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DOI:
10.1016/j.jaad.2018.02.009

Document Version
Accepted author manuscript

Accepted Manuscript

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PII: S0190-9622(18)30186-5

DOI: 10.1016/j.jaad.2018.02.009

Reference: YMJD 12315

To appear in: *Journal of the American Academy of Dermatology*

Received Date: 26 November 2017

Revised Date: 31 January 2018

Accepted Date: 6 February 2018

Please cite this article as: Hernandez I, Alam M, Platt C, Hardman J, Smart E, Poblet E, Bertolini M, Paus R, Jimenez F, A technique for more precise distinction between catagen and telogen human hair follicles ex-vivo, *Journal of the American Academy of Dermatology* (2018), doi: 10.1016/j.jaad.2018.02.009.

Type of article: ***Research Letter***

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Funding sources: This work was supported in part by a basic translational research grant from Unilever, Colworth, UK to R.P., by Monasterium Laboratory, Münster, Germany, and by the National Institute of Health Research (NIHR) Manchester Biomedical Research Centre ("Inflammatory Hair Diseases" Programme).

Conflicts of interest: The authors have no conflict of interest to declare

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Manuscript word count: 500 Figures: 1

Keywords: Methylene blue, hair follicle, hair cycle, telogen, catagen, anagen, follicular unit

Identifying human anagen hair follicles (HFs) *ex vivo* is readily accomplished by stereomicroscopic analysis. However, to reliably distinguish other hair cycle stages, namely late catagen and telogen, by stereomicroscopic analysis alone is difficult, and the "gold-standard" remains histological analysis, which obviously precludes their use for *ex vivo* culture (1,2). In this study, we sought to determine whether methylene blue (MB), a vital stain than can be applied to living cells (3), helps to distinguish late catagen from telogen HFs *intravitally* for subsequent organ culture, thus greatly expanding translational preclinical research into these as yet poorly investigated, but clinically important, human hair cycle stages.

Taking advantage of follicular unit (FU) hair transplantation methodology, when FUs are grouped based on the number of HFs they contain (4), we recorded the number of anagen, catagen and telogen follicles found in 800 FUs from 8 Caucasian male patients (100 FUs per patient) undergoing a standardized FUE hair transplant procedure, with informed patient consent. Since anagen VI follicles are easily identifiable (1), only those FUs that contained catagen and/or telogen HFs were further microdissected, photographed, and immersed in 0.02% MB solution dissolved in saline (~5 minutes), followed by fixation and subsequent evaluation.

As shown in Fig. 1, intravital MB staining greatly enhanced anatomical HF structures that could be visualized by light microscopy alone and permitted correct hair cycle stage classification using accepted, well-defined morphological criteria (2) such as the identification of a prominent epithelial strand (Fig. 1a), a key feature of late catagen HFs, which is absent in telogen HFs. Correct hair cycle stage classification by this method was confirmed by Ki67/TUNEL immunofluorescence microscopy (Fig. 1d).

Importantly, MB staining enabled correct identification of the hair stage in 95.63% of cases, compared to 72.02% in non-MB stained HFs. Thus, this simple, economical, and fast technique constitutes a significant methodological advance in human hair research, since it greatly

facilitates *ex vivo* research on human catagen and telogen HFs without having to resort to histology.

Our analyses revealed a higher percentage of catagen than telogen HFs in all patients (89% anagen, 6.7% catagen, and 3.6% telogen). This supports the previous proposal that the percentage of scalp telogen HFs has been overestimated (2), and questions the accepted 'standard' percentages of 80-89% anagen, 10-20% telogen and 1-5% catagen in the literature based on transversal histologic sections (5) and/or (photo-trichograms), neither of which can definitively distinguish between late catagen and telogen HFs. Although in our study the HFs were from patients with androgenetic alopecia (AGA) and the ratio of anagen:catagen:telogen may differ in comparison to non-AGA individuals, we believe that our data are unlikely to reflect sampling bias, as HFs were harvested from occipital scalp, generally unaffected by AGA. We propose that hair stage distribution in healthy human scalp needs a more systematic re-evaluation, including comparative studies with histological sections. This is important when assessing candidate hair growth-modulatory agents, since minor shifts in the percentage of telogen or catagen HFs can result in major changes in the degree of visible effluvium.

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FIGURE LEGENDS

Figure 1. **Macroscopic analysis of hair follicles isolated from follicular units (FU) is more definitive after Methylene Blue staining**. (a) HF in the late catagen stage with the epithelial strand (arrow) clearly visible after MB (0.02%) staining. (b) A HF which cannot be clearly identified under the stereomicroscope as either late catagen or telogen, followed by MB staining (c) which highlights the small remaining epithelial strand (dotted line) which allows us to identify it as late catagen. (d) Ki67/TUNEL confirms this is a late catagen HF, with the presence of several apoptotic, TUNEL positive cells (arrowheads).