenetic regulation and may require additional antioxidant and proliferation supports provided by the glutathione metabolism. The Over-Representation Analysis (ORA) was performed using the WebGestalt online tool (<https://www.webgestalt.org/>), considering the list of differently transcribed genes between very curly and straight hair follicles (S9 Table) and all mapped EntrezGene IDs from the platform affy_hugene_1_0_st_v1 as the reference list. An ORA was performed for each of the following functional categories: Gene Ontology (GO), KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathways and Wikipathway. Only the significant enriched terms with a false discovery rate below 0.05 are represented in the graph (p -values adjusted by the Benjamini–Hochberg method). (C) The transcription of *CGA*, *FMO1*, *GSTM4* and *FKBP2* genes, which is significantly different between very curly and straight hair follicles, maps to the inner root sheath. The transcripts were detected in sections of human post-mortem scalp samples by *in situ* hybridization, using digoxigenin-labelled probes. A hair follicle drawing, with a red line showing the estimated orientation of the section, is displayed (whenever found to be relevant) next to the brightfield microscopy photograph. (i) *CGA* transcription in a curly hair follicle (a, total magnification 100x) and a straight hair follicle (b–c, total magnification 100x; d, total magnification 400x; e, total magnification 200x). (ii) *FMO1* transcription in curly hair follicles (a, total magnification 40x) and in straight hair follicles (b, total magnification 40x; c–d, total magnification 200x). (iii) *GSTM4* transcription in curly hair follicles (a, total magnification 100x) and in straight hair follicles (b, total magnification 200x; c, total magnification 100x). (iv) *FKBP2* transcription in curly hair follicles (a–b, total magnification 200x) and in a straight hair follicle (c, total magnification 40x; d–g, total magnification 200x). Abbreviations used: CTS – connective tissue sheath; DP – dermal papilla; HB – hair bulb; HM – hair matrix; HS – hair shaft; IRS – inner root sheath; ORS – outer root sheath. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



S9 Table). The fold change of nine genes, selected from both extremes of the list, was further validated by quantitative polymerase chain reaction (qPCR; **S1A Fig**). From the 85 genes, 74 were protein/polypeptide coding genes, five were small nucleolar RNAs, two were processed pseudogenes, two were long non-coding RNAs, and two were small nuclear RNAs. The referred list of significantly different transcribed genes shows a considerable asymmetry in the direction of the fold change: the levels of 68 transcripts were higher in very curly HF while only 17 genes were more transcribed in straight HF. Therefore, in terms of gene transcription, we can already infer that curving the hair fibre is more demanding for the follicle cells.

To have a global picture of the functions associated with gene transcripts that were present in different levels between straight and very curly HF, we performed over-representation analysis (ORA) using two online tools, WebGestalt and DAVID (**Fig. 1B**; **S1B Fig**). For both tools, 80 genes from the total list (**S9 Table**) were recognized as unambiguously mapped to 80 unique EntrezGene IDs. The enrichment analysis results were essentially dominated by the presence in the list of the same two sets of genes: the histone-coding genes (*H4C13*, *H4-16*, *H4C6*, *H3C10*, *H2AC11*) and the genes coding for the enzymes that use the antioxidant metabolite glutathione (*GSTM2*, *GSTM4*, *GPX2*, *GPX3*). In this sense, the results obtained by both tools agree. In terms of gene ontology, DAVID emphasized the histone set as the picked ontology terms were telomere organization, DNA replication-dependent nucleosome assembly, protein hetero-tetramerization (regarding biological process domain); and nucleosome, nuclear chromosome, telomeric region (regarding the cellular component domain) (**S2 Fig**). WebGestalt privileged the glutathione set as the significantly enriched ontology terms were benzene-containing compound metabolic process (regarding the biological process domain) and oxidoreductase activity acting on peroxide as acceptor, antioxidant activity, modified amino acid binding (regarding the molecular function domain) (**Fig. 1B**). In terms of enriched pathways, the WebGestalt was more informative, showing again the enrichment of glutathione and arachidonic acid metabolisms due to the presence of the genes encoding glutathione S-transferases and peroxidases. The fact that histone modifications and glutathione metabolism pathways are both enriched in a gene list that results from the transcriptome analysis in HF cells is not surprising, since these pathways are relevant for several aspects of HF biology. However, their enrichment in a gene list that reflects the transcriptome differences between hair fibre shapes points towards new and interesting research directions.

In the biology of HF stem cells and their directly committed progeny, epigenetic control through histone modifications has an important role in maintaining the vital equilibrium between plasticity and differentiation commitment, and between quiescent and proliferative states [52,53]. HF morphogenesis and postnatal cyclic regeneration depend on this equilibrium as well as on the coordinated activities of transcription factors and epigenetic modifiers acting on HF stem cells [54]. Our ORA results strongly reinforce the possibility that the hair curvature setting is also a cellular process under epigenetic regulation, as suggested by Westgate and co-authors in their review [13].

The list of differently transcribed genes (**S9 Table**), shows that all five canonical histone genes were expressed at higher levels in very curly HF, from which *H4-16* and *H3C10* had fold changes above five. Expression of canonical histone gene clusters is tightly coordinated with the S-phase of the cell cycle. Besides the canonical isoforms, several histone variants are encoded by genes outside these clusters, and their expression and chromatin incorporation are not coupled to cell cycle regulation [55]. It is already known from the published literature that these non-replicative histone variants influence chromatin properties by

affecting the nucleosome stability and local chromatin environment, either directly in nucleosome-DNA interactions, through their primary sequence-specific effects, or by recruiting different chromatin-associated protein complexes [55]. What has been recently acknowledged is that replication-dependent histones also constitute an additional layer of complex epigenetic regulation as reviewed by Singh *et al.* [56]. Therefore, slight differences in the histone primary sequence, as the ones seen between canonical isoforms, can influence chromatin structure, dynamics and its interaction, thus, carrying a potential to confer specialized phenotypic traits. We identified three genes coding for the same H4 isoform (*H4C13*, *H4-16*, *H4C6*), one for the histone 3.1 isoform (*H3C10*) and one for the histone 2A type 1 (*H2AC11*). Though canonical histone 3 (H3) tail post-translation modifications have been the focus of extensive epigenetic studies, two works revealed discrete combinatorial codes of post-translation modifications occurring on histone H4 from human and mouse embryonic stem cells [57,58]. Striking changes in global methylation and acetylation patterns of histone (H4) seem to be associated with epigenetic regulation of pluripotency.

Replication-coupled histones may be transcriptionally regulated as a group [56] or in a cell and context-dependent manner [59,60]. Exactly how the higher levels of these five transcripts, coding replication-dependent core histones, may relate to the epigenetic induction of the very curly hair phenotype is unclear. Of the four canonical core histones, H4 is the most conserved histone without known variants and only two replication-associated isoforms [61], but it remains to be elucidated how it reflects hair fibre curvature phenotype.

Curvature will always imply, at some point in the growing fibre, mechanical stress before it is fixed by cornification [8,62]. Keratinocytes sense and respond to mechanical cues through epigenetic adaptative mechanisms [63]. We cannot exclude the hypothesis that the higher levels of the five histone transcripts in curly HF may be a consequence of the phenotype rather than being part of the cause. However, if so, this hypothesis would imply that the mechanical stress would have to be felt at the bulb's lower level, where cells still have an active nucleus.

In the biology of HF pigmentation, it has long been acknowledged that oxidative stress is linked to the ageing of follicular melanocytes perceived as hair greying reviewed by O'Sullivan *et al.* [64]. Pieces of evidence show a reduction in the ability of follicular melanocytes to neutralize the effects of reactive oxygen species (ROS) with age or in association with premature greying, which involves, among other defence mechanisms, changes in glutathione metabolism. Thirty years ago, Pruche *et al.* had already verified a reduction in the glutathione content as a function of donor age in the matrix, outer and inner root sheaths of plucked human anagen HF [65].

Interestingly, in hair and skin epidermis, cornification involves a complex and coordinated series of events mediated by ROS [66,67]. During corneocyte differentiation, a massive replacement of intracellular content and organelles by a compact proteinaceous cytoskeleton occurs to create space for keratin intermediate filaments. In HF, the sequential destruction of first the nucleus and then mitochondria was proposed. The authors make a suggestive comparison where without the nucleus - the cellular "brain" - the "zombie" keratinocytes are completely focused on respiration and ROS production. ROS generation at mitochondria is spatially controlled and it is an integral event in hair fibre formation, promoting the oxidative environment needed for the cross-linking of keratins and other hair matrix proteins [67,68]. From this perspective, the presence in the list obtained from the transcriptome analysis of HF (**S9 Table**) of four genes coding the antioxidant and detoxifying enzymes using reduced glutathione as a cofactor implies that they may be needed for an effective and coordinated

cornification, preventing premature death of cells due to high levels of intracellular or extracellular physiological oxidative stress. The ORA results we obtained hint at glutathione metabolism as a cellular pathway implicated in the biology of hair curvature. The major antioxidant and detoxifying enzymes using glutathione as a cofactor are glutathione peroxidases and glutathione S-transferases. In our list of differently transcribed genes, from which the ORA results have been drawn (Fig. 1B), we have two genes coding for the selenium-containing glutathione peroxidases (GPxs), *GPX2* and *GPX3*, and two coding for glutathione S-transferases (GSTs) belonging to the Mu class, *GSTM2* and *GSTM4*.

Apart from Phase II detoxification metabolism, GSTs contribute to the detoxification of endogenously produced free radicals and organic peroxides via their selenium-independent glutathione peroxidase activity [69]. *GSTM2* and both *GSTM2* and *GSTM4* enzymes are upregulated by the transcription factor Nrf2, which is a major regulator of cellular redox homeostasis [70,71]. Besides the antioxidant and detoxification functions, both *GSTM2* and *GSTM4* have functions involving the synthesis of prostanoids and leukotrienes, respectively (products of arachidonic acid metabolism). In the human brain, *GSTM2* was demonstrated to catalyse the isomerization of prostaglandin H2 to prostaglandin E2 [72]; it is known that prostaglandin E2 in human HFs has a pro-hair growth activity [73,74].

The GPxs catalyse the reduction of hydrogen peroxide or organic hydroperoxides to water or the corresponding alcohols, respectively, using reduced glutathione as the reductant. According to the literature, all the described functions for *GPX3* and *GPX2* seem to be particularly relevant in highly proliferative epithelial tissues, such as epidermis and HFs, where redox signalling and physiological ROS production play an essential role in the terminal differentiation process [75–77]. The fact that *GSTM4*, *GSTM2*, *GPX3* and *GPX2* are all more transcribed in HFs obtained from donors with very curly hair when compared to donors with straight hair, suggests that follicles producing very curly hair fibres need more protection against oxidative stress and support for cell proliferation. This may be a trait evolutionary preserved from hominids with curly hair for UV-induced oxidative stress [78].

The ORA results imply that epigenetic regulation and glutathione metabolism are mechanisms involved in hair shape establishment. However, there is information lacking on how hair fibre gains its curvature. Of the several studies carried out and proposed mechanisms, recently reviewed by [13,78,79], there is the consensus that an early event of asymmetry is necessary at a molecular level, in the lower follicle bulb, (e.g., a concentration gradient), which would be further propagated as a bulbar asymmetry in cell division rate and cornification. This would then be reflected in corneocyte asymmetric morphology, and so on, jointly creating the mechanical driving force necessary to produce a curved hair fibre. Among all the genes we found to be differently transcribed (S9 Table) one stands out – *SEPTIN3*, more transcribed in the samples of curved HFs. Citing Spiliotis and McMurray (2020) [80], septins are “masters of asymmetry”, assembling into hetero-oligomers on specific intracellular regions, creating micron-scale positive curvatures. Septins promote asymmetry by restricting lateral diffusion, enhancing membrane rigidity and regulating in space the membrane–cytoskeleton interactions; all of which may contribute to hair curvature [80,81]. Little is known about the protein septin 3, but it seems to be developmentally regulated in rat brains [82,83] and it functions in human neurons [84]. Knowing that *SEPTIN3* is transcribed in human HF raises the possible additional involvement of this protein in the establishment of the asymmetry necessary to produce curved fibres. It would be worth verifying if septins, in particular septin 3, can interact directly or indirectly with the major cytoskeleton component of follicle cells – hair ker-

atin intermediate filaments, similar to what happens between septins and other protein members of the cytoskeleton.

The transcription of CGA, FMO1, GSTM4 and FKBP2 genes, which is significantly different between very curly and straight hair follicles, maps to the inner root sheath.

Many genes that we found to be transcribed differently between very curly and straight HFs (S9 Table) had not been previously implicated with the shape of human hair or other aspects of HF biology. The next step was to check if a pool of those genes was indeed transcribed in specific HF cell layers. We selected four protein-coding genes to verify where they were transcribed, by *in situ* hybridization, in *post-mortem* human skin samples from donors with different hair phenotypes. These genes were selected based on their significant variations in expression, either upregulated or downregulated, to increase sensitivity in subsequent *in vitro* screening. *KRT25*, the gene coding for keratin 25, was used as a positive experimental control because, according to the literature, its expression is specific to the HFs, more precisely, to the IRS and medulla [85] (S1C Fig). A slight asymmetry in *KRT25* gene transcription was visible in curly HFs (S1Ci-ii Fig). Asymmetry has been described for the distribution of some proteins [2]: K38 [86], K82 [8], and IGF-binding protein 5 (IGFBP-5) [87]. The quality of *post-mortem* skin samples was validated for the *KRT25* control gene since the signal location was specific to the IRS (S1C Fig).

All the selected genes, *CGA*, *FMO1*, *GSTM4* and *FKBP2*, are transcribed predominantly in the IRS layer (Fig. 1C). This layer is formed at the lower region of the bulb and can support both growth and differentiation of the hair fibre [88]. From the several genes already linked to human hair shape, many of them are expressed in this specific layer [13,89–91].

According to the results from the microarray, the *CGA* gene was the transcript with the highest fold change ($|FC| = 7.5$), more transcribed in very curly HFs. This gene encodes the α subunit common to all the heterodimeric glycoprotein hormones that control major reproductive functions and basal metabolism in the human body, through the hypothalamic–pituitary–gonadal and hypothalamic–pituitary–thyroid axes [92]. Besides the endocrine role, new unconventional functions are starting to emerge for each subunit of glycoprotein hormones, such as the regulation of prolactin in different mammalian cell types, including extra pituitary cells (reviewed by [93]). Remarkably, the human scalp HFs are both a target and a source of prolactin. This hormone affects, at least *ex vivo*, human HF growth and cycling, keratin expression and epithelial stem cell function [94,95]. Further research will be necessary to confirm if the α subunit polypeptide is a new modulator of the extra pituitary prolactin expression in human HFs, along with $TNF\alpha$ and $IFN\gamma$ [96] and if prolactin is indeed related to the *CGA* function as hair shape modulating gene. Fig. 1C_i shows the *CGA* transcription on skin scalp samples of donors of curly (Fig. 1C_ia) and straight HFs (Fig. 1C_ib-e). In straight HFs, the transcription did not occur significantly at the hair bulb level (Fig. 1C_ib, id), it was especially detected in the IRS layers, predominantly above the bulb (Fig. 1C_ic, ie). In very curly HFs, the *CGA* transcripts are easily detected at the bulb level (Fig. 1C_ia), more precisely, in the IRS. Besides the bulb, the *CGA* transcripts are also present in the supra bulbar region. *CGA* transcription seems to be linked to the differentiation process of follicular keratinocytes, in both samples, as it is weaker at the lower bulb, in more undifferentiated cells, intensifying along the axial direction of hair fibre growth and differentiation (Fig. 1C_ia, ib, ic). A faint signal is present throughout interfollicular skin, sebaceous glands and outer root sheath, especially visible in histological sections from very curly HF donors (S1D Fig).

The *FMO1* is one of the six genes composing the *FMOs* gene family which encodes enzymes that catalyse the NADPH-dependent oxidation of a variety of endogenous and exogenous nucleophilic

compounds, known for their role in Phase I detoxification metabolism of xenobiotics [97], and in the regulation of metabolism and cellular resistance to stress [98]. All these functions are important to HF biology and hair fibre formation, as previously discussed. Apart from traditional locations, Janmohamed *et al.* demonstrated that the *FMO1* gene is also expressed in the skin epidermis, including the sebaceous glands and HFs [99], which we have confirmed (S1D Fig). In Fig. 1C_ii, the expression of *FMO1* is visible in both curly (Fig. 1C_iiia) and straight HFs (Fig. 1C_iiib-d) samples. According to Fig. 1C_iiib, in straight HFs stained with the *FMO1* probe, the signal is very faint in the hair bulb, only the upper bulb and supra bulbar regions of the HF appear labelled throughout the area corresponding to the IRS (Fig. 1C_iiic-d). These findings suggest that the *FMO1* transcription is coordinated with the differentiation process of follicular keratinocytes in a similar way to what was observed for the *CGA* gene. The outer root sheath layer is only labelled in very curly HF sections (Fig. 1C_iiia), but this may reflect the higher background in some of these samples (S1D Fig).

In curly HFs (Fig. 1C_iiia), the *GSTM4* transcription signal is detected in the lower bulb, and it intensifies as the IRS layer differentiates towards the apical zone of the hair bulb. In straight HFs, the overall signal intensity is much lower, but the increase of *GSTM4* transcription in the IRS along the axial direction is also visible (Fig. 1C_iiib-c).

The *FKBP2* gene codes for a member of the FK506-binding protein (FKBP) class of immunophilins, a superfamily consisting of highly conserved proteins that possess peptidyl prolyl cis-trans isomerase activity [100]. FKBP2s play an essential role in several cellular processes including protein folding, assembly and trafficking, chaperone activity, receptor signalling and transcription, neurotrophic and neuroprotective roles as well as roles in the development and regulation of apoptosis [100,101]. Ishikawa *et al.* studied the role of endoplasmic FKBP2s as chaperones for collagen biosynthesis [102], affecting the cis-trans isomerization of proline residues and consequent protein compactness and arrangement [103]. The preference for unmodified proline residues by FKBP2 (as present in keratins) and its involvement in protein folding kinetics [101,102] could be important during the massive keratin expression that occurs during keratinocyte differentiation at the bulb and supra bulbar regions. FKBP2 was also found to protect cells from apoptosis (plasma cells) that are exposed to endoplasmic reticulum stress due to protein synthesis burden [104]. In terms of the transcription pattern (Fig. 1C_iv), we verified in both types of HFs that *FKBP2* localizes to the hair matrix and in all the IRS layers, though much more concentrated in the supra bulbar zone. This gene transcript is also clearly present in the outer root sheath (Fig. 1C_ivb, ivf-g).

The evaluation of the transcription location was accomplished. The genes that are transcribed early in the bulb, meaning, near the dermal papilla neck, may probably be more significant in establishing the early cause of a curved HF phenotype. The detection of the selected genes occurred mainly above the critical level of the bulb with a signal increasing at the supra bulbar region where the keratinization and hardening processes take place (*GSTM4* in curly HFs was the exception). Still, their transcription dependence on the keratinization process is interesting. Whatever the early molecular event in the whole biological process of curved hair fibre production, our results reiterate the idea already presented by others that the IRS has the hair fibre moulding function, a determinant for the natural hair shape phenotype. Mapping the transcription to the HF for some of the genes in the list was important to confirm their potential implication in the determination of hair curvature and to indirectly validate the microarray results. In both types of the analysed HF shapes, the location of the transcripts was almost the same, reinforcing that the IRS is the optimal target for the development of safe cosmetic formulations address-

ing the fibre curvature. From the *in situ* hybridization results, it became straightforward that further validation *in vitro* must involve IRS cells.

Part II: The search for new bioactive molecules able to modulate hair phenotype from the follicle

To achieve our ultimate goal of changing hair colour and shape from the inside out, we screened a library of well-known pharmacological compounds for potential bioactive cosmetic ingredients. In our work, we screened Prestwick Chemical Library®, an off-patent collection of 1200 small molecules dissolved in dimethyl sulfoxide (DMSO). Most drugs contained in this library are approved by the FDA, EMA and other agencies, and they already passed all phases of clinical trials, and regulatory scrutiny and have undergone post-market surveillance.

Twenty-three out of 1200 tested compounds were hit modulators of melanin synthesis.

As part of repurposing drugs for colour modulation at the level of hair roots, we screened the chemical library for the ability to alter (by increasing or decreasing) the levels of melanin in a human pigmented melanoma cell line. Fig. 2A_i summarizes the average melanin contents obtained for each compound, normalized to the melanin produced by the solvent control (cells treated with 1 % DMSO). The statistical method for the identification of hits involved selecting a threshold, multiple of the standard deviation (σ), relative to the mean melanin production (μ) of all samples.

About 2 % of the 1200 tested compounds were hit modulators of melanin synthesis. As listed in Fig. 2A_ii, from the 23 detected hits, 10 increased (hit inducers) and 13 decreased (hit inhibitors) above 3σ and below 2σ , respectively, the population average production of melanin after 72 h of incubation. Interestingly, 21 out of the 23 hits were previously unknown as possessing melanogenic effects. All the hits performed better than the respective positive controls. The average melanin content for forskolin (20 μ M), a standard treatment for *in vitro* stimulation of melanogenesis, was 137.6 %. Forskolin is an activator of adenylate cyclase, an enzyme of the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) signalling pathway involved in the positive regulation of melanogenesis. While the average melanin content for kojic acid (2 mM), a competitive inhibitor of tyrosinase activity, and widely used as an inhibitor of *in vitro* and *in vivo* (skin) melanin production was 82.90 %. We selected three of the top four hit melanogenesis inducers and inhibitors for further studies. Hycanthone and sulfapyrazone cannot be included in cosmetic products, as stated in Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. The melanogenic effect of the top-hit compounds was validated using the same compounds from sources other than Prestwick Chemical.

The *in vitro* modulation of melanogenesis by the selected hit compounds was dose-dependent (S2-3 Figs). A cell viability assay was included in this study to avoid skewed outcomes due to a cytotoxicity effect (S2-3 Figs). Both concentration and time of exposition to the drug significantly affected the melanin content; the drug concentration factor had a higher impact on data variability when using melanin inducers, while both factors and their interaction had more equilibrated contributions on data variability when using melanin inhibitors (S10 Table).

Hit inducers promoted a dose-dependent increase in the intracellular level of melanin (S2 Fig). Despite being well tolerated and one of the most effective activators of melanogenesis, phenazopyridine hydrochloride also promoted an almost complete loss of pigment content at the highest non-cytotoxic concentration tested. This phenomenon needs to be extensively studied before cosmetic use in humans can be attempted. The treatment with amodiaquine dihydrochloride dihydrate (AMO) presented a higher weight of the

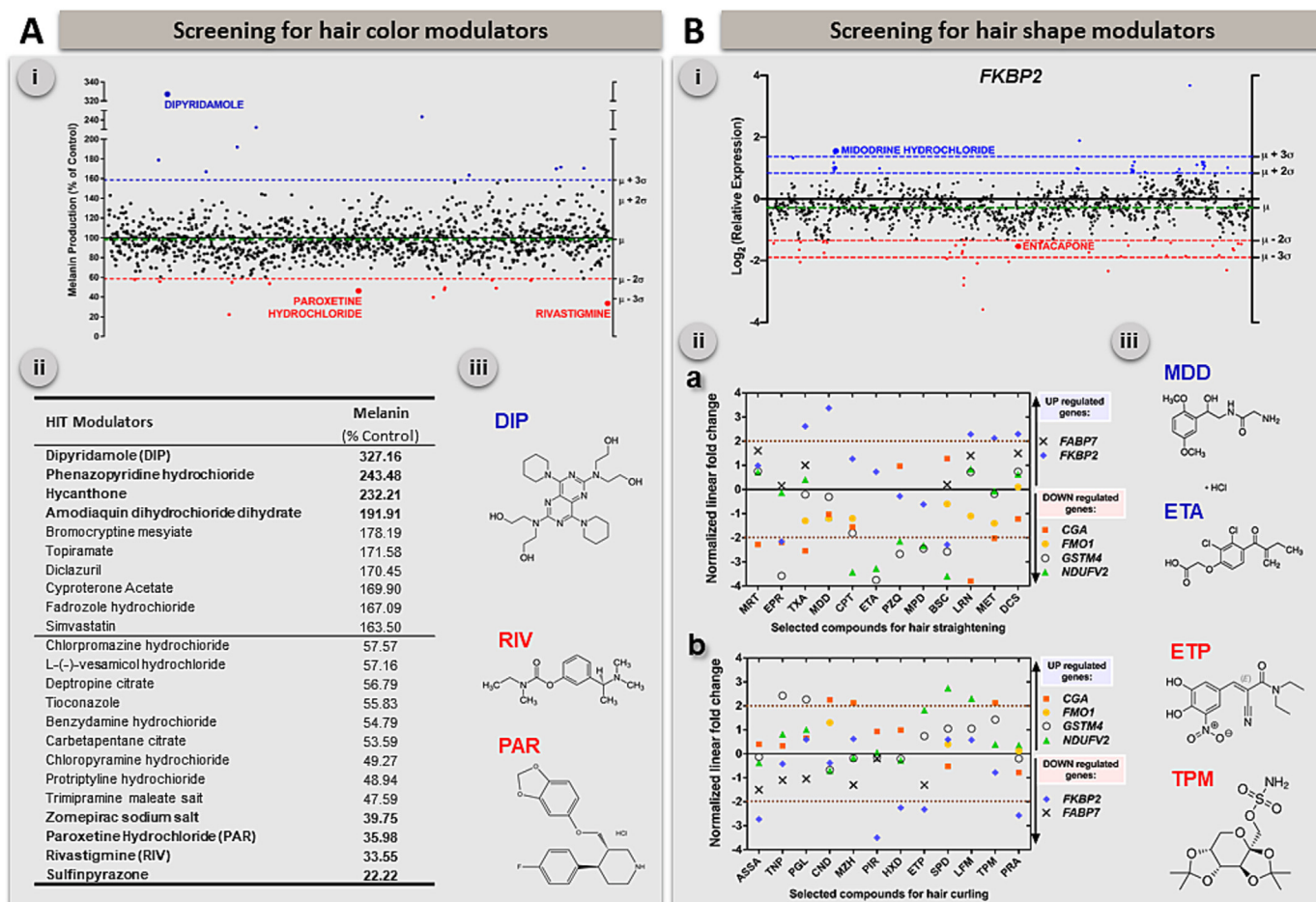


Fig. 2. The search for new bioactive molecules able to modulate hair phenotype from the follicle. **(A)** Twenty-three out of 1200 tested compounds were hit modulators of melanin synthesis. **(i)** SK-Mel-23 cells were treated with 10 μ M of each drug from the library for 72 h and then, melanin quantification was performed using a fluorescence-based method. The melanin contents were normalized by total protein levels in each sample and expressed as a percentage of the solvent control, 1 % DMSO. HTS-Corrector software (version 2.0) was used to analyse data and for hit selection. The cut-off for hit melanogenesis inducers and inhibitors was set at $\mu + 3\sigma$ and $\mu - 2\sigma$, respectively. **(ii)** Hit compounds obtained by HTS-Corrector data analysis of three independent screening assays and their effect on intracellular melanin content of SK-Mel-23, normalized by total protein levels and expressed as a percentage of the solvent control, 1 % DMSO. **(iii)** Molecular structure of the three hit compounds selected for *in vivo* testing: the melanogenesis inducer is represented in blue and the inhibitors are in red. **(B)** Midodrine, ethacrynic acid, topiramate and entacapone were selected as potential hair-shape bioactive agents. **(i)** IRS primary cells were treated with each drug from the chemical library for 24 h under non-cytotoxic conditions (**S4B Fig**). The relative gene expression of FKBP2 is represented as the log₂-linearized fold change, obtained by applying the $\Delta\Delta C_q$ method on qPCR data, normalized for two endogenous genes, the cyclin dependent kinase inhibitor 1A (CDKN1A) and the 18S ribosomal RNA (18S), and using the solvent control (0.5 % DMSO). The cut-off for hit FKBP2 transcription inducers and inhibitors was set at $\mu \pm 2\sigma$. The same analysis was performed for CGA, GSTM4 and NDUVF2 (**S4C Fig**). **(ii)** The best 24 compounds selected as hits in changing the transcription of at least one gene and their combined effect on the transcript levels of the six human genes under study: CGA, GSTM4, NDUVF2, FMO1 (found to be more expressed in very curly compared to straight hair follicles, **S9 Table**), FKBP2 and FABP7 (found to be more expressed in straight compared to very curly hair follicles, **S9 Table**). How each hit affects the gene expression in IRS cells is represented as the linear fold change normalized to the respective σ value. The threshold for a compound to be considered as a transcription inducer or inhibitor hit was established at $\mu \pm 2\sigma$ (dashed brown lines); **(a)** the effect on relative gene expression of the 12 best candidates to potentially induce hair straightening and **(b)** the effect on relative gene expression of the 12 best candidates to potentially induce hair curling. The 24 selected hits were further validated in terms of their biological effect on primary inner root sheath cells regarding the transcription of six genes, using the drugs acquired from other suppliers (**S4D Fig**). **(iii)** Molecular structure of the four hit compounds selected for *in vivo* testing: in blue are the potential hair-relaxing agents and in red are the potential cold-waving agents. Abbreviations used: ASSA – acetyl salicylic acid; BSC – bisacodyl; CND – clonidine Hydrochloride; CPT – camptothecin (S,+); DCS – D-cycloserine; DIP – dipyrindamole; EPR – epirizole; ETA – ethacrynic acid; ETP – entacapone; HXD – hexetidine; IRS – inner root sheath; LFM – leflunomide; LRN – liranafate; MDD – midodrine hydrochloride; MET – metyrapone; MPD – methylprednisolone, 6- α ; MRT – morantel tartrate; MZH – meclozine dihydrochloride; PAR – paroxetine hydrochloride; PGL – pargyline hydrochloride; PIR – piracetam; PRA – pravastatin; PZQ – praziquantel; RIV – rivastigmine; SPD – sulfapyridine; TNP – tranylcypromine hydrochloride; TPM – topiramate; TXA – tranexamic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

factor time in causing the observed variation in melanin production regarding the other two inducers (**S10 Table**). Only dipyrindamole (DIP) (**Fig. 2.A_ii-iii**) was selected as an agent of hair darkening for further studies as it provided a sustained stimulation of *in vitro* melanin production across a wide range of non-cytotoxic concentrations.

In SK-Mel-23 (**S3 Fig**), the profiles obtained with rivastigmine (RIV) and zomepirac sodium salt (ZOM) resembled a U-shaped dose-response relationship (hormetic response) [105]. It is possible that compensatory pathways were activated in response to a

disruption in homeostasis which counteracted the anti-melanogenic effects of these compounds; alternatively, depending on the dose, those compounds might act on different (conflicting) pathways of melanogenesis modulation. Although very uncommon, this type of cellular response towards melanogenesis modulation has been reported before [106]. On the contrary, paroxetine hydrochloride (PAR) exhibited a dose and time-dependent inhibition of melanogenesis over the whole range of non-cytotoxic conditions. Since ZOM has the potential to cause acute dermal toxicity, it was excluded. Thus, RIV and PAR (**Fig. 2.A_ii-iii**) were the hits

selected for further studies as potential agents of hair colour lightening.

Potential hair shape bioactive agents were selected for their regulation of the transcription of selected genes

Contrarily to searching for colour modulators, performing the screening for hair shape modulators is quite challenging as the biological process leading to hair curvature formation and its genetic basis are still little understood. We hypothesized that a good potential curling inducer would promote the simultaneous transcription of the *CGA*, *GSTM4*, *NDUFV2*, and *FMO1* human genes (found to be more transcribed in very curly hair compared to straight HFs, [S9 Table](#)) while repressing the expression of both *FKBP2* and *FABP7* genes (found to be more transcribed in straight hair compared to very curly HFs, [S9 Table](#)). We also hypothesized that a potential hair straightening agent would have the opposite effect on gene transcription.

We searched the chemical library for drugs with the ability to alter, either increasing or decreasing, the transcript levels of the six genes in IRS primary cells. The final concentration of each drug was equal to or below 25 μ M with a final non-toxic solvent volume fraction, previously established at 0.5 % DMSO. Under these conditions, one-fifth of the library still induced a reduction of more than 20 % in IRS cells viability ([S4A Fig](#)). Further dilution of these drugs was set according to the level of toxicity exhibited at 25 μ M, allowing the rescue of 122 additional compounds ([S4B Fig](#)); at this point, more than 90 % of the chemical library was nontoxic to cells, so we proceeded with the screening.

The effect of each compound on the target genes transcription, namely *CGA*, *FABP7*, *FKBP2*, *FMO1*, *GSTM4* and *NDUFV2*, was evaluated by qPCR. For each sample, the amplification of each target gene was normalized to two *housekeeping* genes and compared to the amplification that occurred in the experimental calibrator [cells treated with 0.5 % DMSO]. The linearized relative expression of the *FKBP2* gene for each compound is presented in [Fig. 2.B.i](#) as an example of the analysis performed for *CGA*, *GSTM4* and *NDUFV2* gene transcripts ([S4C Fig](#)). The expression levels of *FABP7* and *FMO1* genes in IRS cells were often too low to be detected by qPCR, even after pre-amplification ([S11 Table](#)). The stochastic amplification for these two genes was used qualitatively, namely *yes* or *no*, whenever needed in hit selection, as a complement to the analysis of the overall drug *curling* or *straightening* effects.

Using the same statistical method as before, but now applying it independently to each collected qPCR data, the compounds that were inducing or inhibiting more than twice the standard deviation (σ) from the average expression level of all samples were selected as hits. Regarding the *FKBP2* gene, we found 32 inducer and 34 inhibitory hits ([Fig. 2.B.i](#)). The mean of linear fold-change in *FKBP2* gene transcription is statistically different from zero (one sample *t*-test, $t = 16.25$, $p < 0.0001$, $N = 1200$) and it is negative (-0.26), meaning that most compounds inhibit its transcription. For the other three transcripts (*CGA*, *GSTM4* and *NDUFV2*) we have identified in the same way other inducer and inhibitor hits ([S4C Fig](#), [S11 Table](#)).

The different groups of hits were assembled in a list of 228 different compounds, and we verified the overall effect of each compound on the expression of all selected genes. After excluding toxic compounds, those which successfully regulated the expression of most of the genes were selected. The 12 best candidates to potentially induce hair curling and the 12 best candidates to potentially induce hair straightening are described in [Fig. 2.B.ii](#). These 24 compounds were hits [$> (\mu + 2\sigma)$; $< (\mu - 2\sigma)$] for at least one of the genes and they did not exhibit a significant contradictory effect on the expression of other targets, as shown by the deviation from the mean relative gene expression ([Fig. 2.B.ii](#)). The results of the

24 candidates were posteriorly validated and the bioactivity of selected compounds was confirmed using re-supplies of the drugs from sources other than Prestwick Chemical ([S4D Fig](#)).

The final identification of the best compounds was achieved by considering all the experimental results apart from the information available for each of the 24 selected drugs: their original clinical purpose, pharmacological, physiological and biochemical properties, and absence from the list of prohibited substances for cosmetic products. Midodrine (MDD) and Ethacrynic Acid (ETA) were selected to induce hair straightening, based on their upregulation of *FABP7* and *FKBP2* and downregulation of *CGA*, *FMO1*, *GSTM4* and *NDUFV2* genes transcription. MDD was the best compound in upregulating the *FABP7* or *FKBP2* genes while ETA was the compound with the highest overall downregulation of the expression of more than one of the four genes, namely *GSTM4* and *NDUFV2*. Topiramate (TPM) and Entacapone (ETP) were selected to induce hair curling, based on their upregulation of *CGA*, *FMO1*, *GSTM4* and *NDUFV2* and downregulation of *FABP7* and *FKBP2* genes transcription ([Fig. 2.B.ii-iii](#)). From the literature, there is no described relation between the action mechanism for the four selected drugs and target genes here analysed, except for ETA. ETA is described as a potent inhibitor of GST enzymes belonging to the alpha, pi and mu classes [[107,108](#)]; here, in primary cells of the IRS, this compound inhibited *GSTM4* transcription, a gene belonging to the mu class of the GST family.

Part III: Changing hair colour and shape from inside out – proof of concept.

At this final stage, we conducted a pilot clinical study with the intervention of cosmetics to disclose the relevance and utility in future cosmetics applications of the hits found in the *in vitro* screening for hair shape and colour modulation. A total number of 33 volunteers participated in this pilot study.

Fourteen volunteers were assigned to the intention-to-change hair colour cohorts: Formulation A (DYP), Formulation B (RIV) and Formulation C (PAR). Nineteen volunteers were assigned to the intention-to-change hair shape cohorts: Formulation D (ET), Formulation E (MDD), Formulation F (TPM) and Formulation G (ETP). The drugs were formulated in an emulsion, prepared with common cosmetic ingredients, here used as a delivery excipient of the compound to be administered and tested. The vehicle formulation was developed for maximal hair follicle permeation and accumulation of compounds [[109](#)] and prepared in compliance with Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Two distinct areas were shaved on the back of the volunteer's scalp, one was treated with one of the seven referred formulations and the other was treated with Formulation O, the placebo. The therapeutic doses and safety profiles of the selected drugs are known. Consequently, this information was used to define their dosage regimen in our study. The doses used were sub-therapeutic and considerably lower (at least 1000 times) than the maximal dose usually prescribed for each one of the selected drugs. Formulations were applied three times a week, for five weeks. At the end of the treatment, no significant differences were detected in hair length from scalp-treated areas between placebo (average length 1.4 ± 0.2 cm) and test formulations (average length 1.4 ± 0.2 cm), according to the paired samples *t*-test performed [$t(31) = 0.239$; $p = 0.813$]. All volunteers completed the study without reporting adverse effects.

Scalp treatment with dipyrindamole, rivastigmine or paroxetine hydrochloride changes the colour of growing hair.

We evaluated the effect of topical scalp application of Formulations A, B and C based on qualitative and quantitative data comparison ([Fig. 3A](#)). As the hair regrew, visually perceptible changes in hair colour were registered and documented by photographs. After

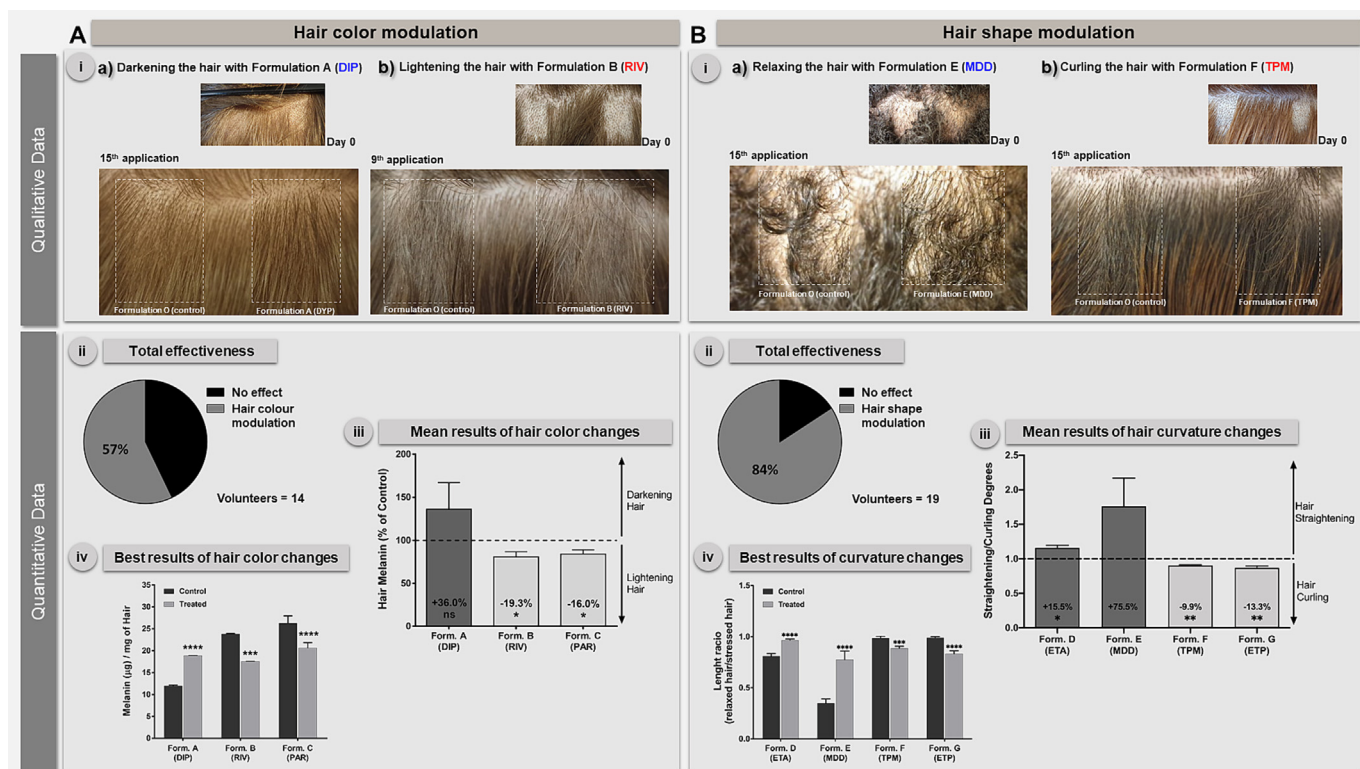


Fig. 3. Changing hair colour and shape from the inside out – proof of concept. (A) Scalp treatment with dipyrindamole, rivastigmine or paroxetine hydrochloride changes the colour of growing hair. Main qualitative (i) and quantitative (ii; iii; iv) outcomes of the clinical study with the intervention of cosmetics, conducted to assess the feasibility of a drug-based approach for hair colour modulation. (i) Representative photographs of (a) hair darkening with formulation A containing dipyrindamole (DYP) and (b) hair lightening with formulation B containing rivastigmine (RIV). The two shaved scalp areas of each donor are shown on day 0 (1st application) for guidance; on the left square, 10 µL of the control formulation while, on the right, 10 µL of the test formulation were applied three times per week, totalizing 15 applications. The large photographs were taken at the application where the differences in hair colour between placebo and test areas were maximized and visible to the naked eye. The photos are shown with a blur filter except in the two application zones, delimited by a white dashed line (original photos shown as **SSA-B Fig**). (ii) Combined effectiveness of the different drugs used for hair colour phenotype modulation. (iii) Relative mean hair melanin contents of positive outcomes, regarding the treatment with Formulation A (DIP), Formulation B (RIV) or Formulation C (PAR). Data were analysed by one-sample *t*-test. **p* ≤ 0.05, when conditions were compared to the hypothetical value 100 %, corresponding to the mean melanin content of hair collected from the placebo-treated scalp areas. (iv) Melanin contents in hairs collected from the scalp of volunteers with the best positive outcomes, regarding the treatment with Formulation A (DIP), Formulation B (RIV) or Formulation C (PAR). The data from each compound was retrieved from the separate two-way ANOVA analysis of all volunteers assigned for each formulation, followed by post-hoc Sidak's test. ***p* ≤ 0.01 or ****p* ≤ 0.001, when melanin contents were compared with the corresponding control (hair collected from the placebo-treated scalp areas). (B) Scalp treatment with midodrine hydrochloride, ethacrynic acid, topiramate or entacapone changes the shape phenotype of growing hair. Main qualitative (i) and quantitative (ii; iii; iv) outcomes of the pilot clinical study with the intervention of cosmetics, conducted to evaluate the feasibility of a drug-based approach for hair curvature modulation. (i) Representative photographs of the (a) hair straightening with formulation E containing midodrine hydrochloride (MDD) and (b) hair waving with formulation F containing topiramate (TPM). The two shaved scalp areas of each donor are shown on day 0 (1st application) for guidance; on the left square, 10 µL of the control formulation while, on the right, 10 µL of the test formulation were applied three times per week, totalizing 15 applications. The large photographs were taken at the application where the differences in hair shape between placebo and test areas were maximized and visible to the naked eye. The photos are shown with a blur filter except in the two application zones, delimited by a white dashed line (original photos shown as **SSC-D Fig**). (ii) Combined effectiveness of the different drugs used for hair shape phenotype modulation. (iii) Mean straightening or curling degrees of positive outcomes, as defined by equation (1) in the Materials and Methods section, regarding the treatment with Formulation D containing ethacrynic acid (ETA), Formulation E containing MDD, Formulation F containing TPM and Formulation G containing entacapone (ETP). Data were analysed by one-sample *t*-test (**p* ≤ 0.05, ***p* ≤ 0.01) when conditions were compared to the theoretical value 1 (no change). (iv) The ratio between the average length of relaxed hair fibres and the length of the same fibres straightened under stress, collected from the formulation and placebo-treated scalp areas of volunteers with the best positive outcomes, regarding the treatment with Formulation D (ETA), Formulation E (MDD) or Formulation F (TPM) and Formulation G (ETP). The data from each compound was retrieved from the separate two-way ANOVA analysis of all volunteers assigned for each formulation, followed by post-hoc Sidak's test. ****p* ≤ 0.001 or *****p* ≤ 0.0001, when the hair length ratios of treated areas were compared with the corresponding control (hair collected from the placebo-treated scalp areas).

the last application, hairs were cut from the treated areas and the melanin content was determined. An overall analysis of the data collected shows that 57 % of the volunteers (8 of 14) exhibited some change in hair colour phenotype by the end of the study (Fig. 3A_ii).

Regarding DYP (Formulation A), the specific effectiveness was 67 %. We could observe a clear hair darkening in two volunteers presenting a natural blond to dark blond (Fig. 3A_ia) while no appreciable effect was detected in the one displaying a brown shade. In responsive volunteers' hair, formulation A increased the mean melanin content of treated areas up to 136 % ± 31 % of that in control areas treated with the placebo formulation (Fig. 3A_ii).

Due to the small cohort size, no statistical significance was achieved comparing the mean to the control [one sample *t*-test: *t* (1) = 1.644; *p* = 0.3479]. However, a trend towards significance was demonstrated. In individual analyses, the melanin content of hairs from the area of the scalp treated with the test formulation was statistically different to the respective placebo-treated area [two-way ANOVA: *F*(1,8) = 368.8; *p* < 0.001]. In the volunteer showing the highest difference (Fig. 3A_iv), the percentage of melanin content in hair from the treated area was as high as 157.0 %, corresponding to a change in hair colour shade from medium to dark brown (11.97 µg and 18.86 µg of melanin per mg of hair, for placebo and DYP, respectively).

DYP exerts its therapeutic effects (pharmacological vasodilation) by inhibiting the phosphodiesterases, the enzymes responsible for the normal degradation of cAMP and cyclic guanosine monophosphate (cGMP) [110]. In melanocytes, cGMP activates Protein kinase C, which in turn enhances tyrosinase activity and, consequently, the production of melanin [111,112]. More importantly, cAMP promotes the activation of Protein Kinase A, which activates microphthalmia-associated transcription factor (MITF). MITF is a master regulator of melanogenesis, boosting various enzymes, such as tyrosinase [113]. Several other inhibitors of phosphodiesterases have already shown positive regulation of melanogenesis: cilostazol, sildenafil, vardenafil, theophylline, and 3-isobutyl-1-methylxanthine [114].

Volunteers with light blond (3), medium brown (1), and dark brown (2) hair were assigned to the treatment with Formulation B (RIV). For three of them (50 % specific effectiveness), the hair growing in areas treated with this formulation displayed a significantly lower melanin content than hair from areas treated with the placebo formulation [two-way ANOVA: $F(1,16) = 11.22$; $p = 0.0041$]; the mean melanin content measured ($81 \% \pm 6 \%$) was also significantly lower than the control patch [one sample t -test: $t(2) = 5.515$; $p = 0.0313$]. In agreement with the numerical data, the brown hair from RIV-treated areas was visibly lighter than the hair from placebo-treated areas in two out of three volunteers responding to RIV (Fig. 3A_{ib} shows one of them). Nevertheless, the most pronounced inhibitor effect of formulation B (Fig. 3A_{iv}) was quantified in one volunteer with black hair who presented a 26.2 % reduction in melanin content, although the difference in melanin content was not visually perceptible as a colour change.

The activity of RIV is associated with the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase. By acting on the muscarinic acetylcholine receptors of melanocytes, acetylcholine is thought to inhibit adenylate cyclase and impair the synthesis of cAMP, causing a subsequent decrease in the synthesis of melanin. RIV also promotes pharmacological activation of the heat shock factor 1, a facilitator of transcription, production, and accumulation of heat shock proteins (HSP) as HSP70 [115], which has anti-melanogenic effects *in vitro* and *in vivo* [116,117]. RIV further inhibits the activity and gene/protein expression of COX-1 and COX-2, enzymes that catalyse the production of prostaglandins, increasing the levels of glutathione [118]. In melanocytes, COX-2 knock-down markedly decreases the expression of several melanogenic factors and prevents the stimulation of melanin by the α -melanocyte stimulating hormone [119].

The treatment with Formulation C (PAR) induced a decrease in melanin content in three of five assigned volunteers but without a lightening effect visible to the naked eye. Melanin contents for the two volunteers with dark brown hair and one with dark blond/light brown hair were statistically lower than the respective controls [two-way ANOVA: $F(1,17) = 43.88$; $p < 0.0001$]. Fig. 3A_{iii} shows that the overall melanin content of positive outcomes ($84 \% \pm 5 \%$) was lower than the 100 % control [one sample t -test: $t(2) = 5.589$; $p = 0.0306$]. As for formulation B (RIV), the highest decrease in melanin content (19.9 % regarding the content in hair from the placebo-treated area) was determined in a volunteer with dark brown hair (Fig. 3A_{iv}).

PAR is used as an antidepressant medication and also in the treatment of various anxiety disorders [120]. The modulation of melanogenesis by drugs with antidepressant properties is well documented in the literature, with both stimulatory [121–123] and inhibitory [124,125] effects being reported. This interlink may be due to the shared embryonic origin of neural cells and melanocytes [126]. Although the inhibition of serotonin reuptake has not been directly implicated in the modulation of melanin production, the ability of such antidepressants to exert agonist or antago-

nist actions towards serotonin receptors has been proposed as the mechanism by which they affect melanogenesis [122,123,127]. Besides that, the ability to depress the intracellular levels of dopa-quinone, an intermediate of melanogenesis obtained from the hydroxylation of tyrosine catalyzed by tyrosinase, can be another mechanism by which PAR exercises its inhibitory effect, similar to 6-nitroguipazine [128]. PAR also regulates mitogen-activated protein kinases (MAPKs) pathways by elevating the levels of phosphorylated extracellular signal-regulated kinases or decreasing the levels of phosphorylated p38 MAPKs [129], which by itself inhibits melanin synthesis [130]. Additionally, PAR showed the ability to decrease the intracellular concentrations of nitric oxide [131], that can promote the synthesis of cGMP and up-regulate MITF [132]. Finally, as for RIV, the inhibition of melanogenesis by PAR can be related to the ability to increase the levels of reduced glutathione and to decrease the activity and gene/protein expression of COX-1 and COX-2 [133].

The pilot-scale clinical study proved that DIP, RIV and PAR can modulate melanin production in HFs, changing hair colour in 15 applications over 5 weeks. The intended alterations observed in melanin were not a result of any physical or chemical modifications to the inanimate hair fibre material. S6A Fig provides evidence that no significant expected changes occurred in the colour of hair shaft samples. Furthermore, the data generated may potentially reveal new therapeutic approaches to deal with melanogenesis-related diseases. Further and larger trials should be performed in wider contexts, but our data already points to the possible use of those topical drugs as safe and effective alternatives or adjuvants of conventional cosmetic treatments to change the colour of the hair fibre from the follicle.

Scalp treatment with midodrine hydrochloride, ethacrynic acid, topiramate or entacapone affects the shape of growing hair.

Regarding the effect of the selected compounds on hair shape (Fig. 3B), we again evaluated the effectiveness based on qualitative and quantitative data. Apart from regular photo documentation of hair regrowth, after the last application, hairs were collected from both treated areas of each volunteer and the length ratios between relaxed and stressed hair fibres were calculated and used to determine the straightening and curling degrees. The data collected indicates good overall effectiveness; 84 % of the volunteers (16 of 19) presented some degree of hair curvature modification by the end of the study (Fig. 3B_{ii}). In general, we observed higher straightening degrees (45.5 %, corresponding to the average percentage degrees of formulations D and E) than hair curling degrees (11.6 %, corresponding to the average percentage degrees of formulations F and G) induced by the topical application of the respective formulations. However, the compounds involved in hair curling were those that showed a higher rate of specific effectiveness among the participants (92 % of total effectiveness for hair curling inducers compared to 75 % for hair straightening inducers).

The maximum hair straightening effect was obtained with Formulation E containing MDD (Fig. 3B_{ia}, 3B_{iv}). The best result corresponded to an increase in hair length ratio of 120 % when compared to the placebo formulation [two-way ANOVA: $F(1,32) = 107.4$; $p < 0.0001$]. The degree of straightening caused by Formulation E was, on average, 76 % (Fig. 3B_{iii}); we obtained a specific effectiveness of 75 % for this formulation. Looking at each volunteer data responding to this formulation, the hair length ratio from areas of the scalp treated were statistically different from those of the respective placebo-treated areas. However, due to the small cohort size and data dispersity, mainly due to the variety in the natural hair curvature of volunteers, no statistical significance was achieved comparing the mean straightening degree to the theoretical value 1, which corresponds to no change of hair curvature [t -test: $t(1) = 1.643$; $p = 0.3480$]. In agreement with the quantitative data, the very curly hair from the scalp area of Volun-

teer #4 treated with Formulation E was straighter than the hair from the placebo-treated area, particularly visible in the hair portion next to the scalp, most “recently produced” (Fig. 3B_ia).

In addition to MDD, we tested ETA with the same purpose (Formulation D). This formulation was also capable of changing hair curvature but to a lower extent than Formulation E. The average length ratio of relaxed to stressed hairs from the placebo-treated areas of volunteers assigned to formulation D (0.829 ± 0.0395) was higher, meaning straighter hair, than the same ratio for volunteers assigned to formulation E (0.725 ± 0.2342). Looking at all data from the volunteers assigned to formulation D, the maximum change in hair length ratio was 19.3 % [two-way ANOVA: $F(1,32) = 59.74$; $p < 0.0001$] (Fig. 3B_iv), which is not very different from the mean straightening degree (15.5 %) of positive outcomes (75 % specific effectiveness) for this compound (Fig. 3B_iii). The hair growing in Formulation D treated areas presented a significantly higher hair length ratio than hair from placebo-treated areas and the mean effect on hair straightening (15.5 %) was significantly higher than the theoretical value 1 [one sample t -test: $t(2) = 7.394$; $p = 0.0178$; Fig. 3B_iii].

Formulation F (TPM) induced a change in hair direction in more than 80 % of the volunteers assigned to this formulation. Fig. 3B_ib exemplifies what we have observed after 5 weeks of TPM treatment. On the scalp region where formulation F was applied 15 times, we saw the start of a left-handed curl, while the hair in the region treated with the placebo formulation remained straight. It should be noted that the curling effect has occurred in both directions, depending on the volunteer. Among all tested compounds, TPM was the most consistent presenting low dispersion of curling degree values. Considering all volunteers, the maximum measured change corresponded to a curling degree of 11 % [two-way ANOVA: $F(1,48) = 74.74$; $p < 0.001$; Fig. 3B_iv], being the average of positive outcomes 9.9 % (Fig. 3B_iii). Even at such modest variations, we can verify from Fig. 3B_ib a visible hair direction change.

The treatment with Formulation G (ETP) gave similar results to Formulation F (TPM), with a specific effectiveness of 100 % as all volunteers assigned to this treatment presented a change in hair curvature. The ratios between relaxed hair to stressed hair lengths, obtained in the placebo-treated hair samples, for both for TMP and ETP arms of the study, have very similar values and are close to 1, which means that the volunteers' hair was straight; there were too few differences between relaxed hair and stressed hair lengths for the placebo formulation. The average curl degree obtained with ETP treatment is slightly higher than the one obtained with TMP, reaching 13.3 % on average (Fig. 3B_iii). Looking at the data of all the assigned volunteers, the volunteer presenting the best result in this group, the formulation G treated hair was 17.4 % more curled regarding the placebo-treated hair [two-way ANOVA: $F(1,36) = 122.7$; $p < 0.0001$; Fig. 3B_iv].

Although the exact mechanisms by which these drugs lead to fibre curvature modification are still to be determined, this pilot study proves that they all can regulate HF after 15 applications in 5 weeks, with quantifiable changes. Again, the expected alterations observed in curvature were not a result of any physical or chemical modifications to the inanimate hair fibre material. S6B Fig provides evidence that no notable changes occurred in the curvature of hair shaft samples. Besides, the results also validate the selected genes as a tool to search for hair shape modulators, indirectly proving the biological function of these six genes on the fibre curvature setting that occurs at the follicle; they also emphasize the IRS as a major cellular player in the process. Functional studies using proper *in vitro* 3D cell and tissue culture, where the genes can be manipulated, should be performed to understand better the mechanistic relationships and their role in fibre curvature set-

ting. When working with animal tissue, any manipulation of genes *in vivo* must be done within the boundaries of fundamental hair shape biology. It cannot be associated with cosmetic research as animal experimentation has been prohibited in Europe since 2013. As mentioned, no significant differences were detected in hair growth rates between the length of hair in scalp areas treated with placebo and test formulations, under the conditions of study. More importantly, no adverse side effects associated with the formulation topical application were reported by the volunteers during the entire duration of the study.

Other clinical trials must nevertheless be conducted to address the major limitations of the present one: small number of participants, unique doses and short duration. Larger and more representative populations will also provide information regarding responsiveness to the treatment according to age, gender, and natural hair pigmentation and shape.

Conclusion

In stark contrast to melanogenesis and hair colour formation, hair shape is far from being well understood. This fact prompted us to use transcriptome analysis to characterise gene expression patterns associated with fibre curvature phenotype. This approach proved to be valuable in gathering more novel information regarding hair shape, which is more difficult to study experimentally. The microarray revealed a pool of new genes whose different levels of transcription can now be associated with different natural hair shape phenotypes. This new pool will certainly open new directions for the fundamental research dedicated to the understanding of fibre curvature setting in the HF. Some genes were selected as key targets for screening possible cosmetic ingredients able to change hair shape phenotype from the follicle. We mapped the location of the expression of these genes to the HF, in particular, to the IRS, which reinforced the importance of this special cell layer in hair fibre curvature establishment.

The pilot cosmetic study was fundamental to obtain a proof of concept that molecules interfering with the most variable genes can induce changes in the phenotype of growing hair. By promoting a coordinated change in the amount of the CGA, GSTM4, NDUFV2, FMO1, FKBP2 and FABP7 the selected compounds led to a change in hair fibre curvature. The results confirm the involvement of the selected genes in hair shape, also validating the microarray results. Likewise, they validate our screening methodology. Other libraries of natural compounds and biomaterials can be screened as they are a valuable source of natural-based cosmetic ingredients. According to the pattern of transcription induction or inhibition, we chose four hit molecules that promoted the changes that were expected based on the *in vitro* test results. The screening methodology developed by us can be extended and improved by others in the search for other molecules able to perform the same task even better: modulate the way that fibre curvature is established in the follicle.

This work also proves the feasibility of topical modulation of hair colour. In a clinical study at the pilot scale, the hair of volunteers became darker or lighter following the treatment of the scalp with formulations containing different molecules. These molecules are repurposed drugs, selected as inducers or inhibitors of melanin biosynthesis in an *in vitro* screening of Prestwick Chemical Library®. This screening is a comprehensive survey of melanogenesis modulation by existing drugs and, besides the reported cosmetic implications, the data generated has the potential to reveal new therapeutic approaches to deal with melanin-related diseases. Our data already supports the use of those topical drugs as a safe and effective alternative to conventional cosmetic treatments to change the colour of hair. The hair phenotype can be modulated

by the topical application of bioactive compounds. These molecules interfere with the cellular biochemical processes responsible for hair colour and shape setting occurring at the hair follicle level and they have the potential of becoming a new class of cosmeceutical ingredients for future hair cosmetics.

The authors ACP, TM, CC and BF are inventors of the international patent application WO 2021/260667 A2, “Composition of hair follicle modulation, methods and uses thereof”, published on 30 December 2021.

ACP is the co-founder of SOLFARCOS – Pharmaceutical and Cosmetic Solutions Ltd, born as a spin-off company of the University of Minho, where he holds the position of CSO.

The authors declare that they have no other known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Teresa Matamá: Investigation, Methodology, Formal analysis, Validation, Visualization, Supervision, Resources, Writing – original draft, Writing – review & editing. **Cristiana Costa:** Investigation, Methodology, Formal analysis, Validation, Visualization, Writing – original draft. **Bruno Fernandes:** Investigation, Methodology, Formal analysis, Validation, Visualization, Writing – original draft. **Rita Araújo:** Resources, Investigation. **Célia F. Cruz:** Resources, Investigation. **Francisco Tortosa:** Resources. **Caroline J. Sheeba:** Supervision. **Jörg D. Becker:** Data curation, Writing – review & editing. **Andreia Gomes:** Methodology, Supervision, Writing – review & editing. **Artur Cavaco-Paulo:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [The authors Artur Cavaco-Paulo, Teresa Matamá, Cristiana Chaves and Bruno Fernandes are inventors of the international patent application WO 2021/260667 A2, “Composition of hair follicle modulation, methods and uses thereof”, published on 30 December 2021. Artur Cavaco-Paulo is the co-founder of SOLFARCOS – Pharmaceutical and Cosmetic Solutions Ltd, born as a spin-off company of the University of Minho, where he holds the position of CSO. The other authors declare no other competing interests].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2023.11.013>.

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