Phototoxic interactions of tattoo pigments with laser and natural light *in vitro*

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II. Abstract

Tattooing, a process in which colorants are permanently embedded in human skin to generate long-lasting images, enjoys a high popularity nowadays. Today, every fifth to fourth person is tattooed. This popularity seems not to be affected by the high number of tattoo related side effects, which especially occur when tattooed skin encounters light. Light induced side effects make up to about 60 % of tattoo associated side effects. Consequently, the interactions of ultraviolet light and laser light on tattoo pigments and the resulting effects on human skin cells were investigated in this thesis to increase the understanding of photo-induced side effects found in tattoos.

Firstly, we treated *postmortem* tattooed pig skin with laser used in tattoo removal. We consequently analyzed cleavage products using gas chromatographic separation coupled to mass spectrometric detection. 3,3'-dichlorobenzidine, a carcinogen produced through cleavage of Pigment Orange (P.O.)13, was found to be cytotoxic and genotoxic in terms of DNA double strand breaks in human fibroblast and keratinocyte cell lines even beneath identified concentrations. While photo-induced cleavage is a key event that leads to tattoo clearing during laser removal, it can also occur on a smaller scale when tattooed skin is exposed to natural light, particularly in the ultraviolet (UV) range. Since light-induced effects are the most common side effects associated with tattoos, our goal was to investigate the underlying pathomechanisms accordingly. However, no *in vitro* model that accurately resembles the architecture of tattooed human skin has been described in the literature so far.

Therefore, we established a 3D *in vitro* 'tattooed' full thickness skin model ($TatS_{FT}$) as an animal replacement model for tattoo research. The uptake of the tattoo pigments used in this study, titanium dioxide (TiO_2) anatase, TiO_2 rutile, Pigment Orange (P.O.)13 and carbon black by dermal fibroblasts was proven by electron microscopy in $TatS_{FT}$. Despite this uptake, the pigments showed no effect on the viability of $TatS_{FT}$ nor its dermal compartment ($TatS_{DE}$). Concordantly, cytokine secretion, general histology, and the expression of important skin homeostasis markers was unaffected by tattoo pigments in $TatS_{FT}$. Contrary to the absence of toxicity in 3D, TiO_2 anatase significantly decreased cell viability and increased interleukin-8 release in 2D monolayer cultured fibroblasts.

Due to the inherent differences in toxicity sensitivity of 2D monolayered cultured fibroblasts and 3D cultured models, we investigated phototoxicity of tattoo pigments in both, 2D and 3D. Concordantly with particle toxicity, phototoxic effects were bigger in monolayer cultured fibroblasts than in 3D. While TiO_2 showed strong phototoxic effects in 2D, these effects were absent in $TatS_{FT}$. However, UVB induced DNA damage marker levels in the dermis of $TatS_{FT}$ were reduced by pigments. Combined with photoprotective effects found in $TatS_{DE}$ concerning viability, these data suggest photoprotective properties of tattoo pigments for the tattooed dermis and its underlying tissue. Contrary to these results, we found P.O.13 to alter cytokine secretion upon UV irradiation in both, 2D and $TatS_{DE}$. While minor

amounts of cleavage products of P.O.13 were identified after UV irradiation, we were unable to proof that these cleavage products might have resulted in adverse effects.

The data in this thesis not only highlight the need for 3D test systems for tattoo phototoxicity research, but also present a highly modifiable 3D *in vitro* test system for this purpose. This work also strengthens concerns regarding TiO_2 anatase and azo pigments like P.O.13 and their use in tattoo inks.

III. Zusammenfassung

Mit dem Tätowieren erfreut sich heutzutage ein Verfahren größter Beliebtheit, bei dem Farbstoffe dauerhaft in die menschliche Haut eingebettet werden, um permanente Bilder in dieser zu erzeugen. Momentan ist jeder fünfte bis vierte Mensch tätowiert. Dieser Beliebtheit scheint die hohe Zahl an tätowierungsbedingten Nebenwirkungen keinen Abbruch zu tun. Viele Nebenwirkungen treten insbesondere dann auf, wenn die tätowierte Haut mit Licht in Berührung kommt. Insgesamt machen lichtinduzierte Nebenwirkungen etwa 60 % der tätowierungsassoziierten Nebenwirkungen aus. In dieser Arbeit wurden daher die Wechselwirkungen von ultraviolettem Licht und Laserlicht auf Tätowierpigmente und die daraus resultierenden Effekte auf menschliche Hautzellen untersucht, um das Verständnis für lichtinduzierte Nebenwirkungen in tätowierter Haut zu erhöhen.

Zunächst behandelten wir *postmortem* tätowierte Schweinehaut mit Lasern, die zur Tattooentfernung verwendet werden. Anschließend analysierten wir die Spaltprodukte mittels gaschromatographischer Trennung gekoppelt mit massenspektrometrischer Detektion. 3,3'-Dichlorbenzidin, ein Karzinogen, welches durch die Spaltung von Pigment Orange (P.O.)13 entsteht, erwies sich in menschlichen Fibroblasten- und Keratinozyten-Zelllinien bereits unterhalb identifizierter Konzentrationen als zytotoxisch und genotoxisch in Form von DNA-Doppelstrangbrüchen. Während die photoinduzierte Spaltung ein Schlüsselereignis ist, das bei der Laserentfernung zum Verblassen der Tätowierungen führt, kann sie auch in kleinerem Umfang auftreten, wenn tätowierte Haut natürlichem Licht, insbesondere im ultravioletten (UV) Bereich, ausgesetzt wird. Da lichtinduzierte Effekte die häufigsten Nebenwirkungen im Zusammenhang mit Tätowierungen sind, war es unser Ziel, die zugrunde liegenden Pathomechanismen entsprechend zu untersuchen. Allerdings wurde in der Literatur bisher kein *in vitro* Modell beschrieben, das die Architektur tätowierter menschlicher Haut genau nachbildet.

Daher etablierten wir ein 3D *in vitro* "tätowiertes" Vollhautmodell ($TatS_{FT}$) als Tierersatzmodell für die Tätowiermittelforschung. Die Aufnahme der in dieser Studie verwendeten Tattoo-Pigmente Titandioxid (TiO_2) Anatas, TiO_2 Rutil, Pigment Orange (P.O.)13 und Carbon Black durch dermale Fibroblasten wurde elektronenmikroskopisch in $TatS_{FT}$ nachgewiesen. Trotz dieser Aufnahme zeigten die Pigmente keinen Einfluss auf die Lebensfähigkeit von $TatS_{FT}$ oder dessen dermales Kompartiment ($TatS_{DE}$). Auch die Zytokinsekretion, die allgemeine Histologie und die Expression wichtiger Marker der Hauthomöostase wurden durch die Tattoo-Pigmente in $TatS_{FT}$ nicht beeinflusst. Im Gegensatz zur fehlenden Toxizität in 3D verringerte TiO_2-Anatas signifikant die Zellviabilität und erhöhte die Interleukin-8-Ausschüttung in 2D-monolayer kultivierten Fibroblasten.

Aufgrund der inhärenten Unterschiede in der Toxizitätsempfindlichkeit von 2D-monolayer kultivierten Fibroblasten und 3D kultivierten Modellen, untersuchten wir die Phototoxizität von Tattoo-Pigmenten sowohl in 2D als auch in 3D. Übereinstimmend mit der Partikeltoxizität waren die phototoxischen Effekte in Einzelschicht-kultivierten Fibroblasten größer als in 3D. Während TiO_2 starke phototoxische

Effekte in 2D zeigte, waren diese Effekte in $TatS_{FT}$ nicht vorhanden. Allerdings wurden die UVB-induzierten DNA-Schadensmarker in der Dermis von $TatS_{FT}$ durch die Pigmente reduziert. In Kombination mit den photoprotektiven Effekten, die in $TatS_{FT}$ hinsichtlich der Lebensfähigkeit gefunden wurden, deuten diese Daten auf photoprotektive Eigenschaften von Tätowierpigmenten für die tätowierte Dermis und das darunter liegende Gewebe hin. Im Gegensatz zu diesen Ergebnissen fanden wir, dass P.O.13 die Zytokinsekretion bei UV-Bestrahlung sowohl in 2D als auch in $TatS_{DE}$ verändert. Obwohl geringe Mengen an Spaltprodukten von P.O.13 nach UV-Bestrahlung identifiziert wurden, konnten wir nicht nachweisen, dass diese Spaltprodukte nachteilige Effekte hervorgerufen haben könnten.

Die Daten in dieser Arbeit unterstreichen nicht nur die Notwendigkeit von 3D-Testsystemen für die Forschung zur Phototoxizität von Tätowierungen, sondern stellen auch ein hochgradig modifizierbares 3D *in vitro* Testsystem für diesen Zweck dar. Zusätzlich stärkt diese Arbeit auch die Bedenken bezüglich der Verwendung von TiO_2 Anatas und Azopigmenten, wie P.O.13, in Tätowiertinten.

IV. Abbreviations

C.I.	Color Index
DT	Diphtheria toxin
ECHA	European Chemicals Agency
IARC	Agency for Research on Cancer
PAAs	Primary aromatic amines
PAHs	Polycyclic aromatic hydrocarbons
P.O.13	Pigment Orange 13
ResAP(2008)1	Resolution on requirements and criteria for the safety of tattoos and permanent make-up
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	Reactive oxygen species
$TatS_{DE}$	Tattooed human dermis model
$TatS_{FT}$	Tattooed human full thickness skin model
TiO_2	Titanium Dioxide
UV	Ultraviolet

1. Introduction

Body modifications – deliberate changes to the appearance or the anatomy of the human physiology – include scarification, removal or change of body parts, piercing, sub- or transdermal implants and piercing. This work, however, is about one of today's most widespread permanent body modification, the tattoo, and deals mainly with the dangers of its coloring agents, the tattoo pigments. It is intended to help uncover potential dangers and long-term influences associated with pigments and to take the first steps towards animal-free tattoo research.

1.1. A brief history of tattooing

Tattooing is an ancient body modification and entwined with human culture for millennia. While tools from the Upper Paleolithic period found during excavations are attributed to have been used for tattooing [1], the definite proof for tattooing on humans is found in form of preserved tattooed human mummies, dating back to the early Neolithic. The 5300 year old ice mummy “Ötzi” for example bears 61 tattoos in various body parts [2]. The colocalization of those primitive geometric-shaped tattoos with pathological findings in the mummy led several scientists to the conclusion that tattooing was performed as a kind of treatment for “Ötzi” [3-5]. While the discussion continues as to whether Ötzi's tattoos were only therapeutic in nature, 5000 year old male and female Egyptian mummies bear the proof of the gender-independent cosmetic or cultural use of tattoos embedded into their skin in form of contemporary art motifs [6]. In addition, tattooed mummies from all over the globe proof tattooing as a worldwide practice [7].

In Europe, tattooing is believed to have persisted through the centuries, with a depression following its prohibition by papal edict in 787, until its reintroduction between the 17th and 19th centuries. The main cause of its reintroduction into Western culture is considered to be the contact with the tattoo art of Polynesia and Japan [8]. Hence, tattooing at that time was mainly limited to sailors. In mid-19th century, members of royal families got tattooed and tattooing thus became socially acceptable for the masses [9].

Tattoos are nowadays an integral part of Western culture: Athletes and other public figures are sometimes excessively and obviously tattooed [10, 11]; TV series [12], movies [13] and songs [14, 15] revolve around tattoos or tattooing. The cultural significance of tattoos led to an increase in the fraction of tattooed people among the population: From 2003 the share of tattooed people grew from 8 % up to 19 % in 2016 [16]. The share of tattooed women was higher than the share of tattooed men. However, the group of tattooed people is highly heterogenous concerning the number and size of the tattoos. An internet survey in German-speaking countries with 3,411 participants showed that 28 % of the tattooed people had more than four tattoos and 36 % had tattoos larger than 900 cm² [17].

Today, the highest share of tattooed people can be found in the age group from 25 – 34 years with up to 44 % people tattooed [16]. While there are only about 24 % tattooed in the younger 14 – 24 age group,

it is unclear if the decline in tattooed people indicates a decreased interest in tattooing. While the share of tattooed people and its importance for the western culture changed over the last centuries, the principle behind tattooing stayed the same throughout the ages.

1.2. The tattooing process and its tools

The tattooing process

Tattooing involves the use of a sharp instrument to wound the skin and introduce a colorant into the resulting wound. Ancient methods used sharp instruments such as flint knives to cut the human skin and then rub the colorant into the inflicted wound. In contrast, modern tattoo artists use disposable needles which are covered with tattoo ink and attached to rotary machines. This allows precise deposition of the pigments in the human skin, thus reducing the injured skin area and enabling more complex tattoo designs. While in some cases, tattooing is also applied to other organs, such as the eyeballs [18], the application of tattooing to skin remains the standard.

Over the eons, tattooing tools evolved from primitive needles, flint knives and thorns to electrically driven tattoo machines. While there are several types of tattoo machines available today, most of them are improvements on Thomas Alva Edison's "stencil pen" patent [19]. Later, first tattoo machines were further developed by Samuel O'Reilly [20], and to the even more advanced coil tattoo machines of Thomas Riley, which's descendants are heavily used today. Despite this revolution in tattooing tools, primitive needles and hammers are still used today: While prison tattooists rely on primitive tools due to the lack of professional tattoo machines, 'traditional' tattoos are favored as a sub-genre of tattooing.

Modern tattoo machines made it possible to use disposable needles and consumables and thus guarantee the necessary hygiene when tattooing. However, a recent study showed that the abrasion of disposable tattoo needles remains in the skin [21]. The abrasion was dependent on the ink used - or more precisely - the tattoo pigments used in the ink. In this case, the abrasion caused by the use of ink containing titanium dioxide pigments was greater than with an ink containing carbon black.

Tattoo inks and pigments

The goal of tattooing is to introduce the water-insoluble coloring agents - the tattoo pigments - into the human skin. Therefore, tattoo pigments are suspended in a complex vector liquid which together form the tattoo ink. Professional inks are designed to achieve high fluidity and richness of pigments while limiting pigment sedimentation and preventing rapid drying of the ink [22]. To achieve these goals, tattoo inks are complex mixtures consisting of pigments, solvents, and additives. Solvents used for tattoo inks include water, ethanol, propylene glycol and glycerin [20]. While tattoo inks with a water activity value below 0.6 inhibit the growth of microorganisms, most tattoo inks use preservatives like benzoic acid, phenol, and formaldehyde to avoid microbial growth [21]. Other possible additives include

thickening agents to inhibit reagglomeration and sedimentation, tensides to support dispersion of tattoo pigments and additives with questionable functions like plant additives and fragrances. The vector liquid of the ink is highly optimized to allow the tattoo artist to deposit the tattoo pigments as easily in the skin as it could be. However, the colorant to be deposited, the tattoo pigments, are themselves a very heterogeneous and diverse group of chemicals.

Tattoo pigments are polydisperse and polymorphous particles with sizes in the nano- or micrometer range [23]. Per definition, they are not soluble in aqueous solutions and therefore show a low bioavailability and reactivity. Tattoo pigments are identified by either their color index number (C.I.) or by the pigment short-cut (Px.y, *i.e.* PO.13 for Pigment Orange 13). Modern tattoo inks contain an average of three different pigments [24]. Tattoo pigments can be of organic or inorganic nature. Inorganic pigments include inorganic salts, metal oxides [25, 26] and carbon black. While inorganic salts were heavily used for colorful tattoo inks for decades, they have been widely replaced by more brilliant organic pigments: Today, 80 % of all colorful tattoo pigments are of organic nature [23, 27].

Organic pigments have become the focus of scientific investigation as they are suspected of being responsible for many tattoo-related side effects. Thus, the results of an online survey from 2010 suggest that colored tattoos inflict more negative reactions than black tattoos [17]. This finding is supported by numerous studies that suggest red colors and shades as the key players in tattoo associated allergic reactions [28-32]. Organic pigments are often manufactured for the intended use in cosmetics, paints or lacquer, not for the use in tattooing [23]. Therefore, pigments are sometimes coated, *i.e.* their surface is modified, resulting in altered bioavailability and physicochemical behavior. Organic pigments can be categorized in a handful major groups by their chemical structure [33]. Today, the main chemical classes are quinacridones, phthalocyanines, and azo [22].

As tattoo pigments are heterogeneous in their chemical structure and thus in their properties and associated risks, it is a difficult but important task to establish meaningful regulation for tattoo pigments This, however, also applies to other ink components used in tattooing.

1.3. Tattoo regulation in Germany

The data collected in tattoo research aims to make tattooing as safe as possible for tattooed individuals by identifying potential threats. However, this data needs to be translated into meaningful regulation at the legislative level.

Until 2009, tattoo inks were only regulated under the Food and Feed Code (German: ‘LFGB: Lebens- und Futtermittelgesetzbuch’). This law does not contain specific requirements for tattoo inks but required their safety in general [33]. In 2003, a resolution on tattoo safety was developed at the European level and refined in 2008 (ResAP(2008)1). It was implemented into German law as the Tattoo Agent Regulation (German: ‘Tätowiermittelverordnung’). From then on, tattoo pigments that released

carcinogenic aromatic amines listed in the Tattoo Agent Regulation and pigments from Annex II and IV of the EU Cosmetics Regulation were banned. The major flaw of this regulation was that all non-listed pigments could be used in tattoo inks. In addition, vector fluids were broadly unaffected by this regulation and no limits were set for impurities and pollutants such as nickel and polycyclic aromatic hydrocarbons (PAHs).

In 2017, the European Chemicals Agency (ECHA) submitted a restriction proposal to the European Commission under the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) process. The aim was to restrict 'the placing on the market of certain chemicals in tattoo inks and permanent make-up' [34]. The restriction process was accompanied by a public discussion on whether Pigment Blue 15:3 and Pigment Green 7, two pigments that would be affected by the restriction, should be banned from use in tattoo inks. Against this background, the German Federal Institute for Risk Assessment (BfR) evaluated potential risks of these two pigments and concluded that there are insufficient data to evaluate them under REACH. However, the BfR also determined that the risk of these pigments should be considered low, especially since they have been used for decades without any known dangerous consequences [35]. In addition, pigments that could replace these pigments could pose unknown risks. BfR also pointed out that even if the data were sufficient for an assessment under REACH, there is no guarantee of safety for these pigments and their use in tattooing, as tattooing is not a registered use under REACH.

The fate of tattoo pigments is of outstanding importance when assessing the risks associated with tattooing.

1.4. The fate of tattoo pigments

As already described, the skin is injured during the tattooing procedure and the ink and the contained tattoo pigments are introduced into the deeper layers of the skin. The amount of pigment deposited in the skin is dependent on various factors, like the concentration of the pigments in the ink, the size of the pigment crystals [36], the color strength of the pigments, the color strength the tattooist wants to introduce into the skin and also most likely of the machines and techniques used during tattooing. A study especially designed to answer the question how many tattoo pigments are deposited in the skin calculated a mean value of 2.53 mg per cm² skin [36]. However, this mean values was calculated using different sources of Pigment Red 22, different needles and tattooists with different skill levels [36]. The mean value of 2.53 mg per cm² skin therefore is a rough estimate of all processes, with the lowest concentration of 0.6 mg per cm² skin deposited by a trained tattooist and the highest concentration of 9.42 mg per cm² skin deposited by a scientist. Finally, the previously mentioned factors affecting the amount of pigment deposited could result in different *in vivo* values for other pigments. However, this estimate is the only available indication of the pigment concentration initially deposited into the skin during tattooing.

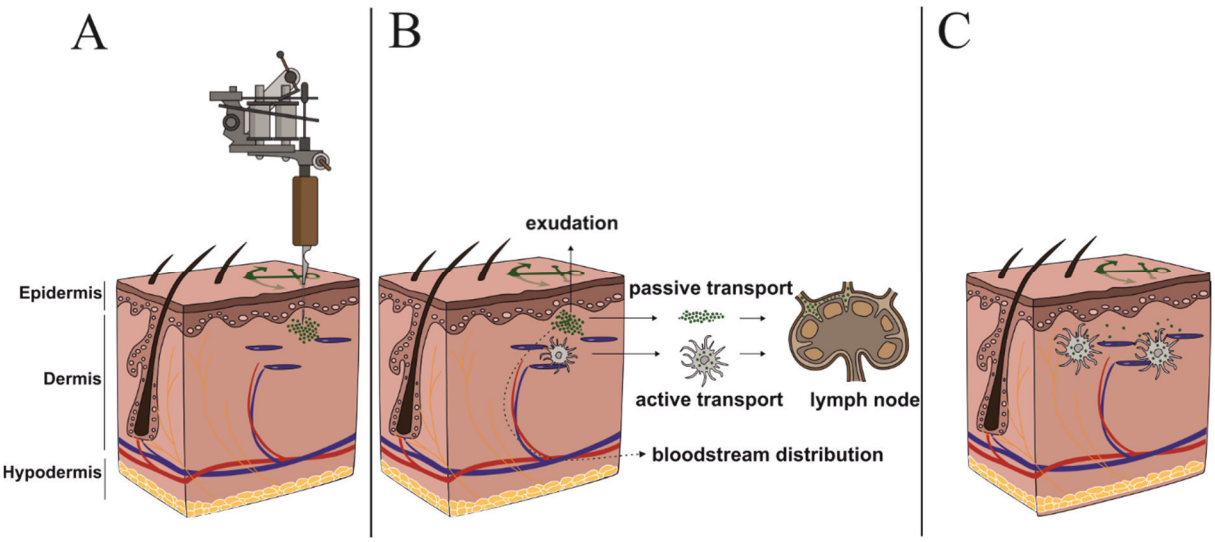

Figure 1: **Fate of tattoo pigments**. The fate of tattoo pigments is illustrated as far as it is understood. A) During tattooing, tattoo pigments are introduced into the dermis. B) Due to the wounding of the skin, immune cells migrate into the tattooed skin area and take up the pigments. Through active and passive transport, tattoo pigments are sequentially transported to local lymph nodes. In addition, some of the tattoo pigments leave the skin by exudation over the injured surface. However, it is not known whether distribution of pigments via the bloodstream occurs after tattooing in humans. C) After wound healing, tattoo pigments persist in human skin, taken up by human dermal macrophages (gray) and dermal fibroblasts (blue). (Figure adapted from [37].)

A follow-up study used this initial tattoo pigment concentration and compared it to concentrations found in red tattoos of deceased tattooed individuals [38]. They found pigment concentrations ranging from 0.002 to 0.15 mg per cm² of skin, with a mean (across all pigments) of about 0.077 mg/cm². Therefore,

they calculated a loss of over 85 % of all tattoo pigments, whether they compared it to 0.6 mg per cm² (87 % loss) or 9.42 mg per cm² (99 % loss). This raises the question of what happens to most pigments after they are injected into the skin.

Human skin histology and early fate of tattoo pigments

Most tattoos today are performed on the human skin. It is the second largest organ and covers the entire human body. Its thickness, microbiome and histology vary not only between individuals and genders, but also within an individual depending on the body site. Human skin plays a critical role in protecting the body from pathogens, chemicals, and (ultraviolet) radiation. It is composed of three layers.

The outermost layer is the epidermis and consists mainly of keratinocytes, which are closely linked together by tight junctions. In addition to keratinocytes, the epidermis also contains melanocytes, Langerhans cells and Merkel cells. It is composed of partial layers of differentiated keratinocytes, with the cornified layer, the *stratum corneum*, facing outward as a result of the differentiation process. On the other side of the epidermis, the *stratum basale* is attached to the basement membrane, which connects the epidermis to the dermis.

The dermis is composed of a complex matrix containing collagens, elastic fibers such as elastin and fibrillin, and an extrafibrillar matrix. It contains fibroblasts, mast cells and macrophages. In healed tattooed human skin, the tattoo pigments are in the dermis.

Below the dermis lies the subcutaneous tissue, the hypodermis. It is heavily supplied with blood and consists of loose connective tissue. Its main function is fat storage. Therefore, adipose cells can be found in addition to fibroblasts and macrophages in the hypodermis.

During tattooing, tattoo ink is injected into the skin by piercing the epidermis and upper dermis with tattoo needles. Immediately after the injection, a fraction of the tattoo pigments leaves the skin through the injured surface by exudation [39]. The wounding causes immune cells to migrate into the wound, and it is likely that they transport some of the tattoo pigments away from the tattooed skin, which is supported by tattoo pigments found in local lymph nodes [21, 37, 40, 41]. In addition, a study in which they found tattoo pigments in liver copper cells from heavily tattooed mice makes the distribution of tattoo pigments via the bloodstream plausible [42]. However, mouse skin is much thinner than human skin and the microscopic images in the study show deposition deep into the dermis and partially into the subcutaneous tissue. Deposition in the highly vascular subcutaneous tissue is an undesirable outcome in tattooing and probably promotes blood flow distribution. However, there is no reliable data on whether blood flow distribution, and thus systemic distribution of pigments, occurs during tattooing or afterwards. The soluble parts of the tattoo ink, on the other hand, are most likely distributed through the bloodstream and lymphatic system and ultimately excreted.

Not all tattoo pigments are initially deposited in the dermis but remain stuck in the epidermis. However, during wound healing, the epidermis is renewed and cleared of tattoo pigments. In contrast, a portion of the tattoo pigments remains in the dermis and forms the tattoo. Overall, a study using a mouse model system estimated a loss of approximately 32 % of the originally applied tattoo pigments over a period of six weeks [46]. While it is not fully understood how tattoo persistence can be explained, there are some theories as to why tattoo pigments are not completely removed from the dermis.

Tattoo persistence

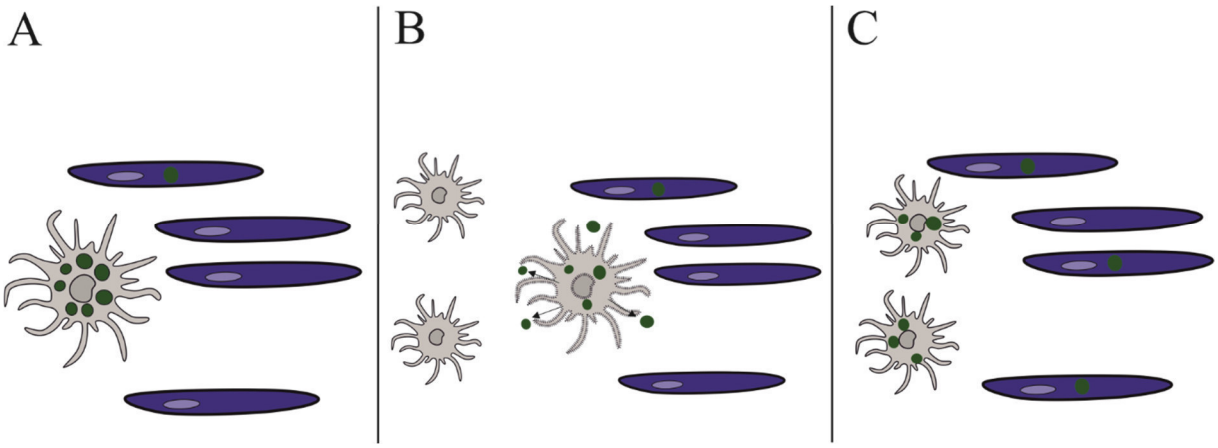

Figure 2: Macrophages and fibroblasts might contribute to tattoo persistence in different ways. Tattoo persistence has generally been explained by longevity of cells. Recent data from rodent model system suggest a capture-release-recapture model for tattoo persistence [43, 44]. A) Tattoo pigments are captured by both, macrophages (gray) and fibroblasts (blue), with macrophages taking up higher amounts of pigments per cell. B) In case of macrophage cell death, tattoo pigments are released from macrophages. C) Tattoo pigments are recaptured mainly by new macrophages, but also by fibroblasts, thus ensuring the persistence of the tattoo.

In the dermis, tattoo pigments are mainly found to be taken up by fibroblasts and macrophages [45]. The persistence of tattoos has generally been explained by the longevity of the cells that take up the pigments, or by the fact that the pigment agglomerates are too large to be removed. In 2018, Baranska and colleagues postulated a model that could explain tattoo pigment persistence by other means [44]: They used a novel mouse model for diphtheria toxin (DT)-induced depletion of macrophages without triggering inflammatory responses. After depletion, they demonstrated that dermal macrophages are completely replaced by blood monocytes from bone marrow. Using this model, they consecutively tattooed the tail of the model mice and were able to show that the pigments were found in the dermal macrophages. Furthermore, they were able to show that after DT-induced depletion, the dermal macrophages were not only replaced by blood-derived macrophages; but that the new macrophages also took up leftover tattoo pigments. No macroscopic change in the tattoo was observed after DT-induced depletion. Using these data, Baranska and colleagues demonstrated that tattoo pigments may undergo consecutive cycles of capture-release-recapture by dermal macrophages.

In 2020, Strandt and colleagues [43] identified a key role for fibroblasts in tattoo persistence using the same mouse model previously used by Baranska and colleagues [44]. While a small number of macrophages take up a large amount of pigment, a high number of dermal fibroblasts take up only a few pigment particles each. After DT depletion of macrophages, the total number of pigment-bearing fibroblasts increased, but not the pigment load per fibroblast. Strandt and colleagues thus demonstrated that fibroblasts and dermal macrophages contribute differently to tattoo persistence.

While future studies need to clarify whether tattoo persistence in humans can be explained by a similar capture-release-recapture model, natural clearance of especially organic tattoo pigments in tattooed human skin has been observed. However, this clearance cannot be explained by the mechanism of tattoo persistence and must be evaluated separately.

Natural clearing of tattoo pigments

While most people do not experience any subjective color change in their tattoo color [17], it has been calculated that over 85 % of originally applied organic red tattoo pigments can be lost over the years [38]. One study showed that up to one-third of applied azo pigment Red 22 can be lost during initial wound healing in the first six weeks after tattooing [46]. Therefore, it must be concluded that tattoo pigments are subsequently removed from the dermis after the initial wound healing process. While there are no information on the time frame in which systemic distribution via the blood or lymphatic system may occur during tattooing, there is evidence to support the clearance of organic tattoo pigments from human skin by photodecomposition.

In general, cleavage of tattoo pigments by (artificial) sunlight has been reported, especially for azo pigments [47, 48] (see Section 1.5, ‘Photo-induced toxicity of tattoo pigments’). Moreover, the same study that calculated the loss of one third of tattoo pigments within the first 6 weeks showed that the loss of pigments is increased to 60 % during the same time when mice were treated with UV light [46]. Therefore, the previously found severe loss of organic (azo) pigments can be attributed to (sun)light-induced cleavage of pigments. Whether this extent of clearance can also be observed in more photostable pigments is unclear. While light-induced cleavage of tattoo pigments may play a critical role in the natural clearing of tattoo pigments, it is an inherent part of the gold standard for tattoo removal: laser treatment.

Laser removal – the gold standard of tattoo removal techniques

Sometimes a tattoo becomes unwanted, leading to the need for tattoo removal. While the actual motivations for removing a tattoo vary widely, in a survey that addressed this issue, at least 6 % reported medical problems [49]. Laser tattoo removal was first used in the late 1960s. Since the early procedure often resulted in significant damage to the surrounding tissue and produced only suboptimal results, it

was not until the use of modern Q-switched lasers that laser removal was defined as the gold standard it still is today.

In modern laser removal, tattoo pigments are irradiated with Q-switched lasers of different wavelengths that emit photons in a nanosecond or picosecond pulse, which are successively absorbed by the tattoo pigments [33]. Although tattoo pigments are in the nanometer range and therefore cool quickly, nano- and picosecond pulses are short enough before thermal relaxation, the time it takes for the pigment to cool to 50 % of its heat, passes. As a result, tattoo pigments are heated with each laser pulse until thermolysis occurs. This process was first described in the 1980s and termed 'selective photothermolysis' [50]. The mechanism leading to clearance of tattooed skin after photothermolysis is not fully understood. While organic pigments may be decomposed at the molecular level, mechanical decomposition of pigments with subsequent removal from the tattooed area by immune cells is also discussed.

While laser tattoo removal is considered the gold standard, 15 % - 33 % of patients are dissatisfied with the results and complete removal can be achieved in only one-third of patients [49, 51]. In addition, tattoo laser removal is painful, with most patients reporting moderate to severe pain during treatment. Small and severe scarring and hyper- or hypopigmentation may also occur afterwards [49, 51]. In particular, if immunologic reactions to tattoo pigments or their cleavage products occur (see section 1.5, 'Immunologic complications'), other tattoo removal techniques that completely remove the pigments from the body, such as surgical removal or dermabrasion, should be considered.

1.5. Tattoo-associated side effects

As the number of tattooed people has increased, evidence of tattoo-related side effects has become more apparent. An Internet survey of 3411 participants in German-speaking countries found that 67.5 % suffered from skin problems after tattooing [17]. In 8 %, complications persisted four weeks after the first tattoo. 6 % reported persistent skin problems and 3 % even showed psychological problems or general light sensitivity [17]. However, recent data have identified photoinduced reactions as probably the most important group of adverse reactions associated with tattooing, with a prevalence of approximately 52 % - 58 % [52, 53].

Photo-induced toxicity of tattoo pigments

In healed, tattooed skin, the tattoo pigments are mainly located in the dermis, where they are exposed to environmental influences such as UV radiation. While a photon with less energy has a longer wavelength and the penetration depth into human skin is antiproportional to the wavelength, roughly 30 % of high-energetic UVA can penetrate deep into the dermis [54] and still reach the tattoo pigments. The interaction of tattoo pigments with light can induce or worsen various side effects like swelling, itching, pain and redness as well as allergic reactions [55-58]. The underlying pathomechanisms that result in

those side effects are not clearly understood. However, there are certain interactions between tattoo pigments and (UV) light known, that could be the cause for those hazardous side effects.

Some organic tattoo pigments, especially azo pigments, were shown to be cleaved by natural sun light into harmful substances [48, 59]. Some pigment UV cleavage products were shown to be similar to those identified after laser irradiation and include carcinogens as well as sensitizers [47]. This might explain, why in some cases, immunologic reactions are described after laser irradiation of another, distant tattoo.

In some cases, irradiation with UVR does not induce photo-cleavage, but leads to the generation of reactive oxygen species (ROS). Depending on the tattoo pigment, ROS can be produced by the interaction of the photon with the pigment itself (see Section 1.6, 'Titanium dioxide') or with common contaminants (see Section 1.6, 'Carbon black'). ROS are a group of small endogenously and exogenously produced reactive molecules, that play key roles in the regulation of important cell and tissue functions [60-62]. Under normal conditions, ROS production by endogenous and exogenous sources and its depletion by cellular antioxidant defense mechanism are in equilibrium. An elevated production of ROS beyond this state is called oxidative stress. Oxidative stress can lead to skin ageing and support carcinogenicity through genotoxic and non-genotoxic mechanisms [63-65]. However, the potential connection of tattoo pigments and (skin) cancer is highly debated.

Contaminations, carcinogens, and cancer

Till today, there is no proven connection between tattoo inks and cancer. A review of published cases tried to render the connection of cancer coincidental [66]. This review, however, was limited to skin cancers co-located to tattoos leaving out systemic carcinogenicity. Nevertheless, tattooing introduces various chemicals into the skin, some of which may be or become bioavailable, leading to an increased risk of (non-skin) cancer.

While the market for tattoo inks has grown over the decades, tattoo pigments are often not specially designed for the use in tattooing. Therefore, contaminations due to educts, by-products and impurities depending on the synthesis are common [39]. Tattoo inks containing various hazardous chemicals have been identified on the European market. These inks contained PAHs (43 %), primary aromatic amines (PAAs, 14 %), heavy metals (99 %) and preservatives (6 %) [27], from which especially PAHs and PAAs are known to support carcinogenicity.

While tattoo pigments should not react under normal circumstances, photoinduced cleavage of tattoo pigments could result in the release of carcinogens into the ink during storage. In addition, manufacturers may coat pigments to enhance their function for their intended use, which is often known only to the manufacturer and can result in purities below 90 % [39]. While the presence of carcinogens in tattoo inks and the release of such from certain tattoo pigments is of great concern, there is insufficient epidemiological data to prove causality. Nevertheless, a toxicological assessment of a 400 cm² tattoo

releasing the potential carcinogen 3,3'-dichlorobenzidine showed a calculated significant increase in lifetime cancer risk [67]. Finally, it is unclear whether and how tattoos affect carcinogenicity, and further epidemiologic studies are needed.

Beside carcinogens, a variety of other harmful substances was identified in tattoo inks. For example, the presence of the reproduction toxin dibutyl phthalate was found in black tattoo inks [68]. In addition, potent metal allergens like nickel, cobalt and chromium were identified in tattoo inks [27, 37]. Again, photoinduced cleavage of organic tattoo pigments could release additional sensitizers and allergens into the ink, leading to immunological complications.

Immunologic complications

Nowadays, allergies are a known side effect of tattooing. Allergic reactions to inks contaminated with chromium, nickel or cobalt have been reported and confirmed by a patch test [69, 70]. In addition to contaminations from tattoo ink, nickel and chromium have also been reported to be released into the skin from the abrasion of tattoo needles [21]. While contamination- and abrasion-induced allergic reactions may be directly facilitated, allergic reactions to tattoos often do occur long after the tattooing process [57]. A study analyzed tattoo pigments used in tattoo inks and concluded that 25 % of the identified pigments were suspected to cause contact dermatitis [24]. In these cases, organic pigments and their cleavage products are heavily discussed as potential allergens, *i.e.* the delayed hypersensitivity against red (azo) pigments in general is reported [58]. Contrary to soluble contaminations, patch testing for tattoos is complicated, especially since potential allergens are only released after UV irradiation. Therefore, photo patch testing is discussed as an promising alternative [71]. In contrast to the patch test, intradermal testing showed positive reactions against a red ink suspected of causing an allergic reaction in a patient [57]. However, intradermal testing is less convenient for subjects than patch testing.

Beside allergies, the auto-immune disease psoriasis, and its connection to tattooing is highly discussed. In literature, there are several case reports of tattoo-induced Köbner phenomenon in patients suffering from psoriasis [72-76].

Infectious diseases

During tattooing, the skin is injured and its barrier function against pathogens is impaired. Under non-sterile conditions, this can lead to microbial infections in the individual being tattooed. A systematic review of the published literature revealed 67 published cases of microbial infections between 1984 and 2015 with a large number of severe bacterial infections ranging from local skin infections such as necrotizing fasciitis to systemic infections such as endocarditis and septic shock [77]. However, it is likely that the dark figure is even higher, since only serious bacterial infections are published.

The main factor for infections is the hygiene applied during tattooing. Although hygiene standards have increased in recent decades, the literature still contains reports of epidemic outbreaks of, for example, tattoo-associated non-tuberculous mycobacterial skin infections [78]. Systematic reviews revealed tattooing as a risk factor for the transmission of hepatitis B and C, especially in prison, where the number of infected people is high and the hygiene standards are low [79, 80].

Hygiene standards also must be applied during production and storage of tattoo inks. Several reports of microbial contaminations of tattoo inks, sometimes even in unopened bottles [27, 77, 81-86] show, that the inks themselves can be sources for infections.

1.6. Associated risks of selected tattoo pigments

As previously described, there are various risks associated with tattooing. While some of them, such as infections, are independent of the pigment used, some pigments pose risks due to their chemical structure, their interaction with UV radiation, or their manufacturing process. Kown risks are described below for the main tattoo pigments studied in this work.

Carbon black

Carbon black is a paracrystalline form of carbon, consisting > 97 % of elemental carbon arranged as aciniform particulate [87]. Its C.I. is 77266 and its size ranges from tens to a few hundred nanometers [88]. While older tattoo inks used metal oxides as pigments, modern tattoo inks mostly use carbon black [22]. Since a survey showed that black tattoo ink is used in about 60 % of all tattoos [17], it is probable that carbon black is the most used (inorganic) pigment today.

The International Agency for Research on Cancer (IARC) lists carbon black as ‘possibly carcinogenic to humans’ [88]. However, this evaluation is mostly based on the possibility of carbon black inducing lung cancer in humans and animal models. The underlying pathomechanism is thought to include impaired clearance resulting in an accumulation of carbon black particles in the lung. This is followed by inflammation, cell injury and finally the production of ROS, which might lead to carcinogenic mutations [88]. While no direct evaluation of the effects of carbon black on (human) skin was performed, there were several data to support skin cancer development due to the dermal or subcutaneous application of carbon black extracts or contaminated carbon black particles. These contaminants are byproducts of the manufacturing of carbon black, the controlled vapor-phase pyrolysis of hydrocarbons [88]. The most common contaminant are the PAHs [89].

PAHs are linked to the mutagenesis of oncogenes in mouse skin [90], the impairment of the functional activation of lymphocytes [91], and the inhibition of macrophage differentiation [92]. In 2010, the IARC classified one PAH as Class 1 carcinogen: Benzo[*a*]pyrene [93]. Its active metabolite, Benzo[*a*]pyrene-7,8-diol-9,10-epoxide, is sometimes seen as ‘the ultimate cancerogen’ [94]. From the other 59

investigated PAHs, 3 were classified as class 2A carcinogens and 11 as class 2B carcinogens, while 45 were classified as class 3 (not classifiable) [93]. PAHs concentrations up to 201 µg/g were found in black tattoo colorants [95]. While PAHs are linked to diverse cancers in lung, bladder, liver and skin, the effect of black ink contaminated with PAHs is unclear.

In 2015, a study tried to unveil the possible impact of tattoo ink contaminated with PAHs on carcinogenicity [96]. In this study, naked C3.Cg/TifBomTac mice were tattooed with black tattoo ink containing high amounts of PAHs [96]. Tattooed and control mice were then separated again into two groups, from which one was consecutively irradiated with three standard erythema doses UV three times a week. In the 4 resultant groups (1. Tattooed and irradiated, 2. Tattooed and not irradiated, 3. Sham-tattooed and irradiated and 4. Sham-tattooed and not irradiated), no development of cancer was seen in unirradiated mice due to the black ink. In the irradiated mice however, the black tattoo ink delayed the onset of cancer by 50 days. Moreover, the tumors were identified as squamous cell carcinomas, which are localized in the epidermis. The epidermis however was clear of pigments. The authors hypothesized that the dermally located carbon black particles reduced the backscattering of incoming light by the dermis, which then resulted in a reduced net-irradiation. In 2017, they tested this hypothesis by measuring the reflection of UVB-irradiation from mouse skin tattooed with the ink used in 2015 and red tattoo ink [97]. In both cases, tattooed skin showed lower UVB reflection than untattooed mouse skin. While this data supports the initial hypothesis, the certain mechanism behind the photoprotection of epidermal tissue by underlying tattoo pigments remains unknown.

Titanium dioxide

TiO_2, also known as Pigment White 6, is an oxide of titanium with the C.I. of 77891. A study revealed titanium dioxide as the most used white pigment [98]. It is not only used in white tattoo inks, but also in colorful tattoo inks to generate different shades of the initial color [23]. TiO_2 can be found in three distinctive major crystal structures: anatase, rutile and brookite. Because brookite is not used industrially, it is not surprising that only anatase and rutile particles can be found in tattoo inks and specimen from tattooed human skin [21, 37, 99]. The IARC lists TiO_2 as possibly carcinogenic to humans (2B) [100]. This classification is mostly based on the ability of TiO_2 to induce respiratory tract cancer in whisker rats exposed by inhalation and intratracheal instillation. The main mechanism TiO_2 can induce carcinogenicity seems to be through secondary genotoxic mechanisms that involve chronic inflammation and oxidative stress [100]. However, nanosized titanium dioxide may directly interact with human DNA, produce reactive oxygen species (ROS) and induce oxidative DNA damages like 8-hydroxyl-2-deoxyguanosine or 8-OHdG [101]. While the generation of ROS was seen by both, anatase and rutile particles, anatase particles produced significantly more superoxides upon UV irradiation.

This difference is caused by the difference in the band gap between anatase and rutile particles: Upon UV irradiation, TiO_2 absorbs photons with energy similar or greater than its band gap (3.0 eV for rutile,

3.2 eV for anatase). This leads to the excitation of an electron from the valence band to the conductor band, creating an electron – hole pair. The excited electrons in the valence band consequently reduce substrates, *i.e.* oxygen, resulting in the formation of superoxide radical anions. Contrary, the hole in the valence band can oxidize substrates such as water or hydroxide ions and generate hydroxyl radicals.

Additionally, TiO_2 is known to have photocatalytic properties [102], which alter the photodecomposition of organic tattoo pigments [33]. This could prove problematic, as TiO_2 is used in a variety of inks to change the color shade.

Pigment Orange (P.O.)13

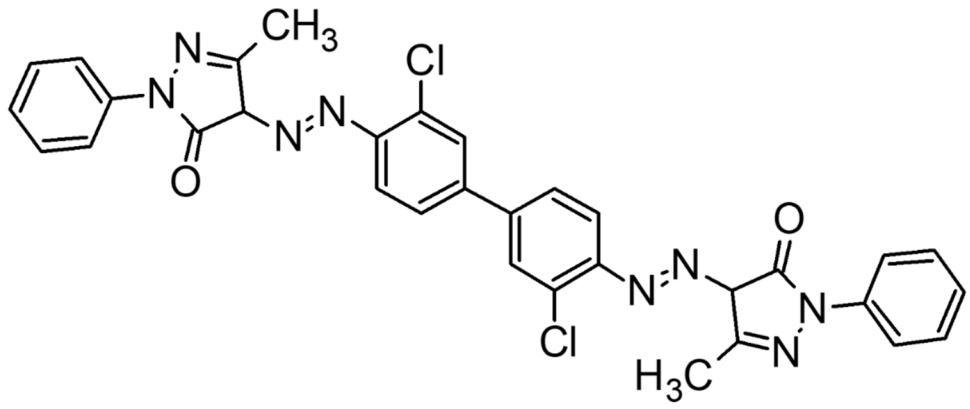

Figure 3: **Chemical structure of Pigment Orange (P.O.)13.** The chemical structure of the diazo P.O.13 reveals its ancestry from 3,3'-dichlorobenzidine, whose backbone is clearly visible between the two azo bonds (N=N).

P.O.13 is an organic diarylide pigment with the C.I. 21110. It is produced by the reaction of 3,3'-dichlorobenzidine with acetoacetanilide derivates, resulting in the formation of two double nitrogen bonds (N=N), which are defining for azo pigments (**Figure 3**). P.O.13 became of special interest after it was identified in dermatome biopsies from patients with chronic allergic reactions against tattoos [32]. Azo bonds are known to be easily cleaved by light. Furthermore, P.O.13 was shown to release 3,3'-dichlorobenzidine and 3,3'-dichlorodiphenyl after irradiation with different light sources and laser [47]. The release of 3,3'-dichlorobenzidine from a 400 cm² tattoo was assessed and the calculated increase in life time cancer risk was significant [67].

Pigment Yellow (P.Y.)138

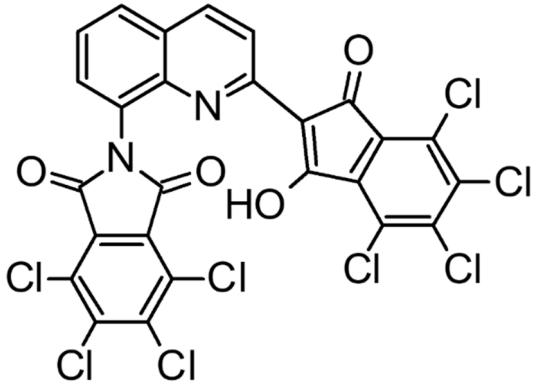

Figure 1: Chemical structure of Pigment Yellow (P.Y.)138.

P.Y.138 is a quinaphthalone with a CI of 56300 and used as tattoo pigment especially in tattoo inks manufactured in Germany [103]. Pyrolysis of P.Y.138 revealed carcinogens as hexachlorobenzene as possible laser cleavage products [103]. Concerning UV-irradiation, quinaphthalone pigments are thought to be photostable, especially when compared to azo pigments.

1.7. Tattoo toxicology research and its cellular model systems

The increase in tattooed people worldwide and the growing knowledge of tattoo-associated side effects shaped tattooing as a field of scientific interest. Since 1999, over 100 publications are published yearly in the highly interdisciplinary field of tattoo research, with increasing numbers of publication each year (**Figure 5**). The identification of possible pathomechanisms associated with tattoo pigments is one of the main research-aims in tattoo research. Therefore, different model systems are used in tattoo toxicology research to mimic human cells or tissues that are in contact with the tattoo inks, pigments, or their cleavage products. Each model system, however, bears different advantages and disadvantages.

Mammalian monolayer cell culture describes a variety of techniques to grow cells under controlled conditions outside of their host species. To do so, cells are isolated from the host tissue and maintained in culture media. Consequently, cells can either be used directly (primary cells) or after immortalization and clonal expansion (cell lines). Mammalian monolayer cell culture is widely used, since it is cheap, easy to handle, fast to perform and the factors of interest (*i.e.*, chemical concentration) are easy to control.

Most tattoo toxicology related studies utilizing monolayer cell cultures use either fibroblast or keratinocyte cell lines derived from either, human or mouse. Independent of origin species, monolayer cell culture has some disadvantages, especially for tattoo toxicology research regarding tattoo pigments. Agglomeration and sedimentation of tattoo pigments might occur in cell culture medium, which negatively affect cell growth. In addition, these effects will increase the concentration of tattoo pigments available to the cells, leading to an overestimation of toxic effects [104, 105]. Finally, the lack of

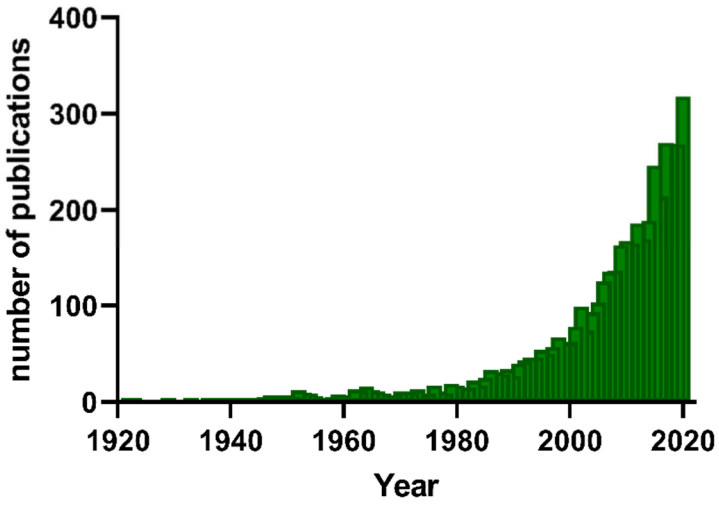

Figure 2: Increase in tattoo related research paper reveals tattoo research as a growing research field. A database search was conducted for scientific publications that contained ‘tattoo’, ‘tattooing’ or ‘tattooed’ in either the title or abstract on PubMed® (https://pubmed.gov). Results are shown as the number of publications for each year.

histological features like the extracellular matrix (ECM), which are present *in vivo*, limits the applicability of 2D cell cultures for tattoo toxicology research. This is particularly the case for phototoxicity studies, in which the dermal placement of the tattoo pigments might be of concern. Generally, monolayer cell culture cannot be used to investigate biodistribution or chronic effects.

To overcome the restrictions of monolayer cell culture and to mimic the *in vivo* situation properly, a high number of studies utilized animal models for tattoo toxicology research. The most used animal models in tattoo research are mice and rats, which are almost exclusively used. While animal models are the only model systems at the moment that can be used to investigate biodistribution, they are expensive and must be considered unethical to be utilized in tattoo research. Beside moral and economic reservations, the limited transferability of animal testing, as revealed by several meta-analyses, questions the general use of animal models for tattoo toxicology research [106]. For tattooing in special, mouse animal models have to be considered suboptimal. As mentioned earlier, mouse skin is thinner than human skin and thus might result in deposition of tattoo pigments in subdermal tissue. This could increase bloodstream distribution and result in overestimation of systemic bioavailability of pigments. Moreover, only 30 % homology between mouse and human skin-associated genes [107], strong differences in wound healing [108] and in the (skin) immune system [109, 110] might have unknown impacts on tattoo toxicology research. However, it has to be noted, that there are several attempts to make the mouse skin more human-like, *i.e.* skin xenografts [111].

3D full thickness human skin models

Somewhere on the scale between 2D monolayer culture and animal models, are *in vitro* organoids and skin explants. Skin explants describe *ex vivo* cultured human skin, mostly obtained as by-product of surgeries. Skin explants therefore are superior models in terms of skin histology and transferability but have a highly limited availability. This limited availability may be the reason why no studies on the toxicology of tattoos using these model systems have been published to date.

On the other side, *in vitro* organoids for human skin are already commercially available. Human skin organoids are also called 3D skin models or 3D skin equivalents, while the attribute ‘full thickness’ shows that they contain both, an epidermal and a dermal layer. While reconstructed epidermis models are widely used in phototoxicity [112] and skin barrier research [113], they are not feasible to mimic *in vivo* tattooed skin due to their missing dermis. However, there are studies published that aimed to estimate phototoxic properties of tattoo pigments with reconstructed epidermises [114].

There are two general types of full thickness skin models: scaffold-free and scaffold-based models [115]. Scaffold-free models are non-adherent cell aggregates of one or more cell types. Dermal ECM is completely produced by fibroblasts, resulting in micro-tissue with small dermises, that are consequently not feasible for tattoo pigment deposition - and therefore for tattoo toxicology research.

In contrast, in scaffold-based models, cells are grown in the presence of a supporting scaffold that allows the models to exhibit structural, functional, and mechanical similarity to human skin [116, 117]. Scaffolds can be compromised of various polymers from natural or synthetic origin. Due to their architectural homology to human skin, scaffold-based models are promising model systems for tattoo toxicology research. However, the mechanical forces that are exerted during tattooing might lead to the destruction of these skin models. Even if tattooing could be done properly, the consecutive wound healing process will most likely exceed their culture lifetime. In addition, reproducible tattoo pigment dosing might be challenging with common tattooing techniques. Even though promising, till today no study was published that utilized tattooed full thickness human skin models.

1.8. Aim of this thesis

Nowadays, tattooing has become one of the most popular body modifications around the globe. Contrary to popular belief, tattooing carries a lot of risks, the consequences of which can occur long after the initial tattooing process. Laser removal of unwanted tattoos has been identified as a possible trigger for adverse reactions against tattoos.

To evaluate risks associated with tattoo laser removal, we irradiated *postmortem* tattooed pig skin and aqueous solutions of P.Y.138 and P.O.13 with laser commonly used in tattoo removal. We consequently identified and quantified their laser cleavage products in pig skin and treated fibroblasts and keratinocyte cell lines with similar concentrations to assess possible cyto- or genotoxicity (see Section 2.1 'Laser Irradiation of Organic Tattoo Pigments Releases Carcinogens with 3,3'-Dichlorobenzidine Inducing DNA Strand Breaks in Human Skin Cells.').

Since the decision to get a tattoo is made for aesthetic reasons in most cases, animal testing for tattoos must be considered unethical. For other aesthetic procedures, such as make-up or hair coloring, animal testing is mostly prohibited in the European Union, which is not the case for tattooing. *In vitro* animal replacement methods are increasingly being used in toxicology research, particularly in makeup research. More dedicated projects utilize three-dimensional organoids, i.e., skin models. However, to date there is no study published that used three-dimensional organoids that considered the specific application, more precisely the dermal localization of tattoo pigments. Therefore, the major aim of this work was to introduce a three-dimensional skin model that considered the localization of tattoo pigments in tattooed human skin, which led to the creation and establishment of the **tat**tooed human full thickness **s**kin model, $TatS_{FT}$ (see Section 2.2 'TatS: a novel *in vitro* tattooed human skin model for improved pigment toxicology research').

Finally, we aimed to use $TatS_{FT}$ and its dermal compartment ($TatS_{DE}$) to investigate the role of tattoo pigments in photoinduced toxicity (Section 2.3 'Phototoxic *versus* photoprotective effects of tattoo pigments in reconstructed human skin models'), since light-induced side effects account for majority of tattoo-related side effects. During both establishment and use in our phototoxicity study, the results obtained with $TatS_{FT}$ were compared with those obtained from monolayer cell culture. Therefore, we were able to show the similarities and differences as well as the advantages and disadvantages of the culture methods employed.

The results of this work should shed a light on phototoxicity of tattoo pigments. Moreover, $TatS_{FT}$ and $TatS_{DE}$ could act as the basis for the replacement of animal testing in (phototoxicity) tattoo research.

2. Results

Results are presented as peer-reviewed publications. They are presented in a logical order to simplify the readability and comprehensibility of the results.

Each publication represents a separate chapter, which is why abbreviations and references are defined within those. While the actual research work, I was involved in is presented in the "Author Contributions" of the publication (if available), my involvement in the particular publication is also presented as a percentage at the beginning of each section for ease of evaluation.

2.1. Laser Irradiation of Organic Tattoo Pigments Releases Carcinogens with 3,3'-Dichlorobenzidine Inducing DNA Strand Breaks in Human Skin Cells.

Hering H., Sung A.Y., Röder N., Hutzler C., Berlien H.P., Laux P., Luch A., Schreiver I.

This chapter was published online on 20.06.2018 in:

Journal of Investigative Dermatology

Volume 138, Issue 12, December 2018, Pages 2687-2690

DOI: https://doi.org/10.1016/j.jid.2018.05.031

Link: https://www.jidonline.org/article/S0022-202X(18)32222-X/fulltext

Involvement of the author within this publication: Project planning (50 %), project execution (50 %), data analysis (50 %), writing of the manuscript (60 %).

Supplementary materials for the following publication are entailed in Annex I.

2.2. TatS: a novel *in vitro* tattooed human skin model for improved pigment toxicology research

Hering H., Zoschke C., Kühn M., Gadicherla A. K., Weindl G., Luch A. & Schreiver I.

This chapter was published online on 13.07.2020 in:

Archives of Toxicology

Volume 94, Issue 7, July 2020, Pages 2423–2434

DOI: https://doi.org/10.1007/s00204-020-02825-z

Link: https://link.springer.com/article/10.1007/s00204-020-02825-z#citeas

Involvement of the author within this publication: Project planning (80 %), project execution (80 %), data analysis (100 %), writing of the manuscript (90 %).

Supplementary materials for the following publication are entailed in Annex II.

2.3. Phototoxic *versus* photoprotective effects of tattoo pigments in reconstructed human skin models

Hering H., Zoschke C., König F., Kühn M., Luch A. and Schreiver I.

This chapter was published online as Journal Pre-Proof on 22 July 2021 in:

Toxicology

Available online 22 July 2021, 152872

DOI: https://doi.org/10.1016/j.tox.2021.152872

Link: https://www.sciencedirect.com/science/article/abs/pii/S0300483X21001955

Involvement of the author within this publication: Project planning (75 %), project execution (90 %), data analysis (100 %), writing of the manuscript (80 %).

Supplementary materials for the following publication are entailed in Annex III.

3. Discussion

3.1. Laser-induced cleavage products of organic pigments pose a challenge for tattoo risk assessment

As the presence of photoinduced cleavage products of tattoo pigments becomes more acknowledged, their importance in the proper safety evaluation of tattoo pigments becomes apparent. We showed the potential of P.O.13 and P.Y.138 to release carcinogens and sensitizing chemicals upon laser irradiation of *postmortem* tattooed pig skin and aqueous solutions. One of the cleavage products, 3,3'-dichlorobenzidine, also induced cyto- and genotoxicity in fibroblast and keratinocytes cell lines (Section 2.1).

In our study, we quantified the laser cleavage products of P.O.13 and P.Y.138 after treatment with Q-switched ruby and neodymium-doped yttrium aluminum garnet lasers commonly used for tattoo removal. Although pig skin is a waste product produced in the meat industry and institutional approval is not required for its use in research studies, future investigations could omit pig skin due to the high similarity of cleavage products in aqueous suspension and pig skin. Omitting pig skin would simplify the process and allow the laser induced cleavage products to be identified in high throughput. Beside laser treatment of tattoo pigments, a pyrolysis-gas chromatography/mass spectrometry method was shown to predict the cleavage patterns of tattoo pigments accurately [103]. This method could also be used to analyze tattoo inks and pigments in high throughput for potentially hazardous cleavage products.

However, the sheer number of pigments that could potentially be used in tattoo inks could render this a Sisyphean task. In contrast, modern technologies may enable the *in silico* prediction of laser- or UV-induced cleavage products and their potential hazards. Unlike *in vitro* and *in vivo* methods, *in silico* methods are less limited by laboratory materials, labor time, and manpower. Moreover, they complete omit animal harm compared to *in vivo* and even *in vitro* methods if the use of animal products such as fetal bovine serum and others is considered in *in vitro* methods. Therefore, *in silico* methods are a promising area of research for risk assessment and are already used to predict absorption, distribution, metabolism, excretion, and toxicity properties in drug discovery and development [118]. Recently, a machine learning model was published that accurately and rapidly predicts bond dissociation enthalpies for organic molecules, the calculation of which is often required to model the thermal stability and drug metabolism of these molecules [119]. Calculating thermal stability might play a crucial role in predicting laser cleavage products and calculating the consecutive metabolism of the resulting cleavage products is indispensable to assess their risks. Therefore, existing advanced models that allow prediction of drug pharmacokinetics and biodistribution in humans [120] could also be modified to model the fate of laser-induced cleavage products and their influence on humans. However, *in silico* models require extensive data both to predict cleavage products and to predict their fate and effects on the individual. To fill these

data gaps, high throughput methods based on our study design could be performed to build the required data bases.

Similar to the data gaps for potential cleavage products formed during laser irradiation of tattoo pigments, the consecutive biodistribution of these substances is largely unknown today. Consecutively, we only investigated the potential effects of aniline, 3,3'-dichlorobenzidine, and hexachlorobenzene on skin cells in our study, using fibroblast and keratinocyte cell lines. While only 3,3'-dichlorobenzidine was able to induce cyto- and genotoxicity in our study, the systemic impact of all compounds remains uncertain. For 3,3'-dichlorobenzidine, an assessment calculated an increased lifetime cancer risk upon the release from a 400 cm^2 tattoo [67]. Similar assessments need to be performed for other cleavage products of tattoo pigments. However, isolated assessments of these substances may underestimate the risks of laser removal, as co-carcinogenic substances such as hexachlorobenzene have been identified as cleavage products. In general, the importance of regulatory assessment of substance mixtures is recognized in the scientific community, and there are several research activities on combined exposure to multiple substances [121, 122]. While in many cases a co-exposure scenario is only a plausibility, laser removal of organic tattoo pigment will most likely produce pigment-specific mixtures of substances, resulting in a definite multi-substance exposure scenario [47, 103]. Ideally, these mixtures should be subjected to a combined risk assessment, the results of which should subsequently be incorporated into the evaluation of the pigment itself. Because this, again, is rendered a Sisyphean task by the sheer number of tattoo pigments, grouping of tattoo pigments is needed. In summary, tattoo pigments must not only be considered as whole particles, but also as their potential cleavage products to fully assess their associated risks after laser treatment. This, however, will not be realized when tattoo pigments are assessed within REACH.

Although laser removal is now considered the gold standard, there are alternatives that may be more advantageous, especially when considering the potential cleavage products of tattoo pigments. Dermatome shaving and surgical removal and their combined use, for example, completely remove tattoo pigments from the organism. Thus, they are especially beneficial when allergic or other chronic reactions against the tattoo have occurred [123]. In general, these alternatives should be preferred over laser removal, especially for smaller tattoos where surgical removal and dermatome shaving are easily feasible. However, a powerful laser removal industry, the daunting thought of having to undergo surgery and lack of patient education about tattoo removal methods force alternative methods to remain in their niches for now.

However, laser treatment is performed on only a minority of tattoos and there are feasible alternatives. Thus, it is at least arguable that the risks associated with laser cleavage products of tattoo pigments should not rather lead to a restriction of laser treatment. However, this limitation can only be drawn for lightfast pigments that are not cleaved by UV light, such as P.Y.138. For certain pigments, especially azo pigments such as P.O.13, irradiation with both laser and UV light have been shown to result in

similar cleavage products [47]. For pigments that are cleaved by (UV) light, a combined assessment is needed to fully understand their associated risk, as described earlier. In the case of P.O.13, however, the release of a mutagenic carcinogen such as 3,3'-dichlorobenzidine [124, 125] upon irradiation with (UV) light precludes its use as a tattoo pigment, as no exposure threshold can be defined for mutagenic substances.

3.2. Chances and limitations of skin models like $TatS_{FT}$ and $TatS_{DE}$ in tattoo toxicity research

While pigment cleavage upon irradiation with (UV) light should be considered a major risk factor in the interaction of light with tattoo pigments, it is not the only one, as previously described (see Section 1.5 and 1.6). Nevertheless, there are large data gaps on the molecular events that lead to the phototoxicity of tattoo pigments. One reason for this gap might be the lack of feasible model systems. We therefore established $TatS_{FT}$ and used it and its dermal compartment $TatS_{DE}$ to assess the phototoxicity of tattoo pigments *in vitro* in comparison to monolayer cell culture (Sections 2.2 and 2.3).

Three-dimensional skin models are potent model systems to mimic human skin *in vitro*, and there is rampant research on these kinds of models to establish animal replacement methods. While skin models were previously used to test the toxicity of tattoo inks and pigments, these tests were limited to the addition of tattoo ink to the growth medium [126] or topical application to the epidermis [114]. While application via the growth medium could be useful to test the acute toxicity induced by soluble ink fractions, it cannot reproduce the effects of tattoo pigments in the skin. In humans, tattoo pigments reside in the dermis and are internalized by fibroblasts and macrophages. When tattoo pigments are applied topically, the epidermal barrier is reintroduced. Therefore, the pigments come into contact only with outer keratinocytes, which is contrary to the *in vivo* situation where the epidermis is pigment-free after wound healing. These shortcomings in *in vivo* like exposure not only limit the transferability of possible toxic effects detected, but rather expose any negative results as possibly false negatives.

In $TatS_{FT}$, we overcame the previously described problems of tattooing a skin model by introducing pigments directly during the manufacturing process. In the resulting skin models, no pigment induced adverse effects were observed and skin homeostasis markers were comparable to the control. Pigment concentrations were chosen to be in range of those published for P.R.22 in *ex vivo* tattooed skin [36]. While initial concentrations of P.O.13 were 0.2 mg/ml, the final concentration is about 0.7 mg/cm² in $TatS_{FT}$. Professionally tattooed *ex vivo* skin bears about 0.6 mg/cm² of P.R.22 [36]. Since TiO_2 is heavier (and carbon black lighter) than P.O.13, we expected these values to be higher or smaller, respectively. Final concentrations of TiO_2 and carbon black were around 1.4 mg/cm² and 0.07 mg/cm². While only P.O.13 and TiO_2 lie within the range of concentration measured for P.R.22 (0.6 mg/cm² to 9.4 mg/cm²), we also expect carbon black to be in within range of *in vivo* concentrations, as $TatS_{FT}$ with carbon black showed a deep black coloring. However, till today there is a huge gap in knowledge regarding on how

much pigments (and ink) a tattooed person is exposed during tattooing. Future research projects should investigate this point in more detail, as all data on this issue derived from a single study that examined a single pigment. Although that study is invaluable for tattoo research, more effort should be made to address the existing gaps in exposure data and weaknesses of the only study for this issue, as addressed earlier.

While the results of establishing $TatS_{FT}$ were very promising, certain characteristics of an ideal tattooed skin model are missing. Especially the lack of dermal macrophages must be considered a disadvantage, since macrophages were shown to take up a majority of pigments in a tattooed mouse model [43, 44]. However, the modular nature of $TatS_{FT}$ will allow for future incorporation of macrophages, as it is already described for similar skin models [127, 128]. While macrophages are essential due to their awarded role in tattoo homeostasis, an immunocompetent skin model is highly preferable to fully understand the phototoxicity of tattoo pigments. As progress towards an immunocompetent skin model is very promising and $TatS_{FT}$ is highly modular, future integration of additional immune cells should be the focus of future research incentives.

Since a proportion of tattoo pigments are within the nanometer range, findings on the biodistribution of nanoparticles should be transferred and applied to tattoo pigments. This is supported by the hypothesis, that the breakdown of tattoo pigments into smaller pigments during laser treatment, especially in cases of inorganic pigments, might lead to their clearance. Especially particles smaller than 100 nm were shown to drain freely to local lymph nodes after intradermal injection. Unfortunately, studies administering nanoparticles intradermally are severely limited. Nevertheless, biodistribution studies of intradermal applicated nanoparticle-coupled vaccines may indicate potential biodistribution of tattoo pigments. In a study, ultrasmall Pluronic-stabilized poly(propylene sulfide) nanoparticles were administered *inter alia* intradermally in mice. In addition to lymphatic drainage to local lymph nodes, those nanoparticles were found in blood and spleen of mice [129]. They also showed that no nanoparticles could be found in blood of mice that lack peripheral lymphatics, proofing that nanoparticles require lymphatic drainage for systemic biodistribution. Similar findings from a study investigating the biodistribution of vaccine conjugated on zwitterionic NIR nanoparticles (V-NIR-NP) support the described distribution pattern: In this study, the authors administered the V-NIR-NP intradermally and intravenously. While they found huge signals of V-NIR-NP in liver, and spleen after intravenous application, they found that most of intradermally V-NIR-NP are transported to the local lymph nodes, but were also found in heart, lung, liver, pancreas and kidney [130]. Again, these findings were supported by a study using nanoparticle-encapsulated rhodamine B after intradermal application via dissolving microneedles, where they found rhodamine B in liver, kidney, spleen and lymph nodes of the mice injected [131]. In humans, the transport of tattoo pigments to the local lymph nodes is common knowledge. While the only study that showed that tattoo pigments could also reach the liver in mice showed methodological weaknesses, as described earlier (see Section 1.4), data derived from

nanoparticle research render the biodistribution of nanoparticulate tattoo pigments into liver, spleen, and kidney plausible. While *in vitro* skin models are limited in the assessment of biodistribution, the lymphatic drainage was shown to play a crucial role in biodistribution of nanoparticles in mice [129]. However, if the capture-release-recapture model, as postulated for tattoo persistence in mice [43, 44], proves to be applicable to humans, pigment transport after initial wound healing will be minimal because dermal macrophages cannot migrate to draining lymph nodes even under inflammatory conditions [132]. While $TatS_{FT}$ and similar skin models are not able to mimic the lymphatic drainage so far, lymph node models with microfluidics exist [133] that may prospectively be used together with skin models to mimic this key event of tattoo pigment biodistribution *in vitro* in multi-organ chip systems [134]. However, to simulate tattoo persistence accurately in skin, if the postulated models are applicable to humans, $TatS_{FT}$ (or similar skin models) must incorporate immune cells capable to drain to local lymph nodes as well as dermal macrophages.

While $TatS_{FT}$ might be the first step to reduce animal harm in tattoo toxicology research, it still utilizes resources produced from animal components, such as bovine serum and collagen. To fully prevent animal harm in future *in vitro* experiments, resources produced from animal components must be avoided completely. In addition to successful serum-free culture of skin explants [135] and reconstructed epidermises [136], a study that investigated serum-free conditions in skin model culture showed no negative impact on lipid organization of the *stratum corneum* nor alteration of skin permeability of benzoic acid an caffeine [137]. Moreover, a study suggest that defined (serum free) medium might not only better recapitulate growth conditions for fibroblasts, but also reduce variability in cell culture inherent to serum-based cell culture [138].

While research on serum-free culture methods needs to be intensified before they are fully operational, several alternatives exist for bovine collagen replacement. While scaffold-free culture is most likely not desirable, as the dermis may be too small to incorporate tattoo pigments [115], scaffolds can be compromised of various polymers from natural or synthetic origin [116, 117]. In addition, the use of human collagen is a promising alternative [139], as most skin models use collagen-based scaffolds to recreate dermal extracellular matrices today [140]. Additionally, there are several synthetic polymers used as dermal scaffolds. Due to their poor cell adhesive properties, natural-synthetic polymer combinations are developed. However, to prevent animal harm, the sources of the natural polymers have to be chosen carefully. Finally, biodegradable esterified hyaluronic acid fibers allow the fibroblasts to construct their own extracellular matrix [141], extending their culture time by an six-fold increase in comparison to collagen based skin models [142].

In summary, $TatS_{FT}$ is now the only skin model that accounts for the dermal localization of tattoo pigments in human skin, representing an important step in *in vitro* tattoo toxicity research. However, future modifications of $TatS_{FT}$ should incorporate dermal macrophages and immune cells able to

perform lymphatic migration into $TatS_{FT}$ to appropriately incorporate current research on tattoo persistence.

3.3. The absence of phototoxicity in $TatS_{FT}$ and $TatS_{DE}$ is no guarantee for pigment safety

In our study we investigated the effects of UVA and UVB irradiation on 'tattooed' and control $TatS_{FT}$ (Section 2.3). In addition, we performed the same experiments with $TatS_{DE}$ and with monolayer cultured fibroblasts, if experimental setup did not require a 3D environment.

Since photo associated side effect make up to 60 % of all tattoo related side-effects, there are several studies focusing on the phototoxicity of tattoo pigments. Mostly, phototoxicity of tattoo pigments is assessed using monolayer cell culture experiments, measuring oxidative stress, the production of ROS or cell viability [143]. However, monolayer cultures are shown to overestimate toxic effects of particulate test substances like tattoo pigments [105]. In our study, TiO_2 was the only pigment that showed concentration and UV-dose dependent phototoxicity. Phototoxicity was also greater for anatase than for rutile pigments, which we expected. Contrary to our expectation, phototoxicity was not measurable in $TatS_{FT}$ and $TatS_{DE}$. The introduction of the third dimension in cell culture even led to photoprotective effect in $TatS_{FT}$ and $TatS_{DE}$.

As $TatS_{FT}$ represent the only skin model with tattoo pigments located in its dermis, there are no direct studies to compare these results with. While there are no *in vivo* data on humans, some experiments have been performed on immunocompetent C3.Cg/TifBomTac hairless mice to investigate phototoxic effect of tattoo pigments. In both studies, mice were tattooed and repeatedly irradiated with UVR. While one study utilized red tattoo ink containing the contaminant 2-methoxyaniline [97], the other study utilized black tattoo ink with high contents of PAHs [96]. While the study utilizing the red ink identified a weak and potentially not clinically relevant cocarcinogenic effect of the red ink, the study utilizing black ink identified photoprotective effects of the ink, as already described. These results are conclusive with our study, which also suggests photoprotective effects of carbon black in $TatS_{DE}$ in terms of viability after UVA irradiation and UVB-induced cyclobutane pyrimidine dimers. The authors of the black ink-study hypothesized, that the black ink decreased backscattering of incident UV radiation. Contrary to this hypothesis, we did not find a protective effect on the epidermis at all, which could have been visible in both, the immunohistological analysis of UVB induced CPDs, or the viability of $TatS_{FT}$. However, we cannot exclude that these effects are present, but under our limit of detection. Yet, we postulated another hypothesis based on our data, which suggest that only cells beneath the pigments profit from their protection. Therefore, we hypothesized that the photoprotection *in vivo* might be a result of a preserved skin homeostasis. Recently, there has been extensive research that highlighted the role of fibroblasts on skin homeostasis and skin cancer development [144-146].

In our study, we were only able to detect photoprotection in $TatS_{DE}$ in terms of viability. As we discussed, the high number of keratinocytes, that are not protected by the tattoo pigments, explain the absence of photoprotection in terms of viability in $TatS_{FT}$. Future research projects using full thickness skin models for tattoo research should therefore take steps to analyze effects on the viability in a spatially resolved manner. The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) method was shown capable to reveal apoptotic cells in skin explants [147] and normal and tumor oral mucosa models [148]. In addition, we hypothesized that the protection of underlying pigments is absorbance driven and thus, it is plausible that cells in the upper pigmented layer might be exposed to phototoxic effects of pigments like TiO_2 anatase similar to those seen in monolayer culture. For TiO_2 anatase, the production of ROS is known upon the absorption of UV photons. Therefore, sensitive and spatial resolved measurement of ROS should be applied on $TatS_{FT}$ after irradiation with UV light to investigate if there is an area of adverse effects in the spatial resolution of $TatS_{FT}$. Generally, ROS generation in tattooed skin models could be measured by electron spin resonance [149, 150]. However, spatial resolution is needed to reveal ROS in a particular layer of skin models. Therefore, oxidative stress sensitive fluorophores like 2',7'-dichlorodihydrofluorescein diacetate can be utilized for spatial sensitive oxidative stress measurement in $TatS_{FT}$. In addition, immunofluorescence staining of oxidative stress products can be performed, such as the staining for 8-hydroxy-2'-deoxyguanosine, an oxidative stress induced DNA damage which was successfully stained in a human skin model [151].

However, despite the lack of influence on $TatS_{FT}$ and $TatS_{DE}$ and the absence of phototoxicity, the pigments investigated in these studies cannot be considered safe: Although concentrations used were within the range of literature data, preselection took place during the establishment of $TatS_{FT}$. In this case, pigment concentrations that prevented contraction of the dermis, which is a natural process in the production of collagen-scaffold based skin models, were sorted out. Additionally, pigments of the highest purity were used. Potential effects caused by frequently occurring contaminants, such as PAHs in carbon black, could therefore not be detected here. Future studies might use tattoo pigments isolated from tattoo inks, to include possible contaminants. In addition, adverse effects like the production of ROS might have taken place in $TatS_{FT}$ unnoted, as described earlier. While we were unable to measure short-term negative effects in $TatS_{FT}$ or $TatS_{DE}$, the possibility exists that long term exposure of tattooed human skin with UV light might cause negative effects. In its present state, we are unable to conclude on chronic or sub chronic UV light exposure from the results produced in $TatS_{FT}$. However, we were able to measure cleavage products of P.O.13 upon UV irradiation, even though the amount of cleavage products were minimal. While we could not distinguish between negative effects of P.O.13 itself (*i.e.* ROS production) and the influence of the cleavage products on $TatS_{FT}$, these cleavage products are concerning, as described earlier.

4. Conclusion and Outlook

The objective of this work was to support the evaluation of risks associated with tattoo pigment phototoxicity. Therefore, we investigated laser cleavage products of tattoo pigments and established $TatS_{FT}$ to facilitate phototoxicity tattoo research *in vitro*.

In this work, the possible effects of carcinogenic, sensitizing, or irritating substances following photocleavage of tattoo pigments were discussed. While the substances produced during laser removal are of most concern, suitable alternatives to laser removal exist in most cases. Therefore, restriction of tattoo pigments due to laser cleavage products is worth discussing. However, tattoo pigment cleavage occurs with some pigments even when irradiated with UV light. To fully assess the risks associated with tattoo pigments, a combined assessment of the resulting mixtures of substances is required. Cleavage product databases in combination with *in silico* prediction models could help to facilitate these tasks. Unfortunately, the evaluation of cleavage products (mixtures) is not realized in the assessment of tattooing pigments under REACH.

In my opinion, animal testing should not be used for research on the toxicology of tattoos since tattooing is a voluntary and cosmetic process. To avoid animal testing, a combination of *in vitro* and *in silico* models should be used whenever possible. However, there are data gaps that cannot be filled with *in vitro* and *in silico* models, such as the question of systemic distribution of tattoo pigments after tattooing. While answering these questions is essential for tattoo risk assessment, this work discussed that findings from related research areas such as nanoparticle biodistribution research should be exploited first.

With $TatS_{FT}$, this work presented the first skin model that properly resembles the architecture of tattooed human skin with the dermal localization of tattoo pigments. However, although $TatS_{FT}$ is an important step towards animal free *in vitro* tattoo toxicity research, it lacks certain characteristics that an ideal skin model for tattoo pigment research should possess. As mentioned earlier, it is essential to include dermal macrophages and other immune cells to properly simulate tattoo homeostasis.

Our results suggest photoprotective effects rather than phototoxic effects of tattoo pigments in tattooed human skin. While photoprotective effects of tattoo pigments are commonly reported in the literature, the high number of photoinduced side effects of tattoos indicates that there are still missing pieces of the puzzle. Therefore, uncovering the deeper mechanisms of photoprotection and phototoxicity must be the focus of future studies.

With regard to the safety of the studied tattoo pigments, this work shows that TiO_2 should be used, if at all, only in its rutile crystal structure. Furthermore, when combining the data from the literature with our results, it is very likely that organic pigments can serve as a repository for hazardous substances not only when cleaved by laser, but also when exposed to UV radiation. While further studies need to investigate the exact dynamics behind UV-induced cleavage of tattoo pigments in human skin, the use of tattoo

pigments that release genotoxic carcinogens such as 3,3'-dichlorobenzidine after UV irradiation should be prevented at all costs. No adverse effects were found for carbon black in our studies, solidifying the position of black, carbon black-based, tattoo inks as potentially the safest if they are not contaminated with PAHs.

5. References

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V. List of Publications

Hering H., Sung A. Y., Röder N., Hutzler C., Berlien H.P., Laux P., Luch A., Schreiver I (2018). Laser irradiation of organic tattoo pigments releases carcinogens with 3,3'-dichlorobenzidine inducing DNA strand breaks in human skin cells. *J Invest Dermatol 138*, *2687-2690*. doi: https://doi.org/10.1016/j.jid.2018.05.031

Hering H., Zoschke C., Kühn M., Gadicherla A.K., Weindl G., Luch A. & Schreiver I (2020). Tats: A novel in vitro tattooed human skin model for improved pigment toxicology research. *Arch Toxicol 94, 2423-2434.* doi: https://doi.org/10.1007/s00204-020-02825-z

Hering H., Zoschke C., König F., Kühn M., Luch A. and Schreiver I. (2021). Phototoxic versus photoprotective effects of tattoo pigments in reconstructed human skin models. *Toxicology* (Pre-proof), doi: https://doi.org/10.1016/j.tox.2021.152872.

VI. Declaration

Declaration

According to § 7 paragraph 4 of the doctoral regulations of the department of Biology, Chemistry, Pharmacy based on the announcements in the Official Gazette of the Freie Universität Berlin No. 21/2018 dated 31.05.2018:

Name of the doctoral candidate: Henrik Hering

I hereby certify that I, Henrik Hering, have written my dissertation entitled "Phototoxic interactions of tattoo pigments with laser and natural light in vitro" independently and have not used any sources or aids other than those indicated by me.

I also certify that I have not previously submitted my dissertation in any other dissertation process.

24.07.2021 Henrik Hering

VII. Annex I: Supplementary material ‘Laser Irradiation of Organic Tattoo Pigments Releases Carcinogens with 3,3'-Dichlorobenzidine Inducing DNA Strand Breaks in Human Skin Cells.’

Due to copyright concerns, Appendix I has been removed from this version.

The contents of Appendix I, i.e., the Supplementary Materials of the paper ‘Laser Irradiation of Organic Tattoo Pigments Releases Carcinogens with 3,3'-Dichlorobenzidine Inducing DNA Strand Breaks in Human Skin Cells.’ are associated with the publication online. To access them, please use the following DOI to access all contents of the publication, such as the Supplementary Materials:

DOI: https://doi.org/10.1016/j.jid.2018.05.031

VIII. Annex II Supplementary material ‘TatS: a novel in vitro tattooed human skin model for improved pigment toxicology research’

Due to copyright concerns, Appendix II has been removed from this version.

The contents of Appendix II, i.e., the Supplementary Materials of the paper ‘TatS: a novel in vitro tattooed human skin model for improved pigment toxicology research’ are associated with the publication online. To access them, please use the following DOI to access all contents of the publication, such as the Supplementary Materials:

DOI: https://doi.org/10.1007/s00204-020-02825-z

IX. **Annex III:** Supplementary material ‘Phototoxic versus photoprotective effects of tattoo pigments in reconstructed human skin models’

Due to copyright concerns, Appendix III has been removed from this version.

The contents of Appendix III, i.e., the Supplementary Materials of the paper ‘Phototoxic versus photoprotective effects of tattoo pigments in reconstructed human skin models’ are associated with the publication online. To access them, please use the following DOI to access all contents of the publication, such as the Supplementary Materials:

DOI: https://doi.org/10.1016/j.tox.2021.152872