Macrophage identification in the different areas of the hair follicle of Androgenetic Alopecia

Supasit Tantiwong[1], Piyaporn Surinlert[2], Saranyoo Ponnikorn[2*]

[1]Division of Dermatology, Chulabhorn International College of Medicine, Thammasat University.
[2]Chulabhorn International College of Medicine, Thammasat University, Pathum Thani, Thailand
[*]Corresponding author, E-mail: saranyoo@tu.ac.th

Abstract

Androgenetic alopecia (AGA) is a polygenetic hair loss disease that causes terminal hair transformation into vellus hair with the progressive miniaturization of the hair follicle. Androgen hormone interaction, imbalance of signaling modulation, genetics, aging, and microinflammation are complex contributions to the pathogenesis of AGA. In the early stage of disease, the peripilar sign represents the sign of microinflammation by showing the infiltration of perifollicular inflammatory cell clusters, including lymphocytes, a few mastocytes, and predominantly macrophages. This study aims to identify the hair follicle macrophage in AGA and comprehensively compare macrophages at various hair follicle compartments. The immunohistochemistry CD68 macrophage markers were stained in 2 healthy and 3 AGA with peripilar signs scalp biopsies. The CD68 positive cell infiltration was not significantly different in each hair follicle compartment of both groups. Furthermore, the infundibulum area seems to have the highest number of CD68 positive cells infiltration, significantly different from the supra-bulb regions of the AGA group and particularly from the bulge and supra-bulb area in the normal group. This study eventually elucidated the immunohistochemical staining for the systematic profile of hair follicle macrophages in normal and AGA hair follicles.

Keywords: *Peripilar sign, microinflammation, macrophage*

1. Introduction

Androgenetic alopecia (AGA), or male pattern baldness, is the most common type of progressive hair loss. AGA is a polygenetic disorder with varying severity, onset age, and hair loss location on the scalp. Hair loss in men usually affects the temporal and vertex regions, sparing the occipital region: the so-called “horseshoe” pattern. Although androgen has caused these changes, most molecular mechanisms have been unknown, limiting the treatments accessible (Lolli et al., 2017). Micro inflammation has been thought to be one of the pathogenesis components of Androgenetic alopecia. According to a previous study, in the hair follicle of early-stage AGA, inflammatory cells were found to be infiltrated in the region of the follicular bulge, which is the putative source of stem cells in the cycling follicle (JAWORSKY, Kligman, & Murphy, 1992). Moreover, in another previous study, peripilar sign was found to be linked to infiltration of perifollicular inflammatory cell clusters (lymphocyte, macrophage, and few mastocytes) (Deloche et al., 2004). Immune cells have been believed to have a role in the hair regeneration process, and microinflammation was one factor affecting the transition phase of the hair cycle and modulating the survival and death of hair follicles by secreting factors from specific immune cells (Eichmüller et al., 1998; Hardman et al., 2019). Perifollicular macrophages are associated with hair cycle inhibition, induction, and even phase transition in murine and human normal hair cycles (Hardman et al., 2019). The number of perifollicular macrophages increased in anagen, decreased in the catagen, and dramatically reduced in the telogen during the hair cycle (Hardman et al., 2019; Paus et al., 1998). Moreover, hair cycle-related variations were seen in macrophage-like cells in rat skin and a switch to a fibroblast growth factor 5+ phenotype that promotes catagen (Suzuki et al., 1998). Furthermore, apoptotic macrophages in telogen hair follicles in mice also secreted Wnt signals, activated epithelial hair follicle stem cells, and promoted anagen (Castellana, Paus, & Perez-Moreno, 2014), precisely as they did in humans (Hardman et al., 2019).

In other inflammatory hair loss diseases, the number of macrophages became higher in the distal hair follicle epithelium and perifollicular mesenchyme in both Lichen planopilaris and Frontal fibrosing

alopecia (Harries, Hardman, Chaudhry, Poblet, & Paus, 2020). Macrophages were shown to be prevalent at the lower infundibulum of hair follicles that showed the peripilar sign compared to other locations in AGA (Deloche et al., 2004).

Although macrophages were involved in inhibition and proliferative stimulation during specific stages of normal hair cycling and were present in various infiltration patterns throughout Lichen planopilaris and Frontal fibrosing alopecia (Harries et al., 2020), including AGA patients (Deloche et al., 2004; JAWORSKY et al., 1992; Kligman, 1988), the pattern of inflammation and macrophage interaction contributing to the etiology of AGA has remained a point of contention. The goal of this study was to use an immunohistochemical test to analyze macrophage interaction in the hair follicle between normal and AGA scalp tissues.

2. Objectives

This study aimed to comparatively investigate the infiltration of macrophage (CD68) in the different hair follicle parts of balding vertex including infundibulum, bulge, supra-bulb, and bulb region of hair follicle from AGA and normal donors by using immunohistochemistry assay.

3. Materials and Methods

3.1 Sample selection

The principles of the Declaration of Helsinki, the Belmont Report, the CIOMS guideline, and the International Conference on Harmonisation-Good Clinical Practice (ICH-GCP) were followed in this study. The study was approved by The Human Ethics Committee of Thammasat University (Medicine) (approval reference COA No. 151/2564). Male patients aged 20-40 years with AGA as diagnosed by dermoscopic examination revealing hair miniaturization of more than 20% at the vertex and less than 20% at the occipital scalp, as well as the presence of brown peripilar sign (brown halo sign found on trichoscopy in AGA patient associated with infiltration of perifollicular inflammatory cell cluster) and physical examination indicating Hamilton-Norwood stage III vertex, were included in the AGA group. Healthy male volunteers aged 20-40 years who had a physical and dermoscopic examination demonstrating normal hair and scalp were included in the normal group. Following informed consent, 6-mm punch biopsies were collected from 3 clinically vertex scalps with the peripilar sign in AGA and 2 vertex scalps of control volunteers. Based on histology, selected samples from AGA patients with relatively inflamed hair follicles (HF).

All collected tissues were preserved as formalin-fixed paraffin-embedded (FFPE) samples. 5-µm paraffin sections of biopsied-scalp samples were prepared for H&E staining to identify hair follicles and surrounding infiltrate. Then, found the appropriate slide to stain for the immunohistochemistry assay. The peripilar sign of AGA was compared with samples from healthy controls.

3.2 Immunohistochemistry staining

5-µm sections of formalin-fixed and paraffin-embedded samples were cut and stained with the following immunohistochemistry marker: CD68 (pan macrophages). Briefly, samples were baked by warming up to 75 degrees Celsius and incubated for 4 minutes, then deparaffined by warming up the slide to 72 degrees Celsius from medium temperature, then warm up the slide to 95 degrees Celsius and incubate for 8 minutes, followed by 20 minutes of Ultra cell conditioning and 36 minutes of Ultra cell conditioning for cell conditioning. Antibody incubation started by warming up the slide to 36 degrees Celsius and incubating for 4 minutes. Then the primary antibody; CD68 (Rabbit monoclonal to CD68, ab213363, Abcam, UK), was applied to the slide and incubated for 36 minutes, followed by applying one drop of HEMATOXYLIN II to each slide, applying the coverslip, and incubating for 8 minutes for counterstaining. Finally, one drop of BLUING reagent was applied on each slide, followed by an application of coverslip and incubation for 4 minutes for post counterstaining.

3.3 Histologic analysis

To assess macrophage profile, macrophage cells were identified as defined HF epithelial compartments: corresponding 30-µm perifollicular mesenchymal compartments (representing the connective tissue sheath, CTS) around each follicle. Further, a corresponding 100-µm perifollicular mesenchymal zone was analyzed to capture the wider inflammation in HFs. Moreover, in each area of HF epithelial compartments, HF was also divided into 4 vertical areas, which are the lower infundibulum, bulge, supra-bulb, and bulb region so macrophages can be localized in each compartment of the hair follicle (Figure 1). A high-resolution scanner was used for all measurements (Motic digital scan).

3.4 Statistic analysis

All HFs were counted in every section, including normal and inflammation appearance by Qupath software. Data were collected as a combination of each disease group and HF compartment. Data were coded, entered, and analyzed using the SPSS software package for statistical science (SPSS for Windows, Version 13.0.1; SPSS Inc., Chicago, IL, USA). Statistical analysis included descriptive analysis as median value and range; Mann Whitney U test was expressed in terms of P-value. A value of $P < 0.05$ was considered statistically significant.

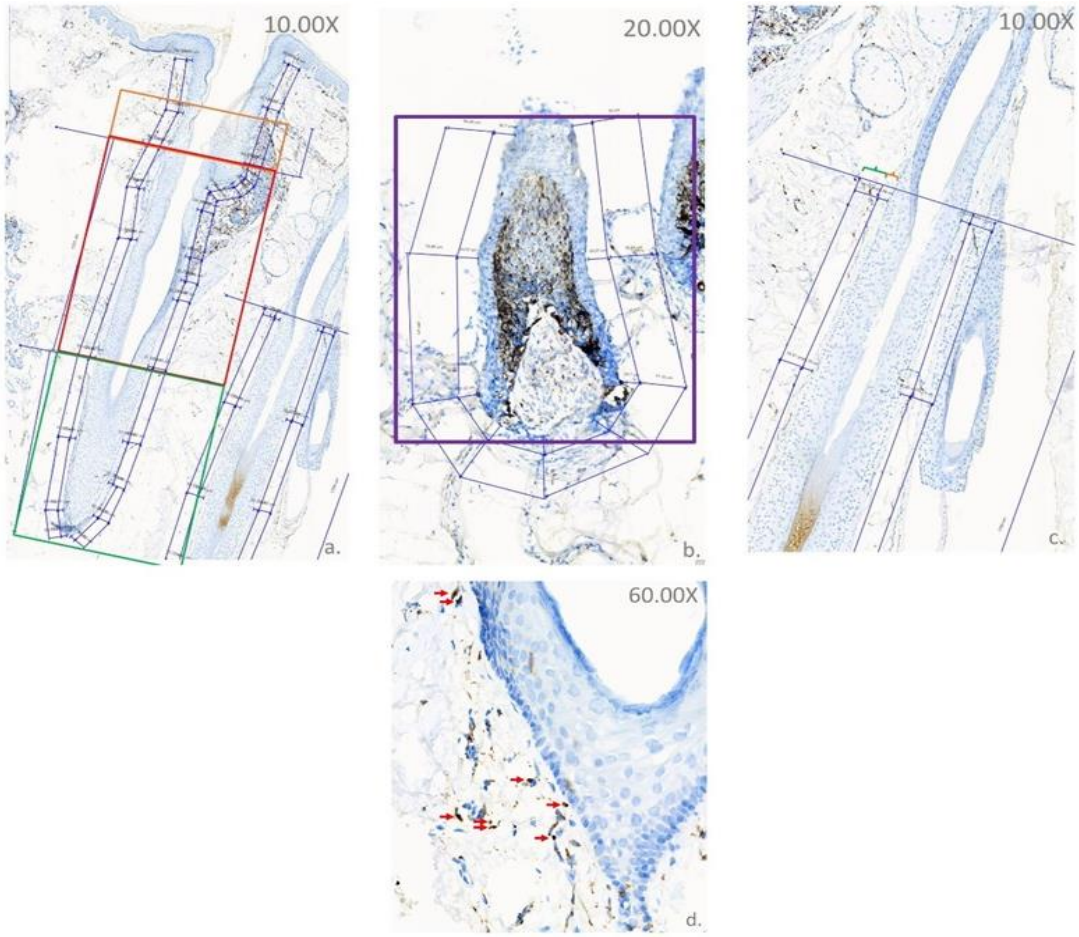

Figure 1 Hair follicle is divided into 4 vertical areas, which are the lower infundibulum: orange box, bulge: red box, supra-bulb: green box (a), and bulb region: purple box (b), HF epithelial compartments are divided into corresponding 30-µm perifollicular mesenchymal compartments: orange brace, and a corresponding 100-µm perifollicular mesenchymal zone: green brace (c), the CD68 positive cell: red arrow (d).

4. Results and Discussion

4.1 Result

Scalp tissue specimens were collected from a total of 5 samples, 3 clinically peripilar signs of vertex scalps in AGA due to macroscopic microinflammation evidence, and 2 vertex scalps of control. In normal hair follicles, the amount of CD68 positive cells in each hair follicle segment was analyzed, and the infundibulum area showed increased CD68 positive infiltration in the normal group (Figure 2) compared to the AGA group (Figure 3). Similar to the infundibulum area, CD68 positive cell infiltration in the normal group (Figure 6) was higher than AGA (Figure 7) at supra-bulb. While CD68 positive cell infiltration in the bulge area was shown to be reduced in the normal group (Figure 4) compared with the AGA group (Figure 5), opposite from the infundibulum and supra-bulb area. However, the difference in each hair follicle segment between the two groups was not significant.

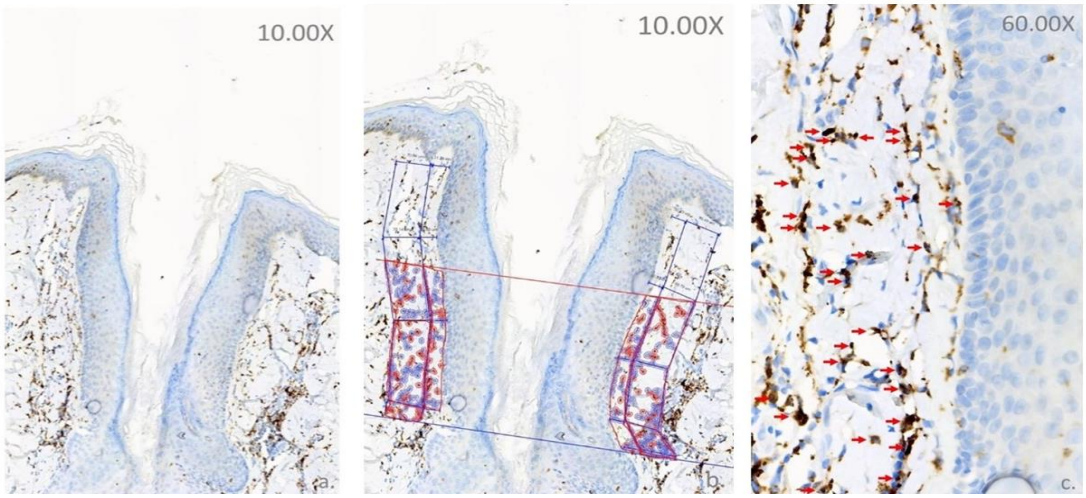

Figure 2 (a) The original images of lower infundibulum stained with CD68 in normal (a), the markup images showing cell detection using QuPath in normal (b), zoom in CD68 positive cells; red arrow, image (c).

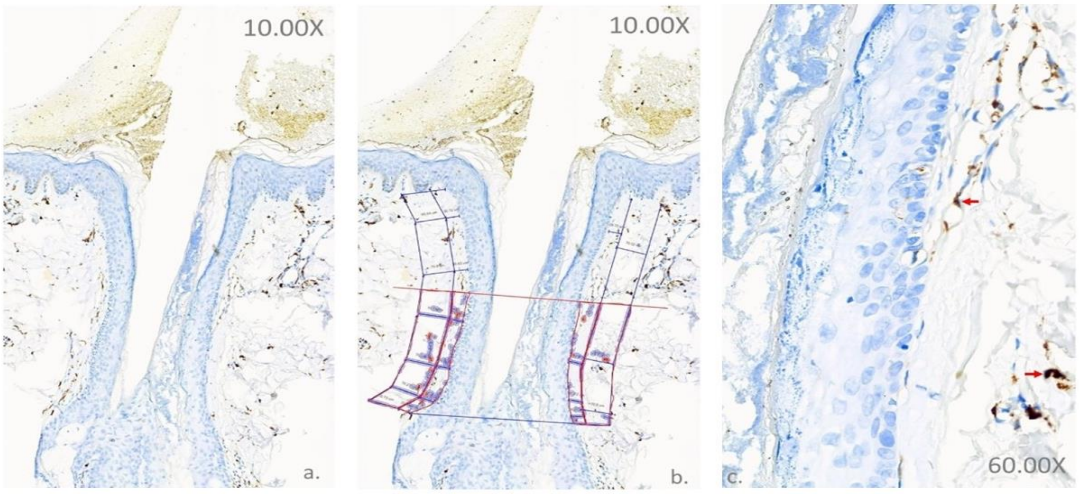

Figure 3 The original images of lower infundibulum stained with CD68 in AGA (a) and the markup images showing cell detection using QuPath in AGA (b), zoom in CD68 positive cells; red arrow, image (c).

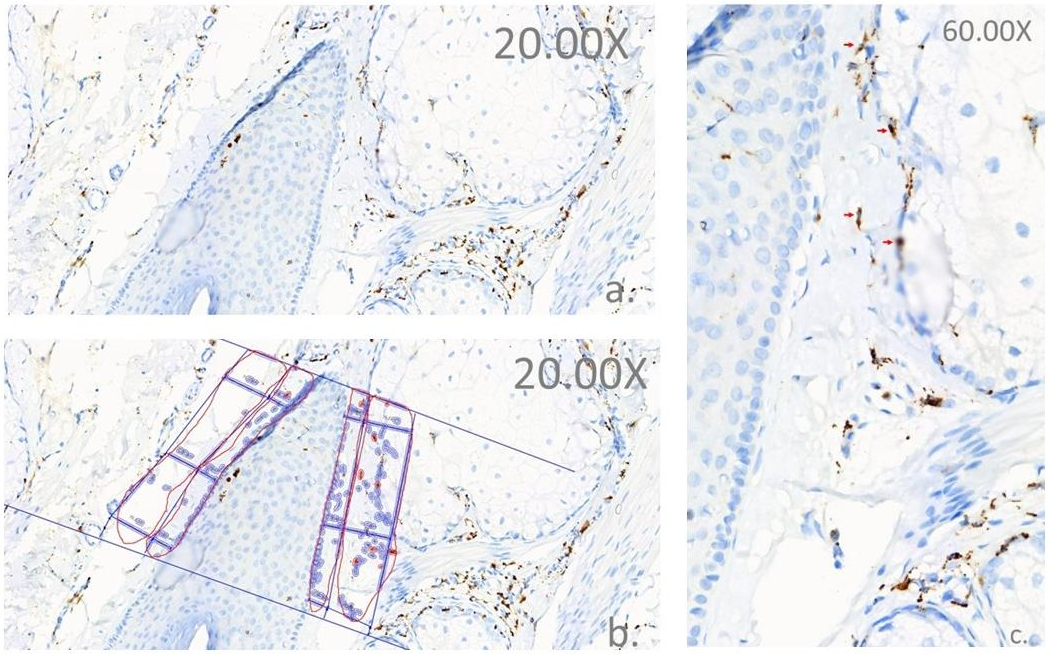

Figure 4 The original images of bulge stained with CD68 in normal (a) and the markup images showing cell detection using QuPath in normal (b), zoom in CD68 positive cells; red arrow, image (c).

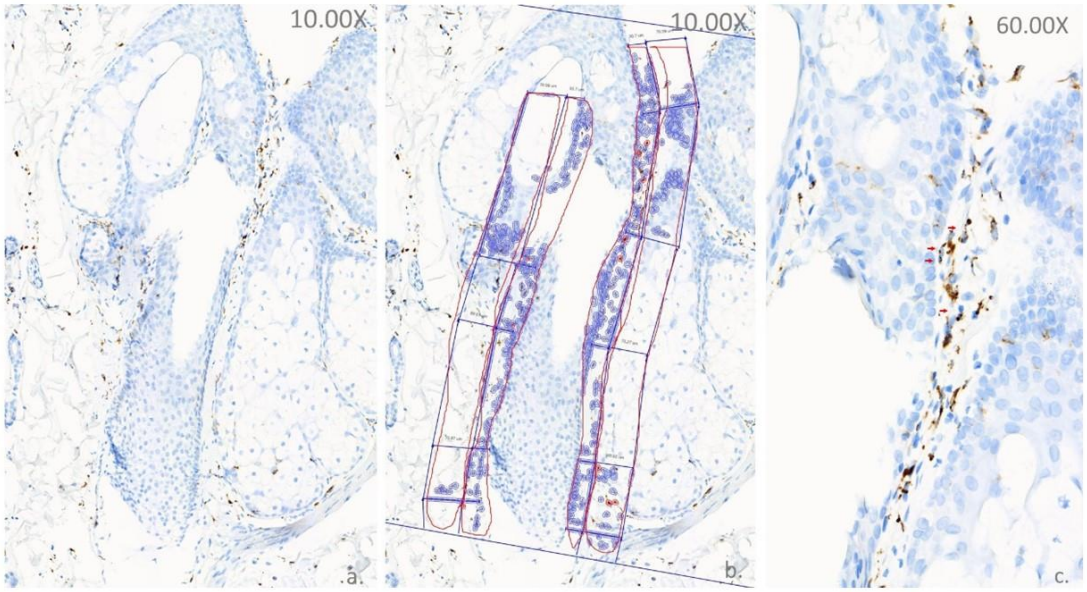

Figure 5 The original images of bulge stained with CD68 in AGA (a) and the markup images showing cell detection using QuPath in AGA (b), zoom in CD68 positive cells; red arrow, image (c).

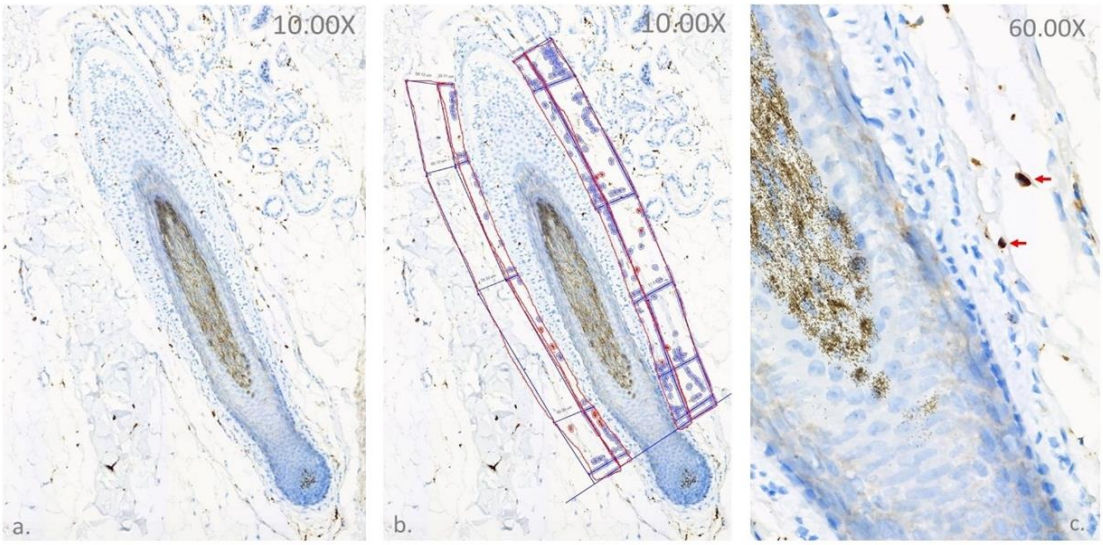

Figure 6 The original images of supra-bulb stained with CD68 in normal (a) and the markup images showing cell detection using QuPath in normal (b), zoom in CD68 positive cells; red arrow, image (c).

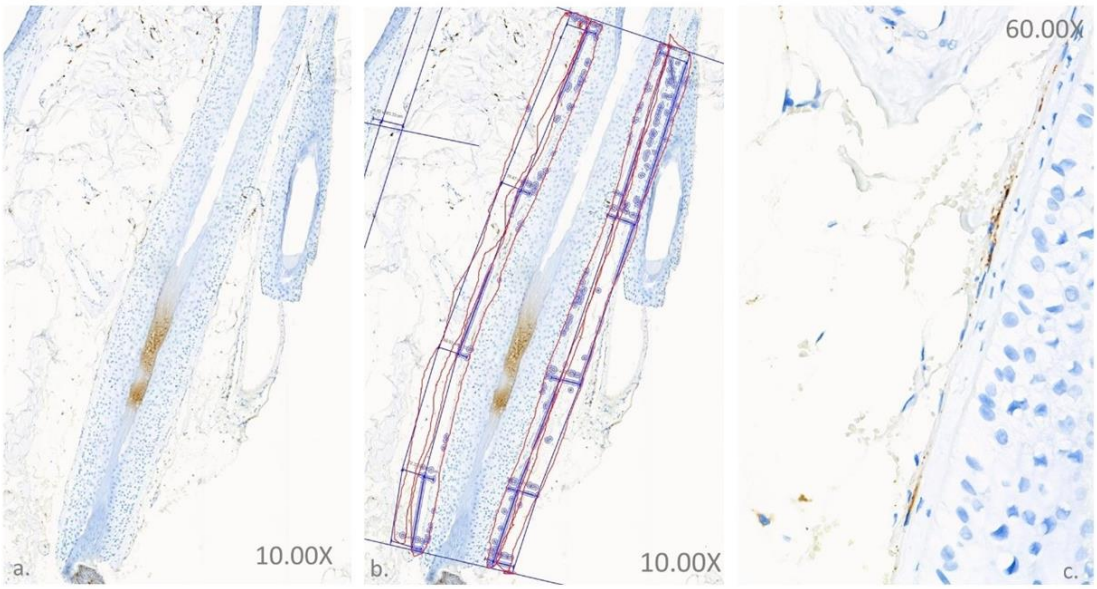

Figure 7 The original images of supra-bulb stained with CD68 in AGA (a) and the markup images showing cell detection using QuPath in AGA (b), zoom in CD68 positive cells; not detected, image (c).

Nonetheless, when each hair follicle segment within the group was compared, the infundibulum had the highest CD68 positive cells infiltration, which was significantly higher than the bulge and supra-bulb areas in the normal group at a p-value of ≤ 0.05. When compared to the bulge and supra-bulb areas in AGA, the infundibulum showed the highest CD68 positive cell infiltration, although the differences were only significant between the infundibulum and supra-bulb areas.

Taken together, when compared to the number of CD68 positive cells in each hair follicle segment. Infundibulum, and supra-bulb, in normal, were found to be higher than in AGA. In both groups, the highest CD68 positive cell infiltration segment was the infundibulum. Except for the bulge area, the number of CD68

positive cells was found to be higher in AGA than normal. However, all of the differences between the two groups in each hair segment were not significant (Figure 8).

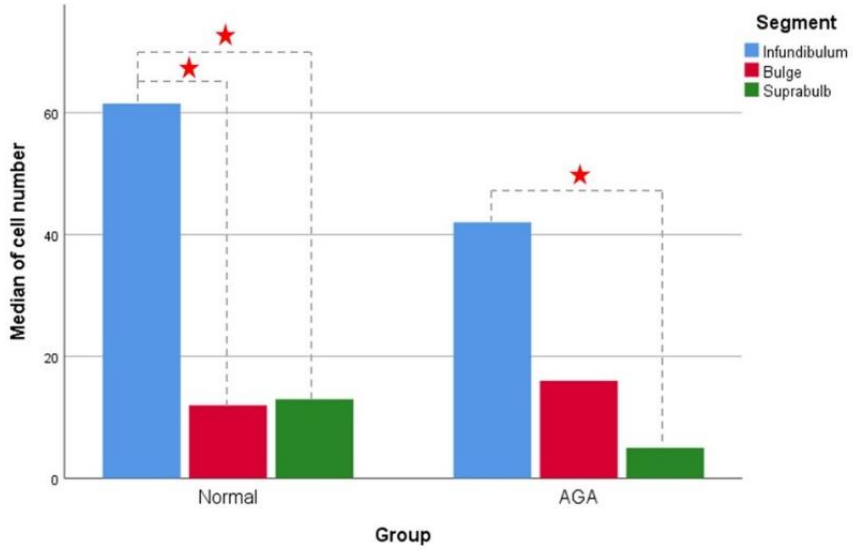

Figure 8 Comparison of the median number of CD68 positive cells in each hair follicle segment in normal and AGA groups.

4.2 Discussion

Besides the hair follicle compartment composes of a heterogeneous population of hair follicle stem cells, dermal papilla, and others, various tissue microenvironment factors also involve the complex dynamic of hair regeneration. Immune cells become one important niche factor having a role in each specific hair cycling stage. Interestingly, in many hair loss diseases, frequent findings demonstrate that increased infiltration of immune cells, especially macrophages, has a deleterious effect on the pathogenesis of the diseases (Harries et al., 2020). The perifollicular inflammatory cell is observed around the lower portion of the infundibulum and isthmus in AGA patients (Abell, 1988; Lattanand & Johnson, 1975; Jaworsky et al., 1992: Kligman, 1988). Suggesting the role of moderate inflammation progressively contributed to the pathogenesis of AGA. Micro inflammation was thought to be one important mechanism associated with the distinct brown peripilar sign finding in the early stage of AGA (Deloche et al., 2004). However, conflicting results also occurred on whether the microinflammation, increased immune infiltration, and hair follicle cell disruption was the most significant molecular pathogenesis inside the early stage of the disease. Moreover, the interaction between immune cells especially the macrophages and hair follicle cell interaction during progressive disease still hasn't been well illustrated. Our finding directly answered whether the CD68 macrophages had an important role in the early stage of the AGA using clinical scalp biopsied for immunohistochemical analysis. Each hair follicle was highlighted in the CD68 positive cell enumerative study including in the infundibulum, bulge, and supra-bulb area. Although the CD68 positive cells were found higher in the infundibulum in the normal scalp than in the disease, the non-significant number of CD68 positive in normal represented the physiological stage of the macrophage contributing to the hair regeneration process specifically the transition stage from telogen to anagen. Macrophages engulfed and degrade collagen fibers of the connective tissue sheath in early ultrastructural photos of catagen follicles. These data suggest that HF degeneration is caused by an increase in macrophage numbers caused by anagen, which then phagocytize cellular debris during the catagen (Parakkal, 1969; Weedon & Strutton, 1981). Rather, macrophages' influence on the HF cycle is due to their large cytokine secretome, which is especially

important during the telogen-to-anagen transition (Harel et al., 2015). This was a possible explanation for why more amount of CD68 positive cells were identified in normal scalp tissues. On the other hand, clinical specimen has more limitation for better evaluation of macrophage interaction in specific hair cycle stage by using anagen hair organ culture as well as a mice model. A recent study from a hair organ culture experiment demonstrated nicely macrophage infiltration pattern and fluctuation through each stage of the normal hair cycle (Hardman et al., 2019). In contrast, the bulge area in AGA showed a higher infiltrating CD68 positive cell count than normal, suggesting that the non-significant number of CD68 positive cells in AGA could be related to HFSC and macrophage interaction, as demonstrated in a previous study (JAWORSKY et al., 1992). Increased macrophage infiltration in the bulge area could imply that macrophage-hair follicle stem cell (HFSC) communication is involved in the disruption of normal hair regeneration. Disruption of the normal hair cycle can cause by many mechanisms, one of them being the prolonged quiescence stage of the hair cycle (Yi, 2017). During the quiescence stage of the hair cycle, TREM+ macrophages are discovered to promote JAK-STAT-mediated control of HFSC proliferation via Oncostatin M (OSM) in a prior study (Wang, Dai, Ferrante, Drake, & Christiano, 2019). HFSC activation and differentiation occur after an apoptotic-driven reduction in OSM secreting macrophages in late telogen (Castellana et al., 2014; Wang et al., 2019). This could reflect why AGA scalp tissues have a higher number of CD68 positive cells at bulge area.

CD68 positive cells only preliminary indicated the macrophage infiltration in the tissue, but an in-depth study of specific macrophage profile activation and pattern of inflammation were unmasking the role of inflammatory processes in AGA. In physiologic hair stage transition, macrophage polarization fluctuates by subtype switching from inflammatory subtype macrophage (M1) in early anagen and early catagen to non-inflammatory subtype macrophage (M2) in the late catagen and telogen (Hardman et al., 2019). Even in hair loss conditions, macrophage polarization exhibit a difference in profile between individuals with the disease and healthy ones (Harries et al., 2020). Therefore, to acquire a better knowledge of the role of each macrophage subtype and polarization dynamics, the different macrophage subtypes, and functional secreting cytokine indicators should be further demonstrated by immunofluorescence staining.

5. Conclusion

This study was the first to compare CD68 positive macrophage infiltration in normal and AGA from human hair follicle tissues. The number of CD68 positive cells in each hair follicle segment showed different distribution in both AGA and normal tissue specimens. Unexpectedly, the infundibulum of normal represented substantially higher than AGA without statistically significant regarding the role of macrophage for hair cycling regeneration in the normal physiological stage. Moreover, the macrophage infiltration was detected higher in the bulge area of AGA possibly demonstrating the inflammatory events like other hair loss diseases. Optimistically, these findings were a good start toward uncovering additional pathogenesis and developing new target therapies for Androgenetic alopecia .However, the significance of the macrophage role remained inconclusive due to the limited number of specimens, requiring further research. Additional research into macrophage polarization and the analysis of relevant cytokines is also required.

6. Acknowledgements

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