Dissertation

Evaluation of Local and Systemic Responses of Tattooing

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Guidelines of the Medical University of Graz on Good Scientific Practice".

Disclosures

The following publication has been published based on the data obtained from this dissertation.

- Tattoo ink pigments biokinetics in vivo in a 28-day porcine model: elements undergo fast distribution to lymph nodes and reach steady state after 7 days. Cambiaso-Daniel et al. Dermatology. 2023 (accepted)

In addition, all co-authors have agreed to include their published data in the dissertation and give therefore the permission to reproduce these, furthermore the illustrious and figures owned by third-party publications permission has been obtained.

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Abstract in German

Hintergrund

Derzeit sind weltweit 20 % der Menschen tätowiert, und Untersuchungen zeigen, dass diese Zahl weiter steigt. Es gibt nur wenige internationale Vorschriften, obwohl die International Agency for Research on Cancer mehrere der in Tattoofarbe enthaltenen Komponenten als krebserregend eingestuft hat. Darüber hinaus gibt es immer noch zu wenig Wissen und Forschungsdaten über die Kinetik der Tattoofarben, sowie langfristige oder systemische Auswirkungen.

Zielsetzung

Ziel dieses Experiments war es, die potenzielle Migration und langfristigen Auswirkungen von Tattoofarben, welche bereits medizinische Anwendungen haben, besser zu verstehen. Sobald die Tattoofarbe in die Haut eingedrungen ist, wurde anhand der Farben Dynamik, die Migration und die Veränderungen analysiert.

Methoden

Es wurden vier weibliche Schweine untersucht. Mit Ausnahme eines Tieres (Kontrolle) wurden alle Tiere an der Innenseite des Oberschenkels jeder Extremität tätowiert. An den Tagen 7, 14 und 28 wurden Biopsien der Haut entnommen. Nachdem jedes Tier am 28. Tag eingeschläfert wurde, sind Homogenate des Gehirns, der Leber, der Milz und der Niere sowie der lokalen Lymphknoten entnommen worden. Nach der Analyse der Tintenzusammensetzung wurden alle Sonden verwendet, um das Vorhandensein von Bestandteilen der Tattoofarbe nachzuweisen.

Ergebnisse

In der Tattoofarbe wurden die folgenden Elemente entdeckt: Zirkonium (1,285 mg/kg), Kupfer (5,681 mg/kg), Titan (211,499 mg/kg), Aluminium (1,195 mg/kg) und Chrom (3 mg/kg). Außerdem wies die tätowierte Haut im Vergleich zur nicht tätowierten Haut statistisch signifikante (< 0,05) Ablagerungen der Elemente der Tattoofarbe auf. Im Vergleich zur Kontrolle stiegen die Werte für Titan um 238 mg/kg, Kupfer (+92 mg/kg), Aluminium (+108 mg/kg), Zirkonium (+23 mg/kg) und Chrom (+0,5 mg/kg).

Außerdem enthielten die Lymphknoten Ablagerungen von Titan, Kupfer, Aluminium, Zirkonium und Chrom, die bei 42±2 mg/kg, 69±25 mg/kg, 49±18 mg/kg, 0,3±0,2 mg/kg bzw. 0,5±0,2 mg/kg statistisch signifikant (< 0,05) waren. Diese Ablagerungen waren in den Lymphknoten von Tieren mit Tätowierung 60-mal höher und waren in manchen Fällen die Hälfte der gesamten verbrauchten Tattoofarbe. Es gab keine nennenswerten Ablagerungen von Tintenbestandteilen in Niere, Leber, Milz oder Gehirn.

Schlussfolgerungen

Unseren Untersuchungen zufolge finden sich in den umliegenden Lymphknoten und in der Haut, erhebliche Konzentrationen einer Reihe gefährlicher Bestandteile von der Tattoofarbe. Im Hinblick auf mögliche Krankheiten, die mit Tätowierungen in Verbindung gebracht werden, werfen unsere Ergebnisse ein Licht auf die Sicherheit von Tätowierungen. Diese Ergebnisse können die zukünftige Forschung leiten, indem sie neue Informationen über die langfristigen Auswirkungen von Tätowierungen liefern. Daher ist weitere Forschung erforderlich, insbesondere in Anbetracht des möglichen Abbaus von Tätowierungspigmenten, wenn diese zur Entfernung gelasert werden.

Abstract in English

Background

Currently, 20% of people globally have tattoos, and research shows that this number is rising. Few international rules have been implemented, despite the fact that the International Agency for Research on Cancer has categorized several of the components included in tattoo inks as carcinogenic. Furthermore, there is still a lack of knowledge and research on data regarding ink kinetics and long-term or systemic impacts.

Objectives

The goal of this experiment was to better understand potential ink migration and long-term impacts by examining the dynamics of tattoo ink once the color has pierced the skin, as tattoos have already found medicinal applications.

Methods

There were four female porcines enrolled. On the inner thigh of each extremity, tattoos were applied to all except one of the animals (control). Biopsies of the skin were obtained on days 7, 14, and 28. After each animal was put to sleep on day 28, homogenates of its brain, liver, spleen, and kidney were extracted, along with local lymph nodes. After the ink composition analysis, all probes were used to detect the presence of tattoo ink components.

Results

The following elements were discovered in the tattoo ink: zirconium (1.285 mg/kg), copper (5.681 mg/kg), titanium (211.499 mg/kg), aluminum (1.195 mg/kg), and chromium (3 mg/kg). Furthermore, tattooed skin had deposits of the tattoo ink elements that were statistically significant (< 0.05) compared to non-tattooed skin. In comparison to the control, the readings for titanium increased by 238 mg/kg, copper (+92 mg/kg), aluminum (+108 mg/kg), zirconium (+23 mg/kg), and chromium (+0.5 mg/kg). Moreover, the lymph nodes contained deposits of titanium, copper, aluminum, zirconium, and chromium that were statistically significant (< 0.05) at 42±2 mg/kg, 69±25 mg/kg, 49±18 mg/kg, 0.3±0.2 mg/kg, and 0.5±0.2 mg/kg, respectively. These deposits were 60 times

higher in the lymph nodes of animals with tattoos and in certain cases accounted for half of the total amount of ink utilized. There were no appreciable ink element deposits in the kidney, liver, spleen, or brain.

Conclusions

According to our research, there are considerable concentrations of a number of dangerous components found in tattoo ink in both the surrounding lymph nodes and the skin. With regard to possible diseases associated with tattoos, our results shed light on tattoo safety and can guide future research by providing fresh information for a more comprehensive knowledge of tattoos' long-term effects. Therefore, much more research is warranted, especially considering the potential breakdown of tattoo pigments once these get lasered for removal.

1. Introduction

1.1 Microanatomy of the Skin

The skin is the outer tissue covering the human body, enabling multiple functions such as protection, regulation and sensation. With a total surface of 1,5–2 m2 and a weight of 3.5-10 kg, the skin forms the largest organ of the body (1).

Microscopically, the skin can be divided into the following three layers: the epidermis, the dermis, and the subcutis. Furthermore, the skin contains multiple derivatives, including hair, nails, sebaceous and sweat glands, in order to fulfill its functions successfully (1).

1.1.1 The Epidermis

The epidermis is a stratified squamous epithelium with a thickness varying from 0.1 mm to 0.8-1.4 mm on the palms and soles. It forms the outer covering of the skin and is mainly composed of keratinocytes, which are arranged in the following four layers: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. Besides keratinocytes, melanocytes can be found in the epidermis to provide an efficient protection against ultraviolet (UV) radiation, whereas Langerhans cells are a part of the adaptive immune system maintaining the barrier against pathogens (1).

1.1.2 The Dermis

The dermis is a tough supportive connective tissue matrix with a thickness varying from 0.6 mm on the eyelids to ≥3 mm on the back, palms, and soles. The dermis is divided into the papillary dermis, which is intimately connected with the epidermis, and in the deeper and thicker reticular dermis. The main components are cells and fibers, which are embedded in a ground substance composed of a semisolid

matrix of glycosaminoglycans. Accounting for 70% of the dermis collagen fibers are the main component of the dermis, providing toughness and strength to the skin, whereas elastin fibers impart elasticity. Being responsible for the production of fibers and ground substance, fibroblasts are most important cells of the dermis. Besides that, histiocytes, macrophages, mast cells, melanocytes, lymphocytes, and Langerhans cells can also be found (1).

1.1.3 The Subcutaneous Layer

The subcutaneous layer consists of loose connective tissue and fat and its thickness can vary between 1-3cm. By forming gliding layers or large pockets of adipose tissue, the main function of the subcutaneous layer is the protection and insulation of the skin. Besides that, the subcutaneous layer plays an important role in adipose homeostasis including lipolysis, adiponectin, and leptin production (1, 2).

1.2 History of Tattooing

The act of tattooing is performed by injecting ink into the skin's dermis layer to create a permanent mark. This type of marking has been used for a variety of purposes and went through numerous meanings during history, for example from identifying persons to impose branding of individuals, or just as a voluntary act commemorating an essential aspect of an individual's personal history (3).

The oldest known tattoos were found on the hands and feet of a 7000-year-old mummy discovered in Northern Chile. More examples of early historical reports on tattoos can be found also in the Old Testament, in writings by the Roman emperor Constantine the Great in 313 AD, and by Pope Hadrian I in 787 AD (3). In the middle ages, during the crusades to the eastern sections of the Mediterranean Sea, sailor man got tattooed with Christian symbols to guarantee that if they died in a foreign land, they would get a Christian burial (3). However, in the 18th century, tattoos were already well known and the word tattoo, deriving from the Samoan word "tatau", which means "to mark", was introduced to Europe only at that time. In these years misfits and convicted criminals, who were brought to and abandoned in the South Pacific, were the first Europeans to receive tattoos from the Polynesian locals at this time. Soon, though, the tattoos began to incorporate more European motifs, such as anchors, women, palm trees, hearts, and weaponry like cannons and swords (3) . From the middle of the 19th century onwards, as tattoos became more prevalent in Europe, the distinction between tattooed and non-tattooed individuals took on a new meaning. Tattoos no longer represented the border of civilisation, but rather referred to certain demographic groupings and at the start of the 20th century, the infatuation with tattoos grew even further, to the point that European aristocrats developed an obsession with tattoos (3).

1.3 Epidemiology

Regardless of whether surveys are conducted in the United States, Europe, or Australia, the general prevalence of tattoos is about 20% of the population (4). These results differed depending on the population investigated, the nationality, and the time-period in which the investigations were conducted. Indeed, currently, the 'older' generations (particularly those over 45 years) have far fewer tattoos than the younger generations (4). According to the Harris Polls, which were conducted in 2003, 2008, and 2012 an overall rise in tattoos in the general population was found, indicating that the prevalence of tattooing in the general population is strongly increasing (4). This observation can be explained by the fact that tattoos in the 1950s–1960s were far from conventional and were certainly not embraced by the American and European middle classes. At that time soldiers, sailors, criminals, prostitutes, and other marginalized groups were all connected with being tattooed (4, 5). In terms of peer influence, 75% of young people with tattoos have at least one close tattooed friend, and 29% have at least one direct tattooed family member (6). Laumann et al., reported that 65% of tattooed people received their first tattoo before the age of 24, while Stirn et al. reported that 77% of tattooed people in Germany got their first one before the age of 35 (7, 8). Furthermore, tattooing has historically been more prevalent among males, or at least more socially acceptable for men than for women. However, also this tendency has gradually shifted during the last 20 years. In fact, women's tattoo rates have quadrupled, and the rates in the early 1990s for the two genders across all socioeconomic classes are now almost equal (9). In some studies, in the United States, tattooed women have even outnumbered tattooed males (4).

1.4 Tattoo Process

Since the beginning of tattooing, the fundamental method has not changed: many needle pricks are used to penetrate the epidermal barrier, allowing the colorant to travel via the needle track and take up permanent residence in the outer layer of the dermis. Initially, tattoos were created by hand with a crude, pointed tool that resembled a pen. Crude substances like soot or naturally occurring minerals were used as colorants. Around 1900, electric machines with a coil engine and an electromagnetism basis began to be widely employed. As a result, they eventually came to rule the global tattoo industry, growing in popularity along with tattoos. Hawk Cheyenne-style motor-driven rotor devices have gained widespread use in recent times. Rotor machines use a single, sterile hand piece with an integrated needle and are simpler to maintain clean. Most tattoo artists, who may or may not have experience, create tattoos. There is a technique and art to it that must be acquired gradually and under guidance. Tattoos can be customized to the customer's specifications or based on examples from a catalogue that are duplicated onto the skin. As previously noted, rotor and electric coil equipment are used by tattoo artists. The standard needle is a straightforward, solid metal stylus, as opposed to a medical injection needle that is hollow and has a channel inside of it. There are two types of tattoo needles: "liners" and "shaders." The first drawing of the tattoo's contour is done using a "liner" and a template. Usually, it is created with three separate stylus needles combined into a single, triangle-shaped final needle. A little cup filled with ink is used to dip the needle into. The tattoo ink is then dosed into the skin when the needle repeatedly breaks through the epidermal barrier by the oscillating motion of the engine-driven needle, which is held between the three needles. Approximately 3,000 needle oscillations per minute are produced by a tattoo machine. The tattoo artist directs the amount of ink injected into the skin with the appropriate intensity of color by continuously cleaning the skin while supervising the procedure. They also manage the depth of the injection, the number of passes, and the total amount of ink on the needle. "Shaders" are made up of four to sixteen or more needles

combined into a single broad needle that functions as a brush. Numerous types and profiles exist. "Shaders" are used to administer the colorant in the skin in an even, horizontal manner while also filling in the spaces between the linings. The tattoo artist, the type of needle used, the tattoo tool, and the tattooed region all have a major influence on the amount of tattoo ink injected into the body. No dosage is set in stone or standardized. Of course, there needs to be a significant variance in the amount of tattoo ink applied per square inch and in the body's overall exposure to a particular ink. Tattooing is neither a continuous or cumulative form of dosage; rather, it is a one-time exposure. It's a single shot with numerous distinct bullets. The technical grade of tattoo needles varies greatly, although this can be readily observed with a surface microscope or magnifying lens. It's possible that some needles have the European quality marking CE on them fraudulently. Some have nickel in them. Poor-quality needles can include hooks that cause trauma and wounds to the skin, making them more harmful to the skin. Tattoo artists who are amateurs, use a needle with or without a hook that traumatizes the skin, or purposefully inject excessive amounts of ink to achieve the desired or even required densely colored tattoo are all likely to have higher tattoo ink dosages and absorption rates. Some tattoos are applied by amateurs in shabby settings at home, while others are done at parties and outdoor marketplaces. Professional tattoo artists create the great majority of tattoos in their parlors. Cosmetologists are typically the ones that execute cosmetic tattoos, also known as permanent cosmetics. They use devices that are fundamentally the same as those used by tattoo artists and operate on the same concept, albeit theirs are frequently better made and more sophisticated. For many years, the permanent makeup industry was dominated by sophisticated rotor machines designed like pencils, featuring an integrated hand piece and needle for single use. The word "micropigmentation" was first used in the USA in 1986. It is equivalent to cosmetic tattooing and permanent makeup per European standards. The instruments and inks that are frequently employed in this industry do not belong to any particular technique or concept, but there are differences in color libraries and certain inks that

are advertised as "semipermanent." There is an infinite variety of tattoo equipment and needles available.

1.5 Tattoo Ink

In the US, tattoo inks are treated as cosmetics by the US Food and Drug Administration (FDA) and the pigments used in the inks are regulated as color additives. Therefore, the admission of tattoo inks doesn't require pre-market review or approval resulting in the development of many new colors, which have not been tested for the use on human skin (10).

Traditional tattoo inks consist of nearly insoluble pigments suspended in a diluent (e.g., water) in combination with additives such as preservatives, fragrances, and formulants. The majority of tattoos are black, with the colorants being composed of soot-related compounds (e.g., carbon black), while colored inks usually contain azo or polycyclic compounds. Traditional tattoo inks, especially colored ones, involve the use of metals, including heavy metals. With the rising awareness of possible adverse health effect, the amount of metals in tattoo inks has been decreasing with the use of soluble dyes and organic pigments. However, the colorants being in use were usually developed for industrial use, not for application in people, and have not been tested for the use in contact with the human body. Despite the use of organic pigments, a significant amount of heavy metals such as titanium, barium, aluminum, and copper can still be found in modern-day tattoo inks as shading additives or contaminants. Furthermore, commonly used preservatives and impurities have been suggested to take up importance in some of the adverse tattoo reactions. However, the analytical focus is on the colorants due to the high concentration in tattoo inks, whereas preservatives and impurities are regarded as less of a problem. With an average of 1 mg of injected ink per cm2 of tattoo and the average tattooed skin surface of a tattooed individual being 100-300 cm2, it is clear, that not only large amounts of colorants but also the accompanying compounds accumulate in the skin and lymph nodes even years after tattooing (10).

Regarding the manufacturing of tattoo ink products, the majority of tattoo ink (70-80%) is produced outside of Europe, with professional artists using primarily ink produced in the United States (US), whereas non-professionals primarily use Asian inks.

Throughout Europe ink producers mostly operate in England, Germany, France, Italy, and Spain (11).

1.6 Regulations

Given the growing popularity of tattoos and the potential existence of dangerous compounds in the inks used for tattoos, obviously, the need for regulations to reduce the dangers caused by unsuitable tattoo inks was necessary (12). As a resolution, criteria for the safety of tattoos and permanent makeup were published first by the Council of Europe in 2003 and later revised in 2008. The criteria included guidelines on package labeling, restriction of hazardous pigments, maximum concentration limits for certain contaminants, and a safety assessment by the producer (12). However, only seven member states of the European Union have produced, afterwards, national legislation in response to this resolution (13). After performing a study, specialists from research and risk assessment for the European Commission's Joint Research Center attempted to provide a legal framework to ensure consumer safety (13). Similar was purposed by the European Chemicals Agency, which submitted a restriction proposition on the materials being used in tattoo inks to the Committees for Risk Assessment and Socio-economic Analysis for evaluation in 2019. This was supported by the evidence, presented by the Joint Research Commission, of the presence of tattoo inks on the European market that did not comply with the limits set by the Council of Europe (14). Finally, on December 14 in 2020, the European Union (EU) established a regulatory requirement for ingredients in tattoo inks, which took effect on January 5, 2022.

The legislative system in the US differs from Europe. Indeed, here the country's Constitution gives the national or 'federal' government distinct authorities (15).
Because the regulations concerning tattoo artists are not established in the US Constitution, each of the 50 states regulates this industry individually. As a result, various regulations apply in different states, with varying degrees of control (15). The fact that tattooing is linked to health hazards is indisputable, and the FDA has stated so. However, tattoo ink and pigment manufacture in the US is a complete uncontrolled sector. Federal authorities have not set any recommendations or criteria. Furthermore,

tattoo pigments are not analyzed before they are put on the market for permanent use in the body (15).

1.7 Tattoo Complications

With the growing popularity of tattooing in the general population, the number of associated complications from tattoo applications and removal has been increasing also (13). According to the Klügl study, a significant amount of people experienced skin problems (67.5%) or systemic reactions (6.6%) directly after tattooing, while 9% of the participants showed persistent health problems. However, only 1% of the tattooed participants conducted a physician because of the occurring complications (16).

Due to the need of breaking the skin barrier, potentially infectious agents, such as bacteria, viruses, and fungi, can penetrate causing a huge variety of infections. The transmission of the hepatitis B (HBV) and C virus (HCV) as well as human immunodeficiency virus (HIV) had been the most feared infectious complication of tattooing, however, due to the improved hygiene standards tattoo-associated infections have been low, especially in developed countries (17).

Furthermore, tattoo inks include sensitizing/hazardous compounds that may induce negative health consequences when applied to and removed from tattoos, and a fraction of the ink may be transferred via the bloodstream (18). Examples of these negative effects are allergic and delayed hypersensitivity reactions presenting as a huge variety of clinical pictures such as allergic contact dermatitis, lichenoid and granulomatous reactions as well as lymphomatoid reactions. However, due to the lack of documentation concerning the composition of especially low-purity industrial products, the mediator of these responses is hard to determine (13). Besides the sensitizing compounds in the ink itself, the deposition of tattoo needle wear particles such as nickel and chromium in the skin and surrounding lymph nodes may have an impact on tattoo allergy formation and systemic sensitization (18, 19).

To this day, it remains unclear whether tattoos may induce local and systemic cancer (13). Tattoo inks have been discovered to include toxic substances such as hexavalent chromium, aromatic amines in azo-colorants, and polycyclic aromatic hydrocarbons in carbon black (13, 14, 19-21). Despite the known cancerogenicity and

genotoxicity of some of the compounds, a causality between tattooing and cancer formation has not been demonstrated yet. This might be due to the multifactorial etiopathogenesis of cancer as well as the formation over a long period of time (13). Cancers suspected to have an association with tattooing include squamous cell carcinoma, basal cell carcinoma, malignant melanoma, leiomyosarcoma, primary non-Hodgkin lymphoma, and dermatofibrosarcoma protuberans (22).

Besides that, other complications include pigmentary disorders and underlying dermatoses, which can be reactivated by tattooing (13).

Since tattoos are not only becoming more prevalent in the general population, but are also widely used in medical applications, yet regulations remain inadequate, and long-term and systemic effects are largely unknown, this study aims to demonstrate that tattoo ink does not remain in the tattooed area but is distributed throughout the entire body.

1.8 Medical Applications

Tattooing is not just rising in popularity among the general public for individual expression, but it is also becoming more significant in medical applications. In the majority of cases, medical tattooing is performed in a reconstructive or restorative context in order to either restore the appearance of affected areas or camouflage them by adding aesthetically pleasing artistic images (23).

1.8.3 Scalp Tattooing

In instances of severe male pattern baldness and alopecia, or when hair does not regrow or cover the region of narrow scars from neurosurgical incisions or facial reconstructive flaps reaching the scalp area a typical medical tattoo application is to provide color to the scalp. The purpose of the tattooing is to create the illusion of hair follicles in locations where there is no more hair (23, 24). Scalp Medical Tattooing (SMT) is particularly suitable in cases where hair transplant options are exhausted and where hair loss is not progressive (23).

1.8.4 Vitiligo and Scar Restoration (Pigmentation blending)

Medical tattooing is a viable therapy option for vitiligo patients who have failed to respond to other therapies. The goal is to restore the look of the original skin color by adding pigment to blend the afflicted regions in with the surrounding skin tone (23, 25).

For hypopigmented scars that aren't suitable for surgical scar revision and can't be improved considerably with laser therapy, medical tattooing can be used to restore the color to a scar for it to blend in with the surrounding skin (23, 26).

1.8.5 Scar Concealment

A surgical restoration approach is unlikely to give a satisfying result when scars are hyperpigmented, have an uneven texture, or cover a significant region. The purpose of medical tattooing in these situations is to hide the scar with an aesthetically pleasing picture that either covers or camouflages it (23, 27).

1.8.6 Nipple Areola Complex Restorative Tattoos

Breast cancer surgery frequently entails the removal of one or both nipple areola complexes (NAC). Medical tattooing provides a solution for the missing NAC, either in conjunction with various nipple reconstruction procedures or as a "tattoo alone" alternative that involves generating a 3D picture instead of reconstructing a nipple surgically (23, 28).

1.8.7 Nailbed Restorative Tattoos and Syndactyly

Another application for medical tattooing can be to recreate the appearance of the nail bed if it has been removed due to amputation or surgical excision, for example (23, 29).

Similarly, medical tattooing can be used as a simple alternative to the surgical treatment of syndactyly. This approach is particularly useful to avoid possible complications associated with syndactyly surgery such as infection, neurovascular damage, scarring, or an unpleasant aesthetic outcome (23, 30).

1.8.8 Others

Besides the use of medical tattoos for reconstructive purposes, the use in radiation oncology to ensure accurate targeting of radiation therapy as well as in laparoscopic

gastrointestinal surgery to facilitate identification of mucosal lesions are further application areas (31).

In addition, medical alert tattooing can be used as a form of medical identification similar to medical alert jewelry in order to make first-responders aware of a medical condition (e.g., allergies and diabetes mellitus) or to indicate health directives such as do-not-resuscitate (DNR). However, this practice currently unregulated by the medical community and it is not an officially recognized procedure (31).

1.9 Tattoo Kinetics

Tattoo inks are made of essentially insoluble pigment as well as a variety of substances and impurities with varying solubility and water as the carrier. It is possible to add alcohols to the vehicle to aid in the solubility of lipophilic compounds. Alcohols, which work well as preservatives, are not introduced in concentrations. It is possible for industrial preservatives to be applied without the product being labeled. A study that looked at 58 different inks revealed that nickel was present in small amounts and, remarkably, in higher concentrations. Nickel is a highly prevalent contaminant. Chrome might occasionally also be available. As previously shown, following tattoo ink injection into the skin, soluble ingredient absorption, pigment displacement, and local metabolism (depending on the ingredient) occur. The blood's ability to absorb soluble compounds can reach a peak in a matter of minutes, hours, or days, after which they are metabolized and eliminated by the kidney and liver. Pigments absorb substances considerably differently, and they do so much more slowly. The desired permanently colorful tattoo is created by the majority of the pigment staying locally entrenched in the dermis between collagen fibers. As previously indicated, pigments are mostly transported by lymphatics and are thereafter kept indefinitely in regional nodes. A tiny percentage of pigments, particularly those with minute particles, can, nevertheless, enter the bloodstream and travel to several organs, most notably the liver, where Kupffer cells are normally responsible for illustrating and deactivating circulating substances. By using phagocytosis, mast cells in tissues and lymph nodes, as well as Kupffer cells in the liver, actively remove particles from tissues. After around six months, the majority of the pigment has likely been absorbed from the skin and distributed into the blood, lymph, and other systems. The assumption is that pigments are similarly resistant to biological degradation and metabolism in the skin and other organs where they are dispersed. The general consensus is that, depending on the particular pigment, exposure to sunlight, and other factors, pigment breakdown and the generation of chemical breakdown products happen very slowly since pigments can last for several years or even a lifetime. Tattoo pigment

is predicted to slowly deteriorate and disappear over decades, exposing the body to daily dose exposures that are below the detection limits of any analytical method applied to critical substances, like the chemicals on the negative list of the Council of Europe resolution ResAp 1. It is crucial to acknowledge that tattooing is a one-time exposure to particles and chemicals rather than a continuous or cumulative exposure when discussing the biokinetics and toxicity of tattoo ink.

1.10 Anatomy of the Vascular and Lymphatic System

The human body's circulatory system is an intricate and critical network that carries two vital fluids throughout the body: blood and lymph. These systems are involved in feeding tissues, preventing infections, and preserving homeostasis in different but related ways. An extensive network of blood vessels, including capillaries, veins, and arteries, makes up the circulatory system of the body. Veins return deoxygenated blood to the heart whereas arteries transport oxygenated blood from the heart to the body's tissues. The minuscule, thin-walled capillaries that link arteries and veins allow waste products, oxygen, and nutrients to be exchanged between the blood and the surrounding tissues. The heart, which has two atria (upper chambers) and two ventricles (bottom chambers), functions as the circulatory system's central pump. Blood that has been deoxygenated by the body is pumped from the right atrium into the right ventricle, which then transports the blood to the lungs to be oxygenated. After returning to the left atrium, oxygenated blood is pushed into the left ventricle, which subsequently expels it into the systemic circulation to provide all bodily tissues with oxygen and nourishment. The circulatory system is responsible for clotting, immunological response, temperature regulation, and transportation. The blood circulatory system and the lymphatic system complement each other as a network of organs, nodes, and vessels. Lymphatic vessels gather extra tissue fluid, or lymph, and resemble veins in structure. Small, bean-shaped structures called lymph nodes are found along lymphatic channels and function as sites for immunological surveillance and filtration. The spleen, thymus, and tonsils are important lymphatic organs that each have unique roles in blood filtration and immunological response. The lymphatic system carries out the following tasks: defense and surveillance, immune system defense, fluid homeostasis, and dietary lipid transportation.

There are several connections between these two systems. While blood arteries provide tissues with nutrition and oxygen, some of the fluid seeps into the interstitial spaces. Lymphatic vessels gather this extra fluid and return it to the bloodstream.

Furthermore, lymph nodes and other lymphatic tissues receive immune cells made in the bone marrow, which move through the bloodstream to these locations where they are activated and take part in immune responses.

The tattoo artist punctures the skin with a needle to introduce ink into the dermis during the tattooing procedure. The circulatory system is inexorably involved in this process in multiple ways:

1. Bleeding: Because tattoo needles penetrate blood vessels in the dermal layer, mild bleeding is frequently the result of tattooing. The blood's function is to transport nutrients and oxygen to the injured area, aiding in the healing process.
2. Immunological Response: The tattoo needle may cause an immunological reaction when it penetrates the skin. The immune system's white blood cells may be attracted to the area to protect against future infections. Localized inflammation, redness, and edema may result from this immunological response as the body's defense mechanisms are triggered.
3. Tattoo Ink Circulation: After the ink is injected into the dermis, immune cells may encapsulate it, giving the appearance of a permanent tattoo. Nevertheless, certain ink particles may enter the bloodstream over time and cause tattoos to gradually fade.
4. Temperature Regulation: The body's temperature is regulated in part by the circulatory system. In response to the painful process of getting a tattoo, the body may increase blood flow to the skin, which could result in blushing or perspiration.

Although the body's reaction to tattooing is also mediated by the lymphatic system:

1. Lymphatic drainage: During the healing phase, any interstitial fluid that builds up at the tattoo site is one of the extra tissue fluids that the lymphatic system

helps to remove. This drainage aids in the localized reduction of edema and inflammation.

2. Ink Particles and Lymph Nodes: A little amount of tattoo ink may be absorbed by lymphatic veins and sent to lymph nodes in the surrounding area. Although this is a typical aspect of the body's waste elimination mechanism, over time it may cause a tattoo to gradually fade.
3. Immunological Response: As vital sites for immunological monitoring, lymph nodes are a component of the lymphatic system. Nearby lymph nodes may momentarily swell during the tattooing process as a result of the procedure's immunological signaling.

1.11 Aims

Since tattoos are not only becoming more common in the general population, but are also utilized in many medical applications, nowadays, yet regulations remain inadequate as well as long-term, systemic effects and tattoo ink kinetic are largely unknown, this project aims to bring more data on tattoo ink migration through a prospective study in a porcine model.

2 Material and Methods

2.1 Animals

After approval by the Austrian Federal Ministry of Science, Research and Economy (GZ BMBWF 2020-0.193.102) our animal study including 4 female swine ("Sus Scrofa Domesticus") was performed in accordance with the 2010/63/EU directive on the protection of animals used for scientific purposes.

The animals arrived for acclimation 10 days' prior study begin at the Institute for Biomedical Research of the Medical University of Graz. Here the animals were hosted in groups of two in an environment equipped with straw, balls and rubber rings. Animals well-being, health condition was monitored regularly by the facility veterinary staff.

2.2 Tattoo Ink

In total 10 tattoo ink colors from three of world most commercialized tattoo ink producer (Intenze Ink, Eternal Ink, Dynamic) were selected. All colors were regularly purchased online directly from the producer and presented no signs of manipulation or damage at delivery. In table 1 all investigated colors are stated.

Table 1.

Analyzed Tattoo Inks.

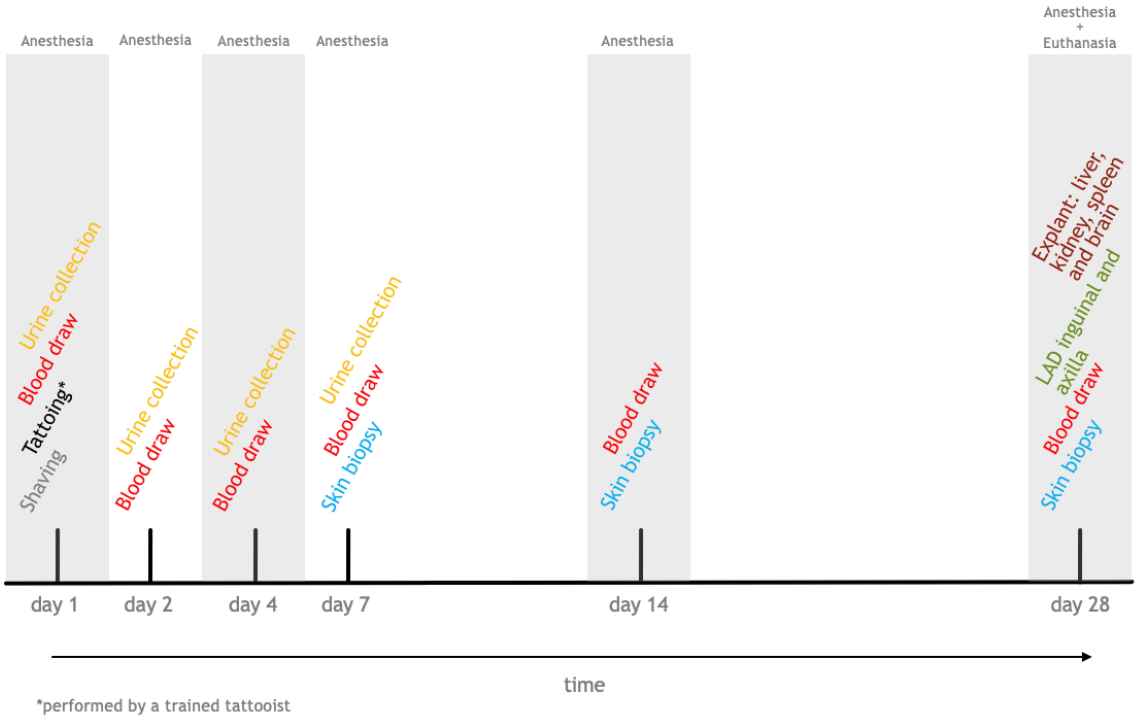
Producer	Commercial name	Color
Eternal Ink	Crimson Red	Red
Eternal Ink	True Gold	Yellow
Eternal Ink	Indigo Dye	Blue
Eternal Ink	Periwinkle	Blue
Eternal Ink	Dark Brown	Brown
Eternal Ink	White	White
Intenze Ink	Red Cherry	Red
Intenze Ink	Golden Yellow	Yellow
Intenze Ink	Zuperblack	Black
Dynamic	Black (BLK)	Black

2.3 Time Schedule

In April 2022, the animals reached the animal facility and after 10 days of acclimation and reaching the desired weight of approximately 13 kg the four-week trial began. An overview of the 4 weeks' time schedule is represented in figure 1.

Figure 1.

Time schedule.



2.4 Anesthesia & Euthanasia

Preoperatively each animal received an intramuscular injection of 0,5 mg/kg Midazolam, 10mg/kg Ketamin, 2 mg/kg Azaperon and 0,2 mg/kg Butorphanol. While the following anesthesia was performed with a standard general tracheal intubation with 2-5 mg/kg/h Propofol, 20 µg/kg/h Fentanyl and 1-2% Sevofluran.

The euthanasia was performed during general anesthesia through intravenous Pentobarbital-Natrium injection.

All procedures were performed by trained veterinary staff of the facility.

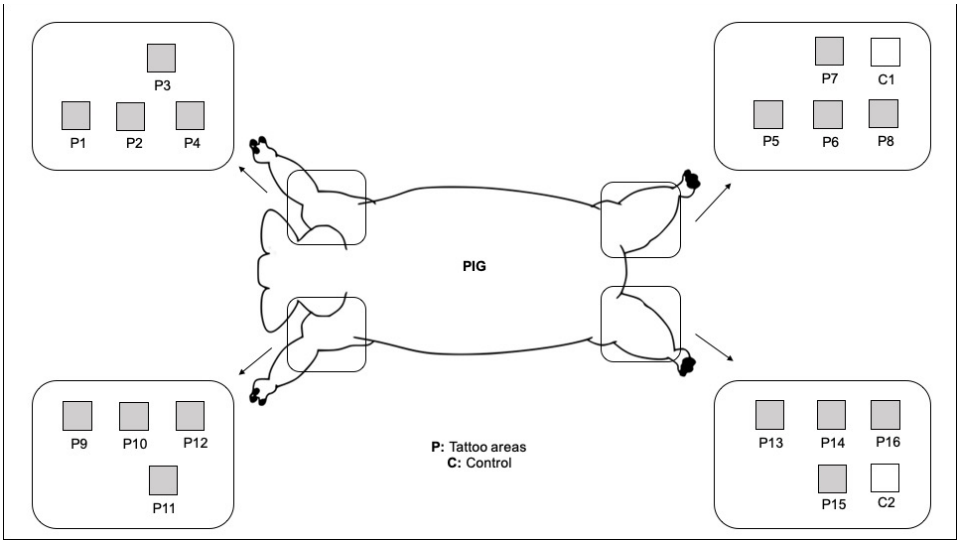
2.5 Tattooing & Dressing

After getting shaved each animal was tattooed by a professional tattoo artist with a Cheyenne HAWK Pen (Cheyenne, Berlin, Germany) tattooo device following standard hygiene rules. Bevor skin disinfection prior to tattooing Intenze Cleanze concentrate (Intenze Ink., Rochelle Park, New Jersey, USA) was utilized to clean the skin and while bevor starting the tattooing all areas were moisturized with Purple Glide (Inkeeze, Newbury Park, California, USA).

Each swine was tattooed in the internal thigh of the anterior and posterior legs bilaterally. In the anterior and posterior thighs four 4x4 cm areas were tattooed per side. In addition, in the posterior thigh one control area per side was marked with small dots at each extremity. A detailed representation of the tattooed areas is illustrated in figure 2.

Figure 2.

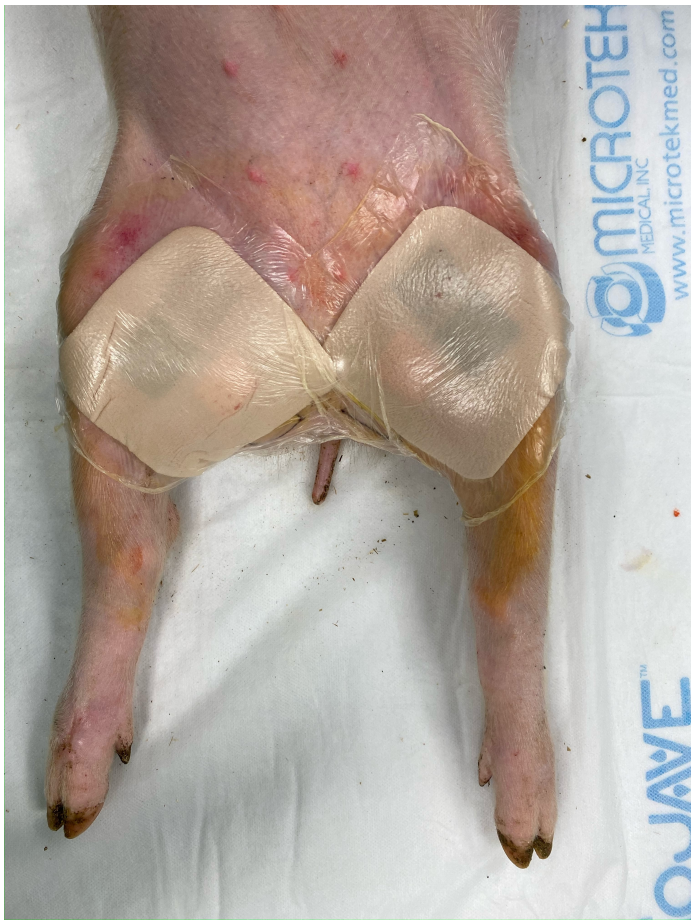
Tattoo areas.



Once completed the tattoos each leg (figure 3) was treated with Purple Glide (Inkeeze, Newbury Park, California, USA) and a dressing with Mepilex® Safetac (Mölnlycke Health Care, Göteborg, Sweden) and Opsite® (Smith & Nephew, Watford, United Kingdom) was applied.

Figure 3.

Dressing.



All products utilized in the tattooing process underwent quantitative analysis for determining eventual cross contamination of the tattoo ink.

2.6 Blood & Urine sample

Each blood and urine sample of approximately 10 ml was taken during general anesthesia, either from peripheral venous access, as well as via suprapubic aspiration. In order to obtain serum specimens, the blood samples underwent spinning at 2000 rpm for 10 minutes. The obtained serum and urine probes where then conserved in a -80°C degree fridge.

2.7 Skin Biopsies

All biopsies were 6 mm punch biopsies (Kai Medical, Honolulu, Hawaii, USA) and were obtained under anesthesia. After obtaining the biopsies after weighting eachpart of these where fixed in Formalin and part native conserved in a -80°C degree fridge. All biopsy sites where sutured with a single stich Dafilon 2/0 (B|Braun, Melsungen, Germany).

2.8 Extracorporeal Perfusion

After heparinization (200 IU pro kg) and euthanasia, the animals underwent a midline laparotomy. After identifying the aorta, the artery was prepared for cannulation direct under the arch. Once ligated the perfusion catheter was inserted and the proximal aorta was cross-clamped and gravity perfusion with cold NaCl was performed.

2.9 Organ Explant & Lymphadenectomy

Once the extracorporal perfusion was finished the liver, spleen, both kidneys and the brain after drilling of the skull were carefully extracted without damaging the primary vessels. Once all organs were extracted these were furthermore cannulated via their primary vessels and perfused with additional cold NaCl solution in order to eliminate any residual blood.

Afterwards, four 6 mm punch biopsies (Kai Medical, Honolulu, Hawaii, USA) were taken from each liver, spleen and kidney segment. After weighting each sample half of the probes were fixed in formalin while the remaining conserved native at -80°C degree after been weighted. Also from the brain four 6 mm punch biopsies (Kai Medical, Honolulu, Hawaii, USA) where obtained from each side respectively from the frontal, temporal and occipital area degree after been weighted. A fresh punch biopsy was utilized for every sample in order to reduce metal cross-contamination. After weighting the remaining organs each was then chopped with an 18 cm zirconium ceramic knife blade (Kyocera, A-Fine GmbH, Hamburg, Germany) on a polyethylene board (Contacto Bander GmbH, Erkrath, Germany). Afterwards each chopped organ was positioned in a sterile cylindrical plastic container (VitaLab GmbH, Pinneberg, Germany) and homogenized utilizing an Ultra Turrax® T 25 (IKA, Staufen im Breisgau, Germany). In order to reduce cross-contamination each organ was homogenized utilizing a new plastic dispersing tool (S 25 D – 14 G – KS, IKA, Staufen im Breisgau, Germany). In addition to do not over heat the probes and improve the process for each probe additional cold NaCl was added. Of each homogenized organ, were than taken four 50 ml probes and conserved at -80°C degree.

Lastly, all lymph node of the anterior and posterior tight were identified and removed. Afterwards of each lymph node package selected nodes were sampled native at -80°C degree after obtaining the weight and in part fixed in formalin.

2.10 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

All sample except the tattoo inks, blood and urine were freeze-dried for at least 72 hours prior analysis. The samples were digested using an Ultraclave IV microwave digestion system (MLS GmbH, Leutkirch, Germany). Therefore, the freeze-dried samples were weighed into 10 ml quartz vessels. The vessels were closed after addition of concentrated nitric acid (65% a.r. Chem-Lab NV, Zedelgem, Belgium, subboiled twice in-house). To build a loading pressure of 40 bar inside the vessel, Argon (5.0, Messer, Gumpoldskirchen, Austria) was introduced. Temperature was ramped to 250°C and held for 30 min before cooling down. After cooling down the solution was transferred into 50 ml polypropylene tubes (Greiner Bio-one, Kremsmünster, Austria) and filled up with ultrapure water to 10 % nitric acid.

For the determination of the total element concentrations an Agilent 7900 ICPMS (Agilent Technologies, Waldbronn, Germany) was used. The elements were determined in collision mode with Helium. Quantification was obtained via external calibration. External calibration solutions were prepared with 10 % v/v nitric acid in a range of 0.01 - 10 µg L^{-1} for V, Cr, Ni, Zr, Nb, Mo, Sn, and Hf, 0.1 – 100 µg L^{-1} for Al, Ti and Cu and 1.0 – 1 000 µg L^{-1} for Fe. Calibration standards were prepared from single element standards (1 000 mg kg^{-1} Carl Roth GmbH, Karlsruhe, Germany and Chem-Lab NV, Zedelgem, Belgium) gravimetrically. Each sample was analyzed three different times and when the obtained value was under the minimal detection range in the results this lower range value was utilized as measurement. For the samples of the tattooed animals mean and standard deviation where obtained from the different probes at the same time point, while for the single control animal only the mean was obtained. Regarding the skin samples mean and standard deviation where obtained from three different samples from the control swine and one for each tattooed swine. Also in this case each sample was measured three times and averages where obtained. For sample preparation ultrapure water (18.2 MΩ cm, supplied by a Milli-Q water purification system, Merck, Millipore, Darmstadt, Germany) and nitric acid (65% a.r. Chem-Lab NV,

Zedelgem, Belgium, subboiled twice in-house) were used. Certified reference materials (CRMs) BOVM-1 (Bovine Muscle, NRC-CNRC, Ottaway, Canada, n=8), ERM-BB186 (Pig Kidney, European Commission, JRC, Geel, Belgium, n=8), and IAEA-407 (Fish Tissue, IAEA, Vienna, Austria, n=3) were digested and measured for quality control and assurance. SRM®1643f (Trace Elements in Water, NIST, Gaitherburg, USA, n=1) was diluted and measured as well. Be, In, Ge, and Lu were used as internal standards and added online via a t-piece in front of the nebulizer to all samples.

2.11 Method Dynamic Light Scattering (DLS)

The particle size and size distribution of the two batches of the Periwinkel ink were analyzed using DLS (Zetasizer Nano-ZS, Malvern Instruments Ltd., Malvern, UK). The ink was four times serial diluted (1:10) with ultrapure water Ultrapure water was obtained using a Milli-Q Advantage A10 water purification system equipped with a Millipore Q-POD Element Unit (both from Merck, Darmstadt, Germany) before measurement. Since we expected at least two main pigments (Pigment Blue 15, TiO_2) an additional pigment separation was carried out. Therefore, 100 µl ink were diluted with 900 µl ultrapure water followed by centrifugation at 16.1 rcf for 15 min (Eppendorf 5412 R, Hamburg, Germany). The supernatant (blue fraction) was deeply blue with a white pallet formed at the bottom. The white pellet was washed until no visible blue residues were left (TiO_2 fraction). The both fractions were serial diluted twice by 1:10 before analysis. Dispersions were analyzed in semi-micro polystyrene UV-cuvettes (Brand, Wertheim, Germany) at 25°C. The measurement angle were set to 173°. Refractive index of 1.38 and absorption of 1.2 were used for analysis of the full ink and blue fraction. Refractive index of 2.61 and absorption of 0.01 were used for the analysis of the TiO_2 fraction. Data analysis was carried out with Mastersizer 2000 v. 5.60 software (Malvern Instruments Ltd.) to report the intensity-weighted diameter of the particles (Z-average) and the polydispersity index (PdI). Good description of the size will be achieved with a PdI below 0.1, PdI below 0.5 Z-average and polydispersity can be used for comparative purposes. The solution refraction index of water was chosen. We therefore also report the peak mean from distribution analysis. Only some samples showed a second peak of agglomerations all below 2% intensity. The data quality report met the criteria for all samples. Only for the blue fraction of Periwinkel II, a warning “cumulant fit error” for polydispersity was given (meaning that z-average results need to be reviewed with more caution). Data are displayed as mean and uncorrected sample standard derivation from three consecutive measurements with automatic run and duration mode.

2.12 Matrix-Assisted Laser Desorption Ionization (MALDI)

LDI-qTOF-MS Method for tissue and ink analysis Samples were analyzed using a MALDI Ultraflextreme time-of-flight (TOF)-MS (Bruker Daltonik, Bremen, Germany). The mass resolution is defined as 40,000 at an m/z of 1200. Spots in close proximity to the sample were used for mass calibration using the xy mass calibration mix. The MALDI unit was used for sample introduction and ionization in LDI mode, without the need for matrix application. The instrument was equipped with a 10 kHz frequency tripled Nd: YAG laser (355 nm). The laser spot size was set to 20 µm acquired in a single shot and a laser focus at 70%. The parameters of the LDI and MS units were optimized to enhance the signal intensities and reduce excessive in-source fragmentation to retain precursor ions. For pigment and ink analysis, single or summed spectra of 1 or 4 pixels, respectively, were investigated. MS2 experiments were manually acquired for the most abundant signals or previously calculated m/z values of interest, for example, of common adducts and in-source fragments. Collision energies were tested, and MS2 fragmentation scans were collected from 10 to 80 eV in 10 eV steps for each precursor m/z, depending on the fragmentation behavior to create an additional MS2 spectral library. 10 µm thick tissue sections on a xy sample holder (Bruker Daltonics) were used for LDI-MS imaging. Instead of point measurements, imaging data were acquired due to an inhomogeneous pigment distribution which might be missed in microscopic images. The laser spot and step size were set to 20 µm each, resulting in an effective spatial resolution of 20 µm. The frequency was decreased to 5000 Hz to reduce sample ablation.

3 Results

3.1 Pre-Trial Tattoo Ink Analysis

The quantitative analysis of the ten-selected tattoo inks from three of the worldwide major companies (Dynamic, Eternal Ink. and Intenze Ink.) confirmed significant differences in composition and what declared in the product information as declared from Wang et al. (19).

Our results show that high quantities of different metals as aluminum (maximal 3,8 g/kg in White from Eternal Ink.), titanium (335 g/kg in White from Eternal Ink), copper (maximal 33 g/kg in Indigo Dye from Eternal Ink.) zirconium (1,3 g/kg in Periwinkle from Eternal Ink.), and hafnium (maximal 36 mg/kg in White from Eternal Ink.) were present in the analyzed products. Furthermore, also non-metals as silicon (maximal 18 g/kg in Crimson Red from Eternal Ink.) From all analyzed inks the blue color Periwinkle from Eternal Ink. showed several elements above g/mg as for example: aluminum 2,6 ± 1,3 g/kg, Silicon 3,9 ± 1,5 g/kg, Titanium 230 ±3 g/kg, Iron 1,6 ±0,1 g/kg, Copper 5,7 ± 0,1 g/kg, Zirconium 1,3 ± 0,1 g/kg. The results of each element in the different inks are represented more in detail in table 2.

In addition, to the quantitative analysis the colors have been burned in a crucible to determine the residual of nonvolatile elements showing residuals until 61%. A detailed representation of all residuals is listed in table 3.

Table 2.

ICP-MS Tattoo Ink Analysis (all values are in mg/kg besides g/kg when *).

	Dynamic	Intenze Ink.			Eternal Ink.					
	BLK	**Zuper Black**	**Red Cherry**	**Golden Yellow**	**Crimson Red**	**True Gold**	**Indigo Dye**	**Periwinkle**	**Dark Brown**	**White**
Na	14 ± 1	606 ± 17	824 ± 127	703 ± 6	192 ± 15	298 ± 11	682 ± 19	340 ± 16	346 ± 12	268 ± 31
Mg		13 ± 1	19 ± 1	59 ± 1	1,7 ± 0,1*					
Al			284 ±8	2,2 ±0,2*	31 ± 8	2,0 ± 0,2*		2,6 ± 1,3*		3,8 ± 0,5*
Si		252 ± 6		7,7 ± 3,4*	18 ± 1*	5,6 ± 0,2*		3,9 ± 1,5*		3,9 ± 1,5*
K	16 ± 1	67 ± 3			55 ± 1					
Ca			59 ± 10		238 ± 8		175 ± 28			
Ti			8 ± 3	28 ± 1*	8 ± 3	77 ± 3*	8 ± 3	230 ± 3*		335 ± 6*
Fe					36 ± 2		169 ± 20	1,6 ± 0,1*	387 ± 11*	
Cu			1,6 ± 0,1*				33 ± 1*	5,7 ± 0,1*		
Zr	1,0 ± 0,1		3,2 ± 0,1	172 ± 2	46 ± 9	447 ± 18		1,3 ± 0,1*	181 ± 6	1,9 ± 0,1*
Mo	0,15 ± 0,01		0,91 (n=2)				112 ± 5	19,7 ± 0,2		
Hf	0,019 ± 0,001		0,061 ± 0,002	3,2 ± 0,1	0,92 ± 0,20	8,7 ± 0,4		24,0 ± 0,3		36 ±1

All values represent mean ± standard deviation. Abbreviations: natrium (Na); magnesium (Mg); aluminum (Al); silicon (Si); potassium (K); calcium (Ca); titanium (Ti); iron (Fe); copper (Cu); zirconium (Zr); molybdenum (Mo); hafnium (Hf)

Table 3.

ICP-MS Tattoo Ink Analysis (all values are in g).

		Color	Tot. m. (g)	Crucible (g)	EW (g)	T + AW (g)	AW (g)	Residue %
Dynamic	**BLK**	Black	4,7299					
Intenze Ink.	**Zuper Black**	Black	4,2848					
	Red Cherry	Red	4,2368	8,1902	0,2409	8,1915	0,0013	0,5
	Golden Yellow	Yellow	4,8162	10,0081	0,1941	10,0198	0,0117	6
Eternal Ink.	**Crimson Red**	Red	4.9395	9,8117	0,2105	9,8134	0,0017	0,8
	True Gold	Yellow	5,4091	9,4251	0,2066	9,4556	0,0305	15
	Indigo Dye	Violet	5,2247					
	Periwinkle	Blue	6,5254	12,4008	0,2279	12,5014	0,1006	44
	Dark Brown	Brown	9,4605	9,9196	0,2321	10,0555	0,1359	59
	White	White	9,1686	10,8654	0,288	11,0401	0,1747	61

3.2 Animals

All four enrolled pigs for the trial were female “Sus Scrofa Domesticus” and came from the same Austrian supplier. The animals had a mean weight at trial begin of 19,6 ± 2,7 kg. the pigs were hosted 2 weeks prior to trial begin and underwent euthanasia at day 28.

3.3 Periwinkle Ink and Tattoo Related Products Analysis

The trial ink lot of Periwinkle was further analyzed and showed very similar results to the pre-trial analysis with: 211499 ± 3795 mg/kg of Titanium, 1195 ± 111 mg/kg of Aluminum, 5681 ± 99 mg/kg of Copper, 3138 ± 63 mg/kg of Iron, 1285 ± 19 mg/kg of Zirconium, 23 ± 0 mg/kg of Molybdenum, 10,0 ± 0,2 mg/kg of Tin, 8,6 ± 0,2 mg/kg Niobium 3,3 ± 1,7 mg/kg of Nickel, 3,00 ± 0,03 mg/kg of Chrome and 2,08 ± 0,03 mg/kg of Vanadium. Regarding the tattoo related products none of these showed high concentrations of relevant elements as represented in table 4.

Table 4.

ICP-MS Tattoo related Products Analysis (all values are in g).

	Edding Pencile	**Intenze Cleanze**	**Purple Glide**	**Mepilex Safetac**	**Opsite**
Na		2695 ± 8	< 5*	1.2 ± 0.1	0.64 ± 0.03
Al		< 5*	< 5*	0.30 ± 0.01	0.15 ± 0.01
Ti	0.79 ± 0,1	< 1*	< 1*		0.070 ± 0.055
Fe	1.49 ± 0,2	< 1*	< 1*	0.75 ± 0.1	0.12 ± 0.03
Cu	0.29 ± 0,1	< 0.1*	< 0.1*	0.066 ± 0.026	0.004 ± 0.002
Zr		< 0.05*	< 0.05*		
Mo		< 0.05*	< 0.05*		
Hf		< 0.05*	< 0.05*		

All values represent mean ± standard deviation. Abbreviations: natrium (Na); aluminum (Al); titanium (Ti); iron (Fe); copper (Cu); zirconium (Zr); molybdenum (Mo); hafnium (Hf)

Furthermore, the DLS analysis, which are summarized in table 5, show the size distribution of particles diameter in the utilized Periwinkle tattoo ink batch.

Table 5.

DLS size distribution of particles diameter in the ink batches. Data are displayed as mean from three consecutive measurements with automatic run and duration mode (SD = and uncorrected sample standard deviation; PdI = polydispersity index; Int = Intensity).

	Mean Z-Average (d.nm)	**Mean Z-Average SD**	**Mean Pdl**	**Mean Peak 1 Mean Int (d.nm)**	**Mean Peak1 Mean Int SD**
Periwinkle	339.40	8.38	0.22	405.37	19.90
Periwinkle (TiO_2 fraction)	389.27	16.80	0.16	426.97	28.34
Periwinkle (Blue fraction)	61.34	2.32	0.26	78.78	3.48

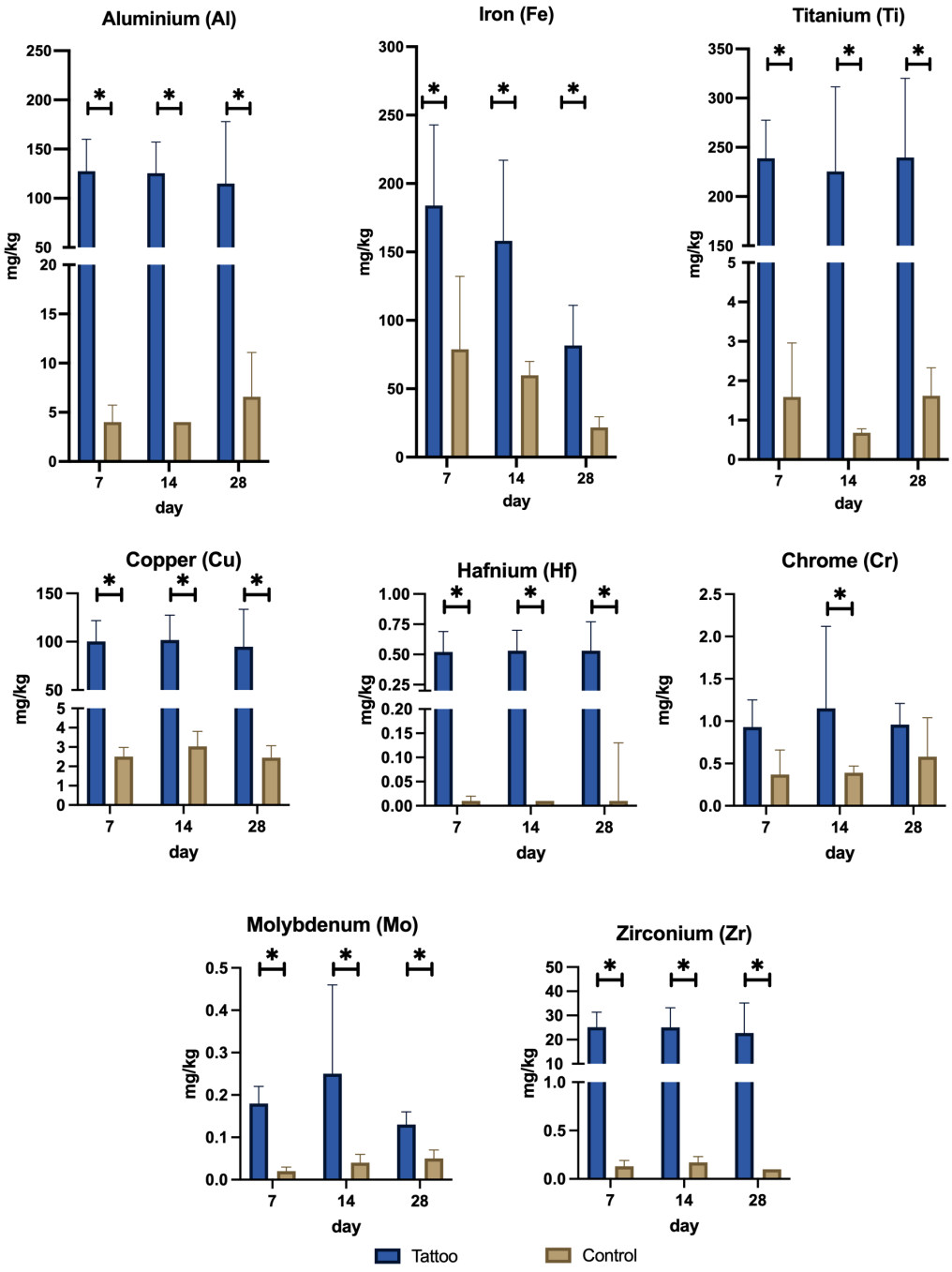
3.4 Skin Analysis

Punch biopsies of tattooed areas showed significant uptake of the elements contained in our tattoo trial ink at each time point (7, 14 and 28 days), and except for the transition metal chromium at day 7 and 28, all concentrations were statistically significant (p value <0.05) higher when compared to the non-tattooed areas. On the euthanasia day the following elements showed the highest mean concentration in skin biopsies of tattooed areas: titanium (239.64 ± 80.47 mg/kg), aluminum (114.96 ± 62.94 mg/kg), copper (94.73 ± 38.70 mg/kg) and iron (81.68 ± 29.35 mg/kg). Less absorption was detected in the elements zirconium (22.72 ± 12.43 mg/kg), chromium (0.96 ± 0.25 mg/kg), hafnium (0.53 ± 0.24 mg/kg), and molybdenum (0.13 ± 0.03 mg/kg). In non-tattooed areas the following mean concentrations of analyzed elements were shown: 21.74 ± 7.77 mg/kg of iron, 6.58 ± 4.50 mg/kg of aluminum, 2.45 ± 0.62 mg/kg of copper, 1.62 ± 0.71 mg/kg of titanium, 0.58 ± 0.46 mg/kg of chromium, 0.10 ± 0.00 mg/kg of zirconium, 0.05 ± 0.02 mg/kg of molybdenum, and 0.01 ± 0.12 mg/kg of hafnium. The highest absolute difference between the mean concentrations in the tattooed areas and non-tattooed areas was detected for the transition metal titanium with a total difference of +238.02 mg/kg in punch biopsies of tattooed areas followed by aluminum (+108.38 mg/kg), copper (+92.28 mg/kg), iron (+59.94 mg/kg), zirconium (+22.62 mg/kg), hafnium (+0.52 mg/kg), chromium (+0.38 mg/kg), and molybdenum (+0.08 mg/kg). These statistically significant differences remained very constant between different time points for all included elements. Regarding the time course of the mean concentrations in the tattooed areas between the different time points, no pattern was recognized. While some elements showed a consistent decrease over time (aluminum and Iron), others showed an initial increase of the mean concentration followed by a subsequent decrease (copper, hafnium, and molybdenum). Different variations in mean concentrations were found for titanium, chromium, and zirconium.

All elements analyzed and respective p-values are summarized in figure 3.

Figure 3.

Element analysis with ICP-MS of skin biopsies at day 7,14 and 28. Statistical analysis were performed between tattooed group vs control using the Mann Whitney U test for non-parametric variables (* = p-value < 0.05).



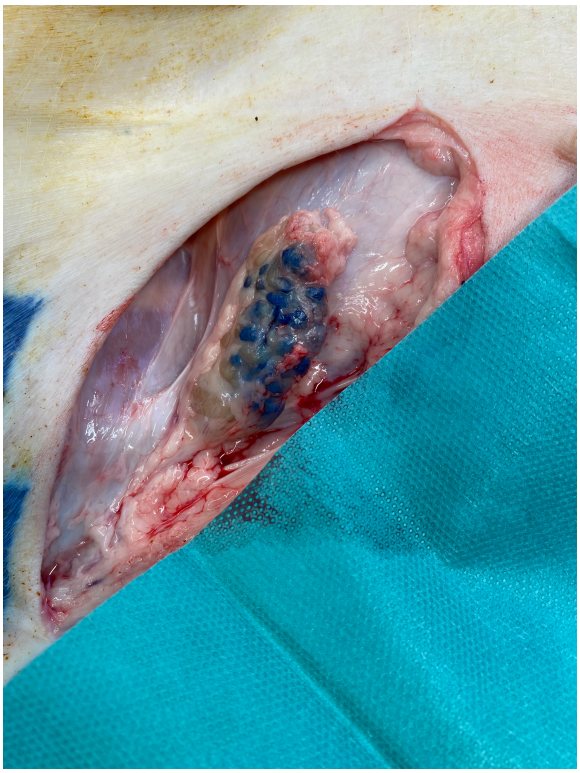
3.5 Organ Analysis

The lymph node samples showed a clearly significant absorption of the elements present in our tattoo trial ink, and except for the transition metal Hafnium, all concentrations were statistically significant (p value <0.05) compared with the lymph node samples of the control animal. No statistically significant absorption of the elements was detected in the organ samples of brain, liver, spleen, and kidney in comparison to the control organ samples.

The following mean concentrations of the elements contained in our tattoo trial ink were detected in the lymph node samples: 239.19 ± 135.22 mg/kg of iron, 68.87 ± 25.35 mg/kg of copper, 49.38 ± 18.34 mg/kg of aluminum, 41.79 ± 2.16 mg/kg of titanium, 0.40 ± 0.15 mg/kg of chromium, 0.26 ± 0.15 mg/kg of zirconium, 0.14 ± 0.03 mg/kg of molybdenum, and 0.01 ± 0.00 mg/kg of hafnium. In comparison, the control lymph node samples showed the following mean concentrations: 20.30 ± 4.72 mg/kg of iron, 0.65 ± 0.12 mg/kg of copper, 1.02 ± 0.06 mg/kg of aluminum, 0.23 ± 0.08 mg/kg of titanium, 0.06 ± 0.00 mg/kg of chromium, 0.05 ± 0.00 mg/kg of zirconium, 0.01 ± 0.00 mg/kg of molybdenum, and 0.01 ± 0.00 mg/kg of hafnium. The highest absolute difference in the mean concentrations between the lymph node samples and the control lymph node samples was detected for the transition metal Iron with a total difference of + 218.89 mg/kg in the lymph node biopsy probes, followed by copper (+ 68.22 mg/kg), aluminum (+48.36 mg/kg), titanium (+41.56 mg/kg), chromium (+0.34 mg/kg), zirconium (+0.21 mg/kg), molybdenum (+ 0.13 mg/kg), and hafnium (+ 0.00 mg/kg). Clinical images of the local lymph knots are shown in figure 4.

Figure 4.

Regional lymph knot package after euthanasia at day 28.



Biopsies of the brain showed the following mean concentrations: 39.48 ± 7.82 mg/kg of iron, 16.01 ± 2.24 mg/kg of copper, 1.17 ± 0.08 mg/kg of titanium, 1.00 ± 0.00 mg/kg of aluminum, 0.18 ± 0.09 mg/kg of chromium, 0.09 ± 0.02 mg/kg of molybdenum, 0.05 ± 0.00 mg/kg of zirconium, and 0.01 ± 0.00 mg/kg of hafnium.

By far the highest mean concentration in the spleen samples was found for the transition metal Iron with a mean concentration of 1280.10 ± 237.78 mg/kg, followed by 2.48 ± 0.35 mg/kg of copper, 1.88 ± 1.19 mg/kg of aluminum, 1.13 ± 0.53 mg/kg of titanium, 0.18 ± 0.27 mg/kg of chromium, 0.05 ± 0.02 mg/kg of zirconium, 0.07 ± 0.07 mg/kg of molybdenum, and 0.01 ± 0.00 mg/kg of hafnium.

The kidney samples showed the following mean concentrations of the elements contained in our tattoo trial ink: 142.12 ± 29.27 mg/kg of iron, 38.34 ± 5.33 mg/kg of

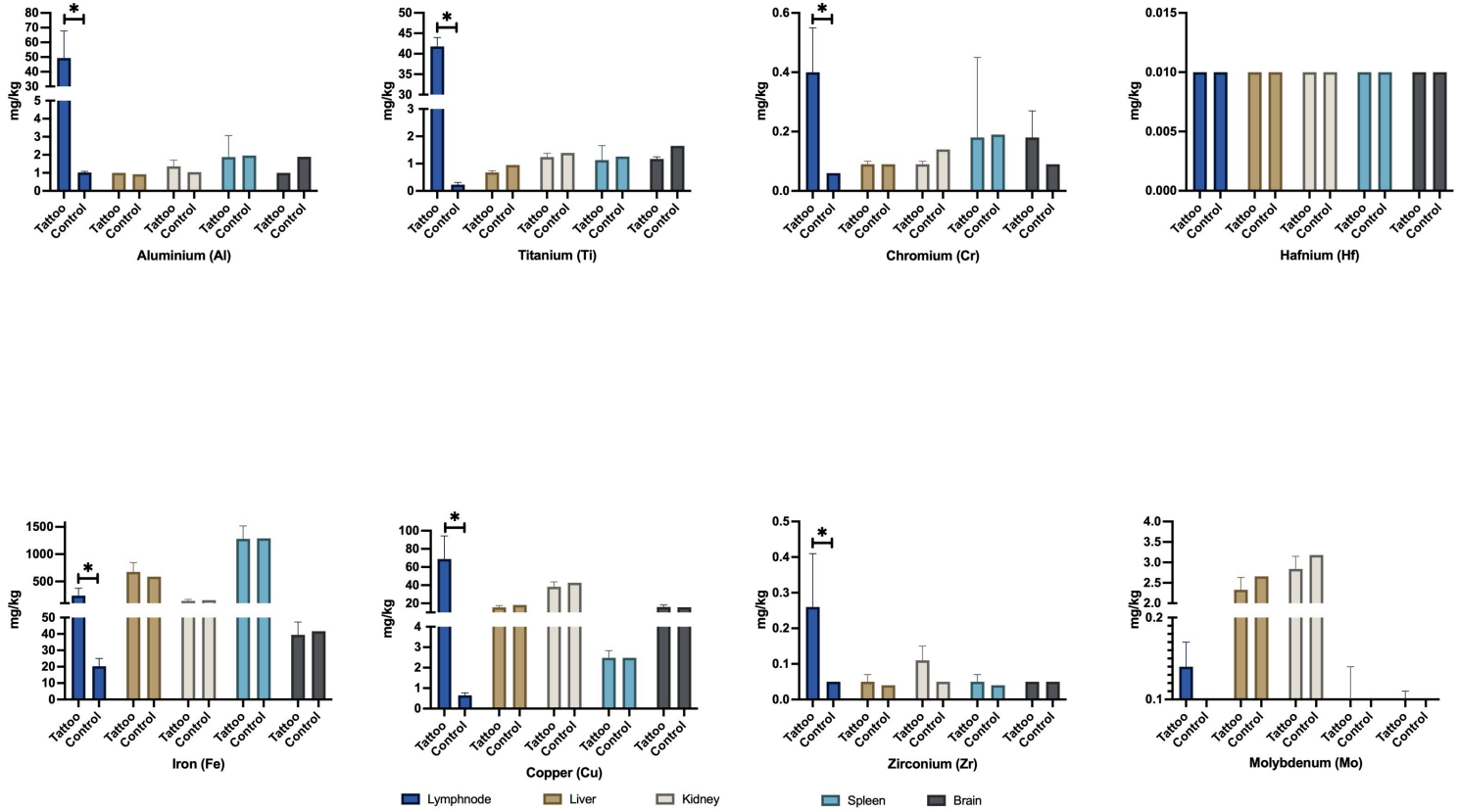
copper, 2.84 ± 0.31 mg/kg of molybdenum, 1.36 ± 0.35 mg/kg of aluminum, 1.24 ± 0.14 mg/kg of titanium, 0.11 ± 0.04 mg/kg of zirconium, 0.09 ± 0.01 mg/kg of chromium, and 0.01 ± 0.00 mg/kg of hafnium.

The following mean concentrations of the analyzed elements were detected in the liver biopsy probes: 675.16 ± 170.58 mg/kg of Iron, 15.59 ± 1.97 mg/kg of copper, 2.33 ± 0.30 mg/kg of molybdenum, 1.00 ± 0.00 mg/kg of aluminum, 0.68 ± 0.06 mg/kg of titanium, 0.09 ± 0.01 mg/kg of chromium, 0.05 ± 0.02 mg/kg of zirconium, and 0.01 ± 0.00 mg/kg of hafnium.

Most elements and p-values of the organ samples and control organ samples on the euthanasia day are summarized in figure 5.

Figure 5.

Element analysis with ICP-MS of peripheral organs after euthanasia at day 28. Statistical analyses were performed between tattooed group vs control using the Mann Whitney U test for non-parametric variables (* = p-value < 0.05).

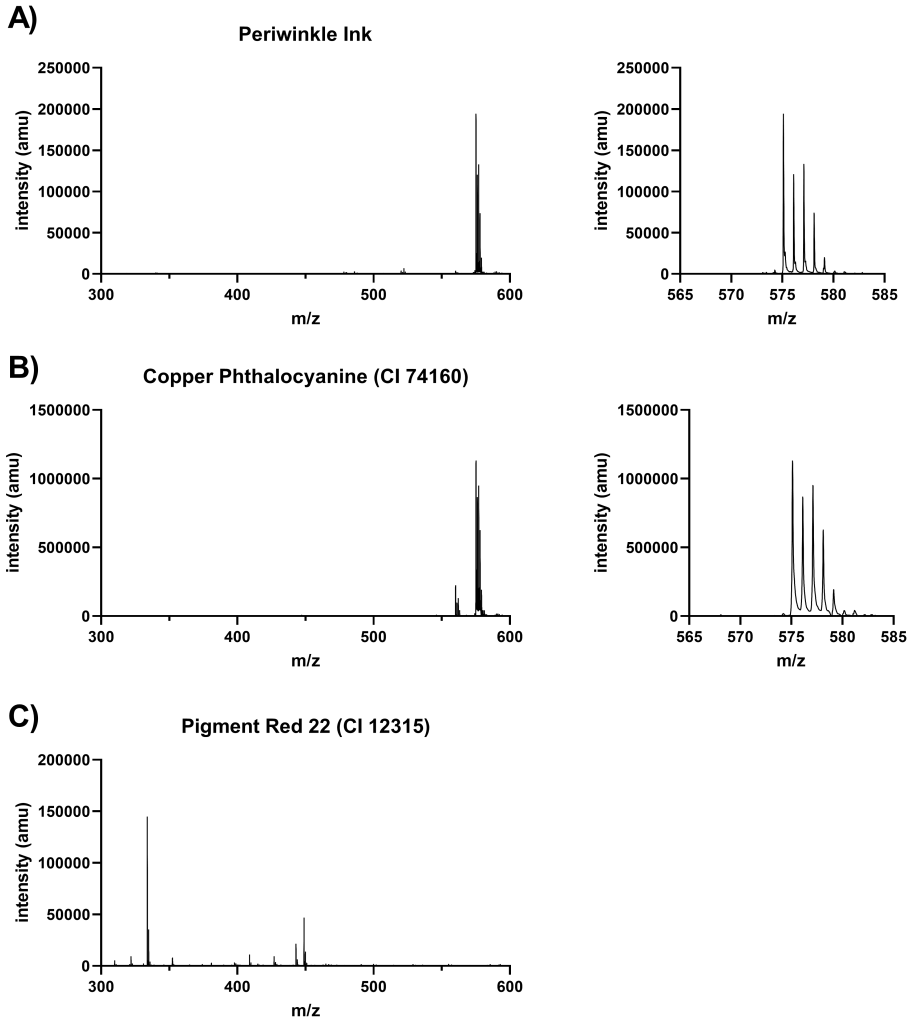


3.6 MALDI Analysis

The label declares the use of the white pigment titanium dioxide, the red pigment 22 and Carbon Black despite the blue appearance of the ink. Although not listed among the ingredients we were able to verify the presence of copper phthalocyanine blue (CI 74160) by MALDI analysis which has been described previously. Furthermore, the same analysis did not detect the declared pigment red 22 (shown in figure 6).

Figure 6.

MALDI analysis for detection of organic pigments. A) Periwinkle ink used for tattooing in the study with zoom to the m/z (mass to charge ratio) between 565 and 585. B) Reference spectrum of copper phthalocyanine blue (CI 74160) with zoom to the m/z (mass to charge ratio) between 565 and 585. The same specific mass pattern can be seen in the ink in A). C) Reference spectrum of pigment red 22 (CI 12315) declared on the ink label. None of the characteristic main peaks appear in the spectrum in A).



4 Discussion

In our comprehensive study, we embarked on a meticulous exploration of the fascinating world of tattoo ink kinetics, unearthing a plethora of intriguing insights that are bound to revolutionize our understanding of this often-underestimated aspect of body art. Our meticulous analysis delved deep into the dynamic interplay between tattoo ink and the human body, shedding light on a spectrum of hazardous elements lurking within the ink's vibrant pigments. This comprehensive investigation not only uncovered the presence of these perilous elements in the regional lymph nodes but also illuminated their silent journey through the intricate layers of the skin. One of the most striking revelations from our research is the unprecedented nature of our findings. To the best of our knowledge, this study represents the pioneering effort to investigate the kinetic behavior of tattoo ink in a living organism, specifically, our porcine subjects. The implications of this groundbreaking research extend far beyond our laboratory walls, as it presents a novel perspective on the behavior of tattoo ink that has remained hidden from scientific scrutiny until now. Our study methodically scrutinized the concentrations of these enigmatic elements within the ink itself, as well as their distribution within the intricate landscape of the skin. Furthermore, our research ventured into uncharted territory by exploring the potential migration of these ink-borne elements to other vital organs, paving the way for a comprehensive understanding of the broader implications of tattoo ink on human health.

Our choice of pigs as the model organism for our study was guided by a profound appreciation of the remarkable parallels that exist between porcine physiology and human biology, with a particular emphasis on skin structure and metabolic processes. Indeed, scientific investigations over the years have consistently demonstrated the striking similarities between porcine and human skin, making pigs an exceptionally suitable model for delving into the intricate dynamics of tattoo ink within the dermal landscape post-tattooing. The utilization of pigs as our experimental subjects opens up a fascinating avenue for comprehending the nuances of ink kinetics as it traverses the intricate layers

of their skin, offering a unique opportunity to bridge the gap between preclinical research and potential insights applicable to human tattooing scenarios. This choice not only enhances the relevance of our findings but also underscores the importance of choosing a model organism that mirrors human physiology to achieve a more accurate and comprehensive understanding of the complex processes at play. (32, 33). Indeed, porcine skin is already utilized since decades as the gold standard model for all type of research in relationship with burns (34).

In our quest for the ideal tattoo ink selection for our trial, we embarked on a thorough and meticulous journey, drawing upon the invaluable insights provided by the pioneering work of Wang et al. as outlined in their 2021 publication in Contact Dermatitis (12). In this groundbreaking manuscript, Wang and their research team conducted an exhaustive examination of approximately 100 distinct tattoo inks, all sourced from renowned global producers. Their analytical focus extended to scrutinizing not only the declared labeling of these inks but also the intricate composition of each individual color variant. From this extensive pool of tattoo inks, a judicious selection process was meticulously undertaken on our end. Rather than relying solely on the data gleaned from Wang et al.'s study, we opted for a cautious approach. We carefully handpicked 10 tattoo inks from their original selection, subjecting them to a rigorous and parallel analysis, employing the same state-of-the-art techniques and methodologies. This methodological congruence ensured that our ink selection process remained insulated from any potential discrepancies arising from different batches, a concern thoughtfully acknowledged and addressed by the very same research group. Our dedication to scientific rigor led us to the culmination of our selection process, resulting in a resounding decision encapsulated in Table 2 of our study. It became evident that the tattoo ink that stood out among the rest, beckoning us for our experimental endeavor, was none other than "Periwinkle" from the renowned Eternal Ink brand. The rationale behind this choice was multifaceted and deeply informed. Notably, Periwinkle exhibited not only a remarkable and unprecedented concentration of numerous elements but, most notably, an exceedingly high content of Titanium—an element of paramount significance

as an ideal tracer in the context of our study, particularly within the realm of peripheral organs. This deliberate and considered selection illuminated the path forward for our experiment, setting the stage for a scientific exploration of unparalleled depth and significance.

In the annals of tattoo history, the prevailing knowledge once encompassed a rather limited spectrum of acute complications associated with the practice, including allergic reactions and infections, as documented in the pertinent literature (17). However, the tattooing landscape has undergone a profound transformation in recent years, with a cascade of emerging evidence that has cast a new light on its potential long-term ramifications. This metamorphosis in our understanding has ushered in a paradigm shift, as tattooing is now being scrutinized for its enigmatic connections to a host of chronic diseases, such as Parkinson's, and an intriguing, albeit unclear, role in the complex terrain of cancer formation (35). Delving deeper into the labyrinth of these long-term complications, it becomes increasingly evident that heavy metals may play a pivotal role in the pathogenesis of these effects. The scientific literature abounds with compelling evidence pointing towards the involvement of heavy metals in these health conundrums. For instance, Chromium has emerged as a suspect, with studies suggesting its potential to increase the likelihood of breast cancer, while other heavy metals have been linked to a myriad of malignancies, encompassing the realms of thyroid, gastric, lung, prostate, renal, and various other forms of cancer (36-41). Moreover, the ominous shadow of cancer extends even to non-Hodgkin lymphoma and cutaneous T-cell lymphoma, conditions that have been tentatively associated with exposure to carcinogenic elements, a revelation reinforced not only by our own study but also by the pioneering work of Wang et al. (19, 42-45). Intriguingly, it's worth noting that most tattoo inks contain elements that have been classified as carcinogenic by the International Agency for Research on Cancer (IARC) for several years, adding another layer of complexity to this multifaceted issue. However, amidst this burgeoning pool of hypotheses and correlations, it's essential to acknowledge that conclusive evidence in the existing literature remains elusive. The precise trajectory of tattoo ink migration within the body

post-tattooing continues to be shrouded in mystery, representing a fundamental knowledge gap. Indeed, the paucity of data on migration and tattoo ink kinetics underscores the pressing need for future studies aimed at filling this glaring void in the literature. These forthcoming investigations hold the potential to either substantiate or refute the aforementioned hypotheses, thus ushering in a new era of enlightenment in the realm of tattoo research and its intricate relationship with human health.

As we traverse the landscape of tattoo-related investigations, a plethora of intriguing reports have surfaced, documenting the occurrence of lymph nodes with unusual pigmentation in individuals adorned with tattoos. Remarkably, these discoveries have often been made serendipitously, arising during a diverse range of medical scenarios such as autopsies, mammography examinations, or even within the confines of the operating room during surgical procedures (46, 47). In instances where these conspicuously pigmented lymph nodes are inadvertently encountered during surgery, a perplexing ethical quandary unfolds. Surgeons, confronted with these enigmatic findings, have grappled with the decision of whether to remove these nodes, which they sometimes classify as potential metastases of an as-yet-undetermined malignant melanoma. The absence of clear and precise guidelines governing the incidental discovery of stained lymph nodes during surgery amplifies the complexity of this ethical dilemma. In the absence of definitive pre-surgical indications for lymph node removal, instances where only pigments are identified through histopathological examination may not necessitate immediate surgical intervention. Yet, the dearth of accurate and comprehensive data concerning the kinetics and potential long-term effects of tattoo ink within the human body lies at the crux of this issue, thwarting the establishment of concrete guidelines in this context. A parallel situation unfolds in the realm of silicone breast implantation, whether for aesthetic augmentation or post-mastectomy reconstruction. In cases where these implants rupture, the leaked silicone gel has the potential to infiltrate and disperse within the locoregional lymph nodes. However, akin to the tattoo ink scenario, the absence of robust data regarding the systemic and long-term effects of silicone gel dissemination following implant rupture looms large. This uncertainty has given rise to

concerns about various autoimmune systemic pathologies, fueling the consensus in favor of removing affected lymph nodes, thus alleviating ethical concerns when confronted with the identification of pigmented lymph nodes (48, 49). This intricate interplay between medical interventions, pigmented lymph nodes, and the dearth of comprehensive data underscores the pressing need for further research in both tattoo and silicone implant contexts to inform future guidelines and decisions in clinical practice

Our groundbreaking research endeavors have unearthed a trove of startling revelations that shed light on the profound impact of tattoos on the human body, ushering in a new era of understanding in this field. One of the most remarkable findings of our study pertains to the concentrations of specific elements, notably chromium and copper, within the lymph nodes of tattooed individuals, a discovery that has sent shockwaves through the scientific community. Astonishingly, our data unveiled that these concentrations could skyrocket to an astounding 60 times higher in comparison to their non-tattooed counterparts, prompting an urgent reassessment of the potential health implications associated with the ubiquitous practice of tattooing (42). The implications of this revelation extend far beyond the confines of our laboratory, as it intersects with the ever-growing global popularity of tattoos, thereby amplifying the urgency of addressing these concerns on a broader scale. Furthermore, it is essential to acknowledge that the prevailing European restrictions on copper levels primarily revolve around the regulation of soluble copper, a category that does not typically encompass compounds that exhibit such prolonged retention within the intricate microenvironment of the skin and lymph nodes post-tattooing. The copper species that we pinpointed in our meticulous analysis is likely to be intricately bound to the copper phthalocyanine pigment present within the tattoo ink, underscoring the importance of distinguishing between various forms of copper exposure. This nuanced distinction carries profound implications, as it raises a poignant question regarding the adequacy of traditional regulatory limits in effectively addressing the unique challenges posed by tattoo-related exposure to metals. It beckons us to reevaluate existing paradigms and consider the need for bespoke guidelines that recognize the intricacies of metal retention within

tattooed individuals. In essence, our research not only unravels the enigma of heightened metal concentrations in tattooed lymph nodes but also serves as a clarion call for a reexamination of the regulatory landscape surrounding tattoo ink ingredients, safeguarding the health and well-being of the ever-expanding population of tattoo enthusiasts worldwide.

Moreover, our comprehensive research has unearthed another layer of complexity in the behavior of tattoo ink within the human body, adding a nuanced dimension to our understanding of this intricate process. Intriguingly, our meticulous investigations into the dynamics of tattoo ink constituents within the skin have unveiled an unexpected pattern. Surprisingly, the concentrations of ink components in the skin exhibited remarkable stability across the time points of days 7, 14, and 28 post-tattoo application. This intriguing finding leads us to surmise that a significant portion of the initial pigment uptake by the lymphatics likely occurs within the first week following the tattooing procedure. In light of this revelation, it becomes imperative for future studies to consider biopsy sampling at even earlier time points, perhaps within the first seven days, to capture the dynamic early stages of pigment migration. However, the intrigue doesn't end here. Our research also brought to light a noteworthy phenomenon – the migration of tattoo ink constituents to distant sites within the body, far removed from the original injection site within the skin. While the concentrations of aluminum and copper found in the tattooed skin were striking, measuring at 115 mg/kg for aluminum and 95 mg/kg for copper, respectively, the mean concentrations detected in the lymph nodes were notably lower, registering at 50 mg/kg for aluminum and 69 mg/kg for copper, respectively. This intriguing migration pattern raises questions about the mechanisms at play that enable such long-distance transport of tattoo ink components. One plausible explanation for this phenomenon appears to be the size of the pigment particles themselves. Our advanced analysis techniques, including dynamic light scattering (DLS), unveiled the presence of pigment particles with dimensions smaller than 100 nanometers, underscoring the pivotal role of particle size in governing the ink's migration dynamics. This revelation opens up a fascinating avenue for future research, where the

interplay between pigment particle size, lymphatic transport mechanisms, and potential long-term health effects can be explored in greater depth, offering a more comprehensive understanding of the intricate journey undertaken by tattoo ink within the human body.

Intriguingly, our investigations into the behavior of tattoo ink constituents within the human body have brought forth a paradoxical revelation – even titanium dioxide, typically characterized by an average particle size exceeding 300 nanometers, demonstrated its ability to embark on a remarkable journey, ultimately reaching the lymph nodes. This phenomenon challenges our conventional understanding of particle transport within the body. While previous research has indeed suggested that larger particles can traverse the complex lymphatic system and find their way to the lymph nodes, the presence of titanium dioxide in these nodes underscores the multifaceted and dynamic nature of this process (18). However, it is important to delve deeper into the nuanced interplay between particle size and migration dynamics. In our quest to unravel the mysteries of tattoo ink migration, we calculated a ratio of copper to titanium concentration within both the skin and the lymph nodes. This meticulous analysis yielded fascinating insights, revealing a stark contrast in these ratios. In the skin, the copper-to-titanium concentration ratio stood at approximately 0.4, whereas in the lymph nodes, it soared to 1.6. This notable disparity suggests a compelling trend – smaller particles are seemingly more inclined to embark on migratory journeys than their larger counterparts, exemplified by titanium dioxide. This revelation carries significant implications, echoing a current trend in the realm of pharmaceuticals, where the utilization of nano-sized particles has gained prominence. Nano-sized particles possess the remarkable ability to traverse biological barriers, including the formidable blood-brain barrier, thereby enhancing drug delivery and therapeutic efficacy. The juxtaposition of these trends in both tattoo ink migration and pharmaceuticals underscores the potential risks associated with the dissemination of nano-sized tattoo ink particles within the body. These intricate findings propel us into an era of heightened awareness, underscoring the need for a more comprehensive understanding of the factors influencing particle migration in the context of tattoo ink. This multidimensional investigation paves the way for future

research endeavors that will unravel the complex interplay between particle size, lymphatic transport mechanisms, and the potential long-term health implications of tattoo ink migration.

Fascinatingly, as we embarked on this journey of scientific exploration, our study yielded results that bear significant implications for our understanding of the intricate dynamics governing the deposition of ink elements within the human body. Intriguingly, our meticulous analysis did not unveil any statistically significant or substantial effect sizes pertaining to the deposition of tattoo ink elements in organs beyond the lymph nodes. While this might initially seem to suggest a lack of widespread migration of ink particles to peripheral organs, it is essential to consider the multifaceted factors at play in our research. One noteworthy aspect that demands consideration is the sensitivity of our analytical method, which, by design, possesses a specific limit of detection. It is plausible that the quantity of ink particles transported to peripheral organs may fall below this threshold, rendering them undetectable through our current analytical approach. Additionally, the process of microwave digestion, an integral step in our analysis, may contribute to the dilution of ink elements within the organs, potentially further obscuring their presence. This phenomenon underscores the need for continuous refinement and development of analytical techniques to enhance our ability to detect trace elements of tattoo ink in peripheral organs. It is worth noting that our findings stand in contrast to those reported by Sepheri and colleagues, who documented the presence of ink pigments in the livers of tattooed mice (50). This intriguing disparity in results may be attributed to several key factors. Firstly, the physiological differences between mice and pigs are quite pronounced, with mice possessing much thinner skin compared to their porcine counterparts. This distinction in skin thickness makes mice more susceptible to ink pigment penetration, potentially facilitating their migration to organs such as the liver. Furthermore, the considerable dissimilarity in skin physiology between mice and humans calls into question the translatability of findings from mouse studies to the human context. In our pursuit of a more clinically relevant model, we consciously chose pigs as our experimental subjects, given their well-documented similarities to humans in terms

of skin physiology and wound healing properties (32, 51). This strategic decision ensures that our research aligns more closely with human experiences and challenges, offering a more robust platform for extrapolating findings to the tattooed human population. However, the complexities of tattoo ink migration within the body persist, urging us to delve deeper into this enigmatic process and its implications for human health. This critical endeavor opens the door to future studies that will refine our understanding of tattoo ink behavior and its potential impact on peripheral organs.

The profound implications stemming from our groundbreaking research transcend the boundaries of the tattooed community, reverberating throughout the broader landscape of public health and medical science. Our findings serve as a clarion call, reigniting a vital debate that has far-reaching consequences as we contemplate the potential emergence of tattoo-related diseases in the years to come. The ever-growing popularity of tattoos among diverse demographics is an undeniable trend, making it all the more imperative to address the underlying health considerations. The striking evidence of metal accumulation within the lymph nodes uncovered by our study raises significant concerns regarding the long-term health ramifications of tattooing. As the number of individuals adorned with tattoos continues to surge, it becomes increasingly critical to establish comprehensive epidemiological studies to monitor the potential systemic effects that may manifest over time. These studies should be designed to encompass a diverse and representative population, allowing for a thorough examination of the relationships between tattooing and a wide spectrum of health outcomes. This proactive approach will enable us to gain deeper insights into the multifaceted interplay between tattoos and human health, shedding light on potential risks and offering a foundation for informed decision-making regarding body art. It is essential to acknowledge that our study was constrained within a specific timeframe, focusing primarily on the healing phase of the tattoo. Consequently, the possibility of excretion or distribution of smaller pigment particles to other organs over a more extended duration cannot be unequivocally ruled out. However, even with longer study durations, the detection of such minuscule quantities in peripheral organs may present an inherent challenge, emphasizing the need

for advanced analytical techniques and vigilant monitoring. An intriguing facet of our study was the utilization of macroscopic staining techniques to visualize pigment accumulation within lymph nodes. While instrumental for our research purposes, this approach also prompts us to reflect on its feasibility and applicability when considering human subjects. The ethical and practical considerations surrounding such procedures warrant careful examination, especially in light of the significant number of individuals with tattoos today. In closing, the sheer magnitude of the global tattooed population necessitates a proactive stance in the field of tattoo research. We must remain vigilant and embark on extensive epidemiological studies in the coming decades to closely monitor potential long-term effects. Only through sustained and rigorous scientific inquiry can we unlock the complexities of tattoo-related health outcomes and provide valuable insights that enhance the well-being of individuals worldwide.

Indeed, as we delve deeper into the multifaceted landscape of tattoo research, the burgeoning popularity of tattoo removal procedures emerges as a critical factor that demands nuanced consideration. These removal procedures, often involving the precise application of lasers to target and break down pigment particles, introduce a dynamic and transformative dimension to the intricate world of tattoo ink within the body. The consequences of this process extend beyond the mere erasure of tattoos, as it gives rise to the formation of smaller pigment particles, each with its own potential trajectory within the body's intricate terrain. These liberated pigment particles, now unbound from their tattoo ink matrix, find themselves at a crossroads. They could potentially accumulate within local lymph nodes, a scenario that echoes the observations made in our study, or they might choose an alternative route, bypassing the lymphatic system and embarking on a journey that leads them to other organs. This complex dynamic underscores the imperative for comprehensive research endeavors that unravel the intricacies of how tattoo removal techniques impact the distribution and fate of pigment particles within the human body (52). Such investigations promise to shed light on the long-term consequences and potential health implications of tattoo removal, adding a layer of

complexity to the already intricate world of tattoos and their interaction with human physiology.

4.1 Limitations

While our pilot project has yielded valuable insights, it is imperative to acknowledge the inherent limitations that come with any scientific endeavor. One of the notable limitations arises from the nature of the trial itself, presenting challenges that could not be entirely eliminated but should not detract from the significance of our findings. Foremost among these limitations is the relatively small number of animals utilized in our study, a constraint that undoubtedly influenced the scope of our results. This limited sample size precluded us from conducting robust statistical analyses to assess potential contamination in organs beyond the skin and lymph nodes. We must emphasize that we were acutely aware of this limitation from the outset and sought to mitigate it by enrolling more animals in our study. However, ethical considerations posed a substantial hurdle in this regard, limiting the number of subjects available for investigation. This ethical imperative underscores the balance between scientific rigor and ethical constraints that often characterizes experimental research. Another salient limitation lies in the duration of our study period, which spanned a mere 28 days. This time frame, while illuminating the snapshot of pigment distribution during the immediate healing phase, may not fully capture the protracted journey of tattoo ink within the body, a process that likely unfolds over decades in humans. The excretion or dispersion of smaller pigment particles to other organs may manifest over a protracted period, well beyond the confines of 28 days. Nonetheless, it is vital to acknowledge that even with an extended study duration, the challenge of detecting trace quantities of elements in other organs may persist. Moreover, the initially chosen worst-case scenario of approximately 9% tattooed body surface area, which mirrored a hypothetical very large tattoo acquired in a single session in a human, introduces its own set of limitations. During the observational period, the animals exhibited a remarkable growth spurt, with their weight and dimensions nearly doubling. This rapid growth led to a decrease in the percentage of tattooed body surface area, a phenomenon that deviated from the initial scenario. While a 4.5% tattooed body surface area may already appear substantial,

it is crucial to understand that our study aimed to simulate a scenario of extreme exposure to the body, with the goal of detecting elements that would have otherwise fallen perilously close to the minimal range of detection with the chosen surface area. In essence, while a larger total tattooed body surface area could conceivably have demonstrated contamination in other organs, this was not discernible within the confines of our study setting. This pragmatic decision to set the initial value at approximately 9% reflected our commitment to maintaining a balance between scientific rigor and the realism of the scenario.

4.2 Conclusions

In conclusion of our pilot study, we conducted a comprehensive examination of the deposition of hazardous elements present in the tattoo ink utilized in our porcine model. The results of our investigation yielded a wealth of significant findings, showcasing a substantial and statistically significant accumulation of these elements within both the skin and the lymph nodes of the tattooed animals. The concentrations observed were staggering, at times reaching levels that were up to 60 times higher than those found in their non-tattooed counterparts. These findings raise critical questions about the kinetics of tattoo ink and its potential long-term effects through organ deposition. To gain a deeper and more nuanced understanding of tattoo ink kinetics, as well as to explore the possibility of long-term health implications associated with ink deposition, it is evident that future studies must build upon the foundation laid by our pilot project. These subsequent investigations should encompass larger cohort sizes and extend the observational time, allowing for a more comprehensive assessment of the journey undertaken by tattoo ink within the body and its potential consequences. The significance of our research extends far beyond the confines of our study, given the potential links between tattoo ink exposure and future pathologies. With the demographic impact of tattoos continuing to rise globally, the fundamental data generated by our pilot study assumes even greater relevance in the current scientific landscape. These findings not only contribute to the existing body of knowledge but also serve as a guiding beacon for future research endeavors. Additionally, they invigorate the ongoing discourse surrounding tattoo safety, prompting a reevaluation of governmental control, from the production of tattoo ink to the tattooing process itself. In essence, our pilot study serves as a vital cornerstone, paving the way for further exploration and heightened awareness regarding the intricate relationship between tattoos and human health. Furthermore, considering the high demand for tattoo removal with laser technologies more data and information are absolutely warranted.

5 Abbreviations

AD	In the year of the lord
Al	Aluminum
Cr	Chrome
Cu	Copper
CRM's	Certified Reference Materials
DNR	Do-not-resuscitate
DLS	Method Dynamic Light Scattering
EU	European Union
FDA	Food and Drug Administration
Fe	Iron
Hf	Hafnium
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Immunodeficiency Virus
IARC	International Agency for Research on Cancer
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
FDA	Food and Drug Administration
MALDI	Matrix-Assisted Laser Desorption Ionization
Mo	Molybdenum
NAC	Nipple areola complex
PdI	Polydispersity Index
Ti	Titanium
TOF-MS	Time of Flight Mass Spectrometry
US	United States
UV	Ultraviolet
SMT	Scalp Medical Tattooing
SD	Standard Deviation
Zr	Zirconium

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