Clinical aspects of scalp cooling in chemotherapy induced alopecia

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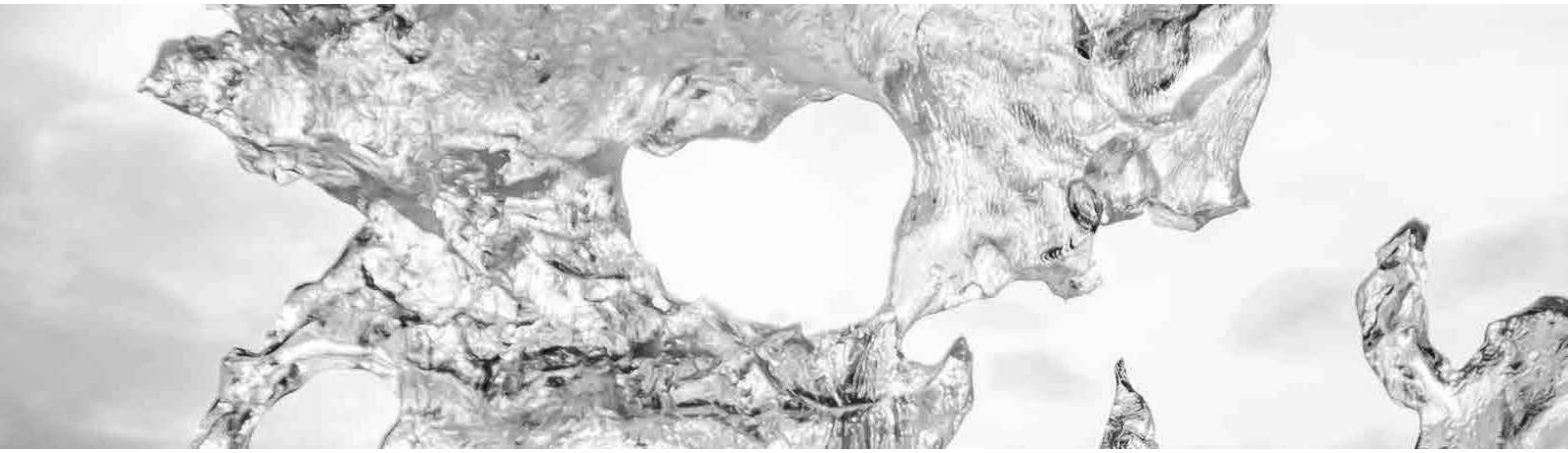
Chapter 7



Translational study with collected hair samples in chemotherapy induced alopecia

Submitted

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ABSTRACT

Purpose: Chemotherapy-induced alopecia (CIA) is generally considered as one of the side-effects with the most impact for patients. To explore the pathobiology of CIA and to improve the efficacy of scalp cooling in preventing CIA, it is important to analyse the molecular damage-response pathways in human hair follicles during and after administration of chemotherapy. We report a single centre explorative pilot study.

Objectives: The objective of this study was to explore damage-response pathways in hair follicles after chemotherapy.

Methods: Hair follicles were collected from ten patients with breast cancer who were planned for treatment with anthracycline-based combination chemotherapy without scalp cooling. Hair follicles were collected before and at different time points after chemotherapy administration and subsequently embedded in methyl-methacrylate. Haematoxylin and eosin (H&E) staining was used to analyse the sections for apoptotic cells. These sections were stained with immunohistochemical antibodies.

Results: Hair follicles were successfully processed according to the protocol. Morphological signs of apoptosis were detected at different time points. Immunohistochemical staining could be successfully carried out in most of the sections. Cells staining positive for p53, caspase-3 and ki-67 could be detected in the hair shaft and the hair bulb. We were not successful in obtaining sufficient numbers of evaluable hair bulbs and therefore could not generate reliable data on markers of apoptosis in the ten treated patients.

Conclusions: These findings stimulate further development of methods to use hair follicles collected from patients as a human-based model for exploring the working mechanism of CIA and to test new potential interventions for hair loss prevention.

INTRODUCTION

Cancer treatment with cytotoxic agents is associated with severe side effects. Chemotherapy induced alopecia (CIA) is one of the most distressing side effects for many patients(1,2) and begins days to weeks after the administration of chemotherapy.(3,4) Chemotherapy interferes with proliferating cells causing cell death or preventing cell growth, but the exact underlying pathobiology of CIA remains insufficiently understood.(3-12) In normal life, the growth cycle of a hair follicle consists of three phases: the anagen, catagen and telogen phase. The hair growth (anagen) phase involves the growth of a hair from a hair follicle and lasts for three to seven years. During the transitional (catagen) phase (2-3 weeks), the hair follicle atrophies and migrates upwards in the skin. During the resting (telogen) phase (3-4 months), the hair does not grow but stays attached to the hair follicle. The telogen phase ends when the old hair is shed and a new hair is regenerated in the anagen phase.(13,14) Since 90% of all hair is in anagen phase and rapidly proliferates, hair follicles are highly at risk to be affected by chemotherapy, resulting in CIA. The most characteristic response of a hair follicle to chemotherapy is an anagen effluvium (shedding of hair follicles in the growth phase). Telogen effluvium also occurs, characterized by increased proliferative activity, presumably as a repair strategy, resulting in diffuse CIA.(15)

Research models for investigating molecular mechanisms of CIA are depicted in Table 1. At present, research in rodent and ex-vivo models is used to explore the pathobiology of CIA and to test novel management strategies in the prevention of CIA. However, these models have limitations in studying the direct effect of interventions on human hair follicles treated with chemotherapy. In rodent models, such as mouse or neonatal rat models, it was found that hair follicle apoptosis largely depended on p53, a key mediator of cellular damage caused by a stress response.(15) However, the clinical relevance of rodent hair follicle models is limited because they do not reflect the cycling rhythm of hair follicles in human beings. Research models with ex vivo cultures of human hair follicles enable direct testing of the damaging effects of defined cytostatic agents.(16-18) Al-Tameemi et al. confirmed with their ex-vivo model that cooling reduced or completely inhibited drug cytotoxicity in human keratinocytes exposed to chemotherapy, supporting the hypothesis of scalp cooling.(17) However, keratinocytes in this model are not in cycle and begin to degenerate after 1-2 weeks in culture. Moreover, it is difficult to imitate the application schedule of standard chemotherapy regimens as human hair follicles damaged by chemotherapy will still be in the recovery phase when they are exposed to the next cycle of chemotherapy. Botchkarev demonstrated that in contrast to wild-type mice, p53-deficient mice show neither hair loss nor apoptosis in the hair follicle keratinocytes after cyclophosphamide treatment.(13) Hendrix et al. and Paus et al.(15,19) showed in mice that hair follicles undergo two distinct pathways of

dystrophy (dystrophic anagen and dystrophic catagen pathway) after chemical damage. During the so-called dystrophic anagen pathway (induced mainly by a lower dose of chemotherapy), the hair follicle undergoes an incomplete primary recovery followed by a retarded secondary recovery during which a normal hair shaft is generated. Hair follicles that undergo the dystrophic catagen pathway (in response to a higher dose of chemotherapy) immediately enter into a dystrophic catagen stage, followed by an abnormally shortened telogen phase. Scalp cooling, possibly forces hair follicles into the dystrophic anagen phase, thereby enabling patients to keep their hair. Two hair follicle damage response pathways (dystrophic anagen and dystrophic catagen pathway) were also reported in a research model of surgical grafting of human hair follicles on immunodeficient mice .(13,15,20) Unfortunately, this technique still does not demonstrate the direct effect of treatment with chemotherapy in hair follicles of patients. Randall et al.(21) reported a method for the fixation, processing, sectioning and immunohistochemical staining on plucked human hair, which allows monitoring of the effects of anti-cancer drugs on cell proliferation Ki -67) and on the upstream signaling molecules that control cell proliferation (p53/ Caspase-3).

So far, there is no effective preventive pharmacological therapy for CIA. Management of CIA primarily consists of counseling, professional psychological support and the recommendation to use a wig when hair loss occurs.(14,22,23) Scalp cooling is the only intervention to prevent CIA, its efficacy depending on the type and dose of chemotherapy. It is assumed that scalp cooling reduces skin temperature, thereby affecting the exposure and metabolism of cytotoxic agents in the hair follicles.(9,24) In vitro models showed that cooling markedly reduced cytotoxicity, in agreement with clinical observations.(17) Sakurai et al. examined the effects of low temperature (32 degrees C) on cells exposed to cytotoxic agents in vitro. (25) Mild hypothermia suppressed induction of apoptosis by p53-dependent and p53-independent mechanisms. P53 activates downstream apoptotic events, leading to a cascade of activation of caspases, including Caspase-3, which induces apoptosis.(26) Ki-67, a cellular marker for proliferation, is down regulated in hair follicles showing apoptotic changes(20), and is therefore an interesting marker in research discovering the mechanism of scalp cooling.

Plucked human hair may offer a minimally-invasive, easily sampled and well-tolerated source of hair follicle cells for the investigation of damage response pathways in patients treated with chemotherapy. In order to improve scalp cooling and to develop new treatment strategies for the prevention of CIA, we set out to explore molecular damage-response pathways in plucked human hair from patients treated with chemotherapy.

Table 1. Research models for investigating the molecular mechanisms of chemotherapy-induced alopecia

Model of chemotherapy-induced alopecia	Strengts	Limitations
Rodent models (neonatal rats and adult mice)(19,27,28)	Rodent models permit investigation of how chemotherapy affects the hair follicle cycling. Similar to in human CIA, hair loss in rodent models generally follows specific patterns.	In neonatal rats chemotherapy affects hair follicles that are still in the final stages of postnatal morphogenesis. In adult mice hair cycling is more or less synchronized across all follicles, whereas in human beings each hair follicle follows its own cycling rhythm.
Organ cultured hair follicles(16,17,20,29)	Enables direct testing of the damaging effects of cytostatic agents and provide the most clinically relevant surrogate model for CIA	Not possible to investigate how chemotherapy affects the hair follicle cycling. No information about the hair loss pattern.
Surgical grafting of human scalp skin on to mice(20,28,30)	Combines benefits of human and rodent models. Resembles key features of CIA that occurs in patients with cancer.	Human hair follicles are exposed to a different milieu of the mouse hormones and local growth regulators.
Hair collection from patients	Minimally invasive technique which enables direct testing of the damaging effects of cytostatic agents in human hair follicles.	No available surrounding scalp tissue to provide more insight in the mechanism of action. Very time consuming and delicate process.

MATERIALS AND METHODS

We conducted an explorative single centre pilot study at the department of Medical Oncology of Northwest Clinics, the Netherlands. Patients with breast cancer who were planned for treatment with at least one cycle of anthracycline-based combination chemotherapy (docetaxel-doxorubicin-cyclophosphamide(TAC), fluorouracil-epirubicin-cyclophosphamide(FEC) or doxorubicin-cyclophosphamide(AC)) without scalp cooling

were enrolled. Patients were excluded if they lacked basic proficiency in Dutch or if they were unable to understand the patient information brochure. The hair follicle analyses were conducted at the department of pathology of the University Medical Centre Utrecht. The study was approved by an independent ethical committee. All procedures were conducted in accordance with the 1964 Helsinki Declaration and its later amendments. Written informed consent was obtained from all patients included in the study.

Sampling and fixation

Patients were asked to collect approximately 30 scalp hairs at six time points after the first cycle of chemotherapy. Collection time points were T0= before chemotherapy, T3=day 3, T5=day 5, T9=day 9, T13=day 13, T17=day 17 after chemotherapy. The first sample collection (T0) took place at Northwest Clinics and was conducted by a research nurse. The following five samples were collected by patients at home. Hairs were plucked from the parietal region of the scalp using a blunt-nosed forceps. Hairs were stored into a tube containing 10% neutral-buffered formalin which was brought home by the patient. The tubes were handed in at the next hospital visit. After collection, the hairs were checked by a research nurse for the presence of a bulb and stored in phosphate buffered saline (PBS) at room temperature at Northwest Clinics and sent to the department of Pathology of the University Medical Centre Utrecht for further analysis.

Processing and embedding

Hairs were trimmed to approximately 1 cm and attached to a small piece of photo sticker, enabling parallel embedding of the hairs and simultaneous sectioning of multiple hair bulbs. Per photo sticker, five hairs were embedded in 3% low melting point agarose. Hairs were horizontally embedded in one agarose block cast in silicone embedding molds. The agarose blocks were trimmed to fit in the processing baskets of the Leica EM tissue processor. The blocks were processed according to the steps as described by Randall et al.(21) After processing, the remaining photo stickers were carefully removed. The processed agarose blocks were embedded in 8 mm, flat-ended, embedding capsules filled with polymerization solution which consisted of catalyzed MMA plus 125 µl N,N-dimethylaniline. The blocks were orientated longitudinally at the bottom of the embedding capsules. To keep the blocks from floating upward after adding the polymerization solution, a horizontally placed shortened pipette tip was gently pushed onto the block. The capsules were then placed in a desiccator at room temperature to flush out the oxygen-free nitrogen for approximately 2,5h during the polymerization of the resin. A layer of water was added to dissipate the heat of the exothermic polymerization.

Sectioning

The polymerized blocks were kept in capsules for another 24 hours at room temperature. When removed from the capsules, the plastic blocks were stored at room temperature before sectioning. The blocks were cut in 2 µm-thick sections on a Leica Ultracut Microsystems microtome (Cell Microscopy Core, Department of Cell Biology at the UMCU) using a glass knife. The sections were picked up with a fine-nose forceps and spread out on a droplet of water on a glass slide. The slide was then transferred to a hotplate to flatten the section out. A toluidine blue staining was performed to confirm the presence of hair follicles in the section. After sectioning, the slides were stored in a refrigerator.

Preparation of slides and Haeamatoxyline and Eosine staining (H&E)

Processed sections were microscopically analyzed and assessed for evaluability. Prior to H&E staining, the slides were incubated at 37°C for approximately 3 hours, followed by overnight deplasticising in xylene at room temperature. Sections were rinsed twice in xylene, three times in 10% methylated spirit, once in 100% ethanol and once in 70% ethanol, before hydration in distilled water. The sections were H&E stained by incubating in haeamatoxylin for 10 minutes and in eosin for 2 minutes. After dehydration, slides were mechanically cover slipped in pertex. H&E stained hair bulbs were analyzed for the presence of apoptotic cells at the Departments of Internal Medicine and Pathology of Northwest Clinics.

Immunohistochemistry

Slides were treated by blocking in 3% hydrogen peroxide for 15 minutes. After being rinsed in distilled water, heat-mediated antigen retrieval took place in an autoclave at 125 ◦C in EDTA, pH 9.0, and cooled down for 20 minutes. IHC staining was performed using the following antibodies: Ki-67 (Dako, clone MIB-1, 1/100), P53 (Dako, clone Do7, or 1/500) and anti-active caspase 3 (Pharmingen Purified rabbit 1/500). Primary antibodies were incubated at room temperature for 1 hour. For p53 and Ki-67 staining, slides were treated pre-and post- antibody incubation with the Novolink kit (Leica, NL). Active caspase 3 staining was treated with the Novolink kit, Bright Vision poly AP-Anti rabbit IgG and Dako liquid permanent red. After counterstaining with haeamatoxylin for 10 seconds, the sections were dehydrated and mechanically cover slipped in pertex. The material was assessed microscopically for evaluability of apoptotic cells. Appropriate positive and negative controls were taken along each staining and consisted of formalin fixed paraffin embedded control sections- and/ or MMA embedded positive control material.

Statistical analysis

Data were collected using standard forms, which were compiled into a database. Due to the small sample size of the pilot study, it was not possible to perform statistical analysis.

RESULTS

Patient characteristics

Between January 2013 and November 2014, ten patients treated with anthracycline-based chemotherapy were included to explore the best time points for hair collection and to optimise the fixation, embedding and staining protocol. Patient characteristics are shown in table 2. The median age of patients was 48 years and all patients were treated for breast cancer. Eight patients were evaluable for hair root analysis after T0. Two patients had not collected hairs at home because of sickness.

Table 2. Patient characteristics

	N
Patients included	10
Patients evaluable for hair root analysis	8
Median age, years (range)	48 (35-57)
Chemotherapy	
FEC	1
TAC	7
AC	2

Hair samples

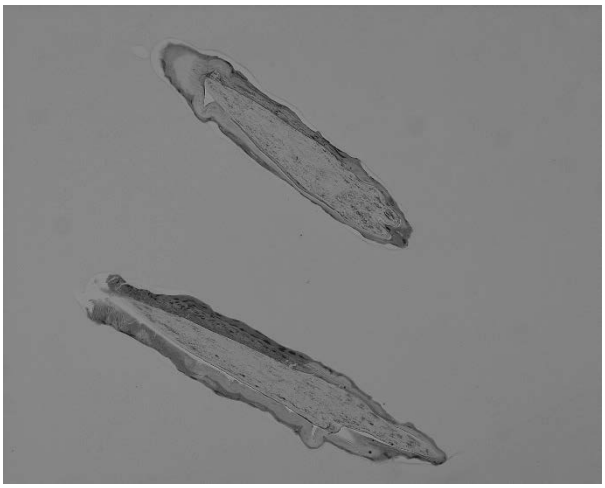
Processed sections were microscopically analyzed and assessed for evaluability. Hair follicles collected are shown table 3. At T0 a total of 61% of collected hair samples was evaluable for analysis. Non-evaluable sections were seen in 39% of the samples and contained hair remnants with or without attached connective tissue or without the presence of any hair follicle epithelium (Figure 1). Unfortunately, a sharp decrease of evaluable hairs was detected over time. At T17 none of the hair follicle samples were evaluable, only hair shafts or connective tissue could be identified.

Table 3. Hair follicles collected at various time points

	T0	T3	T5	T9	T13	T17
No. Patients collecting hairs	10	8	7	6	6	2
No. H&E tissue slides (Each slide containing 1-5 hair follicles)	20	18	14	12	12	6
% Evaluable hair follicles	86%	94%	57%	42%	17%	0%
No. Apoptotic cells in evaluable hair follicles	8	0	2	2	1	0

T0: Before the start of chemotherapy
T3: Three days after chemotherapy
T5: Five days after chemotherapy
T9: Nine days after chemotherapy
T13: Thirteen days after chemotherapy
T17: Seventeen days after chemotherapy

Figure 1. Non-evaluable sections containing connective tissue without the presence of epithelium



Apoptosis

Results of hair root analysis demonstrated the presence of a low frequency of apoptotic cells, characterized by cellular shrinkage, eosinophilic cytoplasm and nuclear fragmentation. Apoptotic cells, found at different time points, seemed to be preferentially located in the root sheath. Isolated apoptotic cells were found as well as areas with several apoptotic cells (Figure 2). The highest amount of apoptotic cells was observed at

T0 and decreased after chemotherapy. In some sections, pink artefacts were present (Figure 3), probably representing dislocation of parts of the inner root sheath resulting from overstretching of the hair follicle when pulling out.

Figure 2. Apoptotic cell in H&E stained section

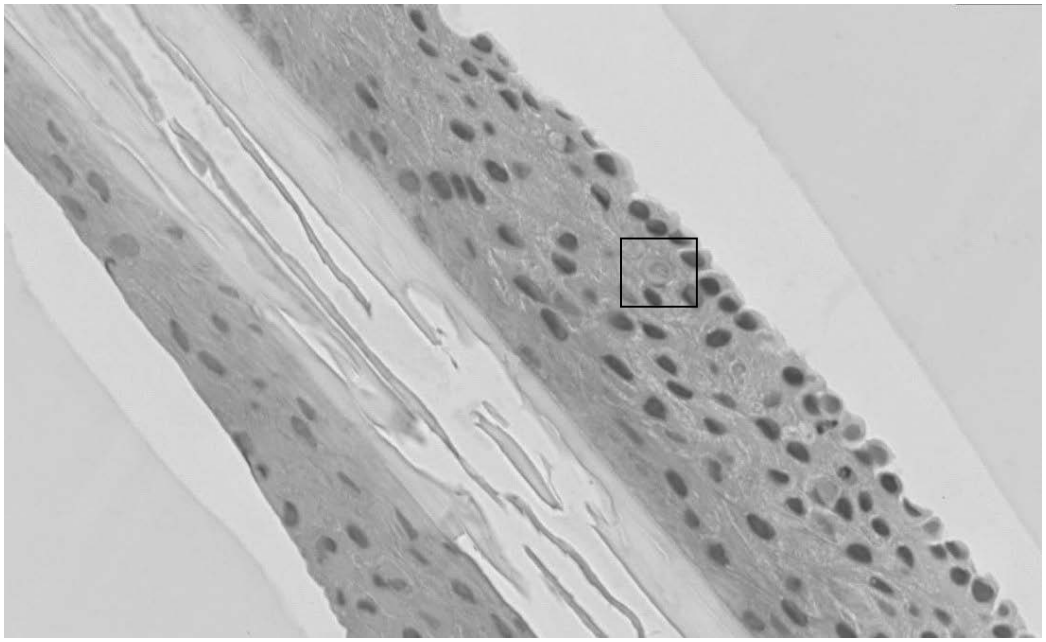
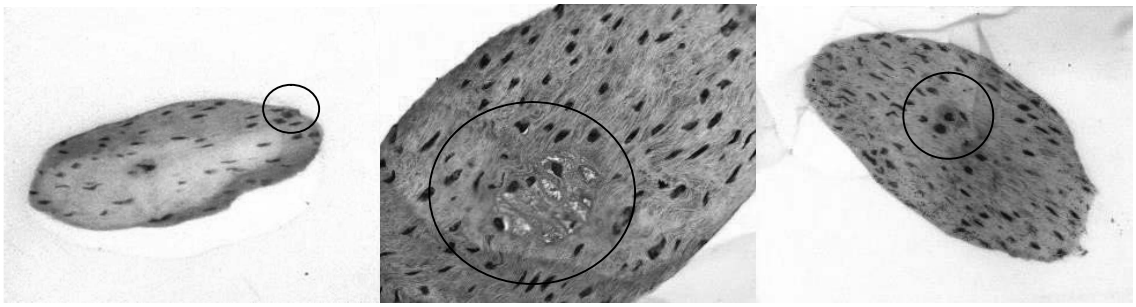


Figure 3. Abnormalities that might be apoptotic cells in H&E stained sections



Immunohistochemical analysis

Immunohistochemical staining was exploratively performed in samples of four patients in whom histochemical sections showed the best quality. P53 and Ki-67 staining showed positive nuclei for p53 (figure 4) and Ki-67 (figure 5) at T0, mainly in the hair shaft and bulbus.

Figure 4. P53 staining (a: T=1, b: T=5)

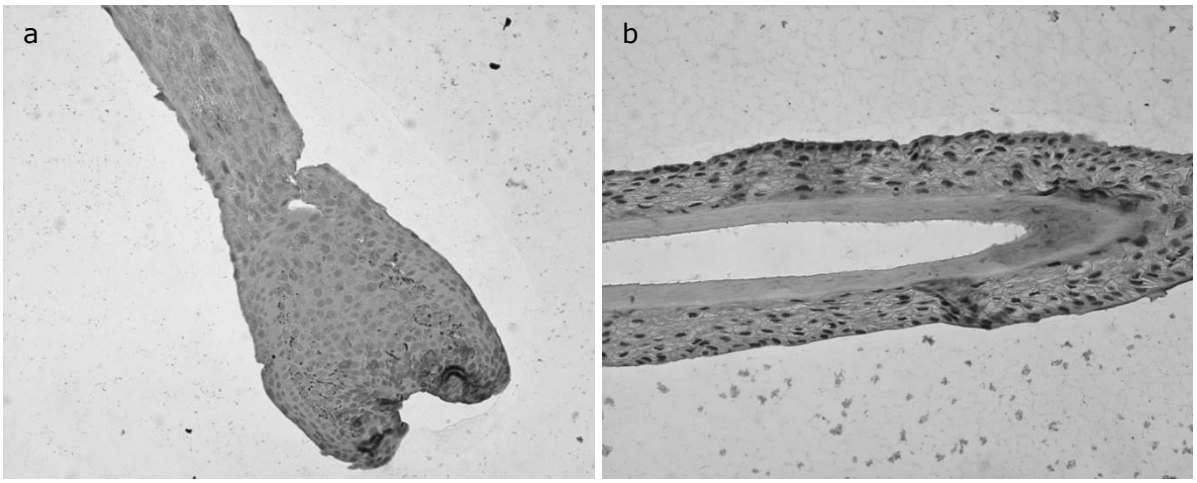
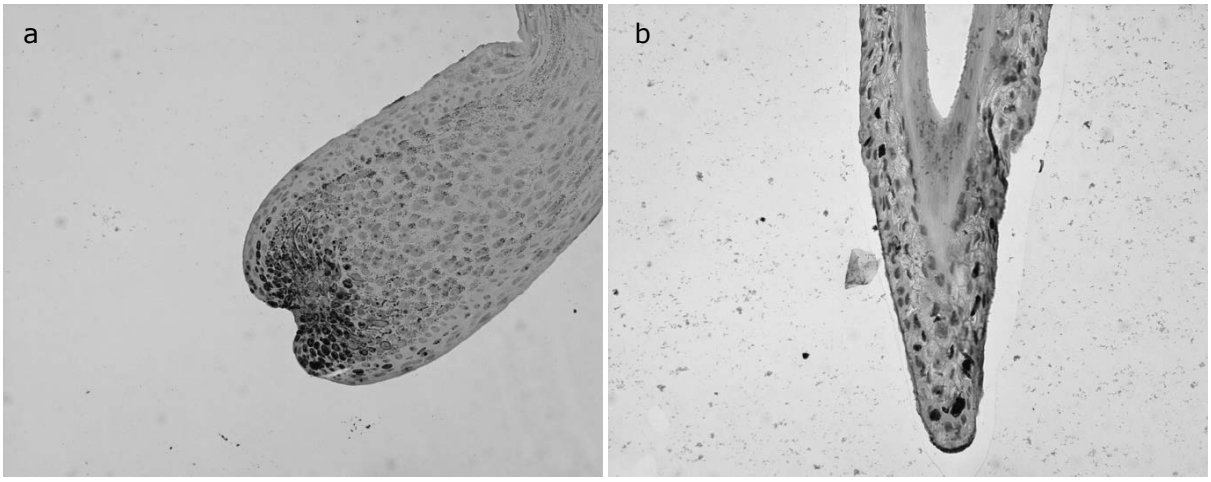
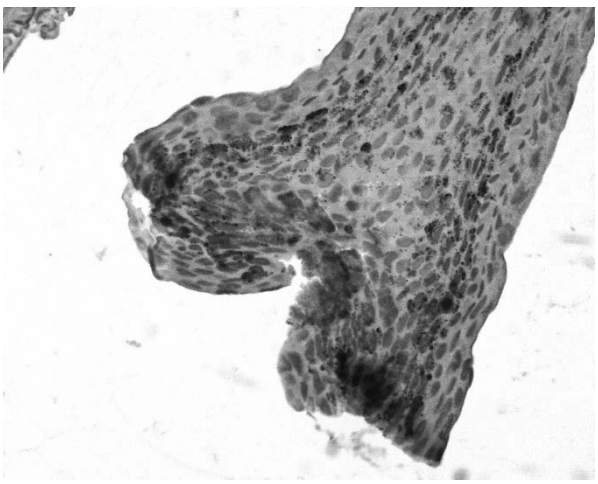


Figure 5. Ki-67 staining (a: T=1, b: T=5)



Ki-67 IHC showed a decreasing brown diaminobenzidine (DAB) precipitate from core to shaft, following the normal proliferation rate of cells in hairs. Samples stained with active caspase 3 antibody showed little positive cytoplasm at T0, mainly in the bulbus section (Figure 6).

Figure 6. Caspase-3 staining (T=1)



DISCUSSION

This study shows results of hair follicle analyses in patients treated with chemotherapy. We optimised the fixation, embedding and staining protocol of hair follicles from patients and explored signs of apoptosis at various time points after chemotherapy. We hypothesized that we could detect signs of apoptosis after chemotherapy administration and it was our intention to confirm this hypothesis in a larger study.
Unexpectedly, as is depicted in table 3, we found a decrease of evaluable sections of hair follicles and shafts over time after administration of chemotherapy. Therefore, we decided to shorten the time interval between hair root analysis in a second group of patients.

Between March 2017 and April 2018 another twenty patients with or without scalp cooling were studied in a similar way to explore the molecular damage-response pathways in human hair follicles after chemotherapy. However, the process of embedding, cutting and coloring was very complicated and time consuming, and there were not enough evaluable hair bulbs for analysis in these 20 patients.
We identified several reasons which can explain why we were unable to confirm our hypothesis (Table 4).

Table 4. Limitations and recommendations

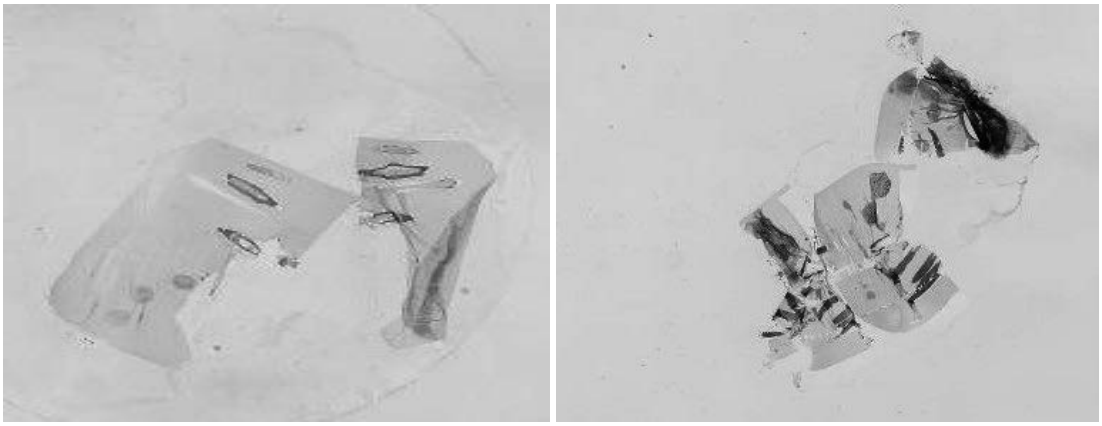
Influencing factor	Limitation	Recommendation for future research
Collection of hairs	Only hair follicles were collected	Take skin biopsies to collect surrounding scalp tissue
Selection of hairs	Hairs were collected by the patient themselves	Controlled collection by an experienced nurse and preselection of hairs with a preparation microscope
Fixation period	Long fixation period could affect the material	Fixation of hair samples maximal 4 hours
Sectioning protocol	Complicated protocol and very delicate sectioning process	3-D scanning or Microscopy with Ultraviolet Surface Excitation (MUSE)

Firstly, hair follicles were collected using a blunt nose forceps. Preferably we would have taken scalp skin biopsies from patients treated with chemotherapy. By collecting surrounding scalp tissue, the effect of chemotherapy on the surrounding skin could have given us more insight into the mechanisms of action. However, when patients have no direct treatment benefit, performing biopsies presents a difficult ethical issue. Due to

these ethical problems, we explored the possibilities to perform immunohistochemical staining on hair follicles directly collected from the patient's scalp. Secondly, the yield of hairs could have been improved by a controlled method for pulling out hairs, and by a preselection of hairs with sufficient follicle epithelium for analysis with a preparation microscope.

Thirdly, the long fixation period of the hair follicles might have caused a disadvantage. According to Randall et al.(21) the optimal period of fixation appears to be 4 hours at room temperature. To prevent patients from daily travelling to the hospital to deliver their collected hair samples, the samples were brought to the hospital with the next scheduled appointment. This considerably delayed the period of fixation but had no negative effect on the morphology of the hair follicles. However, immune histology had to take this into account in the degree of antigen retrieval. Unfortunately, hair follicle research turned out to be a very delicate process and depended too much on the availability of dedicated people. The method of fixation according to Randall et al. was difficult to reproduce and we failed in making a robust model for large scale analysis of hair bulbs. The procedure was too time consuming and despite all effort, too much tissue was not evaluable (figure 7). For a better understanding of the working mechanism of CIA, the method as described by Randall et al. may not be the best option. Solid methods which consistently and reliably can demonstrate molecular damage response pathways are needed.

Figure 7. Non-evaluable hairs due to complicated sectioning



There are some important developments which could be helpful. Multiple hairs could be embedded in an upright position and close to each other to be cut in series and to create a 3-D reconstruction through scanning. This technique enables visualizing the entire hair follicle and suffers less from different levels of depth. Another possibility is the use of Microscopy with Ultraviolet Surface Excitation (MUSE), which uses ultraviolet light to illuminate tissue samples. Commonly used bright-field microscopy requires prior preparation of tissue sections mounted on glass slides, a process that can require hours

or days. The UV microscope removes the need for performing traditional histology and produces high-resolution images of biopsies and other fresh tissue samples within minutes.

A dedicated analytical staff is needed to handle the hair bulbs during the whole process of plucking, preservation, sectioning and staining in order to obtain sufficient slides to perform analysis and to draw conclusions.

CONCLUSION

The aim of this study was to explore molecular damage-response pathways in hair follicles from patients treated with chemotherapy. We found that H&E and immunohistochemical staining was possible on a number of embedded hair follicles. Unfortunately, we were not successful in obtaining sufficient numbers of evaluable hair bulbs and therefore could not generate data on markers of apoptosis in treated patients. However, better and standardized techniques for sampling and analyzing of hair bulbs are needed to study the damaging effects of cytostatic agents and to test new potential interventions for hair loss prevention.

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