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TrichoScan: combining epiluminescence microscopy with digital image analysis for the measurement of hair growth *in vivo*

Hair loss or hair thinning is a common complaint in clinical dermatology, and patients seeking advice for hair loss are not necessarily bald. Also the effects of treatment attempts are hard to measure. Consequently, there is a need for a sensitive tool to monitor hair loss and treatment response. Such a method must be able to analyze the biological parameters of hair growth, which are: 1: hair density (n/cm^2), 2: hair diameter (μm), 3: hair growth rate (mm/day) and 4: anagen/telogen ratio. Here we present the TrichoScan as a method which combines epiluminescence microscopy (ELM) with automatic digital image analysis for the measurement of human, and potentially animal hair, *in situ.* The TrichoScan is able to analyze all biological parameters of hair growth with a so-called intraclass correlation of approx. ninety-one percent with the same TrichoScan operator and an intraclass correlation of approx. ninety-seven percent for different TrichoScan operators. The application of the technique is demonstrated by comparison of the hair parameters in individuals without apparent hair loss with men with untreated AGA and men after treatment with finasteride (1 mg/day), where we were able to detect a significant increase in hair counts and cumulative hair thickness 3 and 6 months after treatment. The advantage of the TrichoScan is that it can be used for clinical studies to compare placebo versus treatment or to compare different capacities of different hair growth promoting substances, it can be used for studying AGA or other forms of diffuse hair loss, and it can be adopted to study the effect of drugs or laser treatment on hypertrichosis or hirsutism. *(Key words: hair, alopecia, computer analysis.)*

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Hair loss or hair thinning is a common complaint in clinical dermatology and patients seeking advice for hair loss are not necessarily bald. In established cases of androgenetic alopecia (AGA) characteristic patterns are easily discernible. However, especially in females the clinician is often challenged by patients with initial stages of AGA where hair loss is reported but alopecia is not recognizable or the effect of treatment attempts are hard to measure. Consequently, there is a need for a sensitive tool to monitor hair loss and treatment response.

Numerous methods have been reported [1] to assess the rate of hair growth. The techniques can be classified as either invasive (*e.g.* biopsies [2, 3]), semi-invasive (trichogram [4, 5], unit area trichogram [6]) or non-invasive (*e.g.* global hair counts [7], phototrichogram [8-13]) methods. Quantitative methods for the analysis of human hair growth and hair loss are necessary to determine the efficacy of hair promoting drugs, and while reviewing the capabilities of the different methods, the common theme emerges that most techniques are of little use to the clinician because they are time consuming, often costly or difficult to perform [14, 15]. Therefore, an operator- and patient-friendly, inexpensive, validated and reliable method is a rational need.

Such a method must be able to analyze the biological parameters of hair growth, which are: 1: hair density (n/cm^2), 2: hair diameter (μm), 3: hair growth rate (mm/day) and 4: anagen/telogen ratio. This paper describes the TrichoScan as such a method which combines standard epiluminescence microscopy (ELM) with automatic digital image analysis for the measurement of human, and potentially animal hair, *in situ.* The application of the technique is demonstrated by comparison of the aforementioned hair parameters of individuals without apparent hair loss with men with untreated AGA and men after treatment with finasteride (1 mg/day).

Materials and methods

Volunteers and patients

A total of 56 persons (25 females, age range 25-48 years, mean 34 years; 31 males, age range 26-39 years, mean 32 years) underwent the study. Ten out of fifty-six volunteers (5 females, age range 25-48 years, mean 34 years; 5 males, age range 26-39 years, mean 32 years) were recruited for the initial experiments to analyse the reproducibility of the method. The measured parameters were hair thickness and hair numbers at the occiput.

Seventeen male patients (age range 25-48 years, mean 34 years), who had recognized progressive thinning of hair and hair loss for more than four years were included to

analyse the progression of AGA with and without treatment. All patients presented the clinical finding of mild to moderate AGA with various degrees of involvement, that were classified according to the Hamilton scale [16] (II-V). Subjects with other forms of alopecia were excluded from the study. Twelve of 17 male patients with AGA were treated with Propecia® (1 mg finasteride/day) for six months. All patients actively treated had had no treatment whatsoever for hair loss at least one month before initiating this study. Eleven of fifty-six healthy male volunteers (age range 28-55 years, mean 36 years) who had experienced no episodes of hair thinning or hair loss, recent illnesses or general health disturbances, were recruited as a control group. Clinical examination revealed no evidence of any hair disorder with either the female or the male volunteers. The measured parameters were hair thickness and hair numbers at the vertex.
For the analysis of daily hair growth and the anagen/telogen ratio, 18 additional volunteers with AGA were recruited.

Clipping of hairs

In individuals affected by AGA, a transitional area of hair loss between normal hair and the balding area was defined and an area of 1.8 cm^2 was clipped (Hairliner, Wella Germany) *(Fig. 1A-D)*. In volunteers without AGA (controls) the vertex was chosen for clipping. All clipped areas were marked with a central, single black tattoo. The tattoo was visible throughout the study. In those 18 volunteers who were recruited for the analysis of the anagen/telogen ratio the scalp was clipped at two locations (vertex and occiput) and was analysed by 2 investigators with the TrichoScan software.

Epiluminescence microscopy (ELM) of clipped hairs

Gray or fair hairs have only limited contrast in comparison to the scalp. Therefore, the clipped hairs within the target area were dyed for 12 min *(Fig. 1E-H)* with a commercially available solution (RefectoCil®, Gschwentner, Vienna, Austria), which is normally used for the coloring of eye brows or lashes. The approach of dyeing the hairs for hair growth studies has been described as giving the same results as uncolored hairs [17]. For the analysis of hair number and thickness the hairs were colored immediately after shaving and for the analysis of the hair growth rate and anagen-telogen ratio the hairs were colored 3 days after shaving.
Thereafter, the colored area was cleaned *(Fig. 1I)* with an alcoholic solution (Kodan® Spray, Schülke & Mayr, Vienna, Austria) and digital images were obtained at 20-fold (analyzed area: 0.62 cm^2) and 40-fold (analyzed area: 0.225 cm^2) magnification by means of a digital ELM system (Fotofinder DERMA, Teachscreen Software, Bad Birnbach, Germany) while the area was still wet *(Fig. 1J)*. This digital camera is equipped with a rigid contact lens which ensures that the images are always taken at the same distance from the scalp. Due the fact that the camera must be pressed onto the scalp, the hairs are always flattened.
Images were taken at day zero immediately after clipping, two and three days after clipping, and three and six months after the initial visit, respectively. Two different investigators each took three images from the same patient at every visit.

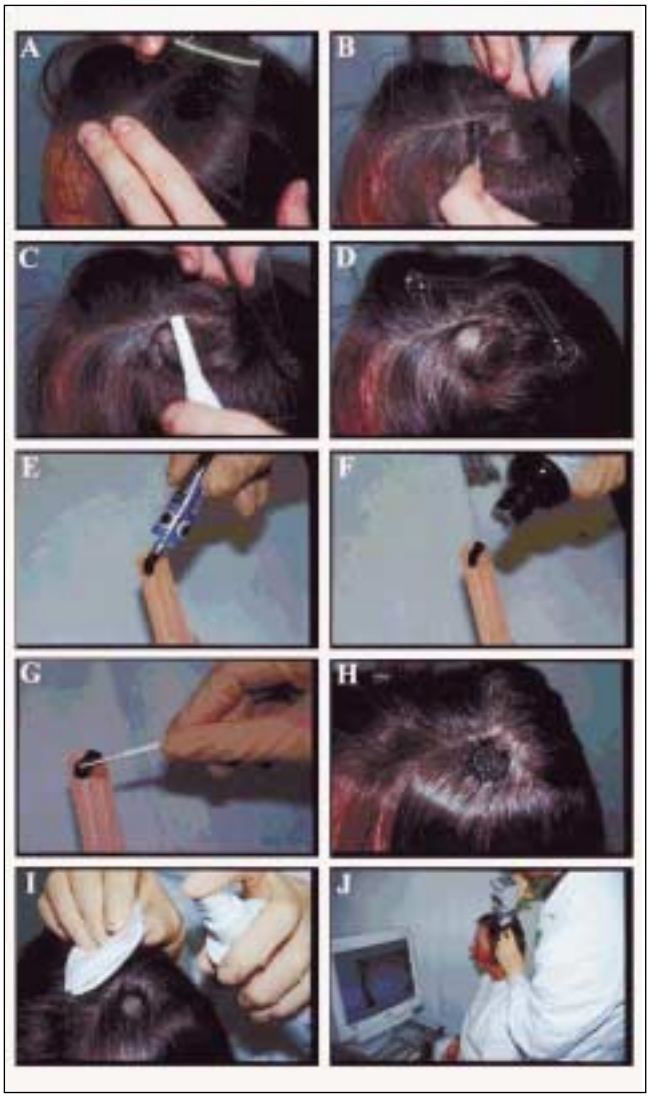


Figure 1. *This is a stepwise illustration of the complete TrichoScan procedure. A: a representative area of the scalp is chosen and the plastic template is applied; B: all hairs are carefully combed through the plastic template; C: the hairs are shaved on the scalp surface; D: the shaved area is 1.8 cm^2 in size; E: 1 cm of dye is applied onto a wooden stick; F: 3 drops of developer are mixed (G) with the dye; H: the dye is carefully applied onto the shaved area; I: after 12 min the dye is carefully removed with an alcoholic solution; J: digital images are taken at 20- and 40-fold magnification while the area is still wet.*

Software for digital image and statistical analysis

For the measurement of hair density (n/cm^2), hair diameter (μm), hair growth rate (mm/day) and anagen/telogen ratio, software was developed (TrichoScan) to analyze these parameters *(Fig. 2)*. The software works step by step through the following algorithms:
1. Selection of color component; 2. Artifact rejection (bubbles and reflections); 3. Determination of threshold; 4. Thresholding; 5. Labeling – Definition of hair regions; 6. Deselecting of small regions (smaller than minimal hair length); 7. Tattoo elimination (works by using the fact that the tattoo is a large, dark region located in the center of the

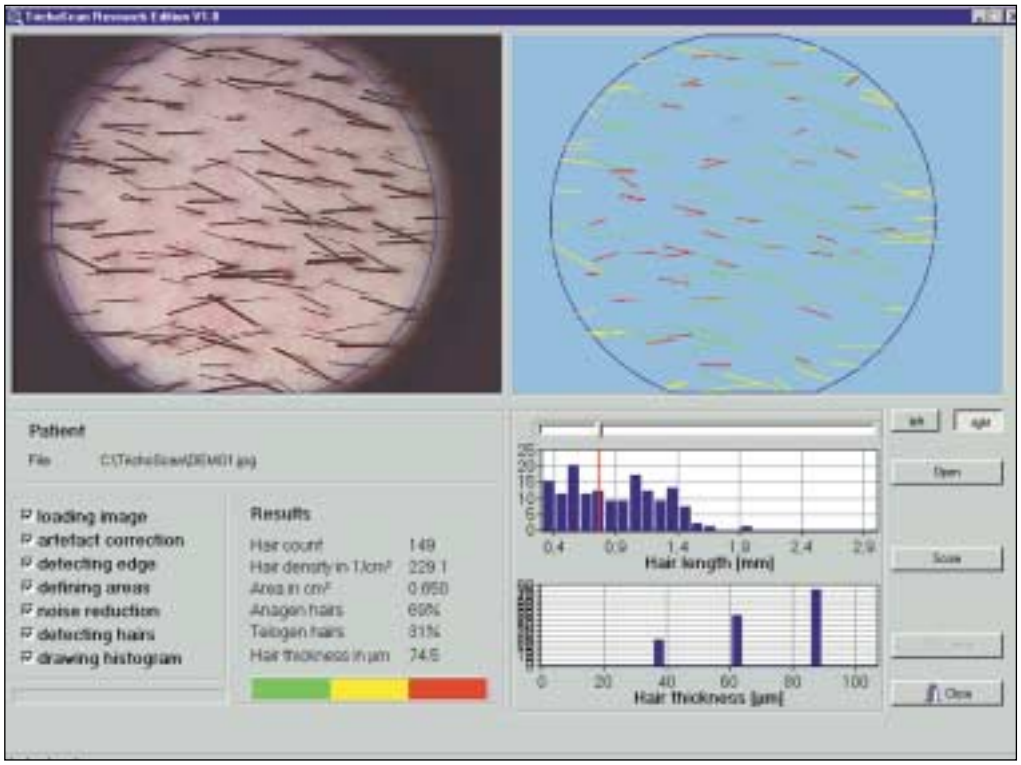


Figure 2. *Example of the TrichoScan analysis of hair number, hair density, cumulative hair thickness and anagen/telogen ratio. The figure illustrates a digital image taken at 20-fold magnification (left side of the image) and shows the area of 0.65 cm² (blue circle) which is analyzed with the TrichoScan software. The TrichoScan results are illustrated on the right side, where the detected hairs are illustrated with different colors. Red hairs are non-growing hairs (telogen), green hairs are growing hairs (anagen) and yellow hairs touch the borders of the circle. The right lower part of the figure shows a histogram of the different hair lengths detected by the TrichoScan software.*

image); 8. Analysis of each hair region: a. Search for the longest straight line (fulfilling several predefined conditions) at the edge of the analysed hair region, b. Reduction of hair region of detected hair; 9. Repetition of steps 8a and 8b until no more hair is found; 10. Repetition of analysis of all hair regions; 11. Calculation of number of hairs, hair density, and mean/median hair thickness/sum of hair thickness. The software was validated by use of more than 500 images, which were taken from the study participants.

Results

Total time "hands-on" for TrichoScan operator

The complete procedure was finished within 15-20 min. The total time "hands-on" for the TrichoScan operator was approx. eight to twelve min *(Fig. 1A-J* and *Fig. 2)*.

Effect of the hair dye

In preliminary experiments we tried to analyse fair or gray hair with the TrichoScan software. However, these hairs produced only little contrast and coloring the hairs resulted in a marked increase in hair detectability and did not interfere with the four basic parameters of hair growth. The dye must be applied for 11-13 min. More than 13 min will unintentionally dye the scalp skin. Less than 11 min results in incomplete staining of hairs.

Effect of the tattoo

In the experiments presented here we used a single black tattoo. During the analysis it became clear that the black ink interfered with the detectability of the stained hairs. Therefore, in future studies we will use red ink for the tattoo.

Precision and sensitivity

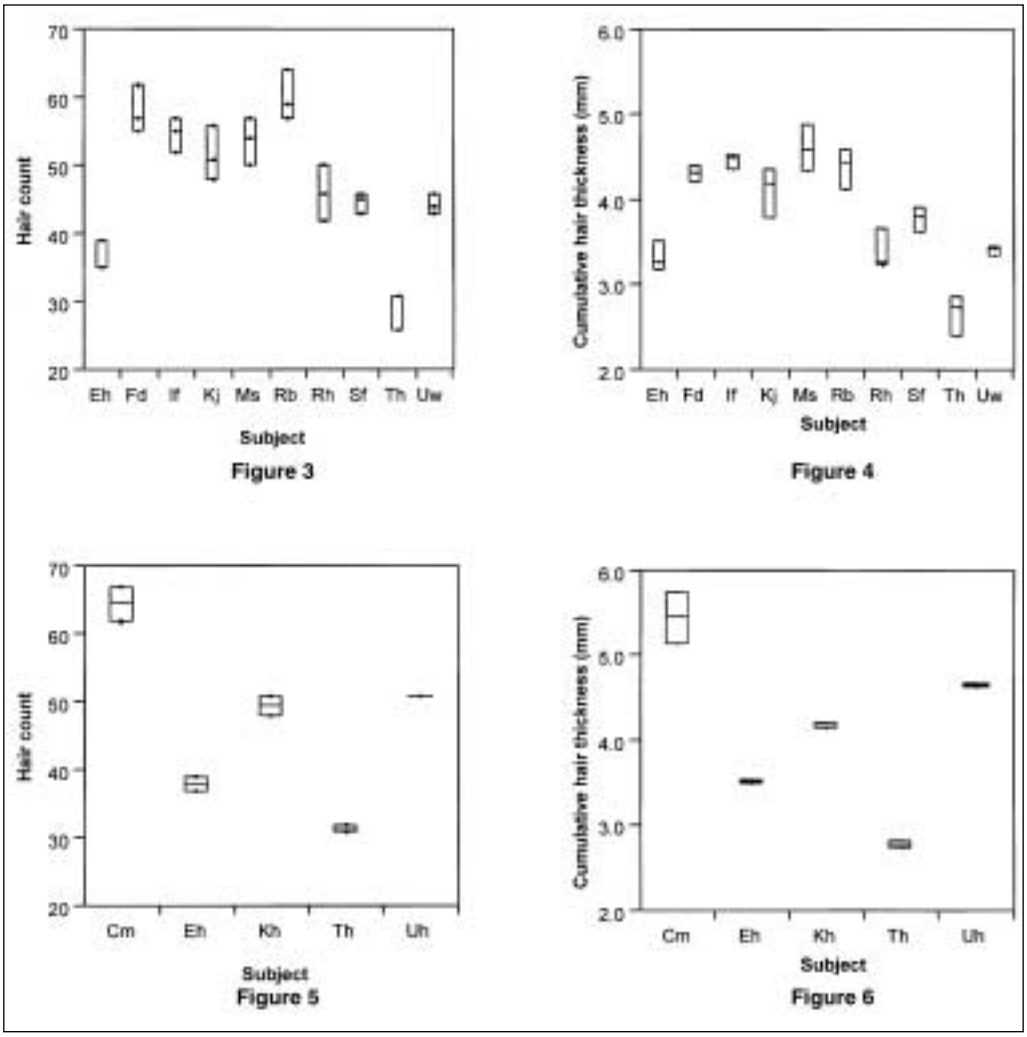
The algorithm excludes all air bubbles, dust, small haemangiomas, nevi, scales, etc., from the calculation without interfering with the number of detectable hairs. In doing so, only hairs are counted and the precision of the method is therefore approximately 100%. The detection limit of the software is 5 µm in thickness. Hairs smaller than 5 µm can therefore not be analysed.

Measurement of hair thickness and hair number

Analysis of intra-operator error
In 10 volunteers the hair number *(Fig. 3)* and cumulative hair thickness *(Fig. 4)* was analysed in the same area, three times by the same investigator. The percentage of variation in hair count between volunteers or so-called intraclass correlation, is estimated at 90.9%. The intraclass correlation for the cumulative hair thickness is 90.6%.

Analysis of inter-operator error
In 5 volunteers the hair number *(Fig. 5)* and cumulative hair thickness *(Fig. 6)* was analysed in the same area once, but by two independent investigators. The intraclass correlation is estimated for the hair count at 97.6%, for the cumulative hair thickness at 96.4%.

Analysis of total hair counts and cumulative hair thickness in volunteers without AGA, with untreated AGA, and AGA treated with finasteride
In individuals affected by AGA, a transitional area of hair loss between normal hair and the balding area was defined and area of 0.225 cm² was analysed at 40-fold magnification. Twelve men were treated with finasteride, whereas 5 men with AGA remained untreated.



Figures 3-6. *The intra-class correlation of three different measurements in 10 volunteers (subjects) from the same investigator is shown for hair counts* (Fig. 3) *and for cumulative hair thickness* (Fig. 4). *The intra-class correlation of one measurement in 5 volunteers (subjects) from two different investigators is shown for hair counts* (Fig. 5) *and for cumulative hair thickness* (Fig. 6).

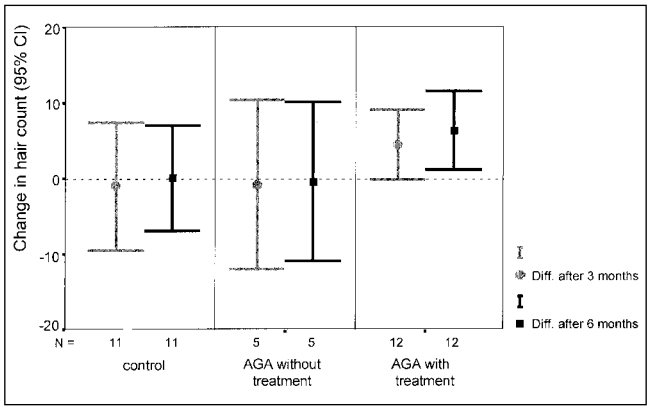
For both variables the differences between the results after 3 months (6 months) and the baseline were calculated. These differences were analysed using a one-sample t-test.

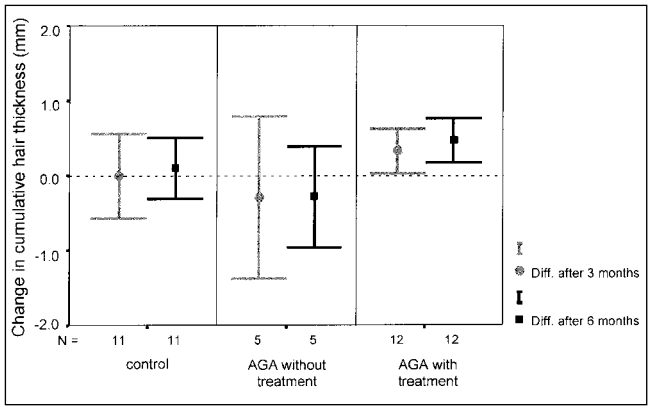
In controls and untreated men we noticed no significant difference in the number of hairs within the observation time of 6 months. By contrast, men treated with finasteride showed a continuous increase at 3 months ($p = 0.055$) and at 6 months ($p = 0.021$) in the number of hairs within the analysed area *(Fig. 7* and *Table I).*

In 11 volunteers without AGA (controls) we observed no significant difference in the cumulative hair thickness within the observation time of 6 months *(Fig. 8)*, whereas untreated men showed a continuous and significant decrease in the overall thickness of hairs 3 and 6 months after the initial visit *(Fig. 8* and *Table I).* By contrast, men treated with finasteride showed a continuous and significant increase in the number of hairs within the analysed area *(Table I)* after 3 ($p = 0.034$) and 6 months ($p = 0.006$) *(Fig. 8)*, compared to the values obtained at baseline.

Table I

Variable	Group	Time	DF	t	p
Hair count	Control	After 3 months	10	– 0.265	0.796
		After 6 months	10	0.029	0.977
	AGA untreated	After 3 months	4	– 0.199	0.852
		After 6 months	4	– 0.106	0.921
	AGA treated	After 3 months	11	2.149	0.055
		After 6 months	11	2.688	**0.021**
Cumulative hair thickness	Control	After 3 months	10	– 0.012	0.991
		After 6 months	10	0.542	0.601
	AGA untreated	After 3 months	4	– 0.739	0.501
		After 6 months	4	1.161	0.310
	AGA treated	After 3 months	11	2.414	**0.034**
		After 6 months	11	3.433	**0.006**





Figures 7 and 8. *Hair counts and cumulative hair thickness were analysed for 6 months in 11 volunteers without AGA, in 5 untreated men with AGA, and in 12 men treated with finasteride (1 mg/day). In controls and untreated men we noticed no significant difference in the number of hairs within the observation time of 6 months (between the values at baseline and after 6 months). In contrast those men treated with finasteride showed a continuous increase (mean with 95% confidence interval) at 3 months (p = 0.055) and at 6 months (p = 0.021) in the number of hairs within the analysed area compared to the values at baseline. Untreated men showed a continuous and significant decrease in the overall thickness of hairs 3 and 6 months after the initial visit (baseline). In contrast those men treated with finasteride showed, in comparison with the baseline visit, a continuous and significant increase in the number of hairs within the analysed area after 3 (p = 0.034) and 6 months (p = 0.006).*

Analysis of anagen/telogen ratio and hair growth rate at the vertex and the occiput in volunteers with AGA

The analysed variables were the portion of anagen hairs (growing hairs) and the hair growth rate (difference of the length of anagen hairs minus the length of telogen hairs divided by the time of measurement after clipping. The telogen hairs are defined as non-growing hairs. Telogen and catagen hairs cannot be differentiated).

For both variables an ANOVA was calculated with the fixed factors diagnosis (AGA/control) and investigator (investigator 1/investigator 2) and the random factor location nested under the diagnosis. The p-values were given for the two-sided problem. *Figure 9* shows the original values of the portion of anagen hairs for the different diagnosis and investigators with 95% confidence intervals of the means. The same is shown in *Figure 10* for the length difference between anagen and telogen hairs.

The model of the portion of anagen hairs explains 96% of the variance, the hair growth 85%. Both models are highly significant. *Table II* shows the model and the single effects. For both variables the diagnosis is highly significant. These results show that an AGA-affected scalp reveals a decreased number of anagen hair follicles *(Fig. 9)*, and these hair follicles grow more slowly *(Fig. 10)*, compared to the hair follicles at the occiput.

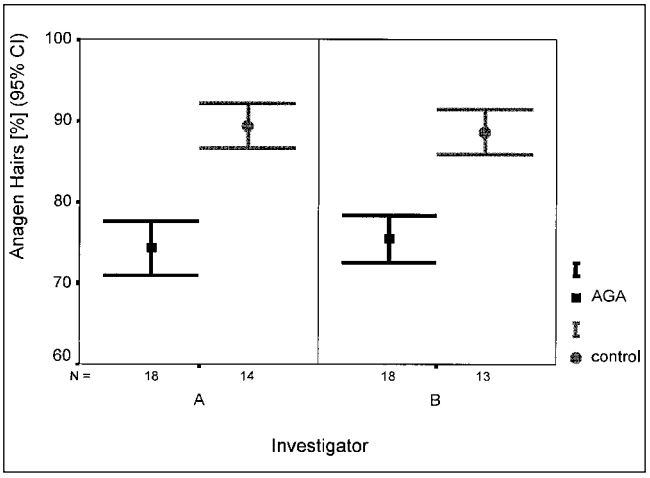
Table II

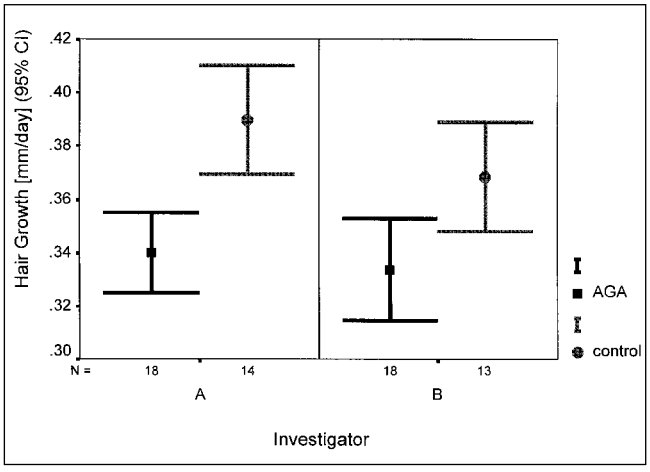
	Effect	DF	F Ratio	p
Anagen hairs	Model	34	17.90	< 0.0001
	Diagnosis	1	57.60	< 0.0001
	Investigator	1	0.15	0.702
	Image [Diagnosis]	31	7.18	< 0.0001
Hair growth	Model	34	4.95	< 0.0001
	Diagnosis	1	15.10	< 0.001
	Investigator	1	2.00	0.168
	Image [Diagnosis]	31	3.40	< 0.001

Discussion

Numerous hair diseases such as scarring alopecias, alopecia areata or trichotillomania, usually do not need a quantitative method to evaluate the amount of hair shedding. Androgenetic Affluvium, however, the most common form of hair loss, is typically difficult to quantify and at present simple but reliable procedures have not been developed. Although scalp biopsies can be justified in that microscopic examination of scalp skin affected by AGA can identify and quantify any changes resulting from treatment, this invasive technique is often not suitable to monitor patients over a prolonged period of time. The classical trichogram is harmless to the patient and easy to use but not reliable.

AGA can be defined as an androgen-dependent process in genetically predisposed individuals, where balding is due to the continuous miniaturization of affected hair follicles, changing large terminal HF into small vellus-like hairs [3, 18, 19]. Any successful treatment should therefore stop or reverse the process of HF-miniaturization and increase the number of terminal HF whilst reducing vellus hair counts. This concept is illustrated by the phase III studies of men with AGA treated with finasteride [20]. In these studies macrophotographs were taken and hairs were counted. This technique produces counts of "visible" hairs, which means that tiny vellus-like hairs cannot be seen or counted. However, during treatment, these vellus-like HF get bigger and subsequently increase the hair count results when the macrophotograph method is used. A major disadvantage of this technique is that it cannot monitor the expected continuous increase in hair thickness during treatment. As a consequence the phase III studies of men with AGA treated with finasteride revealed that the increase in hair counts reaches a plateau after one year of treatment, whereas the hair coverage analyzed by global photographs increased continuously [20]. This increase in hair coverage is due to an increase in hair thickness as shown by histological examination [3], the direct measurement of hair thickness [21] and by the continuous increase in hair weight [22]. Although the Ludwig pattern of AGA in





Figures 9 and 10. *While defining anagen hairs as growing and telogen hairs as non-growing hairs three days after shaving, the TrichoScan is able to calculate a digital trichogram. This figure illustrates the results (mean with 95% confidence interval) from two investigators, who analysed of 18 volunteers with AGA the proportion of anagen hairs* (Fig. 9) *and the hair growth rate* (Fig. 10) *at the vertex and at the occiput (only 14 images). Compared to the occiput, the AGA-affected scalp reveals a decreased number of and slower growing anagen hairs. Both investigators produced similar results.*

women differs in appearance [23] from the Hamilton pattern occurring in men, these pathophysiological mechanims seem to be the same, because female AGA-patients treated with cyproterone acetate [24-26] or minoxidil [27, 28] experience an increase in hair thickness [24-26, 29] or hair weight [27]. Therefore, a reliable hair counting method should primarily be able to calculate the number and thickness of hairs, which is stable within at least 1cm above the scalp [30, 31], in a defined area of the scalp. From a clinical perspective the hair growth rate (mm/day) and the anagen/telogen ratio are of secondary importance.

As early as 1964 Barman *et al.* [32] related a method that used optical contact microscopy to calculate these parameters, and much later Hayashi *et al.* [33] described a similar approach for the measurement of hair growth by the use of optical microscopy and computer analysis. However, these authors were unable to automate the process of calculation and measured the thickness of hairs visually with the cursor on the computer monitor. The authors calculated that the results from different investigators, but from the same image, differ by ± 8.4%, which makes such semiautomatic methods as this unsuitable for clinical practice. A nearly similar approach has been tested with the use of the phototrichogram (PT). The PT has proven to be a suitable and non-invasive tool to monitor the hair growth phases *in situ*. This technique has been improved by the image analysis [12] and later with the use of immersion oil and digital contrast enhancement [34]. However, although a marked improvement of the images and more accurate quantitative data were noted, [34] an automated analysis was not possible.

Until now the analysis of the images has been a tedious and time consuming process. Attempts to automate the process have been performed several times unsuccessfully [33, 34, 39]. This is mainly because the HF on the scalp grow in groups (follicular units) rather than singly and therefore neighboring HF typically overlap or may be aligned in parallel. Furthermore, any photographic analysis software needs good contrast between the HF and the scalp skin to be analyzed, and the fact that many hairs lose their natural pigmentation due to aging or AGA, makes them much more difficult to detect. We have overcome this difficulty by coloring the hairs prior to taking the images, without any negative effect on the collected data. Furthermore, we have created an entirely automatic software for the analysis of the aforementioned parameters of hair growth. Because the described technique is a modified and computerized trichogram we called it TrichoScan. The images were taken with a video system for epiluminescence microscopy (ELM). ELM is a standard procedure for the analysis of melanocytic nevi [35-38], and many dermatologists in Europe already use ELM-systems in daily clinical practice. These devices produce high quality and reproducible digital images, because the images are always taken at the same distance of the lens to the skin surfaces. Our results suggest that ELM-systems can be used for the evaluation of patients complaining from androgenetic effluvium and for monitoring the response of therapy.

Variations that normally occur in hair length, weight, thickness, etc., can be assayed either with reference to standardized values, or by comparing measurements made on two or more occasions over a given period of time. Similar means of assay must be employed to study changes in hair growth which may occur with regard to age or illness. The margin of error of the techniques and the instruments employed should be smaller than the magnitude of the variations to be measured. As our results show, the TrichoScan fulfils these criteria and has advantages over standard procedures used so far for hair measurements. Firstly, it is investigator independent. In other studies using the unit area trichogram, a substantial difference between the collected data from different investigators was noted. In these studies a significantly larger mean total hair count was reported from experienced versus inexperienced observers [40]. Our results show, that this is not the case for the TrichoScan technique. Secondly, many methods are not strictly validated. The hair weight test is a good example where the hair is clipped in a defined target area. However, the sample error for different investigators is unknown. This is mainly due to the methodology itself, because once the hairs are clipped a second investigator cannot clip the same area again to assess the reproducibility of the method. In contrast the TrichoScan is highly

validated with defined values for intra-class correlation between the same and different investigators. This is of crucial importance for clinical studies. In cases where the expected differences between placebo and verum treated patients is known, the minimum number of patients necessary to prove this difference can be calculated. Thirdly, some methods are associated with considerable discomfort to the patient such as the repeated plucking of hairs required by the trichogram technique. The TrichoScan relies on a small analyzed area of the scalp, which is afterwards barely visible. The tiny tattoo is the only discomfort patients will notice. Fourthly, some methods to count hairs are tedious and time-consuming. By contrast, TrichoScan can be performed by experienced hands within 8-12 min "hands on" experience. Fifthly, the number of items of equipment necessary is small. Many dermatologists already have ELM-systems and these physicians would only need the TrichoScan software.

The primary advantage of this technique is that it can be used for clinical studies to compare placebo versus treatment or to compare different capacities of different hair growth promoting substances. This technique can be used for studying AGA or other forms of diffuse hair loss, Moreover, it can be adopted to study the effect of drugs or laser treatment on hypertrichosis or hirsutism. ■

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Abbreviations

HF	Hair follicle
ELM	Epiluminescence microscopy
AGA	Androgenetic alopecia

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