THE ROLE OF STRESS IN THE PATHOGENESIS OF ALOPECIA AREATA: AN OBJECTIVE ASSESSMENT VIA HAIR CORTISOL LEVEL

by

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DEDICATION

Kleidie, Mbbq & Kapi

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DECLARATION

I, Louis Jean Fick, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree to this or any other university.

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THESIS ABSTRACT

BACKGROUND

Alopecia areata (AA) is an autoimmune disease of which the exact pathogenesis is poorly understood. There is conflicting evidence that psychosocial stress causes or exacerbates AA. A systematic literature search was undertaken focusing on studies describing the pathogenesis of AA and Hair cortisol concentration (HCC) as a biomarker of psychosocial stress. HCC is reported to be a reliable biological marker of stress. The estimated scalp hair growth rate of about one centimetre per month may be used to correlate cortisol levels with historic time-points depending on hair length. HCC may advance the debate on the association between stress and AA.

AIM

The primary objective was to determine whether stress acts as a trigger for (i.e. pre-dates) the onset of AA via objective measurement of HCC.

Two secondary objectives were identified:

1. Using validated stress questionnaires to assess whether current stress correlates with HCC.
2. To determine whether there is a difference in HCC of lesional versus peri-lesional scalp in cases.

METHODS

A case control study was performed. Thirteen patients, fulfilling the inclusion criteria were recruited from the Groote Schuur Hopital (GSH) and Red Cross Hospital (RXH) outpatient departments. For each case consent was obtained, a data sheet was filled out, stress questionnaire(s) and two strands of hair, one lesional and one peri-lesional, were collected. Next, 13 healthy controls were recruited from whom a hair strand each was collected.
On the hair samples, the position of onset of hair loss (OOHL) was determined by measuring one centimetre per month after OOHL, from the proximal (scalp near) end. Then three sections of three centimetres each were cut, two distally (representing the six-month period before OOHL) and one proximally (representing the three months post OOHL). In six of these cases a fourth or “current” section was obtained. This represented the section on the scalp and thus reflected current stress

by measuring the most recent HCC. Next, the HCC's of these sections were measured using the Salivary ELISA Cortisol kit©. An additional 44 cases, not meeting the inclusion criteria, was recruited for acquisition of additional stress questionnaires and data sheets.

RESULTS

In all sections (except peri-lesional scalp), on average, the HCC's of cases were higher than those of controls. Cases' HHC's were also more erratic as compared to the relative consistent values of the controls. In analysing the trends of the average HCC within samples over time, the distal sections proved to be the highest. This represents cortisol levels pre-OOHL. This is contrary to what is suggested by the washout effect (1) and could be expected from a spike triggering OOHL.

When looking at the difference between the HCC of lesional versus peri-lesional sections, all lesional HCC readings were higher than that of the perilesional sections, except the mid-section. The trend of HCC over time with in a sample also seemed to differ. Lastly, the questionnaires showed the factors that significantly and positively correlated with stress were smoking and age.

LIMITATIONS

The number of cases recruited was small and likely the reason for non-significant differences in all the above HCC comparisons. The extreme HCC values could have influenced average values, despite the methods that was followed to assure correct measurements.

CONCLUSIONS

Higher and more erratic HCC's of cases relative to controls could suggest that cortisol plays a role in AA. The higher HCC's in distal sections (pre-OOHL) could reflect the true HCC pre-OOHL (at least in some cases). This is because the exact OOHL is difficult to determine and probably earlier.

Higher HCC in lesional versus peri-lesional sections and also different trends within these samples suggests that local cortisol produced in the hair follicle could play a role in the presence or absence of disease in different areas of the scalp. Lastly, the results of the questionnaires could indicate that smoking is likely a

failed coping mechanism as a result of stress and older patients probably have more insight into the disease, better coping mechanisms and other, more significant, stressors.

The data does suggest elevated HCC both pre OOHL and to a lesser extent versus normal scalp. This then lays the foundation for a larger study to significantly validate these results.

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ABBREVIATIONS

AA	Alopecia areata
PUVA	Psoralen and Ultraviolet A
MHC	Major Histocompatibility Complex
NKG2D+	Natural Killer Cell Activating Receptor Group 2D
GSH	Groote Schuur Hospital
RXH	Red Cross Children's' Hospital
OOHL	Onset of hair loss
CRH	Corticotropin-releasing hormone
CRF	Corticotropin-releasing factor
MSH	Melanocyte-stimulating hormone
UCT	University of Cape Town
HPA	Hypothalamic-pituitary-adrenal axis
HCC	Hair cortisol concentration
ELISA	Enzyme-Linked Immunosorbent Assay
K10	Kessler Psychological Distress Scale
SRQ-20	Self-Reporting Questionnaire 20-Item
LCMS	Liquid Chromatography Mass Spectrometry

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Alopecia areata (AA) is a form of lymphocyte mediated non-cicatritial alopecia. It is an autoimmune disease that targets the hair follicle where both genetic and environmental factors play a role in its susceptibility and severity.

More than three quarters of patients affected with AA believe it is caused by stress (2). Quality of life is adversely influenced by the AA as well as the social aspect of the affected individual's life (3). Although there are validated questionnaire based tools for the diagnosis of stress, their use in patients with alopecia is complicated by the fact that hair loss itself also causes significant anxiety and depression (4). Further, the assessment of past stress is subject to significant recall bias (5).

Most questionnaires are only validated for stress experienced at present or the recent past (mostly within 6 months). It can therefore not measure stress for a specific time in the past. Thus, questionnaires are more useful for measuring current or ongoing stress and inevitably the results will include causes of current stress such as that caused by AA. So, the only useful questionnaire-based tool in retrospective assessment of stress would therefore be based on recalling verifiable stressful historic events occurring prior to the time frame of interest. This, however, does not allow for assessment of the resultant *perceived stress* on the individual. This makes the interpretation of results difficult - did the hair loss cause stress or was it the other way around (a chicken-and-egg situation).

If a study had to be designed using only questionnaires, the only useful way would be to assess a population and prospectively look for disease. A more reliable measure of chronic stress is therefore needed in the form of objective biological marker(s) which would aid in evaluating the role of stress in the pathogenesis of disease. Recently, the validity of measuring cortisol in hair as a bio-marker of chronic stress has been documented (6-12).

2. Alopecia areata

2.1. History

The first description of AA was by Celsus, Aulus (Aurelius) Cornelius, a Roman physician and medical writer (ca. 30 B.C.-45 A.D.). He described discrete and circular patches that progressed to complete hair loss. The first written use of the actual term, however, was by Sauvages in his "Nosologica Medica" in 1760. The

word “alopecia” is derived originally from Greek *alōpekia*, literally ‘fox mange’ and “areata” from the Latin meaning “area or patch”.

2.2. Epidemiology

Alopecia areata is the most prevalent autoimmune disorder with a lifetime incidence of approximately 2% worldwide (13) and represents 2-3% of skin disorders (14). It occurs in both men and women and in all age groups (15), but in occurs mostly in 30-59 year age group (16). Females are more commonly affected in a 2.3:1 ratio (17). Epidemiological data in Africa is not available, but it is commonly seen at our facility and in a study of 113 children with hair loss in South-East Nigeria, 43 (31%) has been found to have AA (18).

2.3. Clinical

Alopecia areata mostly manifests as round to oval alopecic patches on the scalp (hence the layman’s term “spot baldness”) with the affected skin otherwise clinically normal. It can, however, affect any hair bearing area on the body, the scalp being predominant in 90% of cases (19). When the whole scalp is involved, it is known as alopecia totalis, the whole body is involved, alopecia universalis and when the scalp margin is involved it is termed ophiasis. The natural history of this disorder is characterized by spontaneous remission, reoccurrence and exacerbation. Disease activity can be measured via the hair pull test (20) and the presence of regular round yellow dots on trichoscopy (dermoscopic imaging of the scalp and hair) can also indicate active disease (21). Clinical response can be measured via the severity of AA tool (22) (see figure 1). Cutaneous manifestations of AA other than the hair involvement include nail changes. The most common nail finding is fine pitting and other changes include ridging, nail plate thinning, twenty nail dystrophy, distal onycholysis, striate leukonychia and coarse pitting (23). However, nail changes are only seen in 10% of cases (21).

2.4. Differential diagnoses

In the case of the classic localised disease dermatoses mimicking AA may include resolving tinea capitis, trichotillomania, temporal triangular alopecia, traction alopecia, loose anagen syndrome, pressure-related alopecia, healed aplasia cutis and “burnt-out” cicatricial alopecia as well as systemic diseases like secondary syphilis and lupus erythematosus (21).

When dealing with the diffuse variant initial presentation may be misdiagnosed as telogen effluvium and androgenetic alopecia.

2.5. Investigations

In most instances, the history and clinical examination (with trichoscopy) is sufficient to confirm AA amongst these differentials.
In less classic presentations, a scalp biopsy may prove useful.

Trichoscopy
The features of AA on trichoscopy include the presence of black dots, tapering hairs, broken hairs, yellow dots, and clustered short vellus hairs (24).

Histology
Pathological features in AA depend on the duration of the disease.In the early phase, the dermatopathologist will find a normal *total* number of hairs. The inflammatory component is that of a peribulbar mononuclear cell infiltrate often referred to as a “swarm of bees” (with occasional eosinophils). The inflammation predominantly is found affecting terminal anagen and catagen hair bulbs, but occasional exocytosis into bulbar epithelium is seen. Other findings include degenerative changes of hair matrix, more terminal catagen and telogen hairs as well as miniaturized hairs, trichomalacia and narrowing of hair shafts.

In longstanding and stable disease, most hairs are found to be in catagen or telogen phases. Multiple miniaturized, “arrested”, rapidly cycling hairs are seen, known as nanogen hairs with mild peribulbar mononuclear cell infiltrate of anagen-like or catagen-like bulbs.

Additional investigations may include special stains on histology and where indicated fungal cultures and serology for lupus erythematosus or syphilis. The increased association of autoimmune disease in patients with AA is not an indication for routine screening and should be indicated by clinical examination. Although one small case series found that iron deficiency is more common in females with AA (25), this was not confirmed in two subsequent studies (26, 27) and thus routine testing is not recommended.

2.6. Treatment and prognosis

Because of the facts that AA runs an unpredictable course and can improve spontaneously, treatment guidelines have been formulated to facilitate treatment trials. The latest guidelines are from the British Association of Dermatologists' of 2012 (21). These state that AA does not pose any ill effects to general health and the level of aggression of treatment must be weighed up against the resultant psychological impact the disease has on the individual.

For limited patchy hair loss, the current suggested treatment is super-potent topical steroids or intralesional corticosteroids (strength of recommendation C). For extensive patchy hair loss, alopecia totalis or universalis suggested treatment is contact immunotherapy (strength of recommendation C) and/or camouflage a wig or hairpiece (strength of recommendation D) (21).

Continuous or pulsed systemic corticosteroids and PUVA currently are not recommended due to the potential for serious side-effects and inadequate evidence of efficacy. Dithranol (anthralin) and minoxidil lotion are safe, but there was previously little convincing evidence proving efficacy, however recently higher concentrations (0.5% versus 3%) of dithranol have been shown to be effective even in severe cases (28).

The prognosis of AA is variable. Good prognosis is usually seen in cases of rapidly progressive AA regardless of the treatment as well as in those with persistent vellus hairs regardless of disease severity (29). Earlier onset and longer duration of disease were significantly associated with higher relapse and lower cure rates (29).

2.7. Pathogenesis

2.7.1. Autoimmunity and genetic predisposition

Alopecia areata has been shown to be an autoimmune disorder (30). It is associated with various other autoimmune disorders including vitiligo, lupus erythematosus, psoriasis, atopic dermatitis, autoimmune thyroid diseases, and allergic rhinitis (31). The exact aetiology and pathogenesis of AA are currently unknown, but genetic (32) and environmental factors have been implicated (33, 34). The hair follicle enjoys relative immune privilege via mechanisms including the down regulation of MHC classes I and II, less Langerhans cells and expression of immunosuppressive cytokines. In genetically predisposed

individuals, physical/emotional stress, infections, and hormones likely play a role in breakdown of this privilege (35-38). Vitamin D deficiency can aggravate AA severity (39, 40). Further, AA is characterized by a Th1-mediated immune response where CD8 cells infiltrate the follicle while CD4 cells are found in the perifollicular region (41-44). In a recent development a fully humanized animal model was achieved by transferring normal human NKG2D+ peripheral mononuclear cells onto human scalp transplanted onto severe-combined immunodeficient mice (30). The exact function of NKG2D+ peripheral mononuclear cells is an area earmarked for future research (34).

2.7.2. Psychosocial stress

The literature on the relationship between mental health and AA is contradictory ranging from studies finding that 93% of AA cases having a serious mental disorder (45) to studies reporting that psychological factors do not play any role in triggering AA (46).

2.7.2.1 Subjective

Environmental factors seem to play a role in triggering onset of AA and/or exacerbating the disorder. Cases have been found to have a higher number of stressful life events prior to onset of hair loss (47). Cases of stress-reactive AA have also been found to potentially suffer from depressive disorder (48).

2.7.2.2 Objective

Skin biopsies of alopetic skin shows increased staining for type 2 beta Corticotropin-releasing hormone (CRH) receptors around hair follicles which could have proinflammatory effects (49). In an experiment to prove that elevated Corticotropin-releasing factor (CRF) leads to cushing syndrome in mice, the CRF gene was expressed in transgenic mice showed bilateral symmetric hair loss (50). Similar models used by Wang also demonstrated diffuse hair loss and histology showed follicular atrophy and increased telogen hair (51).

Plasma levels of Melanocyte-stimulating hormone (MSH) and cortisol were found not to be statistically different in AA cases as a potential measure of abnormal HPA functioning although there was a trend toward lower MSH and an increased cortisol (52). This once again is a short-lived value and does not necessarily correlate with past levels (i.e. preceding the onset of hair loss).

3. Hair cortisol as a biomarker of psychosocial stress

Until recently, substrates for cortisol measurement have been serum, urine, and saliva. These only measure acute changes (past few hours to days) and thus do not reflect the stress response over prolonged time periods or a specific time period in the past. In addition, due to the diurnal variation in cortisol secretion samples either has to be collected at specific times of the day (saliva and serum), or require a laborious collection method (24 hour urine collection), making them unsuitable for population analysis (53).

Apart from the ultradian and circadian rhythms that influence current cortisol levels it is also affected by nicotine (54), alcohol (55), food (56), exercise (57), injuries or hypoxemia (58) and acute psychosocial stress (59, 60).

In the past few years, a new and uniquely different method to determine chronic cortisol exposure in man has been developed namely the measurement of cortisol in mammalian hair, with first evidence thereof in 2004 (61). Hair is a stable substrate that can be used to test for various exposures (drugs, toxins, nutrients, etc.) months-to-years before, depending on the length. The examination of cortisol in a specific hair segment is believed to provide a retrospective index of cumulative cortisol secretion over the time period during which the hair segment has grown (62). Recent studies have demonstrated that hair cortisol can reliably be measured. The validity of hair cortisol as a retrospective index of long-term cortisol secretion has now been supported by research using a range of different paradigms, both in animals (63, 64) as well as in human participants (7, 65-70). In addition, evidence has been reported confirming marker qualities of hair cortisol levels with regard to chronic stress and anxiety-related measures (6-12). Evidence for whether stressful life events raise cortisol levels have been conflicting (71, 72).

The reference range for cortisol levels in hair of healthy non-obese individuals have been found to be 17.7-53.2 pg/mg of hair with a median of 46.1 pg/mg (65).

Lipophilic compounds in the blood circulation (including cortisol), can primarily be incorporated into the hair following diffusion from capillaries supplying the hair into the growing hair follicle. When the hair then emerges from the scalp, the compound stays trapped within the inner hair shaft (73-75). Sebum and sweat secretions, together with external contamination post formation of the hair strand may represent other mechanisms of incorporation (73, 74).

In the only meta-analysis up to date on hair cortisol concentrations, Stalder et al. showed a hair cortisol concentration (HCC) 21% higher in male versus female subjects. They also found HCC to increase with age and a decrease with time. When the proximal three centimetres section of hair was compared with the next three-centimetre section, a decrease of 29% was demonstrated. The deduction here was that different segments of hair in a particular case cannot serve as an internal control and that separate controls should be tested matching the same hair section of interest. Interestingly that although bordering on statistical significance neither hair washing frequency, hair treatments nor oral contraceptive use affected HCC's and it is suggested that in most circumstances their influence is negligible. With regard to the physical laboratory testing enzyme-linked immunosorbent assay (ELISA) protocols tested a higher HCC than liquid chromatography-based analyses. Inter-laboratory differences have been reported for various reasons, however good inter-laboratory correlations have been reported with the ELISA methods (1).

With regards to sociodemographic variables and potential confounders, evidence is conflicting suggesting that neither age, gender, hair colour, hair products nor oral contraceptive significantly alter HCC. However, there is weak evidence that smoking may cause elevation in HCC and that HCC may decrease over time (washed out) (76) and with the dying or bleaching of hair (70).

In recent study it has also been shown that elevated finger nail cortisol levels correlates with stressful events experienced (72) and so this could then prove to be another way to objectively measure past cumulative stress.

4. Conclusions and research questions

The exact pathogenesis of AA is poorly understood and multifactorial. There seems to be a strong belief and conflicting evidence that stress causes or exacerbates AA. Hair cortisol measurement has been proven to be a reliable biological marker for cumulative prior stress. This measurement has up to date not been performed in AA cases and may provide convincing evidence in the debate of stress and AA.

Because hair on average grows one centimetre per month (77), it is possible to estimate based on hair-length how long ago specific cortisol levels were present, thus objectively determining whether the stress predated the alopecia.

The primary aim of this project is therefore, to analyse the relationship between AA and stress (as measured HCCs); in cases versus controls and in cases pre-dating onset of hair loss.

Three secondary objectives were identified:

- To determine whether there is a difference in HCC of lesional skin versus peri-lesional skin in cases.
- To determine whether there is a correlation between disease activity (as determined by the hair pull test) and HCC.
- To determine whether validated stress questionnaires correlate with HCC.

5. Figures

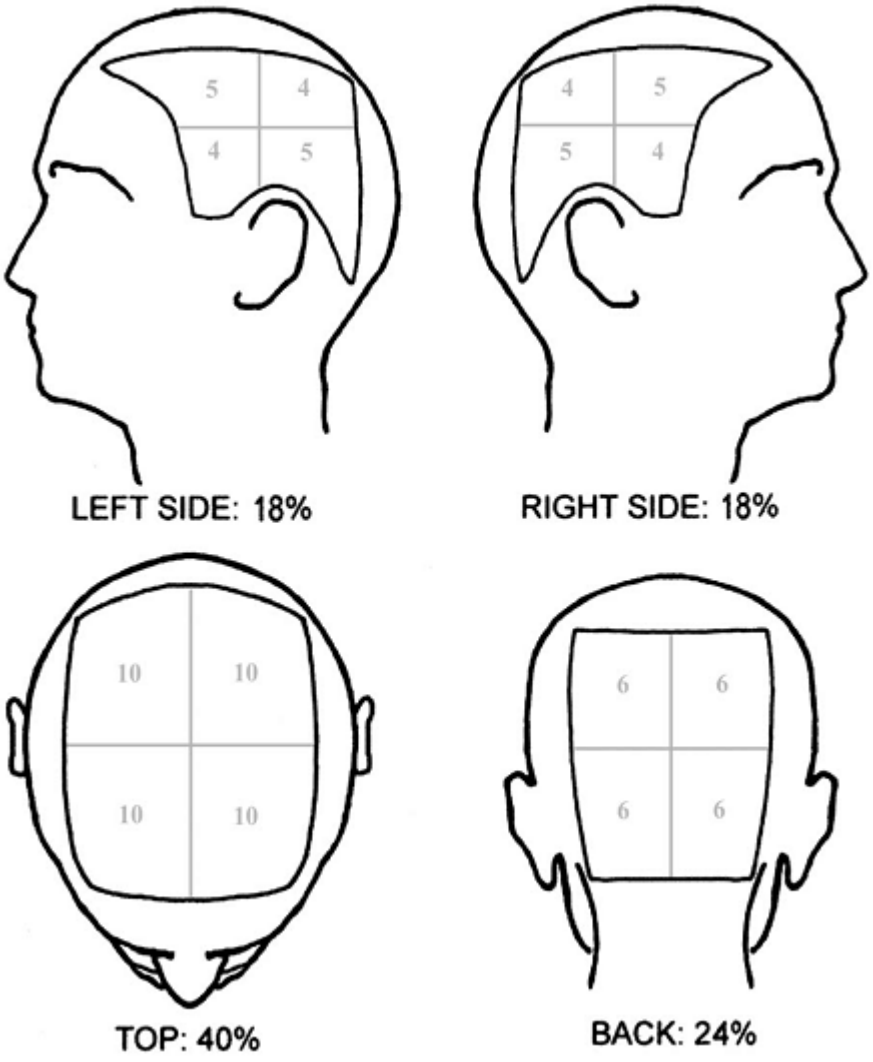

Figure 1: Severity of AA tool.

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CHAPTER 2

PUBLICATION-READY MANUSCRIPT FOR SUBMISSION TO THE BRITISH JOURNAL OF DERMATOLOGY

THE ROLE OF STRESS IN THE PATHOGENESIS OF ALOPECIA AREATA: AN OBJECTIVE ASSESSMENT VIA HAIR CORTISOL LEVEL

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Statements (70 words):

• What is known about topic?

The pathogenesis of alopcia areata is multifactorial and poorly understood. It is widely believed that stress is associated with onset of disease, but this remains to be proven.

• What does this study add?

Objective measurement of stress via hair cortisol concentration was assessed for the first time in alopecia areata cases to determine if stress could trigger onset of hair loss.

ABSTRACT

BACKGROUND

The pathogenesis of alopecia areata (AA) is poorly understood and multifactorial. There seems to be a strong belief, but conflicting evidence that stress causes or exacerbates AA. Hair cortisol measurement has been proven to be a reliable biological marker for cumulative prior stress. This measurement has up to date not been used in AA cases and may provide convincing evidence in the debate of stress and AA.

OBJECTIVES

The primary aim of this project was to determine whether stress triggers onset of hair loss (OOHL) in AA by analysing the relationship between hair cortisol concentrations (HCCs) pre-OOHL in cases vs controls.

Three secondary objectives were identified:

- To determine whether there is a difference in HCC of lesional skin versus peri-lesional skin in cases.
- To determine whether there is a correlation between disease activity (as determined by the hair pull test) and HCC.
- To determine whether validated stress questionnaires correlate with HCC.

METHODS

A case control study was performed. Fourteen patients, fulfilling the inclusion criteria were recruited from the GSH and RXH outpatient departments. For each case consent was obtained, a data sheet was filled out, stress questionnaire(s) and two strands of hair, one lesional and one peri-lesional, were collected. Next, 14 healthy controls were recruited from whom a hair strand each was collected.

On the hair samples, the position of onset of hair loss (OOHL) was determined by measuring one centimetre per month after OOHL, from the proximal (scalp near) end. Then three sections of three centimetres each were cut, two distally (representing the six-month period before OOHL) and one proximally (representing the three months post OOHL). In six of these cases a fourth or "current" section was obtained. This represented the section on the scalp and thus reflected current stress by measuring the most recent HCC. Next, the HCC's of these sections were measured using the Salivary ELISA Cortisol kit©. An additional 44 cases, not meeting the inclusion criteria, were recruited for acquisition of additional stress questionnaires and data sheets.

RESULTS

HCC's on average were higher in cases than in controls (before, during and after OOHL). The difference in HCC's, however, was not statistically significant. There was no statistical difference between HCC's in lesional and peri-lesional scalp samples. Distal section HCC's were the highest. HCC's correlated positively with disease activity, but was non-significant. There was no statistically significant relationship between HCC's and stress questionnaires.

CONCLUSIONS

Although the result was not statistically significant, likely due to small sample size, stress as measured by HCC may trigger OOHL in AA. HCC does not play a role in whether an area of the scalp is affected or not. Disease activity may be cause for stress. A larger study is warranted to validate these findings.

1.1 Introduction

Alopecia areata (AA) is a form of lymphocyte mediated non-cicatritial alopecia. It is an autoimmune disease that targets the hair follicle where both genetic and environmental factors play a role in its susceptibility and severity.

More than three quarters of patients affected with AA believe it is caused by stress (2). Although there are validated questionnaires for the diagnosis of stress, their use in AA is complicated by the fact that hair loss itself also causes significant anxiety and depression (4). Further, the assessment of past stress is subject to significant recall bias (5).

Recently, the validity of measuring cortisol in hair as a bio-marker of chronic stress has been documented (6-8). The primary objective of this study was to determine whether stress pre-dates (i.e. can act as a trigger for) the onset of AA via objective measurement of hair cortisol concentrations (HCC's).

1.2 Materials and methods (or Patients and methods)

1.2.1 Sample

Approval for this case control study was obtained from the University of Cape Town Human Research Ethics Committee (HREC/REF 174/2015). Fourteen patients, fulfilling the inclusion criteria were recruited from the Groote Schuur and Red Cross Hospitals' outpatient departments.

Inclusion criteria:

- Patient with AA on scalp.
- OOHL within the past 12 months.
- Remaining scalp hair at least three centimetres longer than the OOHL (one centimetre per month, reflecting three months after OOHL).

Exclusion criteria:

- On systemic steroid treatment.
- Cushing's syndrome/disease or clinically cushinoid.

On each case, the following was obtained

- Written consent
- Data sheet
- Stress questionnaires

- On cases, younger than 18; The Strengths and Difficulties questionnaire.
- On cases, older than 18; the Kessler Psychological Distress Scale (K10) and the Self-Reporting Questionnaire 20-Item (SRQ-20) questionnaires.

- Hair samples

  As can be seen in Figure 1, two strands of hair were shaven off the scalp. One at the periphery of the alopecia patch ("lesional") and the other on the vertex in a clinically unaffected part of the scalp ("peri-lesional"). Hair was pulled through a one-centimetre hole in a plastic sheet, bound with tin foil, and shaven off the scalp with hair clippers.

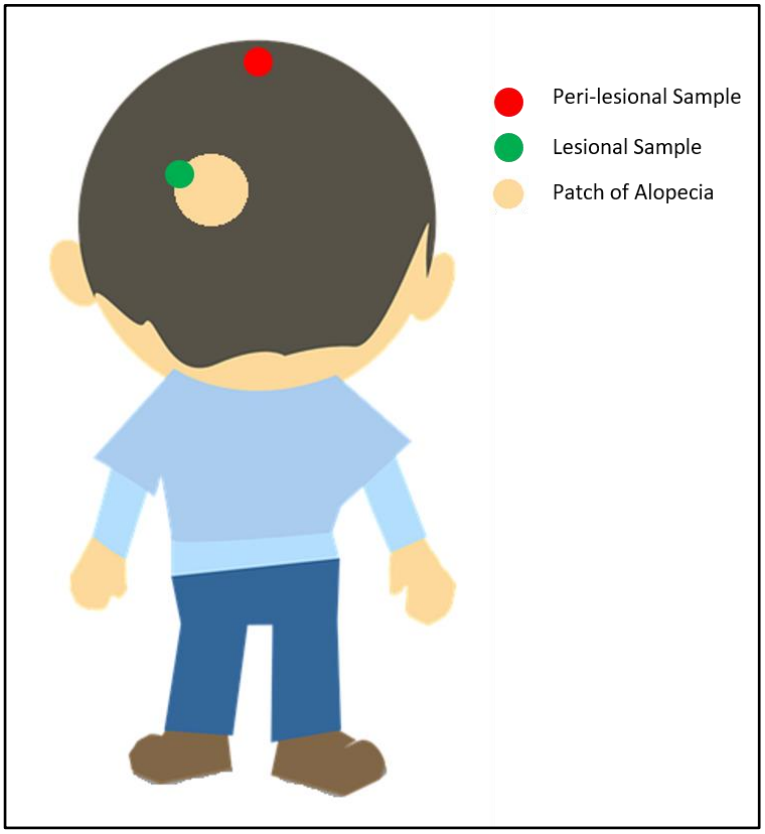

Figure 1: Locations of hair sample collection

An additional 44 cases, were recruited to obtain additional data from questionnaires. As can be seen in Figure 2, the final sample thus consisted of 14 cases HCC's and questionnaires and 44 cases with questionnaires only.

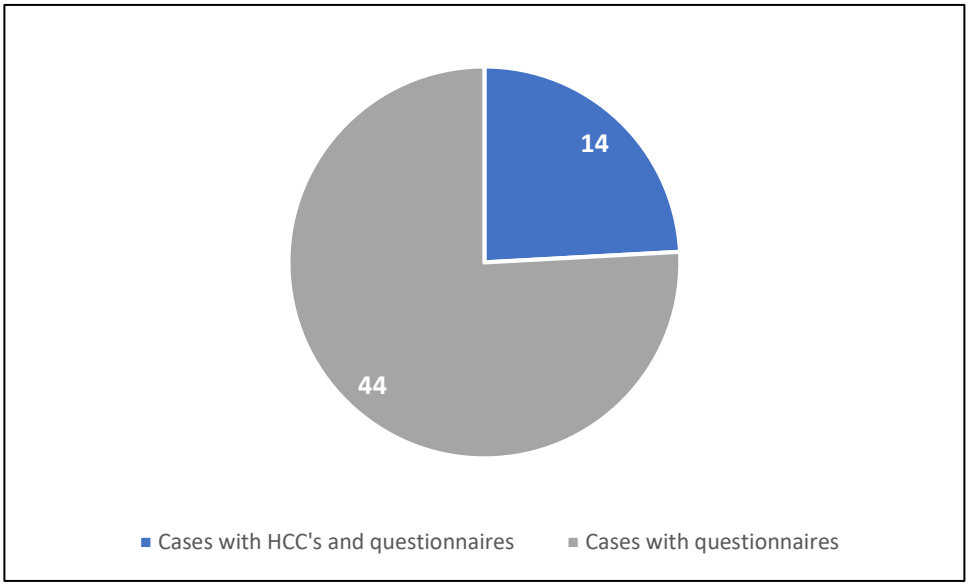

Figure 2: Composition of sample

Fourteen healthy controls were recruited from whom a hair sample each was collected from the vertex. They were controlled for age, gender and length of hair.

On the hair *samples*, the position of onset of hair loss (OOHL) was determined by measuring one centimetre per month after OOHL, from the proximal (scalp near) end (figure 3). Then three *sections* of three centimetres each were cut, two distally (representing the six-month period before OOHL) and one proximally (representing the three months post OOHL). In six of these cases a fourth or “current” section was obtained. This represented the section on the scalp.

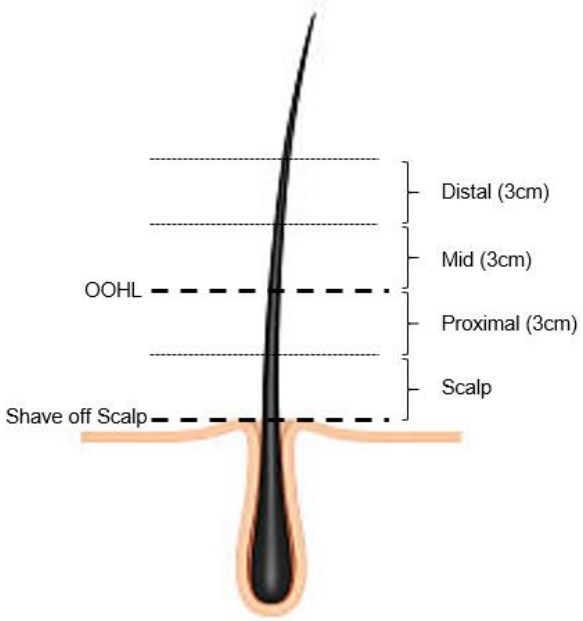

Figure 3: Cutting of hair samples into *sections*.

The hair samples were rinsed in warm water (37°C for 15 seconds) to remove any water soluble and solid particles. Then, each sample was subjected to three, consecutive 3-minute wash cycles with isopropanol. The hair was put in tissue paper and allowed to dry overnight. The dried hair was pulverized using an Omni Bead Ruptor 24 (Omni International). For the cortisol extraction, approximately 20mg of hair was incubated in a glass tube with 1,5ml of methanol for 24 hours with shaking. The tubes were centrifuged 5minutes at 13000rpm and the cortisol extract collected and filtered through a 0.22um filter.

1.2.2 Hair cortisol analysis

The cortisol in the extracted samples was measured using the Salivary ELISA Cortisol kit© according to the manufacturer's instructions with the reagents provided. The ELISA plates were read with a micro plate reader (Thermo Scientific Varioskan, USA) at 450 nm. A subset of the samples was validated using liquid chromatography mass spectrometry (LCMS).

1.2.3 Data analysis

The ***primary objective*** was to determine whether stress acts as a trigger for the onset of AA objectively via measurement of HCCs. In this regard, the difference

between lesional HCC's of cases versus controls were assessed in all sections pre- and post OOHL. To allow for possible inaccurate dates of OOHL, the highest positive difference in HCC of the mid and distal segments for each case was also analysed to evaluate if statistical significant differences were possible. In this regard, to test for differences, the two-sample t-test was used.

The ***first secondary objective*** was to determine whether there is a difference in HCC of lesional skin versus peri-lesional skin in cases. In this regard, once again, a two-sample t-test was employed.

The ***second secondary objective*** was to determine the relationship between disease activity (as determined by the hair pull test) and lesional HCC. To assess this relationship, Point-biserial correlation analysis was employed. Since the hair pull test is measured by means of a dichotomous variable (1 = positive pull test, 0 = negative pull test), and the HCC can be classified as ration data this method deemed appropriate.

The ***third secondary objective*** was to determine the relationship between stress scores (as obtained through validated stress questionnaires) and lesional HCC's (as determined by HCC in proximal sections). To assess this relationship, the Pearson correlation coefficient was employed. The Pearson correlation coefficient deemed appropriate given the nature of the underlying data (ratio data). Ratio data are numerical scales with order and exact differences between values. Ratio data also have an absolute zero.

Three validated questionnaires were used for the third secondary objective: the K10, SRQ-20 and Strengths and Difficulties questionnaires. The K10 questionnaire is used to measure psychological distress in adults. The numbers attached to the patients' 10 responses are added up and the total score is the score on the Kessler Psychological Distress Scale (K10). K10 scores range from 10 to 50. The SRQ-20 questionnaire was designed to screen or identify cases for psychiatric disturbance. Each of the 20 questions is scored 0 or 1. A score of 1 indicates the symptom was present and 0 indicates the symptom was absent. The maximum score is therefore 20. The Strengths and Difficulties questionnaire (SDQ) is considered a valid, rapid measure for emotional and behavioural problems in children. The 25 items in the SDQ comprise of 5 scales of 5 items each. The total difficulties score is generated by summing scores from all the scales except the prosocial scale. The resultant score ranges from 0 to 40.

1.3 Results

Demographics

As can be seen in Table 1, a total of 58 cases were included in the study of which 41 were female. 50 subjects were coloured, six black, one indian and one was white. The ages varied between four and 58 years old. The highest number of cases was in the third decade of life and the lowest the fourth and sixth, although the spread was relatively even with regards to the females. Males tended to be younger as were black patients.

Table 1: Demographics of sample

Age	Number	Gender			Race		
		F	M	C	B	I	W
≤10	10	6	4	6	3	0	1
11 - 20	11	6	5	10	1	0	0
21 -30	13	8	5	12	1	0	0
31 - 40	7	6	1	6	1	0	0
41 - 50	10	9	1	9	0	1	0
51 - 60	7	6	1	7	0	0	0
Total	58	41	17	50	6	1	1

With regards to HCC's, samples on 14 cases with controls were obtained. As can be seen in Table 2, 7 (6) had lesional (peri-lesional) scalp samples, 13 (12) had lesional (peri-lesional) proximal samples, 12 (12) had lesional (peri-lesional) mid samples and 13 (10) had lesional (peri-lesional) distal samples available. On case 9 only a proximal HCC's could be obtained since the other samples were too small to obtain a reading. Case 13 did not have a peri-lesional sample and with case 14 all the samples were too small to get a reading. Case 14 was omitted from any further HCC analysis.

Table 2: HCC Measurements (pg/mg hair)

Case	Lesional				Perilesional				Control	Scalp	Proximal	Mid	Distal
	Scalp	Proximal	Mid	Distal	Scalp	Proximal	Mid	Distal					
1		1.52	-	1.74		1.46	1.64	-	**1**		5.69	5.30	3.34
2	7.12	2.68	1.16	0.82	2.53	0.83	0.88	0.79	**2**	2.23	3.17	1.11	3.46
3		2.38	5.96	4.62		11.42	24.29	2.14	**3**		9.82	-	0.00
4	110.78	90.09	6.53	4.19	2.51	12.37	39.15	3.26	**4**	2.20	1.43	1.35	2.30

5		8.15	21.12	25.29		9.42	25.65	66.19	**5**		7.09	4.88	4.50
6		6.65	4.00	2.10		3.93	1.06	-	**6**		3.45	1.54	3.32
7		15.72	24.44	212.85		11.16	18.47	35.80	**7**		3.38	4.24	2.10
8		1.77	1.05	0.68		13.68	3.03	1.94	**8**		3.86	-	-
9		0.37	<LOQ	<LOQ		0.58	<LOQ	<LOQ	**9**		1.52	1.91	0.68
10	1.82	5.03	5.81	7.95	2.07	3.09	3.54	4.72	**10**	11.77	11.24	22.84	48.47
11	3.54	-	-	-	2.78	-	-	-	**11**	2.96	5.27	6.32	7.17
12	2.75	2.50	3.10	2.28	3.83	3.05	3.14	1.83	**12**	4.27	6.07	6.49	3.30
13	14.61	3.05	3.19	1.64	-	-	-	-	**13**	1.89	0.87	0.75	1.07
14.	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**14.**	2.75	1.89	3.49	4.38

For the ***primary objective***, to determine whether stress acts as a trigger for the onset of AA, the HCC's of cases relative to that of controls were assessed. In 5 instances (38.46%), the HCC of the cases were all higher than their matched controls and in 6 instances (46.15%) those of the controls were all higher. When combining the four sections (scalp, proximal, mid and distal), 19 (55.88%) of the case samples had higher HCC's than the controls.

As can be seen in Table 3, in all sections, on average, the HCC's of cases were higher than those of controls. However, none of these differences were statistically significant. The highest difference was found to be scalp observation of cases vs controls followed by that of distal, proximal and mid sections.

When specifically looking at the two sections corresponding to HCC prior to OOHL (mid and distal), the above non-significant finding holds true. In an attempt to accommodate the fact that OOHL is difficult to accurately determine and in some cases, may represent the mid- and in other the distal segments, we used the maximum positive difference in these two sections in all 13 cases. However, the deference was still not statistically significant.

Table 3: Primary objective, two-sample t-test results

	Lesional Case vs Control				
	Scalp	**Proximal**	**Mid**	**Distal**	**Greatest Difference (Mid and Distal)**
Average Difference (Case- Control)	19.22	6.86	3.27	19.16	22.66
Std	18.13	7.59	4.07	21.82	66.72
t-stat	1.06	0.90	0.80	0.88	1.07
p-value	0.34	0.39	0.45	0.40	0.31

Average trends, as per figure 4, within lesional samples were also analysed. In cases, the highest HCC was the distal section, followed by the scalp, proximal and mid sections. Control samples showed very little difference within the sections.

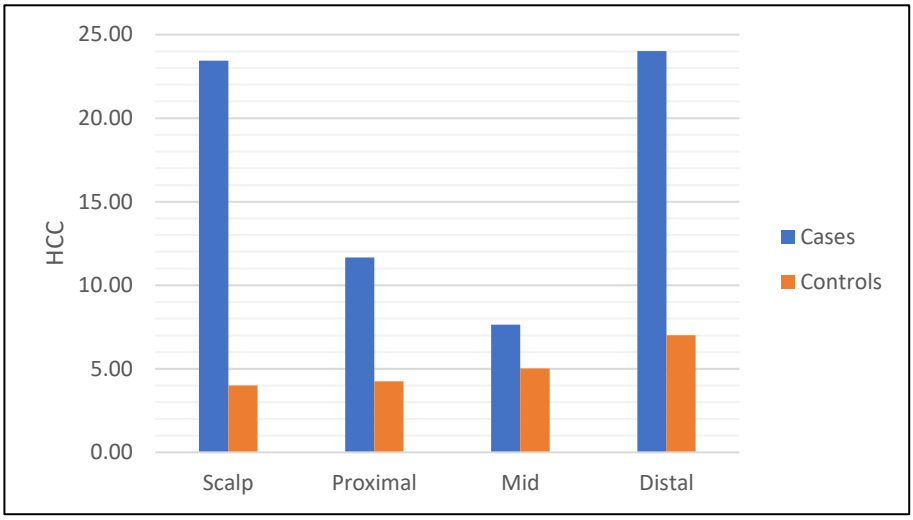

Figure 4: Average HCC trend analysis by section in hair samples.

For the ***first secondary objective***, the difference between the HCC of lesional skin versus peri-lesional skin in cases was assessed. As can be seen in Table 4 the average sections of lesional skin had higher HCC's than their peri-lesional counterparts for all but the mid-section. The results of the two-sample t-tests were, however, non-significant for all the sections.

Table 4: First secondary objective: two-sample t-test results

	Scalp	**Proximal**	**Mid**	**Distal**
Average Difference (Lesional – Peri-Lesional)	22.46	5.99	-5.11	17.75
STD	21.48	7.33	4.15	23.34
t-stat	1.05	0.82	-1.23	0.76
p-value	0.35	0.43	0.25	0.47

For the ***second secondary objective***, the relationship between disease activity (as determined by the hair pull test) and stress (as determined by HCC in proximal sections) was assessed.

Eleven cases were available to perform this test. Ten had lesional proximal HCC's, but for one case the scalp HCC was used. Six cases displayed a positive pull test and five did not. On average those cases with positive pull had higher HCC's than those without. However, one of the positive pull HCC observations (case 4) seemed to be an extreme outlier in the dataset.

Table 5 indicates the data and correlation between HCC and disease activity. The correlation coefficient was 0.35. However, this correlation coefficient was not statistically significant.

Table 5: Relation of disease activity and HCC

Case number	Pull Test	Lesional Proximal HCC
2	Neg	2.68
3	Neg	2.38
10	Neg	5.03
12	Neg	2.50
13	Neg	3.05
Average		**3.13**
4	Pos	90.09
5	Pos	8.15
6	Pos	6.65
7	Pos	15.72
9	Pos	0.37
11	Pos	3.54
Average		**20.76**
Difference		**17.63**
Point biserial correlation		**0.35**
t-stat		**1.14**
p-value		**0.28**

For the ***third secondary objective***, the relationship between stress scores (as determined by validated stress questionnaires) and HCC's (as determined by HCC in proximal sections) were assessed and scores were analysed with validated references.

The scores obtained in the three validated stress questionnaires by the 58 cases surveyed can been observed in Table 6.

Table 6: Questionnaire results

	Case #	K10	SRQ20	Strengths & Difs
Cases with HCC's and Questionnaires	**1**	35	#N/A	#N/A
	2	13	3	#N/A
	3	23	10	#N/A
	4	13	6	#N/A
	5	16	3	#N/A
	6	17	#N/A	#N/A
	7	14	2	#N/A
	8	16	3	#N/A
	9	20	7	#N/A
	10	13	4	#N/A
	11	24	5	#N/A
	12	36	11	#N/A
	13	#N/A	#N/A	13
	14	#N/A	#N/A	13
Cases with Questionnaires	**15**	20	5	#N/A
	16	21	8	#N/A
	17	23	#N/A	#N/A
	18	14	0	#N/A
	19	15	#N/A	#N/A
	20	32	11	#N/A
	21	13	3	#N/A
	22	23	6	#N/A
	23	23	9	#N/A
	24	17	3	#N/A
	25	17	#N/A	#N/A
	26	13	3	#N/A
	27	40	11	#N/A
	28	14	4	#N/A
	29	37	16	#N/A
	30	42	11	#N/A
	31	12	4	#N/A
	32	30	13	#N/A
	33	30	11	#N/A
	34	28	10	#N/A
	35	16	3	#N/A
	36	15	5	#N/A
	37	25	6	#N/A
	38	#N/A	13	#N/A
	39	#N/A	2	#N/A

	40	28	11	#N/A
	41	33	15	#N/A
	42	#N/A	#N/A	2
	43	#N/A	#N/A	6
	44	#N/A	#N/A	8
	45	#N/A	#N/A	15
	46	#N/A	#N/A	9
	47	#N/A	#N/A	10
	48	#N/A	#N/A	24
	49	#N/A	#N/A	4
	50	#N/A	#N/A	21
	51	#N/A	#N/A	13
	52	#N/A	#N/A	14
	53	#N/A	#N/A	21
	54	#N/A	#N/A	10
	55	#N/A	#N/A	18
	56	#N/A	#N/A	25
	57	#N/A	#N/A	14
	58	#N/A	#N/A	28

The ranges of scores, interpretation and results with regards to the sample of 37 cases who completed the K10 questionnaire can be seen in Table 7.

Table 7: K10 interpretation

Range	Patient likely to	# of participants	% of participants
< 20	be well	17	45.95%
20 – 24	experience mild stress	8	21.62%
25 -29	experience moderate stress	3	8.11%
30 +	experience severe stress	9	24.32%
		37	**100%**

Most cases fell in the category of being well and second most of the cases fell in the category of being severely stressed. On average, the K10 stress score was 22.19 which falls in the category of experiencing mild stress.

The ranges of scores, interpretation and results with relation to the sample of 34 cases who completed the SRQ-20 questionnaire can be seen in Table 8.

Table 8: SRQ-20 interpretation

Range	Threshold	# of participants	% of participants
< 8	below	20	58.82%
8 +	above	14	41.18%
		34	**100%**

Most cases fell below the threshold. The average SRQ-20 score was 6.97 which is slightly below the threshold.

The ranges of scores, interpretation and results with regards to the sample of 19 cases who completed the SDQ questionnaire can be seen in Table 9.

Table 9: SDQ interpretation

Range	Interpretation	# of participants	% of participants
0 - 16	Normal	13	68.42%
17 - 19	Borderline	1	5.26%
20 - 40	Abnormal	5	26.32%
		19	**100%**

Most cases fell in the category of being normal and second most of the cases fell in the category of being abnormal. On average, the SDQ score was 14.11 which falls in the normal category.

As can be seen in Table 10, the relation between HCC and the K10 were negative, but non-significant. The correlation between the SRQ20 score and HCC indicated very close to no relation at all.

Table 10: Pearson correlation analysis of stress questionnaires and HCC.

		Proximal
K10	**Coefficient**	-0.34
	t-stat	-1.13
	p-value	0.29
SRQ20	**Coefficient**	-0.02
	t-stat	-0.05
	p-value	0.96

1.4 Discussion

Demographics

The demographics are skewed by the fact that significantly more women than men were included in the study when compared to the actual incidence. This can likely be ascribed to the fact that women tend to wear longer hairstyles which were searched out to fulfil the primary objective. Another factor could be that it is likely more of a cosmetic concern for women and thus they probably tended to seek out medical attention more aggressively and was probably also more willing to participate in the study in an attempt to remedy their situation. The aim of the study was not incidence, though. The high number of coloured cases included in the sample is due to the demographic of the drainage area. The higher number seen in their third decade of life is in keeping with international incidence.

With regards to the ***primary objective*** the average HCC of the four sections of cases proved to be higher than those of controls, with no statistical difference. Together with the scalp section the highest difference was actually the distal section, corresponding to HCC pre OOHL. This could possibly signify stress triggering OOHL. The high difference in the scalp section could then reflect the resultant stress caused by the AA.

Interestingly, though, in terms of trends within each hair sample, the average distal HCC's were the highest of the four (figure 4). This could signify a HCC spike before OOHL, once again pointing to stress triggering OOHL. It has been shown in controls as well, however, to a much lesser degree. This is contrary to the decline that is found the more distal sections are from the scalp, the so called "wash-out effect" (75). Another interesting observation is that although neither the cases nor the control HCC's over time differed significantly, one can appreciate the fluctuation in HCC's of the cases versus the relative constant HCC's of controls (figure 4).

With regards to the ***first secondary objective*****,** there was no difference shown between the HCC of lesional vs non-lesional sections, meaning that local cortisol is probably not the reason why in an individual with AA certain areas of the scalp are affected and others not.

When looking at the correlation of disease activity and HCC's under the ***second secondary objective***, it proved to be positive as expected, but non-significant. This shows that stress could possibly trigger disease activity or active disease cause stress as measured.

In the ***third secondary objective***, the relation between HCC and validated questionnaires was negative. Although non-significant, this was unexpected and could be explained by an extreme outlier as well as the fact that there were few contact points.

Due to the small sample size and the pilot nature of the study, statistical significance is less relevant than showing a negative or positive correlation.

1.5 Limitations

Although AA is commonly seen in our facility, the number of cases recruited is small. This is because OOHL tended to be more than 12 months prior in most instances at the time of first assessment. The likely explanation is the fact that this is a referral institution where the load is quite significant leading to a delay with initial contact with the division. Many potential candidates also had non-shed hair that was too short to obtain segments predating the OOHL. OOHL is also difficult to precisely determine. This is due to the fact that patients often don't discover the problem themselves since unlike conditions like telogen effluvium the hair that is lost is often not so dramatic and the bald patch gets pointed out by someone else, i.e., their hairdresser. The condition is also asymptomatic.
With regards to the HCC's extreme values occurred which could have influenced average values, despite the methods that were followed to assure correct readings.

1.6 Conclusion and recommendations

The number of cases recruited was small and likely the reason for non-significant differences in all two-sample t-tests performed to do HCC comparisons. Stress could possibly trigger OOHL in AA as suggested by the following: 1) HCC's of cases were higher than controls, 2) HCC pre-OOHL was the highest section in the hair samples of cases and 3) the difference between the HCCs of cases versus controls were also highest pre-OOHL (together with the scalp near section, possibly signifying the expected stress caused by AA). Cases' HHC's were erratic as compared to the relative consistent values of the controls. This then lays the foundation for a larger study to validate these results.

1.7 References

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Appendices

1. Ethics Approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

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26 March 2015

HREC/REF: 174/2015

A/Prof N Khumalo
Dermatology
G23
NGSH

Dear A/Prof Khumalo

Project Title: THE ROLE OF STRESS IN THE PATHOGENESIS IN ALOPECIA AREATA (AA): AN OBJECTIVE ASSESSMENT VIA HAIR CORTISOL LEVEL (MMeD-candidate-Dr L Flick)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above mentioned study.

Approval is granted for one year until the 30 March 2016.

Please submit a progress form, using the standardised Annual Report Form, if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

We acknowledge that the following student:-Louis Flick is also involved in this project.

Please note that the on-going ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the HREC REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

HREC REF 174/2015

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

HREC REF 174/2015

2. BRITISH JOURNAL OF DERMATOLOGY: AUTHOR GUIDELINES

Original articles

Original articles are the Journal's primary mode of communication. Original articles must include a structured abstract (maximum 250 words), and should not exceed 3000 words of body text. Original articles must include bulleted statements (maximum 70 words per question) in answer to the following questions: What's already known about this topic? and What does this study add? Please refer to the specific category section for any bulleted statements that must be included.

The categories for original articles within the Journal are as follows:

- Clinical Trials
- Epidemiology
- Translational Research
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- Evidence-Based Dermatology (including Systematic Reviews)
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- Surgical Dermatology
- Paediatric Dermatology
- General Dermatology