THE USE OF FRACTIONATED ABLATIVE LASERS AS A TRANSDERMAL MICROPORATION TECHNIQUE

By

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A Thesis Submitted for the Degree of

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From

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Dedicated to my parents:

Chief (Mr) Olusola Oni MBBS, FWACS, FRCS, FRCS(eng), MD, MSc, LLM, GDL

and

Chief (Mrs) Merle Oni RGN, BA (Hons), LLM

for their unfailing, ever constant guidance and support

The work on which this thesis is based is my own independent work except where acknowledged.

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Abstract

Fractionated lasers have acceptable aesthetic outcomes with reduced side effects for the patient. This technology could be applied for other uses such as laser assisted transdermal delivery (TDD). This technology has been demonstrated as feasible in an *in vitro* setting. The purpose of this thesis was to investigate if this could be translated from the laboratory bench into clinical practice.

The histopathological mechanism generated by fractional lasers for facilitating TDD was elucidated with a series of *in vivo* studies utilizing a porcine animal model. Abdominal skin of a pig was subject to different laser settings and biopsy samples taken for histological analysis. The histopathological pattern generated by the laser was analyzed to determine optimum laser settings. The same animal model was then used to demonstrate systemic absorption of a topically applied study drug (lidocaine). The effect of laser setting in relation to drug absorption was examined by serum blood sampling. In addition, adipocyte derived stem cells were delivered to the dermis. Subsequently a human clinical model was used to prove that laser assisted TDD can be clinically applicable. Patients were subjected to a facial rejuvenation laser procedure prior to application of the study drug and serial blood serum levels were taken. Laser type and influence on systemic absorption of the topically applied study drug (lidocaine) was then investigated.

Histopathologically fractionated lasers partially ablated the *stratum corneum*, and perforated the underlying epidermis/dermis creating 'microchannels' which allowed absorption of lidocaine and stem cells. The depth and thermal injury of these microchannels could be determined by changing the laser settings. Enhanced systemic absorption of a topically applied study drug was successfully demonstrated in both animal and human clinical models. The amount of drug absorbed could be manipulated by altering laser settings, but also influenced by factors such as intrinsic enzymatic rate.

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PEER REVIEWED PUBLICATIONS ARISING FROM THE WORK WITHIN THIS THESIS

Oni G, Rasko Y, Kenkel J. Pre treatment with a laser – a safe and effective method of analgesia for facial rejuvenation. (Accepted for publication ASJ, February 2013)

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PRESENTATIONS ARISING FROM THE WORK WITHIN THE THESIS

Oni G, Rasko Y, Kenkel J. Pre treatment with a laser - safe and effective method of analgesia for facial rejuvenation. ASAPS, Vancouver, May 2012 (oral)

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Oni G, Brown S, Kenkel J. Can a fractional laser assist transdermal absorption of lidocaine in an in vivo animal model? ASMLS, Grapevine Texas, April 2011, BMLA, Woburn UK, May 2011. (oral)

Oni G, Robbins D, Kenkel J The effect of double-pulsed compared to single pulsed Deep FX Ablative Laser on Human Facial and Abdominal Skin – a histological study. Postgraduate research symposium, Dallas, February 2011 (poster)

Oni G, Brown S, Kenkel J. Serum Lidocaine and MEGX concentrations after application of 4% topical lidocaine to the face. ASAPS, Washington, April 2010 (oral)

LIST OF ABBREVIATIONS

ASC	adipocyte derived stem cells
ALA	5-Aminoaevulinic acid
BrdU	Bromodeoxyuridine (5-bromo-2'-deoxyuridine)
cm	Centimetre
CO_2	Carbon dioxide
conc'n	concentration
DNA	Deoxyribonucleic acid
Er:YAG	Erbium- doped yittrium aluminium garnet
FDA	Food and Drug Administration
g	gram
H&E	Haematoxylin and eosin
Hz	Hertz
IACUC	Institutional Animal Care and Use Committee
IPL	intense pulsed light
IRB	Institutional Review Board
J	joules
J/cm^2	Joules per centimetre squared
kg	kilogram
LA	local anesthetic
Laser	Light Amplification by Stimulated Emission of Radiation
MAL	methyl 5-aminolevulinate
MEGX	monoethylglycinexylidide
min	minutes

mJ	millijoules
mg	milligram
mmol	millimoles
mm	millimetres
ml	millilitres
MTZ	microscopic treatment zones
Nd: YAG	Neodymium-doped yttrium aluminum garnet
nm	nanometers
OTC	Over the counter
PDGF	Platelet derived growth factor
PDT	photodynamic therapy
SVF	stromal vascular fraction
TDD	transdermal drug delivery
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UTSW	University of Texas at Southwestern Medical Center
USA	United States of America
µg/ml	micrograms per millilitre
µm	micrometers
µmol	micromoles
VEGF	Vascular endothelial growth factor
5 Fu	5-fluorouracil

CONTENTS

SYNOPSIS

The work contained in this thesis has moved the *in vitro* work already published on laser assisted transdermal drug delivery, which is summarized in Chapter 1, into *in vivo* studies and potential clinical applications.

The effects of both the erbium- doped yittrium aluminium garnet (Er:YAG) and carbon dioxide (CO_2) lasers on the microstructure of porcine skin was explored using a porcine animal model. Chapters 2-4, focus in particular on these lasers and their ability to create 'microchannels' within the skin that permit the passage of substances. These chapters look at the particular settings that create these channels and the depth to which these channels go in the skin. It is known that when these channels are made, thermal injury occurs and that injury can vary dependent on the setting of the laser. However there is no published work in the literature, which looks specifically at the relationship between type of laser, exposure and histological changes for the purposes of drug delivery. The aim was to optimize the laser settings so that the thermal effects can be minimized whilst theoretically maximizing the amount of drug absorption.

The subsequent chapters of the thesis, Chapters 5-9, explore how this technology can be used clinically, using the parameters determined by the histological analysis, firstly, through animal studies and secondly, in a clinical setting using human subjects.

Chapter 10 summarizes the work, its limitations and its current applications and future experimental/clinical directions.

CHAPTER 1 - General introduction, background and literature review

1.1 General Introduction

This thesis aims to explore how the use of laser technology could assist the transdermal delivery of drugs. In order to investigate this hypothesis, several phases of studies were undertaken, both in animal and human clinical experiments. This introduction looks at the background to this topic covering several areas. Firstly, the literature regarding transdermal delivery of drugs has been reviewed, secondly the role of cutaneous lasers is covered and thirdly this is linked together by reviewing the published literature into laser assisted transdermal drug delivery.

1.2 Background

The transdermal route of drug delivery was first popularized in the USA in 1979 when the first patch was approved for use in motion sickness. Since then a variety of medications have been available for delivery by this route, ranging from hormone replacement, to pain relief to nicotine replacement to name a few. The popularity of this type of drug delivery system is three fold; it can be patient administered, it is painless and the inherent risks associated with hypodermic injections are removed. In terms of bioavailability it is often superior to the oral route in medications that undergo first pass metabolism in the liver. Because of these advantages much research has gone into methods of delivering drugs through the transdermal route. However, the intrinsic properties of skin have meant that there are relatively few drugs that can be delivered this way.

Skin is composed of several layers. The outer most layer is the *stratum corneum* which is formed largely from dead cells which are bound together by keratin produced by corneocytes embedded in lipid regions. This layer is relatively thin at 10-15μm and is responsible for the barrier function of the skin. The major lipid classes in human *stratum corneum* are ceramides, cholesterol and long chain free fatty acids. This means that this layer is hydrophobic and lipophilic. Therefore in order for drugs to penetrate through this layer, they must be able to diffuse through the lipid areas between the corneocytes before they can reach the epidermis. The composition of the *stratum corneum* is important, as this is the first interface that the drug must penetrate in order to be absorbed within the more vascular dermal layers. Because of this, current transdermal preparations are limited to drugs that are lipophilic in nature, and also of low molecular weight of less than a few hundred Daltons (around 500-600 Daltons) or encased in a lipid carrier such as a liposome.

Techniques for stratum corneum perforation:

Given that the *stratum corneum* is the main barrier to transdermal drug delivery there has been a move towards techniques that can disrupt this barrier in order to enhance its permeability and allow the passage of a greater variety of drugs. Methods that have been used include tape stripping/dermabrasion, chemical enhancers, iontophoresis, ultrasound, microneedles, electroporation and thermoablation with varied success. Most of these techniques however remain experimental with the exception of tape stripping and microneedles.

Tape stripping has been widely used in drug pharmokinetics and is commonly used in dermatological research, as it is a simple method of removing the *stratum corneum* (Tojo and Lee, 1989). It involves the repeated application of adhesive, but there is no linear correlation to number of strips and amount of *stratum corneum* removed (Escobar-Chavez et al., 2005). This technique has been used for the investigation of many topically applied drugs such as anesthetics e.g. lidocaine (Padula et al., 2007), disinfectants, anti-inflammatories and corticosteroids (Escobar-Chavez et al., 2005, Pellanda et al., 2006), anti-virals (Jarvis et al., 2004), as well as fragrances and dyes (Jacobi et al., 2006).

There is a wealth of published works on microneedles as a method of piercing the *stratum corneum*. These needles can be solid or hollow, and there has been a recent interest in exploring the feasibility of dissolvable microneedles (Migalska et al., 2011, Garland et al., 2012). The technique is said to be painless, but this may be related to the shallow depth of penetration of the needle in relation to the pain fibres and diameter of the needle (Prausnitz, 2004). This technique show great promise and it has been shown *in vitro* and *in vivo* to increase transdermal delivery of different drugs. However, the technology is still being developed and needs to address issues such as method of delivery of the microneedles, ensuring the microneedles are safely extracted, drug dosing and stability as well as factors related to effect on patient's skin such as irritation (Migalska et al., 2011, Kim et al., 2012, Donnelly et al., 2010).

The ideal method for *stratum corneum* disruption would involve a technique, which is painless to the patient, consistent in its delivery, and would leave no residual

scarring/discomfort. Attention has therefore has turned to the potential role of the established and widely available modality of ablative lasers as a *stratum corneum* poration technique.

Drug preparation – facilitation of transdermal diffusion:

The method of drug preparation has an effect on its ability to permeate through the skin. As the *stratum corneum* is a lipid filled layer, drugs that are lipophilic in their composition have greater penetrance through the skin than hydrophilic-based preparations. Liposomes facilitate drug penetration through the skin because they have a lipid bilayer construct that encapsulate the drug (de Leeuw et al., 2009). Numerous drugs are now prepared utilizing a liposomal carrier in order to improve their bioavailability when delivered transdermally. Other preparations such as using an alcohol base also enhance the penetrative effect of the drug. This is because alcohols act as skin penetration enhancers, by removing lipid from the *stratum corneum* and therefore increasing its permeability (Lachenmeier, 2008).

1.3 LASER: definition and cutaneous applications

Gould first coined the term ‘laser’ in 1959. It is an acronym for Light Amplification by Stimulated Emission of Radiation. This refers to a process whereby energy is harnessed from excited electrons returning to their unexcited state to produce a beam. The characteristics of laser light, which differentiates it from white light, are that the beam is coherent (all emitted photons are ‘in step’ and have a definite phase relation to each other),

monochromic (the laser light is of the same wavelength and therefore, the same colour) and does not diverge (the laser light beams are all in parallel). The 'light' produced is electromagnetic radiation of any frequency not just in the visible spectrum, for example there are infrared lasers, ultraviolet lasers, x-ray lasers etc.

A laser consists of 5 principal components: a gain medium, a pumping energy source, a highly reflective optical cavity (which contains mirrors at either end), output coupler and the laser beam, which is emitted through the mirror in the optical cavity. The type of laser beam produced is related to the gain medium, which can be a solid, liquid or gas.

There are also various laser radiation emission modes: continuous, pulsed and superpulsed. Continuous laser radiation emission occurs when there is continuous emission of laser radiation. In contrast, for the pulsed emission mode, the laser is emitted intermittently; the energy is 'stored' between pulses and delivered in short bursts. In other words the laser has 'on' and 'off' periods and the tissue has the chance to dissipate any heat accumulated between one pulse and the next. A superpulsed emission mode is where the laser radiation emitted in very short bursts to allow an even greater build up of energy, but the time between pulses is much longer than pulsed lasers.

Broadly speaking there are three main groups of lasers: non-ablative, full ablative and fractional ablative lasers.

i. Non-ablative lasers:

There are numerous examples of non-ablative lasers utilized in day-to-day clinical practice, for example the pulsed dye laser or the alexandrite laser. Non ablative cutaneous lasers tend to target the epidermis and the superficial dermis. The benefit of these lasers is the energy delivered while still targeting a specific chromophore does not cause damage to the epidermis/dermis and vaporization of tissue. Types of chromophores targeted by these types of lasers include haemoglobin and melanin. Thus they are typically used for procedures such as port wine stain/superficial vascular naevi removal (haemoglobin) or hair removal/superficial lentigines/melasma removal (melanin). Non-ablative lasers are also used in conjunction with topical drugs for skin cancer removal treatment also known as photodynamic therapy (PDT).

ii. Ablative lasers:

Ablative lasers are more aggressive and target all the layers of the skin, generating thermal injury, which, in essence vaporizes the skin. The carbon dioxide (CO_2) laser and erbium-doped yittrium aluminium garnet (Er:YAG) laser are the two ablative lasers that are used for skin rejuvenation. They possess different properties. The CO_2 laser targets water as its chromophore and operates at the 10600nm wavelength. This laser can be used in multiple ways including as a scalpel. It has been an effective device for skin rejuvenation but the positive clinical outcomes come at the expense of patient morbidity following treatment. Patients suffer prolonged oozing, pain and swelling as well as persistent redness. However, further reduction in downtime was preferable and there were subsequent efforts to produce a laser system that gave the benefits of full ablative treatments without the

prolonged healing times. In order to address the side effects of ablative treatments with the CO_2 laser the 'gentler alternative' Er:YAG laser was introduced. The target chromophore for this laser is again water, and it operates at 2940nm wavelength. However, due to its shallower depth of absorption there is less residual thermal damage, which in turn leads to a reduced amount of collagen remodeling. The upside of this reduced thermal damage however, is a reduction in the post treatment side effects and faster healing times (so called 'downtime' for the patient). There are other differences between the CO_2 and the Er:YAG lasers. The interaction between the Er:YAG laser and its target tissue involves a photomechanical reaction. This absorption of energy causes immediate ejection of the desiccated tissue from its location, creating a characteristic "popping" sound. This translation of Er:YAG laser energy into mechanical work aids in protecting the surrounding tissue; minimal thermal energy remains to dissipate and cause collateral damage. In contrast the CO_2 laser produces a photothermal effect in the skin converting the laser energy into heat, which is dissipated into the tissues.

iii. Fractional ablative lasers:

Fractional ablative lasers were first introduced as an alternative to full ablative lasers, which are used for ablative skin resurfacing. As with full ablative lasers, the target chromophore is water and is therefore, are also powerful lasers in terms of the amount of tissue damage they can cause.

Rox Anderson *et al.* (2004) initially described the concept of fractional photothermolysis. They stated that this method created 'microscopic thermal wounds', which spared the tissue surrounding each of those wounds (Manstein et al., 2004). They went on to define

new terms that they had introduced into laser literature. They are noted to cause 'microchannels' or so called zones of ablation penetrating the *stratum corneum* and extending into the epidermis and dermis. A zone of coagulation, which effectively represents necrosed thermally damaged cells, surrounds these channels. Importantly the zones of ablation are surrounded by normal skin that has not been affected by the laser beam. In particular these microscopic treatment zones (MTZs) or microchannels will be referred to throughout this thesis. The theory of micro-island damage and fractional photothermolysis has led to the development of new laser devices and technology. Both the Er:YAG and CO_2 lasers histologically have been shown to generate coagulated columns in the skin, with a single pass treatment.

Fractional ablative lasers were designed to achieve similar cosmetic rejuvenation results but with a shorter recovery time. This is in contrast to full ablative lasers. Full ablative lasers whilst effective at facial rejuvenation were complicated by long recovery times for patients, which would involve weeping, crusting, burning discomfort and persistent erythema. This was due to the fact that full ablative lasers removed not only the *stratum corneum*, but also the epidermis and part of the dermis too in order to stimulate neocollagen synthesis. The theory behind fractional lasers is that instead of removing the epidermis and part of the dermis in its entirety, the laser would 'punch' holes in the skin partially ablating part of the tissue. In this way, there were islands of normal skin around the zones of ablation from which the skin would heal faster. This also reduced the weeping, crusting and erythema that patients would have previously suffered from if they had undergone a full ablative treatment.

1.4 Review of literature: Lasers and transdermal delivery – *ex vivo* systems

It has been demonstrated in preclinical *ex vivo* models that the pretreatment of skin with a laser enhances transdermal delivery of drugs. Several studies have looked at the effects of the numerous different lasers with particular reference to this. These studies have shown that these lasers enhance permeability of the skin to substances. They postulate numerous mechanisms centered on ablation of the *stratum corneum* which they theorize can effectively enhance permeability of skin because it is this layer that is the greatest barrier to drug diffusion. These studies have demonstrated the increased passage of molecules and have proposed two mechanisms for this; firstly, via the skin appendages i.e. hair follicles and secondly, by enhancing intracellular pathways. This effect of ablation has been shown in numerous studies to be reversible within a few days. Although the carbon dioxide laser has also been shown to effectively ablate the *stratum corneum* of the skin, the concern with this laser is the resulting collateral thermal damage, the so-called zone of coagulation.

In vitro studies of laser enhancing drug diffusion

There are several *in vitro* studies from a group from Taipei Medical University (Lee et al., 2002, Lee et al., 2001, Fang et al., 2004, Lee et al., 2007, Lee et al., 2008). Their studies used animal models (pigs and nude mice) and looked at the Er:YAG laser as a method of enhancing drug diffusion across the skin. Full thickness skin was taken from the animal subject. They were able to demonstrate the enhanced diffusion of macromolecules,

vaccinations, 5 fluorouracil (5-Fu), and lipophilic and hydrophilic drugs across the skin membrane after pretreatment with the laser.

Further, work has been done by Gomez *et al.* in 2008, looking at the use of the Nd:YAG laser as a method of controlling transdermal delivery of 5-Fu. This group found in a rabbit skin *ex vivo* model that altering the fluence of the laser led to changes in absorption of the 5-Fu (Gomez et al., 2008). Latterly utilizing the same animal model, the same group compared Er:YAG and Nd:YAG lasers on the effect of 5-aminoaevulinic acid (ALA) absorption. From their studies they concluded that both lasers enhanced ALA penetration but that lower energies were required to facilitate this using the Er:YAG laser (Gomez et al., 2011).

Forster *et al.* in 2012, demonstrated in a porcine skin *ex vivo* model that laser pretreatment with the Er:YAG laser enhanced the skin penetration of ALA which is commonly used in combination with photodynamic therapy (PDT) to treat a number of skin conditions such as skin cancers (Forster et al., 2010). A group from Taiwan, has demonstrated an increase uptake in Vitamin C derivatives across skin take from the nude mouse, with both the Er:YAG and the CO_2 lasers (Hsiao et al., 2011). They found similar enhancement with pig skin, again for both the Er:YAG and the CO_2 lasers (Huang et al., 2012). Most recently, Haak *et al.* (2012) have shown in their work that the uptake of both large and small molecules through the skin can be facilitated by altering the number of ablative microchannels formed in the skin (density) due to the fractional laser up to a certain point (Haak et al., 2012a). This work was conducted using human skin taken from the abdomen of donors and Franz cells. It did not look at the effect of charge, physical structure or lipo- and hydrophilicity of the molecules.

1.5 Review of literature: Fractional ablative lasers and transdermal delivery – *in vivo* systems

There are very few published works in the literature, which look at the effects of laser on transdermal drug delivery in an *in vivo* animal model, and fewer still in humans.

Animal studies:

Haedersdal *et al.* in 2010 investigated the use of the fractional ablative CO_2 laser and its effect on the transdermal delivery of methyl 5-aminolevulinate (MAL), a drug that is used for photodynamic therapy (PDT). Utilizing an *in vivo* porcine model, they pretreated the porcine skin with the CO_2 laser prior to applying MAL (Haedersdal et al., 2010). They found that the fractional laser treatment facilitated delivery of the topically applied drug deeper into the skin and concluded that the fractional laser created deep channels within the skin that could be used for the direct penetration not only of MAL but for drugs in general. Hsiao *et al.* from Taiwan have demonstrated both *in vitro* (with a porcine animal model) and *in vivo* (with nude mice models) that fractionated lasers can assist transdermal delivery of vitamin c molecules (Hsiao et al., 2012). Their study however, was not able to comment on depth of penetration and potential side effects of this method. The reason for choosing two different animal models was also not clear. Further, a study by Liu *et al.* using rats as the animal model and the Nd-YAG laser demonstrated that glycerol uptake is increased dramatically following laser pretreatment but that this effect (albeit an attenuated one) is maintained over a period of one week (Liu et al., 2013).

Human studies:

A study from the Wellman Laboratory demonstrated an enhanced efficacy of topical anaesthetic when pre-treated with the Er:YAG laser (Yun et al., 2002). This study consisted of 12 patients undergoing facial resurfacing. Half of their faces were pretreated with Er:YAG laser, the other half of their faces were not. Subsequently a topical lidocaine (ELA-Max) cream was thickly applied to both sides of their faces with occlusion for 60 minutes. They found that 'Er:YAG laser ablation of the *stratum corneum* substantially improved the effectiveness of topical anaesthesia'. The paper did not elucidate the mechanism for this.

As can be seen from the literature review, there is a paucity of *in vivo* work in this area. This thesis will therefore, investigate the feasibility of utilising existing fractionated laser technology to enhance transdermal drug delivery, utilising both animal and human *in vivo* models.

CHAPTER 2 - Histopathological injury to porcine skin with fractionated lasers – a qualitative analysis

2.1 Introduction:

This thesis aims to explore how the use of existing laser technology can assist the transdermal delivery of drugs. The review of the literature has shown that this is an emerging field that could potentially have a wide-ranging field of applications if proven to be successful. Most of the published works are studies utilizing *in vitro* models. The work for this thesis will move from the laboratory bench to a clinical model in order to assess the efficacy of laser as a tool for enhancing drug delivery.

As discussed in Chapter 1, there are numerous different fractionated lasers that have an ablative effect on tissue. The choice of laser to use in this thesis was dependent on those that were available clinically within the plastic surgery department. The two types of fractional ablative lasers available were the erbium-doped yittrium aluminium garnet (Er:YAG) and the carbon dioxide (CO_2) lasers. At the time of writing, the CO_2 laser was most commonly used in the department for facial resurfacing procedures, however the merits of the Er:YAG were increasingly being explored at the time of commencement of the laboratory work.

Animal model

In order to investigate this further, several phases of studies were undertaken both in animal and human clinical experiments. Animal models are indispensable to wound

research because it is not possible to simulate biological situations of problematic wounds using *in vitro* models. As can be seen from many of the papers referenced, several different animal models have been used. These range from mice, to rabbits to pigs. For the purposes of this thesis, all preclinical studies were performed using porcine animal models. The pig was selected because it has been demonstrated that pigskin is structurally similar (Figure 1) to the human skin (Sullivan et al., 2001, Davidson, 1998).

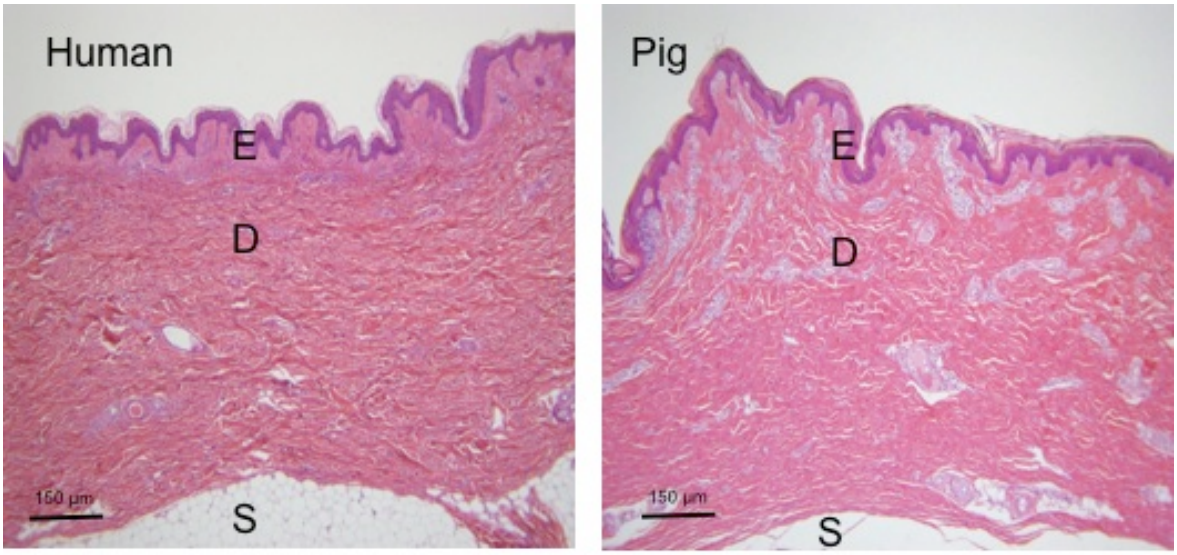


Figure 1: Comparisons between human and porcine skin. Note human skin has slightly thinner *stratum corneum*/epidermis (E) with more tightly packed subcutaneous tissue (S). The dermal component (D) is similar between the two.

The porcine model has been used to study a wide variety of wound types including burns, radiation injuries and pressure ulcers (Kjellstrom et al., 1997, Hyodo et al., 1995, Lefaix et

al., 1996, Constantine and Bolton, 1986). Given all these factors, a porcine model has the closest concordance with human skin and is, therefore, ideal for preclinical studies.

In this preliminary study, the effect of two different lasers on porcine skin was evaluated to decide which laser was most suitable for use in the subsequent animal studies.

2.2 Method:

Ethical approval for the experiments was sought and granted by the Institutional Animal Care and Use Committee (IACUC) at University of Texas (UT) Southwestern Medical Center, Dallas, Texas, USA (appendix 1). One adult (200-250lb) female domestic pig was used. The animal was acclimatized for one-week prior to use. Feeding, sleeping and change in behaviour were monitored daily by a veterinarian. The animal was kept nil per mouth the night before the procedure.

Laser type and setting:

Under general anesthesia the skin of the belly of the pig was shaved. Four areas (2 for each of the 2 lasers, there were two settings) measuring 3cm x 3cm were marked on the skin with a permanent marker separated by 10 cm gaps. This was arranged into 2 rows and 2 columns (related to the different fluences/spot sizes):

Row 1:

Fractionated Er:YAG (Profile, Sciton Inc, Palo Alto, CA), 22% density, 250 μm spot size

Column 1: 250 j/cm^2 (1000 µm ablative depth)

Column 2: 125j/cm^2 (500 µm ablative depth)

Row 2:

Fractionated CO_2 laser (Deep FX, Lumenis, Santa Clara, CA), 300Hz, 10% density, 250 µm spot size

Column 1: 15 mj (1.1J/cm^2)

Column 2: 30 mj (1.5J/cm^2)

Histological analysis:

8 mm punch biopsies were taken from each of the treated areas immediately post treatment. In addition an 8 mm punch biopsy was taken from untreated skin as control. The specimens were fixed in 10% buffered formaldehyde solution at pH 7.4 for at least 48 hours, passaged thorough graded alcohol, embedded into paraffin wax and cut into 6 µm sections with a microtome (Shandon Finesse E, Thermoscientific, Kalamazoo, MI). Each section was stained with haematoxylin and eosin (H&E) and then examined under the light microscope to identify any tissue damage or reaction.

Data analysis:

Where ablative columns (microchannels) were visible, 5 columns were randomly selected and measurements were taken of column depth, and ablative width using QCapture Pro 6.0 software (Q Imaging, BC, Canada). An average was taken and tabulated. Statistical

differences between the laser settings for each group was tested for using Student's t test, with $p<0.05$ taken as significant (Excel 2011, Microsoft Corp, Redmond, Wa). For the normal control, 5 areas were randomly selected and measurements taken for the thickness of the epidermis and dermis. The dermis was taken as the area from dermal-epidermal (DE) junction to the junction between the dermis and subcutaneous tissue (Figure 2).

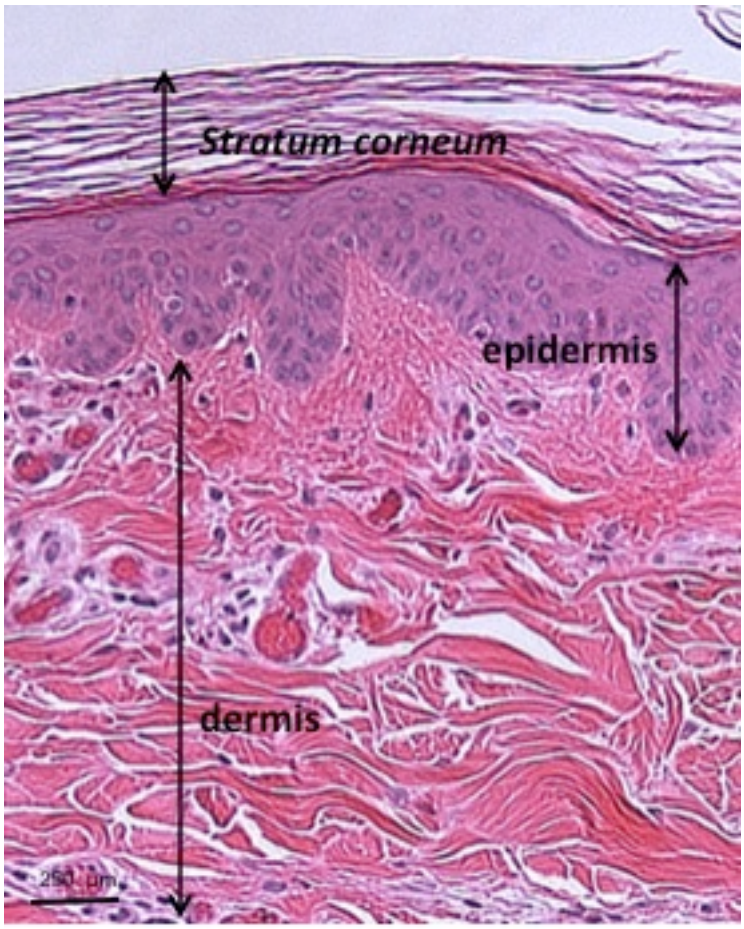


Figure 2: Normal control porcine untreated abdominal skin

2.3 Results (Table 1):

Normal control:

The epidermis was on average 85.9 µm in thickness (range 24.78-116.65 µm). The dermis was on average 1967.0 µm in thickness (range 1321.1 - 2873.0 µm).

Er:YAG (Figure 3):

There was an appreciable difference between the two laser settings in terms of both depth of the microchannels and the width of the collateral thermal damage lining the microchannel (see Tables 1 and 2). The average microchannel depth at the 125 j/cm^2 (500 µm ablative depth) laser setting was 501.7 µm (range 431.6 - 559.6 µm). The average microchannel depth at the 250 j/cm^2 (1000 µm ablative depth) was 884.3 µm (range 725.1 – 1041.0 µm). Therefore, doubling of the energy setting resulted in a concomitant 43.3 % increase in the ablative column depth (p=0.005). The collateral thermal injury generated was 55.6 µm (range 39.32 - 74.56 µm) at the 125 j/cm^2 setting and 80.9 µm (range 73.6 - 91.9 µm) at the 250 j/cm^2 setting. This represents a 31.3% increase in thickness, when the energy delivered is doubled (p=0.053).

Table 1: Comparison of ablative microchannel generated by CO_2 and Er:YAG fractional ablative lasers

	Laser and laser settings			
	CO_2 15 mJ	**CO_2 20 mJ**	**Er:YAG 500µm**	**Er:YAG 1000µm**
Average ablative depth (µm)	561.8	787.6	501.7	884.3
SD +/-	152.8	110.6	46.1	101.8

Table 2: Comparison of collateral thermal injury generated by CO_2 and Er:YAG fractional ablative lasers

	Laser and laser settings			
	CO_2 15 mJ	**CO_2 20 mJ**	**Er:YAG 500µm**	**Er:YAG 1000µm**
Average ablative depth (µm)	88.1	103.7	55.6	80.9
SD +/-	24.9	17.9	11.7	9.3

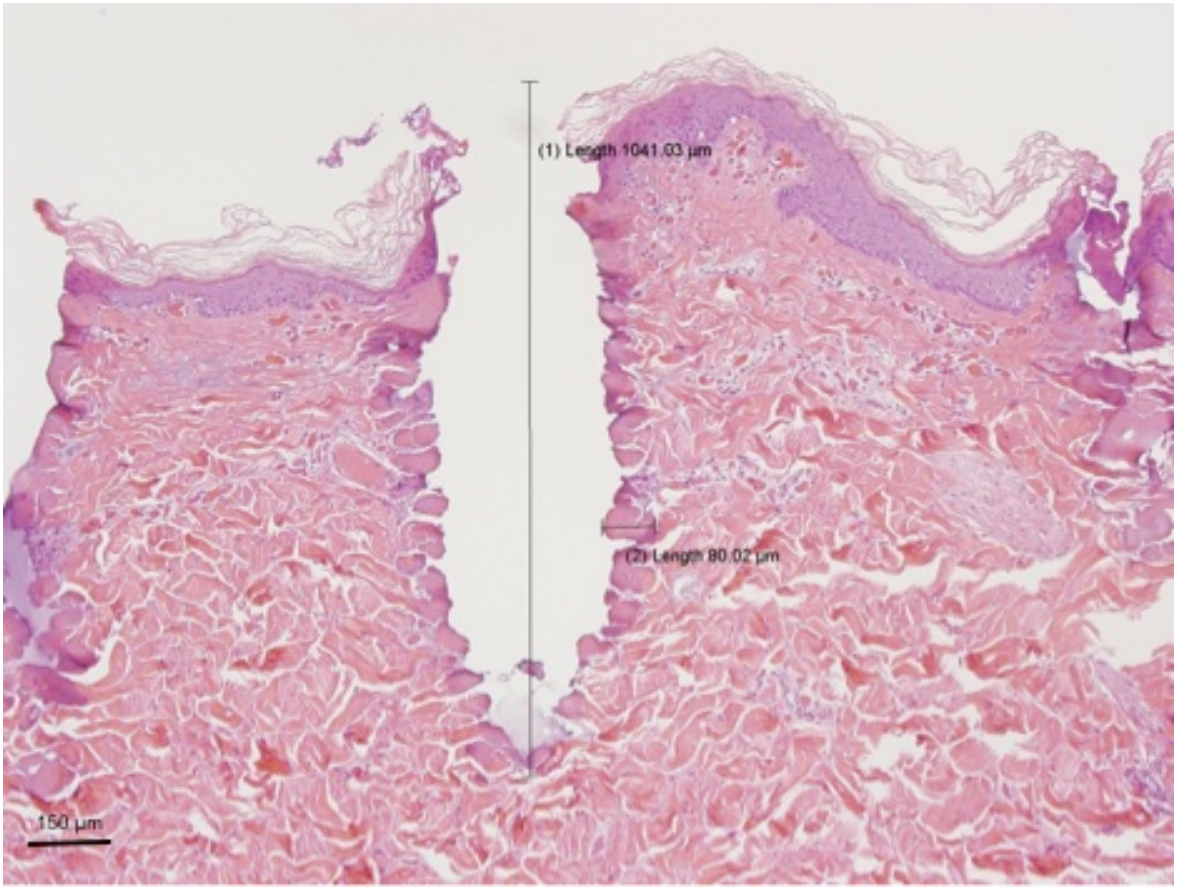


Figure 3 Er:YAG laser 250J/cm^2 (1000µm ablative depth). Note minimal exudates at base of microchannel.

CO_2 fractional ablative laser, Deep FX (Figure 4):

There was an appreciable difference between the two laser settings (see Tables 1 and 2). Ablative columns were easily visualized. The depth of ablative column increased with energy setting, as did the width of collateral thermal energy. At the lower 15 mJ setting the average microchannel depth was 561.8 µm (range 378.2 - 785.84 µm). In comparison, at the higher 20 mJ setting the average microchannel was 787.6 µm (range 633.4 - 961.4 µm). This represents a 29% increase in column depth when the energy delivered is

increased by 25% (p=0.08). In terms of collateral injury the lower 15 mJ energy setting resulted in a width of 88.1 µm (range 47.2 - 121.73 µm). At the higher 20 mJ setting the average width was 103.8 µm (range 70.1 - 121.3 µm). This represents an increase in collateral injury width of 15.2% when the energy delivered is increased by 25% (p=0.329).

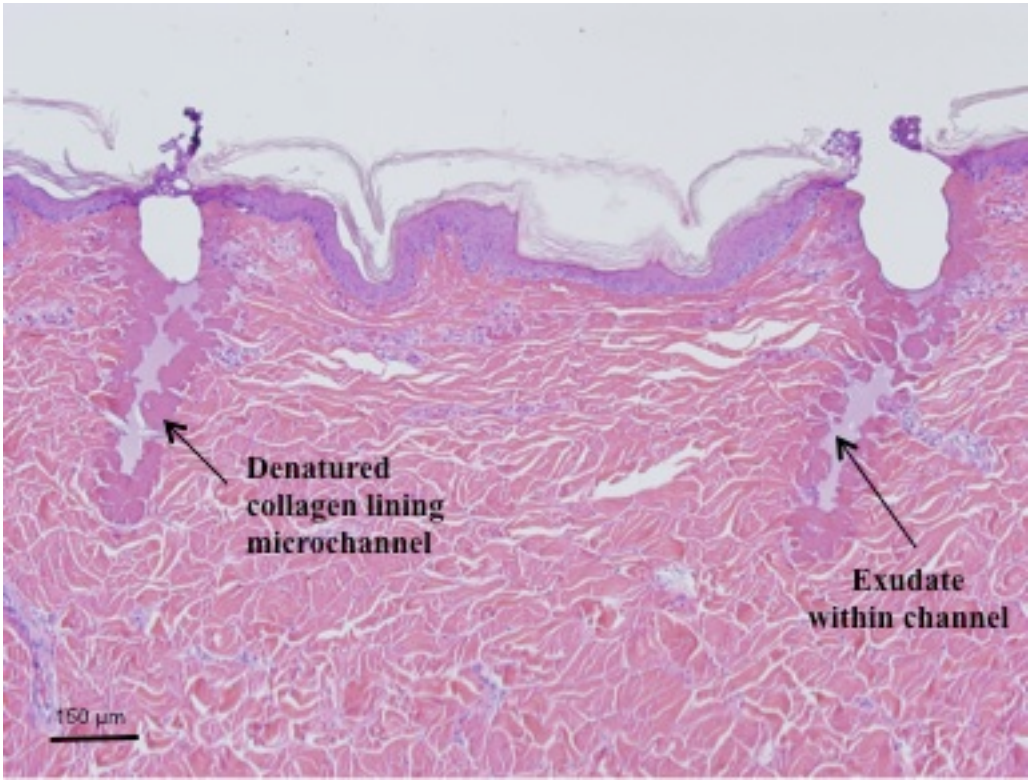


Figure 4: Deep Fx, Lumenis, fluence 20J, note exudate within channels, as well as lining of microchannel by denatured collagen

2.4 Discussion:

This preliminary study demonstrates the very different histopathological profiles of fractionated ablative lasers. The depth and width of ablation is related not only to the energy setting, but also to the type of laser used and the properties of that laser. Both the Er:YAG and the CO_2 lasers have an affinity for water. However, the Er:YAG laser has a wavelength of 2490 nm which is closer to the maximum absorption of water, therefore, giving it the theoretical benefit of higher precision (Karsai and Raulin, 2010, Alster and Lupton, 2001) and less collateral damage. The CO_2 laser has a wavelength of 10600 nm and whilst it too has a high affinity for water, the CO_2 laser's primary effect is photothermal whereas the Er:YAG is primarily photomechanical (Alster, 1999). This photothermal effect results in greater thermal injury to the surrounding tissue. This was reflected in this study, where the fractionated CO_2 laser produced very clear microchannels but with a greater collateral tissue damage profile than the Er:YAG laser. In addition, for a smaller increase in energy there was on average a greater amount of collateral damage. It is difficult to compare the two different lasers exactly because the energy settings were not equivalent but what the results suggest is that the Er:YAG laser would have a greater range over which the microchannels could be manipulated compared to the carbon dioxide laser.

Another consequence of the greater thermal injury caused by the fractionated CO_2 laser was the microchannels produced had a considerable amount of exudate within them. Exudate within the microchannels would prove problematic for drug delivery. This is because, the hydrostatic forces pushing the exudative fluid out of the tissue into the channels would directly compete with the diffusion of the substance applied topically. This therefore, reduces any potential for transdermal diffusion.

In terms of column depths, the fractionated CO_2 laser however, created comparable collateral thermal injury both in terms of depth and width at both 15 mJ and 20 mJ of energy compared to the Er:YAG laser. Observing the pattern of injury the fractionated CO_2 laser tended to create more collagen denaturation distal to the micro-channel compared to the Er:YAG laser (Figure 5). While this would be advantageous for aesthetic reasons in facial resurfacing, the emphasis of this study was to select the laser that would generate micro-channels with the least amount of tissue damage.

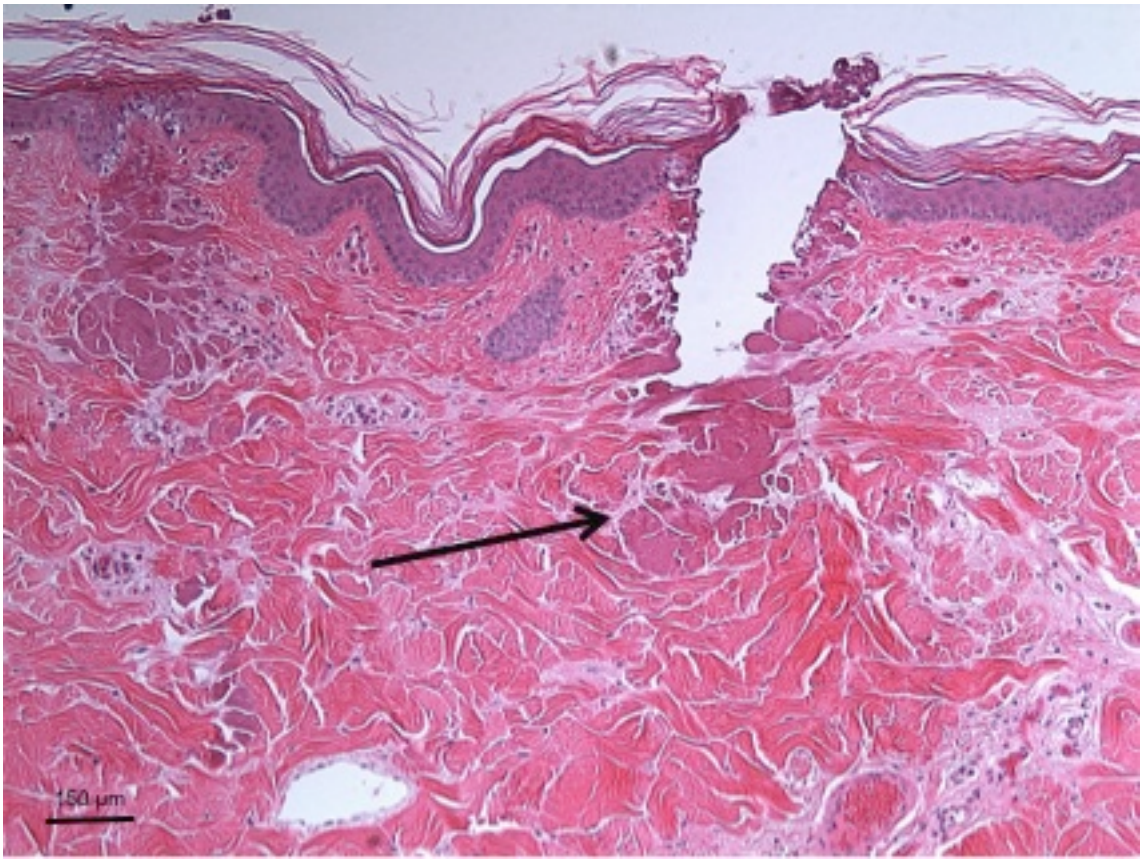


Figure 5: Deep FX, Lumenis CO_2 laser, injury pattern. Note the large amount of collagen denaturation distal to the micro-channel

As has been previously mentioned, there are other methods such as tape stripping, microneedles, electroporation and iontophoresis. All these alternative methods are largely experimental. Tape stripping while simple and cheap, is not a precise technique for removing *stratum corneum*. In addition there are issues with patient compliance. A study by Chen *et al.* compared the use of fractional laser versus tape stripping as a microporation technique (Chen et al., 2012, Escobar-Chavez et al., 2008). They found that the fractionated laser was a superior method for vaccine delivery.

Iontophoresis increase the permeability of the stratum *corneum* by providing a driving force directly onto the drug. It is a non-invasive technique, which permits the passage of charged and uncharged molecules across the skin by using a small electric current. The downside of this technique is that molecules have to be of small size and there may be minor skin irritations. This technique is used clinically in the treatment of hyperhidrosis and plantar fasciitis as well as other musculoskeletal complaints (Choi et al., 2013, Osborne and Allison, 2006).

One of the most promising investigated microporation techniques is the microneedle. It penetrates to the *stratum corneum* and is said to be painless. It is also a more consistent method of microporation compared to other techniques such as tape stripping. There are some manufactured handheld devices, which utilize microneedles for transdermal drug delivery. At present this technology is still in the experimental stage with both numerous *in vitro* and *in vivo* studies (Park et al., 2013, Milewski et al., 2010). The side effect profile is minimal compared with the laser, with erythema and infections rarely being reported. In contrast, depending on the laser setting, the side effects for ablative lasers, could range from mild erythema and oedema, to crusting, weeping and herpes infections,

to longer-term effects on pigmentation. A study by Kumar *et al.* combined iontophoresis with microneedles to investigate if there was a synergistic effect (Kumar and Banga, 2012). They found that combining the two techniques significantly enhanced the transdermal delivery of human growth hormone.

There is a wealth of studies on the use of microneedles, these produce similar microchannels within the skin but involves inserting a foreign body into the skin. Work is still being carried out looking at solid versus hollow microneedles, as well as dissolvable microneedles. Microneedles are said to be painless, but this is due to the fact that they only penetrate the *stratum corneum*, which have no pain fibres (Prausnitz, 2004). Cutaneous laser technology is widely available and in clinical use. Depending on the laser setting and the patient's pain threshold could be potentially also painless.

2.5 Conclusion:

This study has demonstrated that the fractionated laser can be used as a method for microporation of the *stratum corneum*. The aim is to use this existing technology to facilitate transdermal drug diffusion. From these results it was felt that the greater the tissue damage the less likely that drug diffusion would occur. In addition, clinically, the greater the tissue damage the greater the potential for an increase in complications. Therefore, it was felt that for the following studies it would be better to use a laser in which a wide range of energy settings could be used and controlled for and which could be correlated easily to depth with the minimum of collateral damage to the surrounding tissue. The Er:YAG allows this because the energy settings range from 12.5 j/cm^2 (which

correlates to a 25 μm ablative depth) to 375 j/cm^2 (which correlates to a 1500 μm ablative depth).

CHAPTER 3 - Er:YAG fractional ablative laser: histopathological injury as a function of increasing fluence

3.1 Introduction:

From the work carried out in Chapter 2, the erbium-doped yittrium aluminium garnet (Er:YAG) laser produced deep channels that were easily detectable histologically with the least amount of collateral thermal injury to surrounding tissue compared to the carbon dioxide (CO_2) laser. The Er:YAG laser also has the feature of being able to set the laser either by fluence/energy or by depth of ablation. This allows the possibility of altering fluence/energy in order to manipulate depth of injury. What is not clear is how this would change histologically depending on the amount of energy/fluence used. The following study looks at the change in histopathological tissue injury generated by the laser as a function of increasing fluence. In this way it is possible to validate the laser settings in the porcine model. For example, if the laser were set to say 250µm depth, one would expect a margin of error. This study assessed what that margin of error would be and whether it is consistent over the energy range of the laser. In addition, this study sought to illustrate whether collateral thermal injury surrounding the ablative column is proportional to the laser energy or independent of it. This information could be useful when deciding what settings to use for later experimental work.

3.2 Method:

Ethical approval for the experiments was sought and granted by the Institutional Animal Care and Use Committee (IACUC) at University of Texas (UT) Southwestern Medical Center, Dallas, Texas, USA (appendix 1). One adult (200-250lb) female domestic pig was

used. The animal was acclimatized for one-week prior to use. Feeding, sleeping and change of behaviour were monitored daily by a veterinarian. The animal was kept nil by mouth the night before the procedure.

Laser type and setting:

Under general anesthesia the skin of the belly of the pig was shaved. Five areas (A-E) measuring 3cm x 3cm were marked on the skin with a permanent marker separated by 10cm gaps. The Er:YAG (Profile, Sciton Inc, Palo Alto, CA, USA) was used for this study, each area had a different fluence/energy setting tested, as follows (Table 1, Figure 1). The Er:YAG laser also allows the operator to set the density of the energy delivered. This refers to the percentage of tissue that is targeted by the laser for a given area. For the purposes of this study, at all energy levels, the density was set at 22%, which was the maximum density that the laser operates at. This was to help ensure adequate columns would be visualized in biopsies with brightfield microscopy.

Table 1: **Fluence used to treat each area on the pig**

Area	Energy/Fluence (J/cm^2)	Manufacturers ablative depth (μm)
A	25.0	100
B	62.5	250
C	125.0	500
D	187.5	750
E	250.0	1000

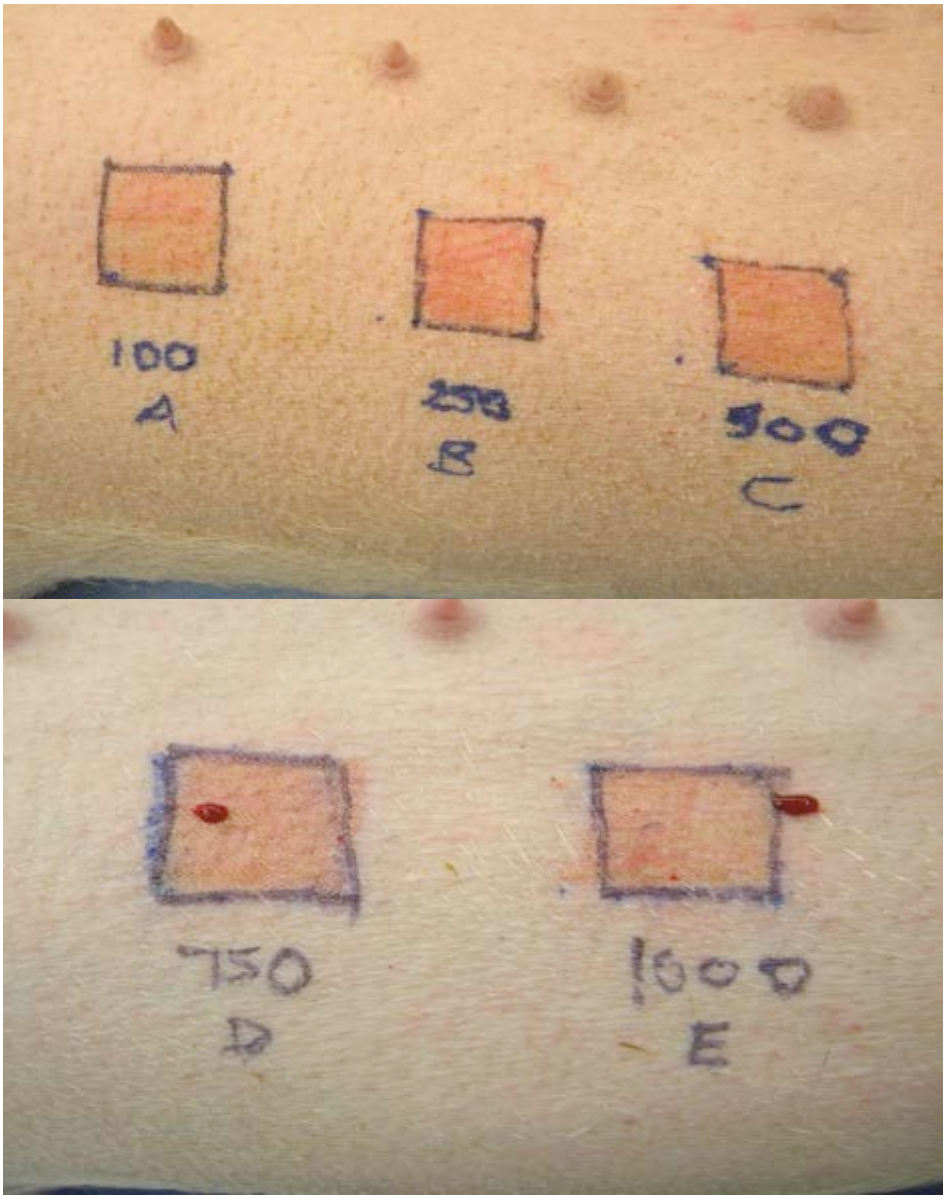


Figure 1: Each area treated with different laser fluences. Note all fluences cause some hyperaemia post treatment

Histological analysis:

8 mm punch biopsies were taken from each of the treated areas immediately post treatment. In addition an 8 mm punch biopsy was taken from untreated skin as a control. The specimen were fixed in 10% buffered formaldehyde solution at pH 7.4 for at least 48 hours, passaged thorough graded alcohol, embedded into paraffin wax and cut into 6 μ sections with a microtome (Shandon Finesse E, Thermoscientific, Kalamazoo, MI). Each section was stained with haematoxylin and eosin (H&E) and then examined under the light microscope to identify any tissue damage or reaction.

Data analysis:

Where ablative columns (microchannels) were visible, 5 columns were randomly selected and measurements were taken of both column depth, and ablative width (maximal ablative injury as shown by collagen denaturation, Figure 2). The software used for this was QCapture Pro 6.0 software (Q Imaging, BC, Canada). An average was taken and tabulated. Statistical differences between the laser settings was tested for using Student's t test, with $p<0.05$ taken as significant (Excel 2011, Microsoft Corp, Redmond, Wa).

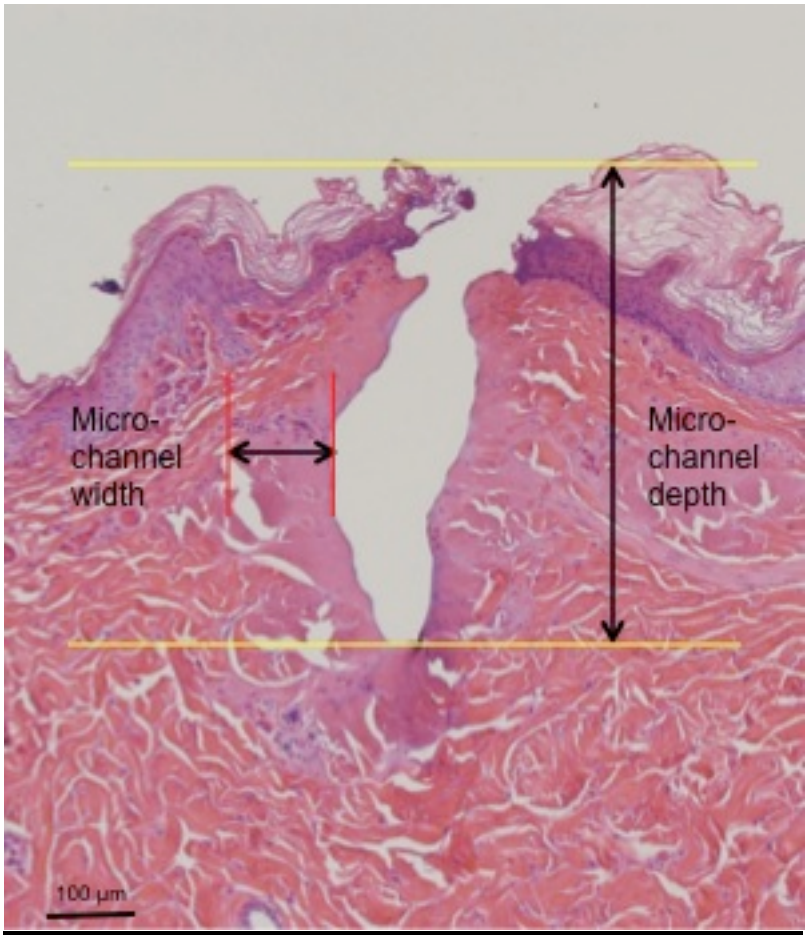


Figure 2: Measurements that are taken when assessing depth and width of micro-channels and collateral thermal injury

3.3 Results:

For each laser setting there were definite ablative columns seen within the tissue. However, in order to see the laser generated microchannels multiple sections were taken. This is because the microchannels are laid down in rows. Each microchannel is bridged by normal skin (Figure 3 & 4). As the laser energy setting increased, the amount of normal tissue bridging the micro-channels decreased.

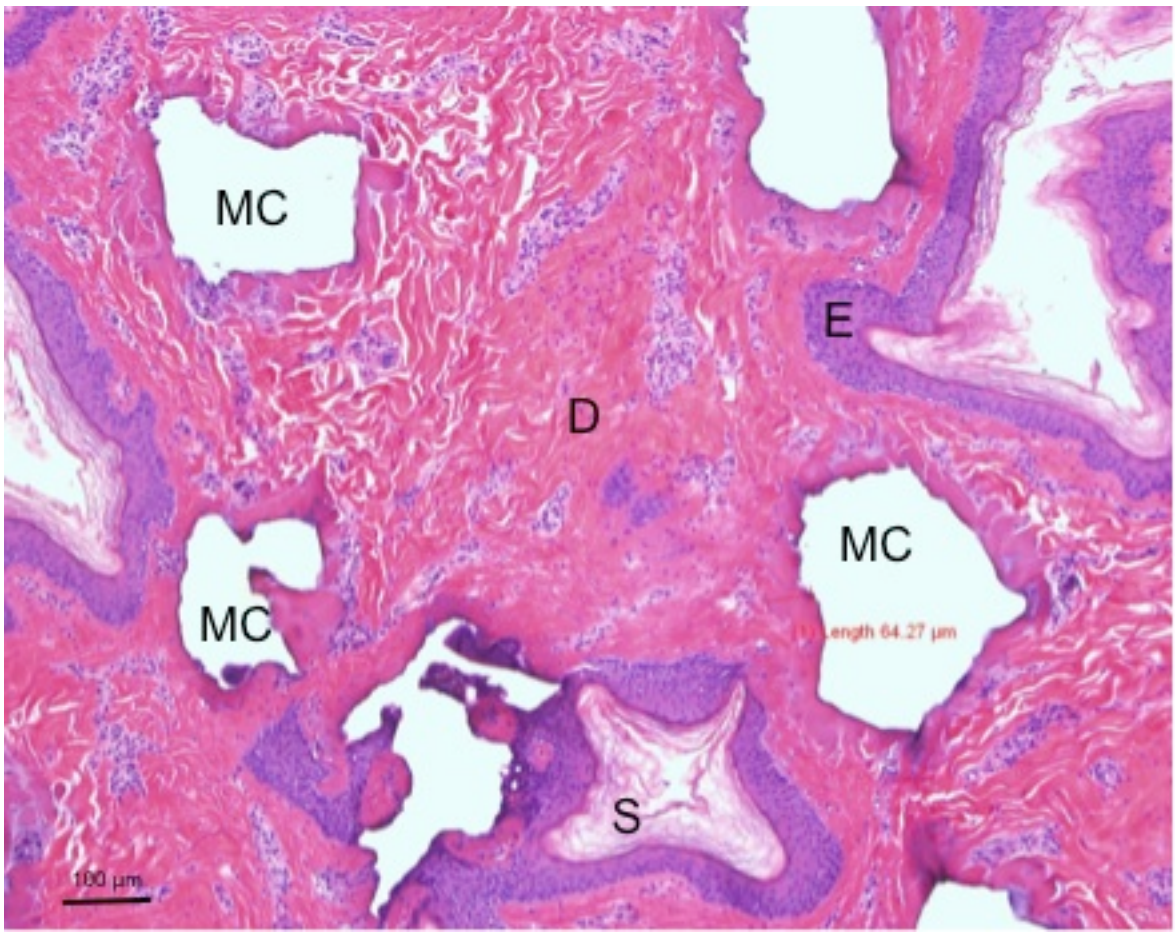


Figure 3: Horizontal section 200 µm through porcine skin treated with the Er:YAG laser (laser setting 125 J/cm^2). Note *stratum corneum* (SC), epidermis (E) and dermis (D) are all visible in this cross section. The microchannels (MC) are bridged by normal tissue.

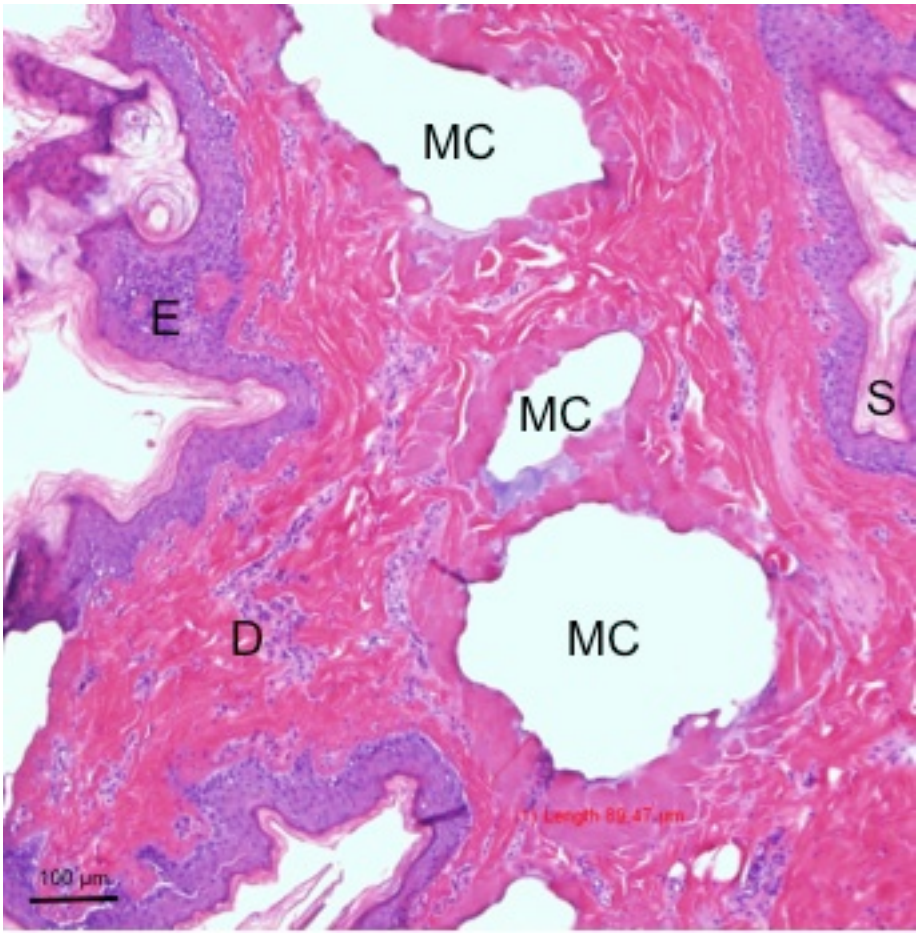


Figure 4: Note amount of normal tissue bridging the columns is reduced at the higher energy setting (laser setting 250 J/cm^2). Thickness of microchannel's coagulated wall is 64.3 µm for the lower laser setting, and greater for the higher power at 89.5 µm.

The amount of normal skin varied depending on the amount of energy delivered to the skin. Therefore, some histological sections contained no microchannels at all, or partial columns (Figure 5).

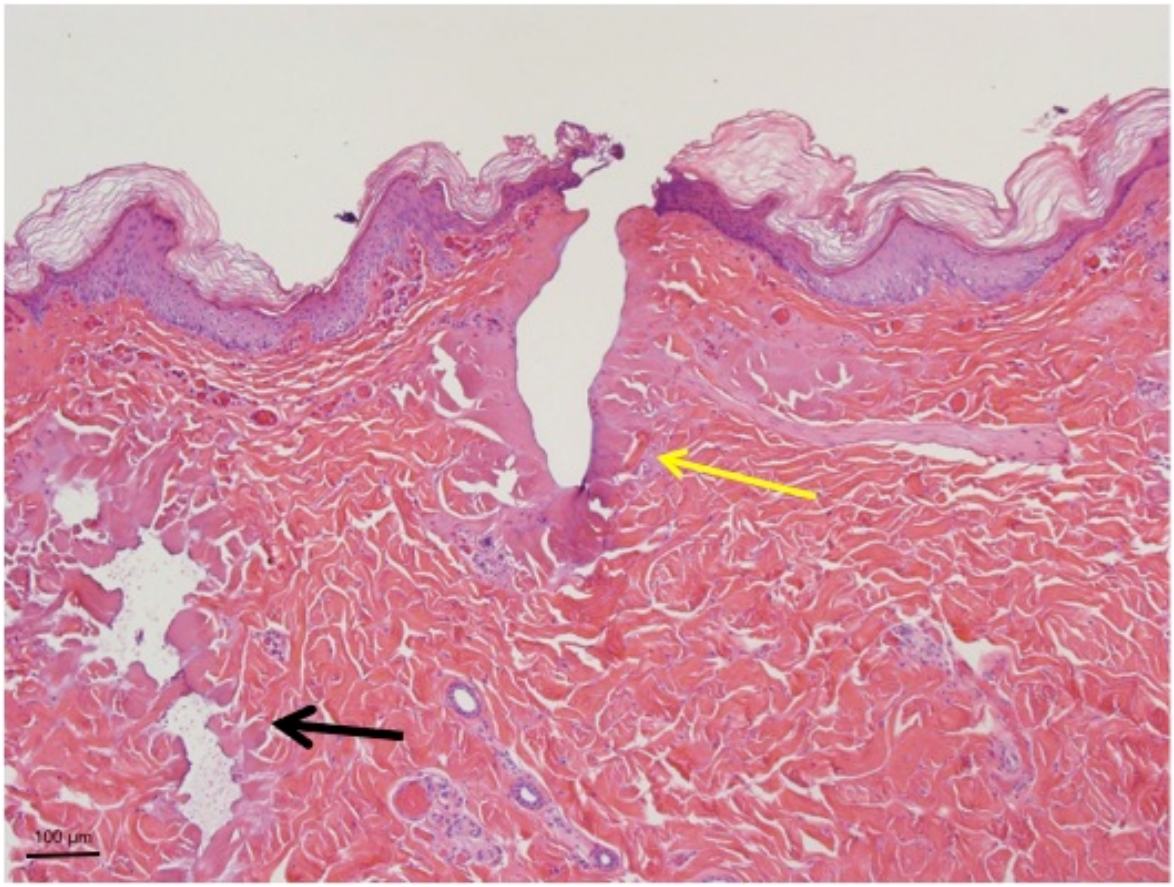


Figure 5: Visible microchannel in center of picture (yellow arrow) but note partial column in dermis to left (black arrow).

Microchannel depth and width:

With increasing fluence the ablative microchannels also increased in depth (Table 2, Figure 6). Apart from the lowest ablative depth setting, the measured depths of microchannels were greater than the laser setting. The actual ablative depth of the microchannels for any given setting was numerically close to the ablative depth setting of the laser (Table 2, Figure 6).

Table 2: Comparison of ablative microchannel generated by differing energy settings of the Er:YAG fractional ablative laser

	Ablative depth by laser setting				
	100 µm	250 µm	500 µm	750 µm	1000 µm
Average microchannel depth (µm)	92.66	281.92	573.64	828.65	1052.35
SD +/-	18.26	74.65	81.20	121.71	155.28
p value	0.42	0.39	0.11	0.22	0.49

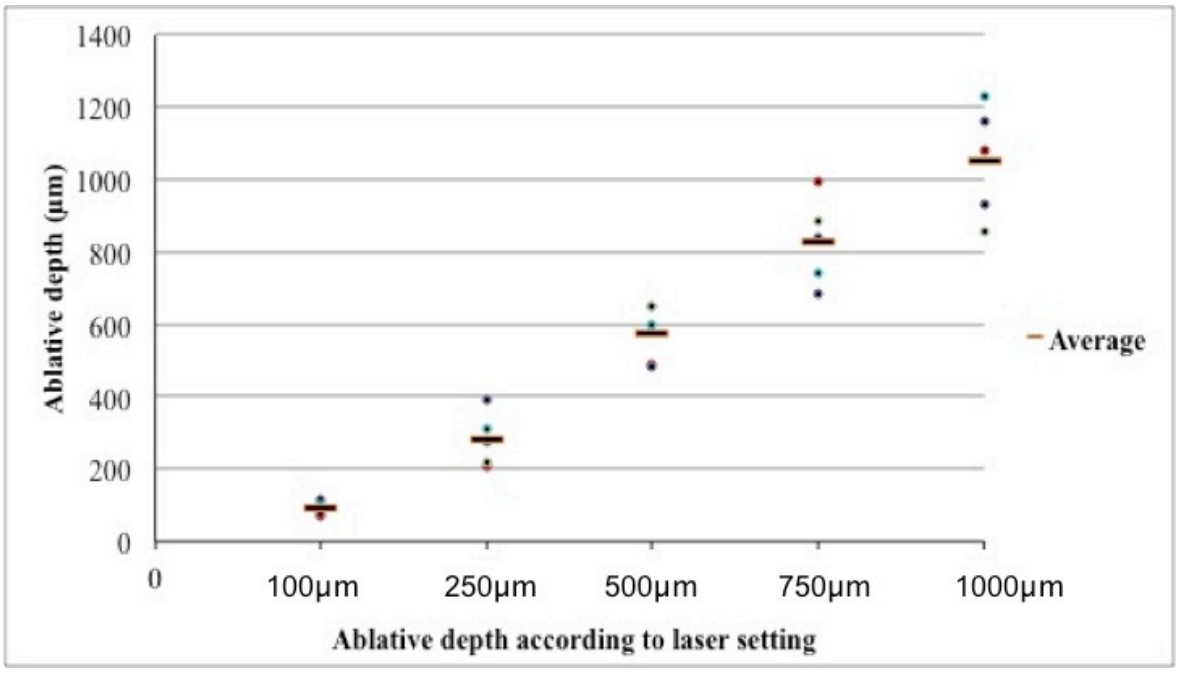


Figure 6: Relationship between laser setting and actual laser depth as measured histopathologically. The observed ablative depths were not significantly different from the laser setting.

Collateral thermal damage lining the microchannel:

A doubling of laser energy did not result in a doubling of collateral thermal injury. The width of denatured collagen lining the ablative microchannel did not follow a linear pattern (Table 3, Figure 7). While overall the width of denatured collagen lining the micro-channels increased with increasing energy setting of the laser, the actual percentage increase of denatured collagen decreased with increasing energy. For example, increasing the laser energy from 25J/cm^2 to 125 J/cm^2, gave rise to a 5-fold increase. However the average increase in denatured collagen width went from 18.76 µm to 58.53 µm, which represents a three-fold increase (p=0.97). Increasing the laser energy from 25 J/cm^2 to 250 J/cm^{2,} which represents a 10 fold increase only increased the average denatured collagen

width from 18.76 µm to 82.78 µm, which represents a 4.5 fold in collagen damage (p=0.95). When comparing the collateral thermal injury widths across all groups, there was no statistically significant difference (ANOVA, p=0.84).

Table 3: Comparison of collateral thermal injury generated by increasing fluence of the Er:YAG fractional ablative laser

	Ablative depth by laser setting				
	100 µm	250 µm	500 µm	750 µm	1000 µm
Average microchannel depth (µm)	18.76	36.64	58.53	75.02	82.78
SD +/-	5.35	8.28	10.75	24.41	23.93

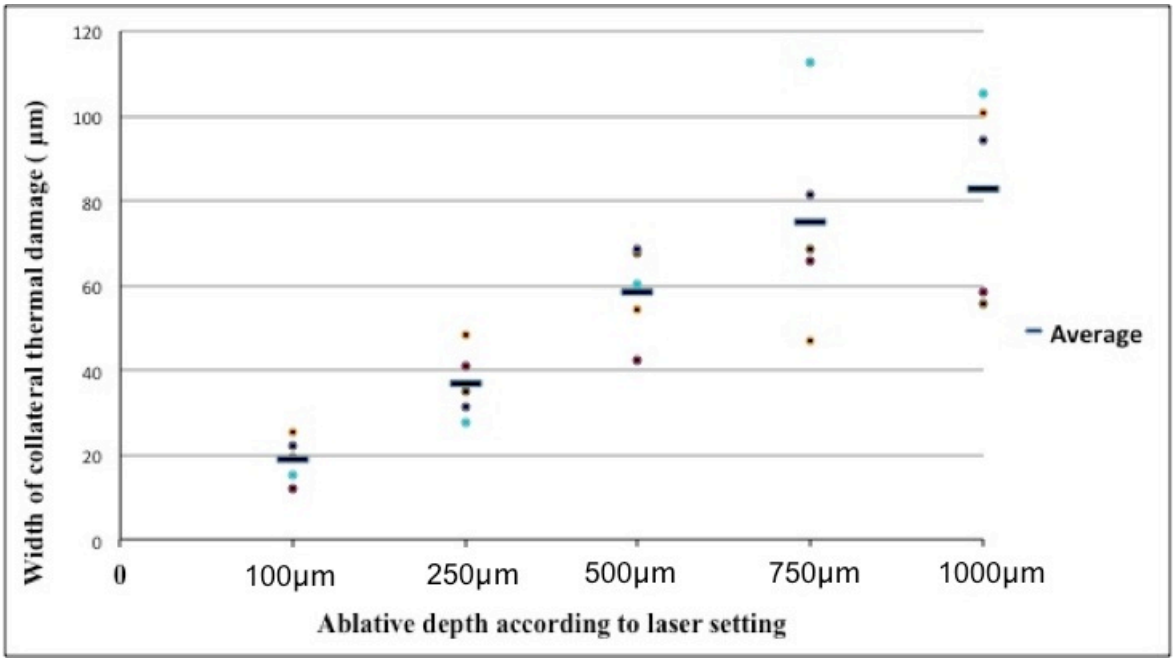


Figure 7: Relationship between thickness of denatured collagen lining the microchannel and increasing fluence.

3.4 Discussion:

The fractionated Er:YAG laser was chosen as the ablative laser to study in further depth because of the ease with which the laser energy could be converted to ablative depth and the fact that it caused the least amount of collateral thermal damage. In this study it has been demonstrated that as expected, the depth of ablative micro-channel increased proportionately to the increase in laser energy delivered and that this is fairly consistent with the stated settings of the laser. What is interesting to note is that the amount of collateral thermal damage does not increase proportionally with the laser setting but appears to be increasing at a *reducing* proportion as the laser energy delivered *increased*. The difference between collateral thermal injury widths across all groups was not found to be statistically significant which was surprising. One of the reasons for this could be that the laser energy was being attenuated by skin appendages such as blood vessels and hair follicles. The dermis being well vascularized would have the ability to absorb some of the laser energy and carry it away along the blood vessels, as opposed to the energy being deposited within the tissue causing collagen denaturation. In some sections one could see the ablative channel being truncated by blood vessels or a hair follicle (Figure 8).

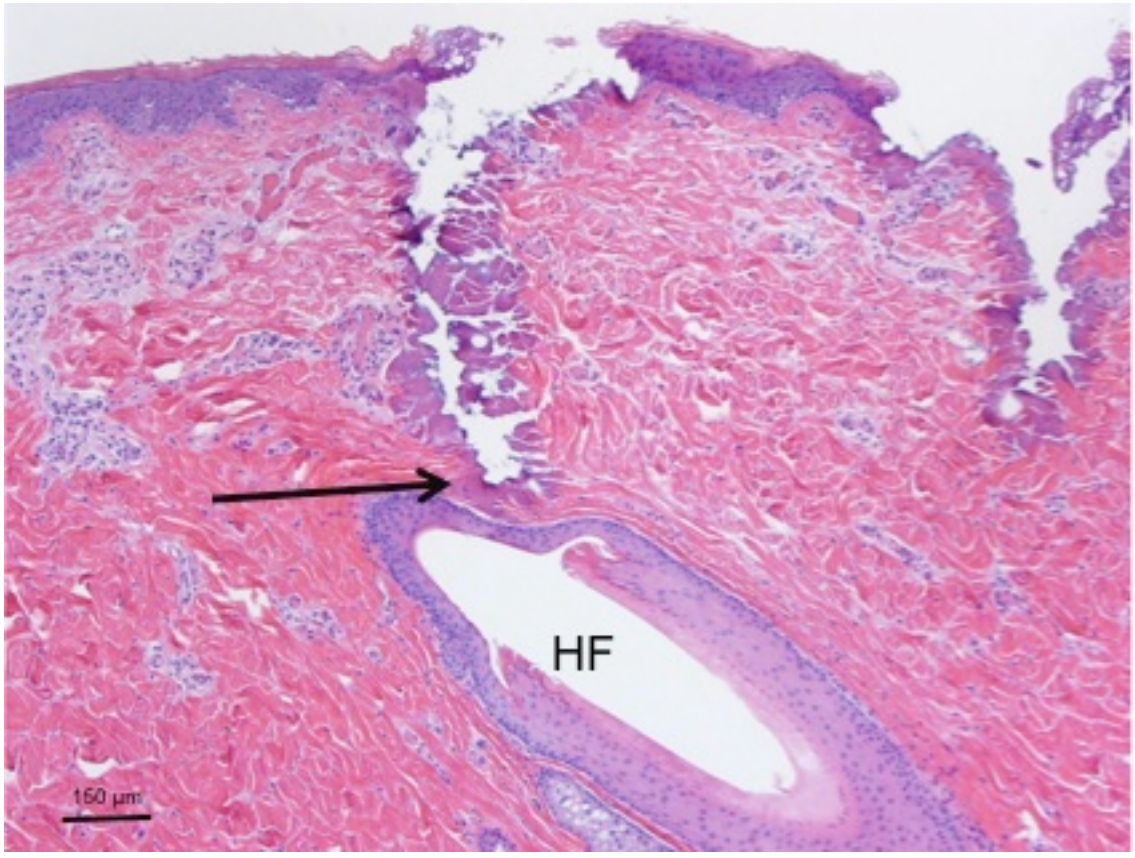


Figure 8: Micro-channel (black arrow) prematurely truncated by a hair follicle (HF).

For the purposes of exploring laser assisted transdermal drug delivery, these features are extremely useful. From the studies performed here, with this laser it is now possible to target the layer in the skin that one would wish to deliver the drug. For example, if a drug is to be targeted to the epidermal layers/superficial dermis, then a laser setting of 100-250 µm would be appropriate. If for example, a drug were to be delivered deeper into the dermis then settings around the 500-750 µm would be appropriate. The effect of collagen denaturation is yet to be determined in relation to drug delivery. However, it is now known from this study that the amount of denatured collagen does not double with double the energy as one might expect but that the effect is attenuated.

Problems with histology:

It was found that when processing and analyzing the specimens there were numerous problems that were encountered along the way. Firstly, the process is lengthy and time consuming. Secondly, at the time of laser treatment the hand piece had to be perfectly perpendicular to the epidermis. This in itself is challenging because the skin undulates, and does not lie perfectly flat. Thirdly, punch biopsies, which were used to collect the tissue, are not ideal. When a punch biopsy is taken the tissue tends to come out not as a perfect cylinder but more like a cone (Figure 9).

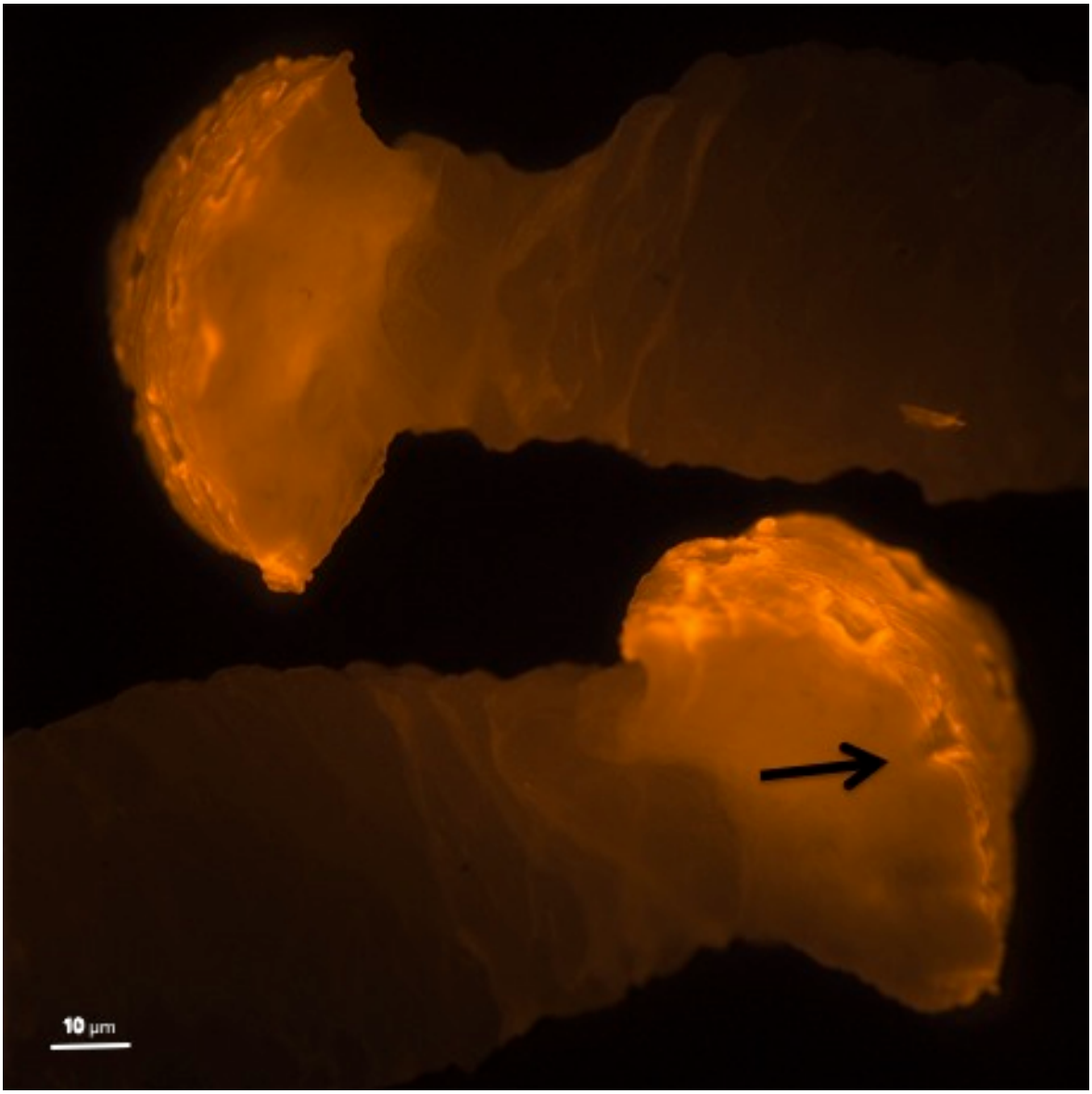


Figure 9: Eosin stained under fluorescent microscopy. Note the mushroom shaped morphology to the punch biopsy. Microchannels are just visible in the specimen (black arrow)

This means that even if the laser beams were perfectly perpendicular to the skin, the microchannels generated would bend with the natural contraction of the skin when it is

biopsied. The tissue is then fixed in formalin and then embedded in paraffin. If the tissue is angled when it is embedded this will affect the angle of the micro-channels when sectioned as they will be sectioned obliquely (Figure 10).

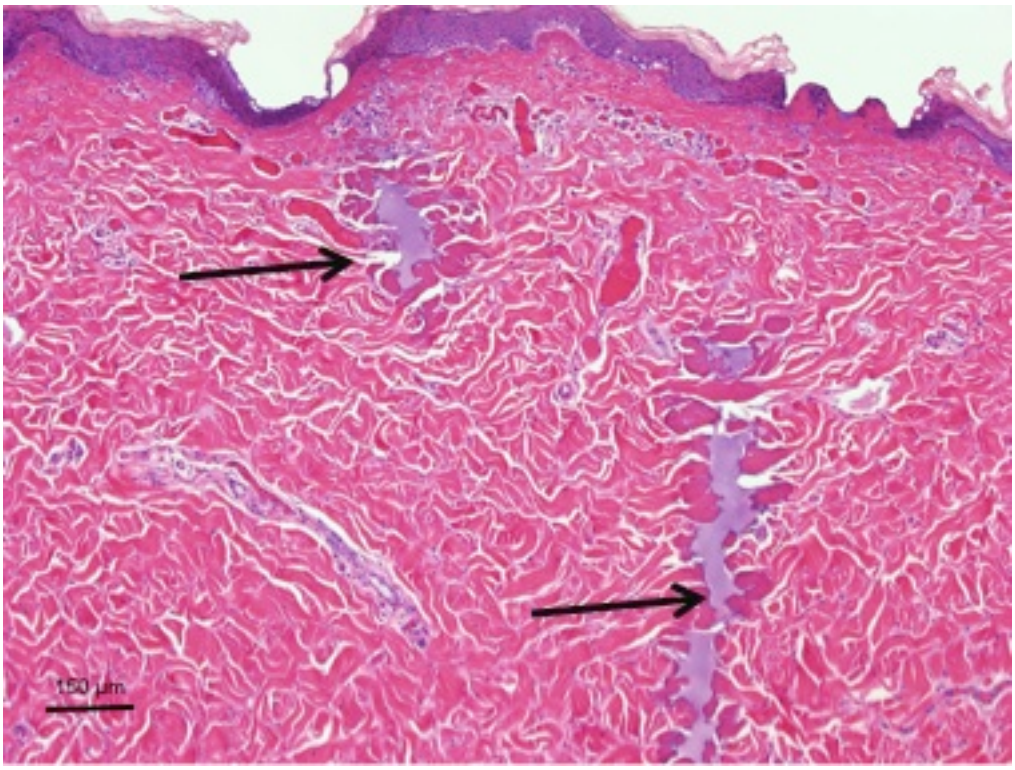


Figure 10: Micro-channels within the tissue (black arrows), they do not communicate with the epidermis. This suggests that the tissue has been embedded at an angle.

Lastly, during sectioning, serial sections have to be taken to be able to get maximal column depths. Each histological section is 5 µm in width, and each spot size generating the micro-channel is 250 µm in diameter, and as we pointed out above, there is normal tissue

separating the microchannels. It was found that in some sections there were no micro-channels at all.

Electron microscopy may have given more histological information from the biopsy samples. It is however, a costly technique. In addition the biopsy still needs to be performed and also the tissue sectioned, therefore, some of the drawbacks discussed above would still be applicable using this technique. Optical coherence tomography is a technology that may have been useful to eliminate some of the problems described above with histological analysis (Enfield et al., 2010, Donnelly et al., 2012). This technique utilizes near infra red light to produce a micrometer resolution three-dimensional image. Using this technique, punch biopsies would not be required and assessments of depth of channel, thermal injury and secondary effects such as oedema could all be measured *in vitro* with the animal still under anesthesia. Unfortunately this technology was not available in the laboratory but is a consideration for future studies.

3.5 Conclusion

The next step is to determine whether substances applied topically to the pretreated laser irradiated area are absorbed into the microchannels and whether they diffuse out of the microchannel given the amount of denatured collagen that lines those channels.

CHAPTER 4 - Investigation of diffusion of dyes from laser generated 'micro-channels' into porcine skin tissue (*in vivo* analysis)

4.1 Introduction:

The previous chapter demonstrated the histopathological injury that the fractionated erbium-doped yittrium aluminium garnet (Er:YAG) laser made in porcine skin. It was found that the channel depth could be manipulated by altering the energy setting on the laser. The concern with channels was that they were lined with denatured collagen, which might be a hindrance to diffusion of substances through the microchannels into adjoining tissues. Therefore, the aim of this study was to determine whether substances could move into these microchannels as well as diffuse into the surrounding tissues. The relevance of this study is to demonstrate *in vivo* in a porcine model, which dye/marker could be used to demonstrate diffusion of a substance from the microchannels produced by the laser into the surrounding tissues. To this end, a selection of tissue dyes, which could be detected in histological sections, was used. The following dyes were investigated: tissue marking dye, methylene blue, and a fluorescent dye (Alexa fluor dextran 488).

4.2 Method:

Ethical approval for the experiments was sought and granted by the Institutional Animal Care and Use Committee (IACUC) at University of Texas (UT) Southwestern Medical Center, Dallas, Texas, USA (Appendix 1). One adult (200-250lb) female domestic pig was

used. The animal was acclimatized for one-week prior to use. Feeding, sleeping and change of behavior were monitored daily by a veterinarian. The animal was kept nil by mouth the night before the procedure.

Laser treatment:

Six areas measuring 5 cm x 5cm were marked on the abdomen of the pig, separated by 10 cm gaps and pretreated with the Er:YAG, set at 500 µm (125J/cm^2) in three rows of two columns. Column 1 had the laser pretreatment; column 2 served as the control.

Each area had a different dye applied:

1. Tissue marking dye (black, Triangle Biomedical Sciences, Durham, NC). This dye is commonly used for edging pathological specimens for orientation and therefore, should not be affected by histological processing.
2. Methylene blue (Sigma Aldrich, St Louis, MO). This is a commonly used drug in medicine and therefore, is not systemically toxic to the animal. It has a lower molecular weight (of around 320 MW) than the tissue marking dye, and so may be able to diffuse through the tissue more easily. This is a water-soluble dye, so it is possible that it could be leached away during histological processing.
3. Alexa fluor dextran 488 (Invitrogen,Grand Island, NY). This is a photostable fluorescence marker dye (molecular weight 10,000), which appears green on fluorescent microscopy. It is used commonly for *in vitro* cellular work, for labeling molecules and cell analysis. Although it is clear to the naked eye this dye should

be fixed in formalin and so if there is any penetrance into the tissues it will be fixed during the processing of the tissue, and not washed away.

The tissue dye having a thick consistency was painted onto the laser irradiated area. Blotting paper was then applied over the area and held in place with an occlusive dressing (Tegaderm™, 3M St Paul, Minnesota) for 30 minutes and then removed. For the methylene blue (which has a runnier consistency) the blotting paper was soaked in the methylene blue solution prior to application as a dressing over the laser irradiated area. The blotting paper was then held in place with an occlusive dressing again for 30 minutes to allow the material time to diffuse or be transported into the skin. The fluorescent dye also had a runny consistency and was applied in the same manner as the methylene blue solution.

Biopsy:

After removal of the dressings at 30 minutes, samples were obtained from control and irradiated areas using a 6mm punch biopsy.

Tissue analysis by Histological examination

The specimens were fixed in 10% buffered formaldehyde solution at pH 7.4 for a maximum of 48 hours, passaged thorough graded alcohol, embedded into paraffin wax and cut into 6 μ sections with a microtome (Shandon Finesse E, Thermoscientific, Kalamazoo, MI).

a. Each section was stained with H&E and examined under ordinary light microscope to identify any tissue damage or reaction.
b. Each section was examined under the fluorescent microscope.
c. Any passage of dye into the columns/tissues was noted.

4.3 Results:

Macroscopic appearance:

The only specimen in which dye could be seen was the one in which tissue marking dye had been applied. Most of the methylene blue was found on the blotting paper. The fluorescent dye was clear in nature when applied so it was difficult to determine the macroscopic presence of the dye.

Microscopic analysis:

Tissue marking dye:

For the control, the tissue marking dye was visible on the *stratum corneum* however, there was no penetration into the tissue. The tissue marking dye being black in colour was easily visible within the laser-generated microchannels (Figure 1). There was no dye found outside of the microchannels.

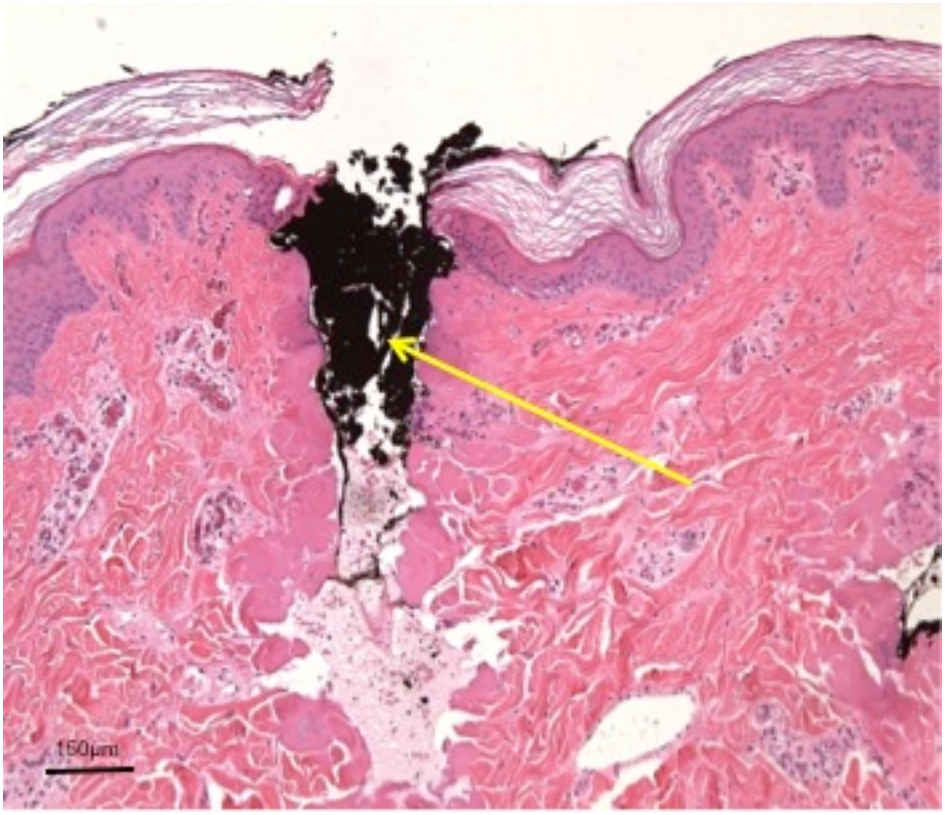


Figure 1: Tissue marking dye visible within the microchannel, note no diffusion of the dye into surrounding tissues

Methylene blue:

For the control there was no methylene blue visible on the *stratum corneum* or diffused into the tissue below. For the laser treated specimens, there was very little dye seen within the microchannel or on top of the *stratum corneum* (Figure 2) and no dye was seen within the tissue.

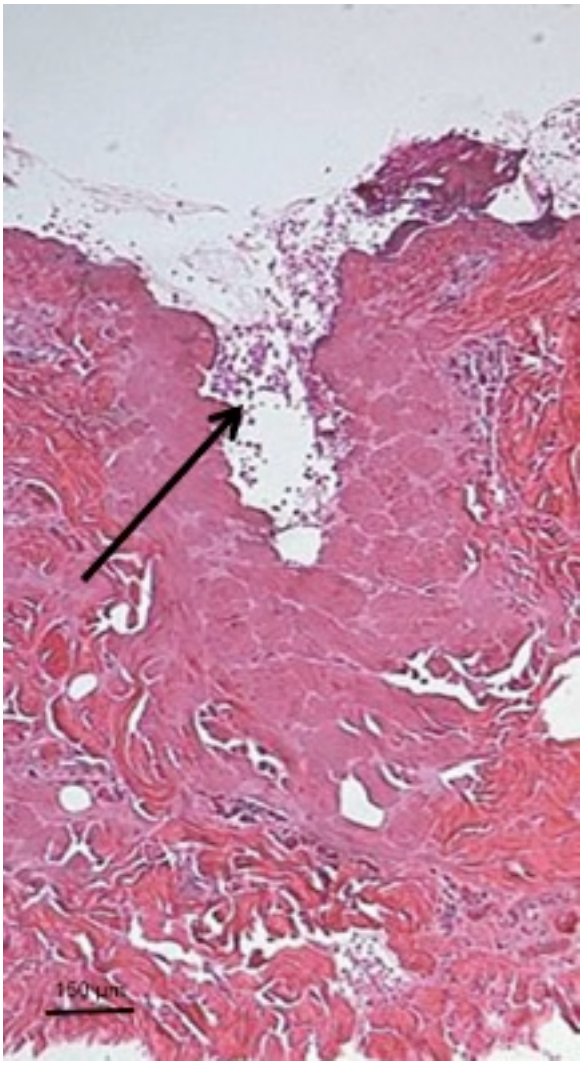


Figure 2: Very little methylene blue dye visible in the microchannel. No dye within the microchannel walls or in the tissue.

Alexa fluor 488:

For the control there was some auto-fluorescence of the *stratum corneum* and blood vessels within the specimen. Weak fluorescent signal was seen lining the microchannel on fluorescent microscopy (Figure 3). There appeared to be some increased intensity of

signal, which corresponded to blood vessels. However the auto-fluorescence of the blood vessels had a stronger signal in areas away from the microchannels (yellow arrows).

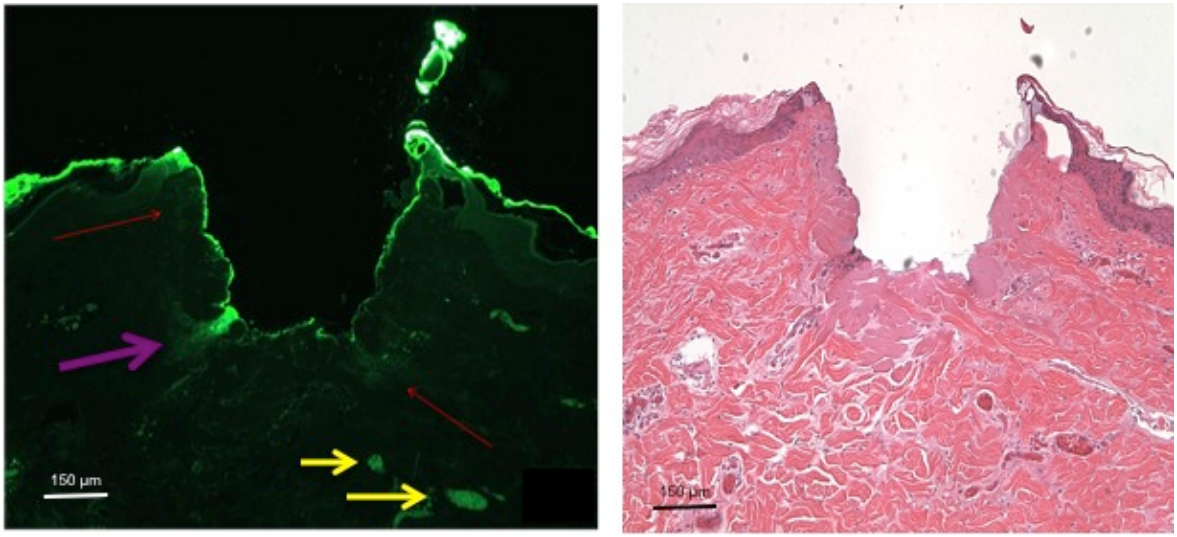


Figure 3: Image left - H&E stain, note no evidence of tissue staining with the fluorescent dye. The laser microchannel is easily visible. Image right - same section but seen under a fluorescent microscope. Alexa Fluor 488, note green fluorescence seen lining the microchannel (red arrows), with possible diffusion along blood vessels (purple arrow). Some auto fluorescence for distal blood vessels (yellow arrows).

4.4 Discussion:

The laser pretreatment produces numerous microchannels within the tissue. The aim of this study was to determine whether substances could be delivered through those channels into the surrounding tissue. Multiple dyes were tested in this experiment to see if they could convincingly demonstrate the potential of these microchannels as conduit for drug

delivery. Unfortunately it was not possible to demonstrate this unequivocally with the selection of dyes used.

Tissue marking dye is very visible within the micro-channels on histology however, because it has a high molecular weight it could not diffuse through the tissue. Methylene blue is known to be able to diffuse through tissue however, very little was seen on histology. This could be due to the dye being washed away as part of the processing prior to embedding and sectioning. The tissue is fixed in formalin for 48 hours, and then washed through graded alcohol before being embedded in paraffin. The dye is more than likely washed away as part of this process. The fluorescent dyes were chosen because they are fixed in the tissue at the point that it is placed in formalin. However, the downside of using the fluorescent dye is that the tissue also has an innate auto-fluorescence and this makes it difficult to distinguish between the dye and the tissue.

There are different techniques for assessing transdermal drug delivery in the literature. For example quantum dot (QD) are engineered nanoparticles that have fluorescent characteristics (Zhang and Monteiro-Riviere, 2008). They have been used to assess if hydrophobic drugs such as ketoprofen can cross the skin (Degim and Kadioglu, 2013). They can be combined with other methods such as microneedles to enhance their absorbance (Gittard et al., 2011). This technology is still in the experimental stage but shows great promise particularly in the field of directed cancer treatments and vaccinations (Singh and Lillard, 2009, Bolhassani et al., 2011).

This study utilized an *in vivo* animal model; however, one of the more common methods of investigation of transdermal drug delivery is the Franz diffusion cell system. A Franz

diffusion cell consists of two glass components: an upper donor chamber and a lower receptor chamber. A membrane separates the two chambers; this is the membrane through which the study drug/molecule must pass. This is an *ex vivo* technique which although effective at demonstrating transdermal movement of molecules, does not necessarily replicate what would occur in an *in vivo* setting. The membrane used in the chamber consists of skin removed from the animal or human model or could be synthetic.

In vivo studies, which look at the feasibility of microporation techniques, employ the use of numerous study drugs. The drugs utilized may have fluorescent potential such as MAL and can be visualized using fluorescent microscopy (Haedersdal et al., 2011, Haak et al., 2012b). Other methods include the use of low molecular weight drugs that are detectable in the blood after absorption such as diclofenac or propofol (Kasha et al., 2012, Juluri et al., 2013).

4.5 Conclusion:

Despite the shortcomings of these dye experiments, they still raised the possibility that substances could penetrate laser-generated channels. This possibility could be further explored using topical application of substances which ordinarily are not absorbed through the skin in large quantities but which could be detected in the blood should enhanced absorption occur. This is in keeping with many of the studies in the literature in which drug penetrance is used to assess the feasibility of new transdermal delivery techniques.

CHAPTER 5 - Can fractional lasers enhance transdermal absorption of topical lidocaine in an *in vivo* animal model?

5.1 Introduction:

The transdermal route of drug delivery was popularized in the USA in 1979 when the first patch was approved for use in motion sickness (McCauley et al., 1979). Since then, a variety of medications have been available delivered by this route, ranging from hormone replacement, to pain relief to nicotine replacement to name a few. The popularity of this type of drug delivery system is three fold; it can be patient administered, it is painless and the inherent risks associated with hypodermic injections are removed (Torin Huzil et al., 2011). In terms of bioavailability it is often superior to the oral route in medications that undergo first pass metabolism in the liver. Much research has gone into methods of delivering drugs through this route because of these advantages. However, the intrinsic barrier protective properties of skin, namely the *stratum corneum*, has posed limitations to drugs delivered in this way (Barry, 2001), with only 20 drug formulations in around 40 products approved by the FDA (Prausnitz and Langer, 2008, Subedi et al., 2010). There has been a move therefore, to techniques, which can disrupt the *stratum corneum* in order to enhance its permeability and allow the passage of a greater variety of drugs. Methods that have been used include chemical enhancers, iontophoresis, tape stripping/dermabrasion, ultrasound, microneedles, electroporation, and thermoablation with varied success (Prausnitz and Langer, 2008, Wang et al., 2005, Subedi et al., 2010, Guy, 2010).

It is thought that the pretreatment of skin with a fractional laser enhances transdermal delivery of drugs, and there are published works both with *in vitro* and *in vivo* models that attest to this (Lee et al., 2001, Haedersdal et al., 2010, Lee et al., 2011c). The theory for mechanism of action has not yet been fully elucidated, in particular, whether the drug is lipophilic or hydrophilic has an effect on absorption even with the *stratum corneum* removed, but several studies have looked at the effects of the erbium-doped yittrium aluminium garnet (Er:YAG) and carbon dioxide (CO_2) lasers with particular reference to this (Lee et al., 2007, Haedersdal et al., 2010, Yun et al., 2002, Lee et al., 2002). These studies have shown that these lasers enhance permeability of the skin to substances by ablating (partially or fully) the *stratum corneum* which is the greatest barrier to drug diffusion across the skin. This ablative effect has been shown to be reversible within a few days depending on the laser used (Hantash et al., 2007). These lasers most likely enhance drug delivery as a result of 'fractional photothermolysis'. The concept of fractional photothermolysis was introduced by Rox Anderson's group in 2004.(Manstein et al., 2004) This method creates 'microscopic thermal wounds', which spared the tissue surrounding each of those wounds, thus prompting a more rapid patient recovery following laser treatment. Histologically, fractional lasers produce what we have termed 'microchannels' in the skin (Figure 1).

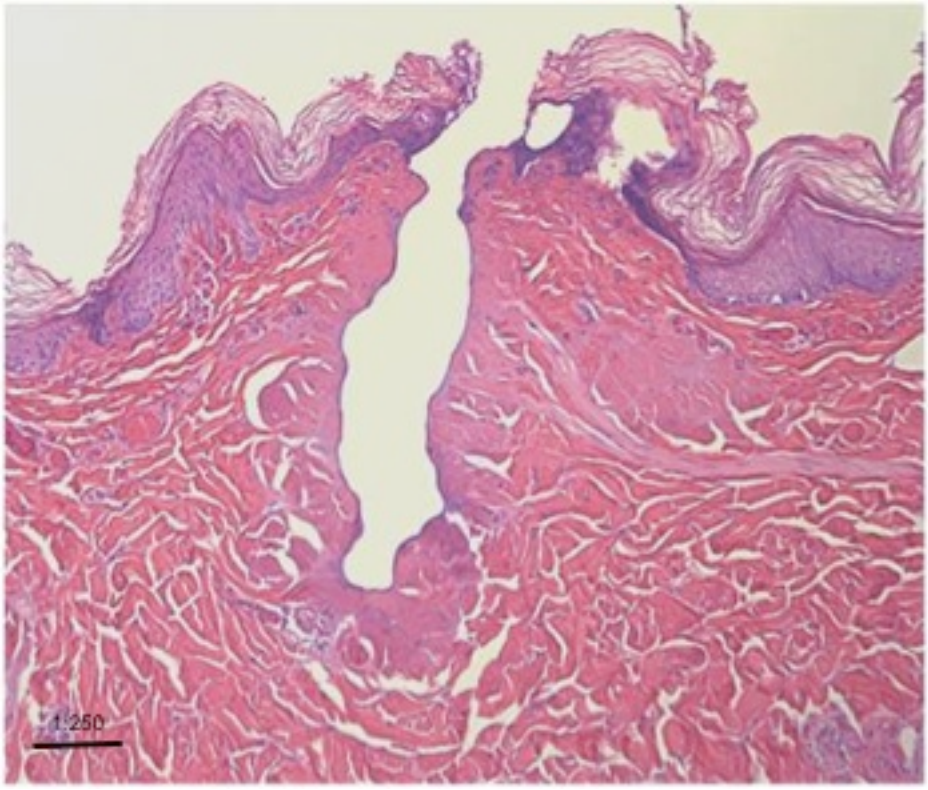


Figure 1: Microchannel' generated in porcine skin following fractional Er:YAG laser treatment

The aims of this study were two-fold, firstly to investigate if these microchannels can be utilized to enhance transdermal delivery of the study drug, topical lidocaine, in an *in vivo* porcine model. Topical lidocaine is a widely available drug, which can be bought over the counter. It has a well-known pharmokinetic profile. Lidocaine and its metabolite monoethylglycinexylidide (MEGX) can be measured in blood serum. Secondly, this study looks at how changing laser energy effects absorption of the drug for a given density of the laser beam.

5.2 Method:

Ethics approval for the study was sought and granted by the IACUC board at UT Southwestern (appendix 2). The animals were acclimatized for one-week prior to use in the pilot study, and kept nil by mouth the night prior to surgery.

Day 0:

Four animals were included in this study. Under general anesthesia the skin of the belly of each pig was shaved. A 400cm^2 area was marked on the abdomen of each animal. Topical lidocaine (LMX-4, Ferndale laboratories) was then applied at time 0 and covered with a Tegaderm™ occlusive dressing (Figure 2). After 60 minutes the dressing and the topical lidocaine was removed. The area was cleansed with normal saline. Bloods were drawn at the following time points: 60, 90, 120, 180 and 240 minutes. After the last blood draw, the animal was allowed to recover from the anesthesia

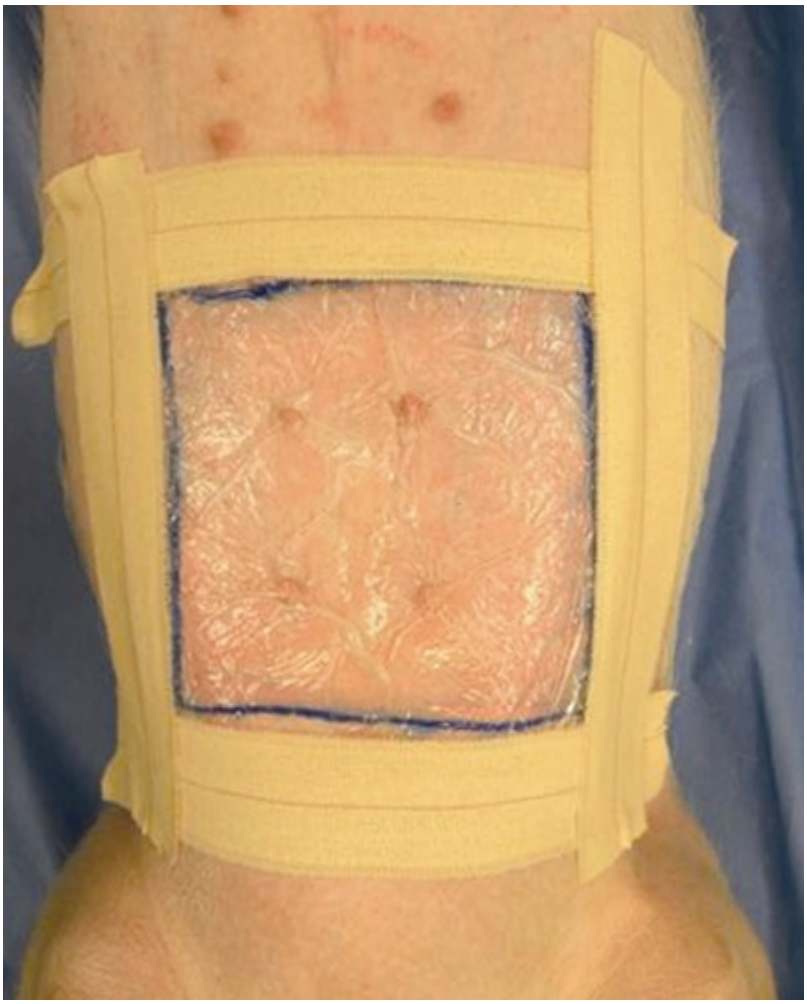


Figure 2: Lidocaine applied to the abdomen of the pig and covered with an occlusive dressing

Day 7:

Under general anesthesia the skin of the belly of each pig (N=4) was shaved. A 400cm^2 area was marked on the abdomen of each animal prior to laser pretreatment.

Laser type and settings:

Fractionated erbium-doped yittrium aluminium garnet (Er:YAG) laser (Profile, Sciton, Palo Alto, CA) was used for this study. The laser energy settings used varied from 500 µm ablative depth (125 j/cm^2) to 25 µm ablative depth (12.5 j/cm^2), at 22% density. A 1cm^2 scanning hand piece was used to deliver the laser energy (table 1, figure 3). For each of the groups, 2 animals were included (N=2). Group 1 was pretreated with 500 µm ablative depth (125 j/cm^2), Group 2 with 250 µm ablative depth (62.5 j/cm^2), Group 3 with 50 µm ablative depth (25 j/cm^2) and Group 4 with 25 µm ablative depth (12.5 j/cm^2). Immediately following the laser treatment topical lidocaine (LMX-4, Ferndale laboratories) was then applied and covered with an occlusive dressing for 60 minutes. After 60 minutes the dressing and the topical lidocaine was removed. The area was cleansed with normal saline. Bloods were drawn at the following time points: 0, 60 (at time of removal of occlusive dressing), 90, 120, 180 and 240 minutes. The animals were allowed to recover and the same process was repeated 7 days later on an untreated area on the abdomen. This time after the last blood draw the animals were euthanized.

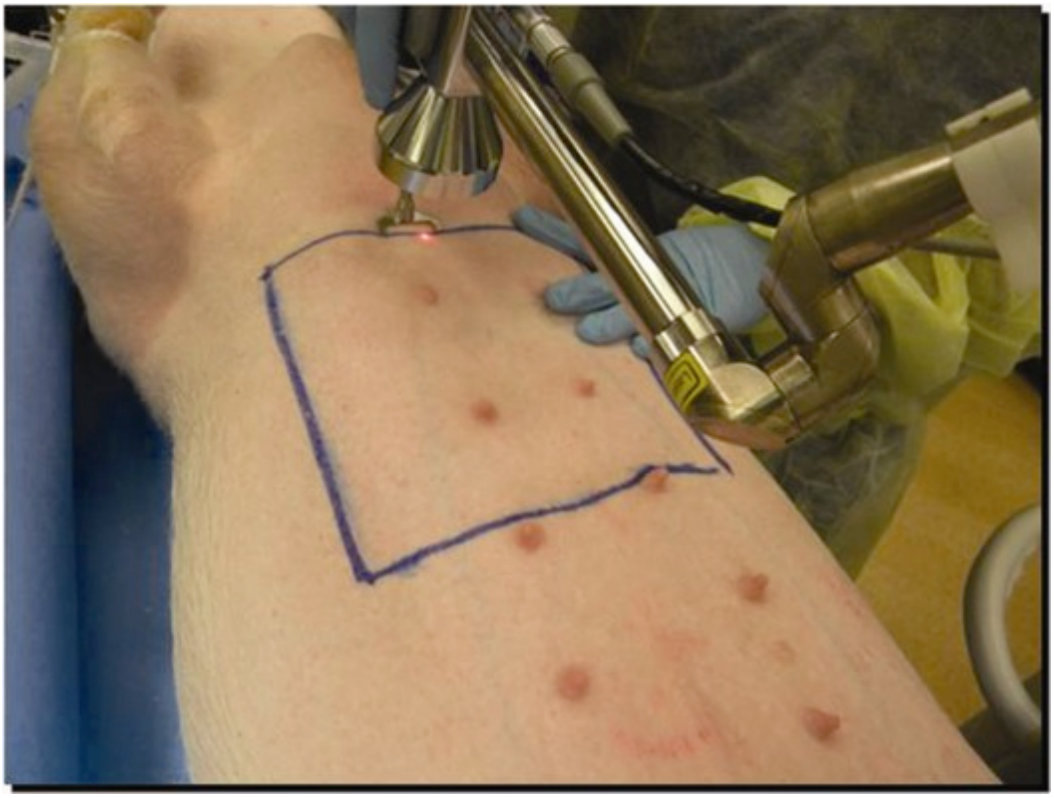


Figure 3: Laser treatment to the pig abdomen prior to application of the lidocaine.

Table 1: Laser settings per group

Group	Ablative depth (µm)	Fluence (J/cm^2)	Density (%)	Scanning hand piece size (cm^2)
1	500	125	22	1
2	250	62.5	22	1
3	50	25	22	1
4	25	12.5	22	1

Blood sample procurement and analysis:

Whole blood samples (approximately 15ml of blood) were collected in ethylenediaminetetraacetic acid (EDTA) containing vials and allowed to clot. The blood samples were then centrifuged (3000 rpm for 10 minutes at 4°C) and the serum stored at minus 80°C until analyzed. The Department of Clinical Chemistry, George-August University (Goettingen, Germany) analyzed lidocaine and MEGX in serum using liquid chromatography-tandem mass spectrometry (LC-MS-MS). This is a published validated technique that is simple, sensitive, reproducible and rapid (Streit et al., 2001).

Statistical analysis:

The serum concentration-time courses of lidocaine and MEGX were characterized using Microsoft Excel (Microsoft Corp., Redmond, Wash.). A repeated measures ANOVA test for non-parametric data was used to analyze differences between the groups. Associations with $p<0.05$ were considered statistically significant.

5.3 Results:

Absorption of topical lidocaine – no laser treatment (control):

For all animals and at all time points there were **no** detectable levels of lidocaine or its metabolite MEGX in the blood serum.

Absorption of topical lidocaine – laser pre treatment:

For all animals and at all time points there were detectable levels of lidocaine or its metabolite MEGX in the blood serum. Peak serum levels did not exceed 1µg/ml at any time point. The absorption varied with the energy setting. Table 2 summarizes for each time point the serum levels of lidocaine and MEGX for each animal.

Table 2: Effect of laser energy on absorption of lidocaine and its metabolite MEGX

Laser Setting (µm)	Lidocaine (µg/ml)	MEGX (µg/ml)	Time to peak concentration Lidocaine (min)	Time to peak concentration MEGX (min)
25	0.30	0.0144	90	120
50	0.48	0.036	120	120
250	0.62	0.0477	90	120
500	0.45	0.0182	90	120

Serum lidocaine levels in relation to laser energy setting (Table 3, Figure 4):

There was a significant difference between the serum levels of lidocaine (p=0.0002) and MEGX (p<0.0001) between the four groups. The greatest absorption of lidocaine, 0.62 µg/ml, occurred with the 250 µm ablative depth (range 0.59-0.71 µg/ml), at 90 minutes after application. The lowest absorption of lidocaine occurred with the 25µm ablative depth (0.14 – 0.46 µg/ml). The absorptions of lidocaine at 500 µm ablative depth (0.29 – 0.66 µg/ml) and 50µm ablative depth (0.31 – 0.68 µg/ml) were similar. For all laser settings on average, except 50 µm ablative depth, peak serum levels of lidocaine occurred at 90 minutes. For the 50 µm ablative depth peak levels occurred at 120 minutes.

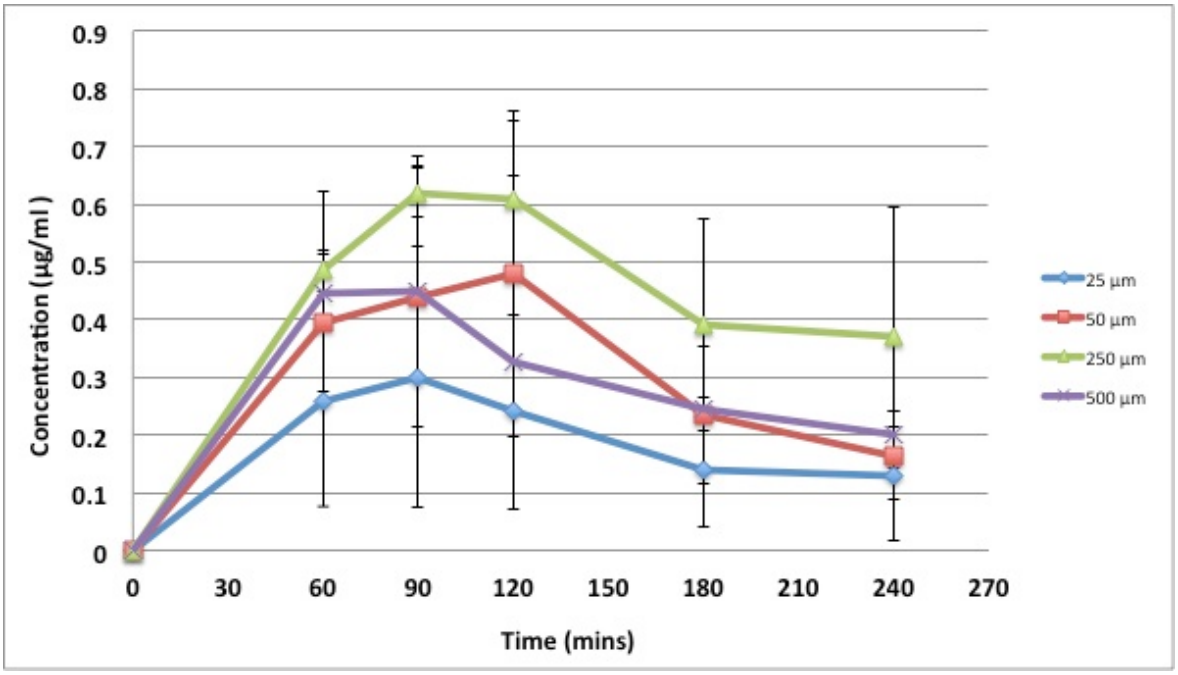


Figure 4: Average serum lidocaine levels in relation to laser energy setting (N=2 for each laser setting, p=0.0002)

Table 3: Individual animal results for serum lidocaine

		Time (min)						
Ablative depth (µm)	**Pig**	**0**	**60**	**90**	**120**	**180**	**240**	***p* value**
25	1 2	0 0	0.13 0.39	0.14 0.46	0.12 0.36	0.07 0.21	0.05 0.21	0.008*
50	1 2	0 0	0.31 0.48	0.28 0.6	0.28 0.68	0.15 0.32	0.11 0.22	0.021*
250	1 2	0 0	0.51 0.46	0.59 0.65	0.52 0.71	0.26 0.52	0.21 0.53	0.085
500	1 2	0 0	0.23 0.48	0.27 0.60	0.22 0.68	0.29 0.32	0.20 0.22	0.143

Serum MEGX levels in relation to laser energy setting (Table 4, Figure 5):

There was a significant difference between the serum levels of MEGX ($p<0.0001$) between the four groups. The greatest serum levels of MEGX, 0.0477 µg/ml, occurred with the 250 µm ablative depth (range 0.0446-0.052 µg/ml), and this occurred at 120 minutes after application. The lowest serum levels of MEGX occurred with the 25 µm ablative depth (0.0111 – 0.0197 µg/ml). The absorption of MEGX at 500µm ablative depth (0.013 – 0.0265 µg/ml) and 50µm ablative depth (0.008 – 0.0674 µg/ml) was similar. For all laser settings, on average the peak serum levels of MEGX occurred at 120 minutes after application of the topical drug.

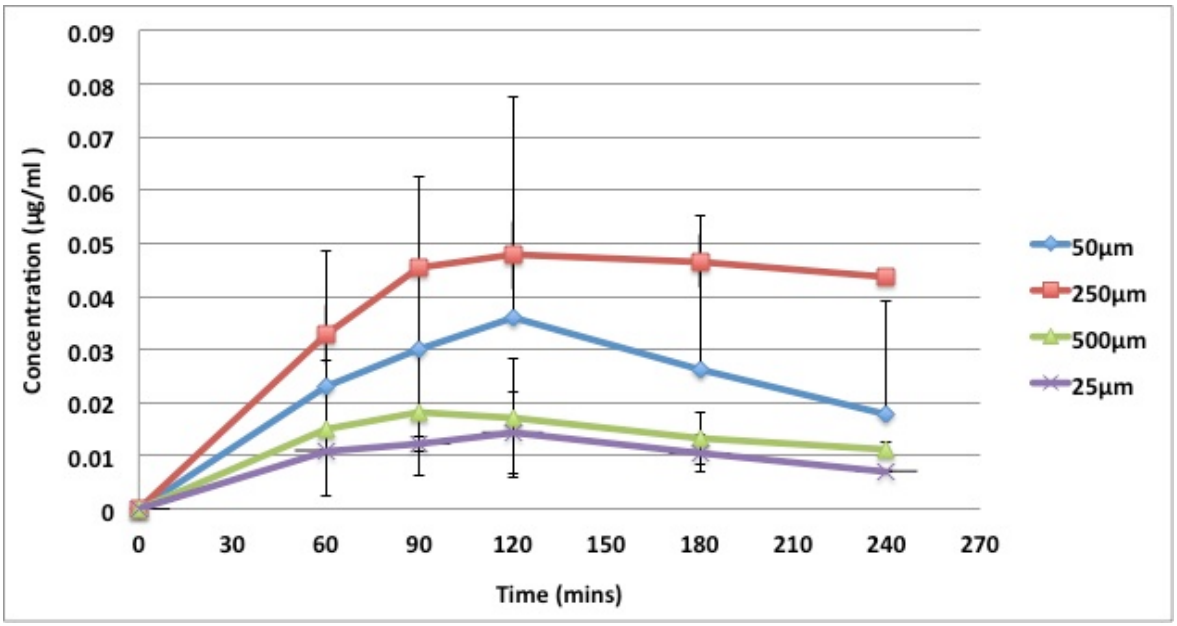


Figure 5: Average serum MEGX levels in relation to laser energy setting (N=2 for each laser setting, *p*<0.0001)

Table 4: Individual animal results for serum MEGX

		Time (min)						
Ablative depth (µm)	**Pig**	**0**	**60**	**90**	**120**	**180**	**240**	***p* value**
25	1 2	0 0	0.011 0.011	0.013 0.011	0.020 0.009	0.013 0.008	0.007 0.007	0.140
50	1 2	0 0	0.005 0.041	0.007 0.053	0.008 0.067	0.006 0.047	0.003 0.033	0.008*
250	1 2	0 0	0.037 0.029	0.052 0.039	0.052 0.043	0.050 0.043	0.043 0.045	0.051
500	1 2	0 0	0.006 0.024	0.010 0.027	0.009 0.025	0.010 0.017	0.013 0.011	0.055

5.4 Discussion:

This study demonstrates that laser pretreatment of skin enhances the absorption of topical lidocaine and therefore, its serum levels in an *in vivo* model. Without laser pre-treatment there were no detectable serum levels of lidocaine or its metabolite at any time point. Following laser treatment for any given laser setting, systemic absorption of lidocaine was achieved. This enhanced systemic absorption of a drug by laser pretreatment has not been shown before in the literature. The barrier to drug absorption through the skin is the outer most layer, the *stratum corneum*, which is formed largely from dead cells which are bound together by keratin produced by corneocytes embedded in lipid regions (Groen et al., 2011). In humans this layer is relatively thin at 10-15μm and is responsible for the barrier function of the skin. The major lipid classes in human *stratum corneum* are ceramides, cholesterol and long chain free fatty acids (Bouwstra et al., 2000). This means that this layer is hydrophobic but lipophilic. Therefore in order for drugs to penetrate through this layer, they must be able to diffuse through the lipid areas between the corneocytes before they can reach the epidermis. This study was performed in a porcine model and it is known that the *stratum corneum* in pigs is functionally similar to humans (Gray et al., 1982). In addition, porcine skin closely resembles human skin in terms of epidermal thickness, dermal-epidermal ratio, collagen composition, and adenaxal structures, making this species suitable for preclinical studies (Sullivan et al., 2001).

The fractional laser perforates the *stratum corneum*, facilitating passage of molecules through the skin. The benefit of using a fractional laser is that it treats only a percentage of the skin, in the case of this study only 22%, which was the greatest density setting for this laser. The remaining normal skin allows rapid healing of the treated area compared to

more traditional full ablative lasers (Jih and Kimyai-Asadi, 2008). It may well be that using a lower density would also facilitate transdermal delivery of lidocaine, but because of the reduced epidermal/dermal injury, this would decrease the associated down time and lead to a more rapid recovery. In other words, what would be the minimum threshold density laser setting required to facilitate transdermal passage of a drug? More studies would need to be done to investigate this further.

This study has also demonstrated that absorption of the drug could be manipulated by altering the laser energy setting, and that even the lowest of fluences facilitated the transdermal absorption of the study drug. One could postulate therefore, that manipulating both fluence and density setting of the laser could also have an effect on drug absorption, which could possibly be predicted. Again further detailed work would need to be done to see if a dose-response curve can be established.

Laser energy and drug absorption:

The expected trend was that as the energy of the laser increased so too would the absorption of the drug, however this was not the case. With the energy range used in this study maximum absorption occurred at the 250µm ablative depth and not at the 500µm ablative depth as expected. There could be several possible explanations for this. Firstly, based on previous studies by this group, between 100-300µm depths in porcine skin, there is a vascular network within the papillary dermis (data not shown). In humans depending on the anatomical location, the papillary dermis is at a depth of around 100-150 µm (Koehler et al., 2010). The presence of blood vessels therefore, enhances the absorption of

the drug into the bloodstream, and hence the greater absorption at 250 microns as opposed to 500 microns. Secondly, the histopathological injury created in the epidermis and dermis by the ablative laser is a series of microchannels surrounded by normal tissue. However, coagulated cells line these microchannels. As the energy setting increases so too does the thickness of the coagulated lining of the micro-channel, and thus this could act as a barrier to drug absorption. This has previously been demonstrated by our group with the carbon dioxide and the Er:YAG laser (Farkas et al., 2010a, Farkas et al., 2010b). Thirdly, it was observed that increasing the laser energy led to visible punctuate bleeding in the treated areas, edema and exudate. These three factors alone would make it difficult for lidocaine to be absorbed into the skin.

Effect of surface area/amount of drug applied:

This study used a fixed area of 400cm^2 and a fixed amount of topical anesthetic, 5g of 4% lidocaine. However, surface area and amount of drug applied will have an effect on the serum levels. At no time did the serum levels of lidocaine exceed 1µg/ml (the lower limit of therapeutic range of intravenous lidocaine for the treatment of cardiac arrhythmias in human studies). The level at which toxic effects are said to occur is 5µg/ml. From a previous study performed by this group, 400cm^2 is the average surface area of the face and neck in human subjects (Oni et al., 2010). This same study showed that topical application of 5g of 4% lidocaine to normal untreated facial skin resulted in systemic absorption. However, typically more than 5g of the topical drug would be used in a clinical application such as facial resurfacing. In addition the face is more vascular than other parts of the

body (Bailey et al., 2011a). Therefore, it is possible that serum levels of lidocaine could increase dramatically with increasing amounts of topical anesthetic applied following laser pretreatment. Further studies would need to be carried out to ascertain the safety profile.

Ramifications for clinical practice and future directions

This study has ramifications for clinical practice. There has already been a series of studies which look at ex vivo absorption of drugs following pretreatment of skin with the Er:YAG laser. There are several *in vitro* studies from a group from Taipei Medical University (Lee et al., 2002, Lee et al., 2001, Fang et al., 2004, Lee et al., 2007). Their studies used animal models (pigs and nude mice) and looked at the Er:YAG laser as a method of enhancing drug diffusion across the skin. Full thickness skin was taken from the animal subject, pretreated with the laser and then diffusion of molecules was looked at using a Franz diffusion cell. They were able to demonstrate the enhanced diffusion of macromolecules, 5 fluorouracil, and lipophilic and hydrophilic drugs across the skin membrane after pretreatment with the laser. They showed that lipophilic drugs had a greater diffusion across the skin membrane than the hydrophilic drugs; the study drug that was used was lipophilic in composition.

An *in vivo* study by Haedersal *et al.* demonstrated, in a porcine model, increased penetration of methyl 5-aminolevulinate (MAL), a porphyrin precursor, following pretreatment with the CO_2 ablative laser (Haedersdal et al., 2010). In addition there has been a human study from this group demonstrating subjectively an enhanced efficacy of topical anesthetic when pre- treated with the Er:YAG laser (Yun et al., 2002). In this study

12 patients underwent facial resurfacing, half of their face was pretreated with Er:YAG laser the other half was not. They then had topical lidocaine (ELA-Max) cream applied to both sides of their face with occlusion for 60 minutes. They found that 'Er:YAG laser ablation of the *stratum corneum* substantially improved the effectiveness of topical anesthesia' as measured by a subjective pain score assessment by the study participants. Ruiz-Rodriguez *et al.* also showed that applying PDT-ALA immediately after fractional resurfacing improved the clinical outcome when compared to fractional resurfacing alone (Ruiz-Rodriguez et al., 2007). It may well be that this is a technique that could be used to deliver a variety of drugs transdermally not just topical anesthetics.

This study adds to this body of work by demonstrating that not only can there be enhanced penetration/absorption of a drug following laser pretreatment but that this can lead to systemic absorption. Extrapolating what we have learned in this study, it would follow that if a similar study were performed in humans a depth of 100-150 microns would be ideal for the papillary plexus (Koehler et al., 2010). This can have both its risks and benefits depending on where the drug needs to act, locally or systemically and which drug is being evaluated. For example, the action of topical lidocaine should be confined to the dermis/epidermis with systemic absorption being minimized to prevent undesirable toxic side effects. However, our study has demonstrated that the *amount* of absorption of the topical lidocaine is related to the laser setting for a given amount of topically applied drug. No assessment was made clinically of the anesthetic effect in this study. Singer *et al.* in their randomized control trial demonstrated that laser pretreatment prior to topical anesthetic application increased the speed of onset compared to no laser treatment, a conclusion that has been supported by a number of other clinical studies (Singer et al.,

2006), (Koh et al., 2007, Baron et al., 2003, Shapiro et al., 2002). A balance can therefore be made between enhancing anesthetic effect and speed of onset of anesthesia against systemic absorption of lidocaine, simply by manipulating the laser energy. This is the subject of future work to be carried out by this group, as the clinical application for this could range from laser resurfacing of the face to skin graft harvesting as long as the systemic safety profile is acceptable.

5.5 Conclusion:

This study demonstrated that transdermal absorption of topical lidocaine could be enhanced by pre treating skin with a fractional laser. The amount of absorption of the drug could be manipulated by altering the energy setting and thus ablative depth of the laser. This technique has ramifications to clinical practice, and further studies need to be carried out to ascertain how this can be utilized for an efficacious transdermal drug delivery system.

CHAPTER 6: The use of a fractional ablative laser to deliver adipocyte derived stem cells transdermally - a feasibility pilot study

6.1 Introduction:

Chronic wound healing problems can be a challenging clinical problem to solve and can represent significant morbidity for some patients. In addition multiple dressing changes over a protracted length of time can place an enormous drain on healthcare resources in terms of hospital admissions and long term care. As we look for ways to improve these often recalcitrant debilitating wounds, it is natural to consider the potential of stem cells as a possible solution.

The role of stem cells in cutaneous wound healing is the subject of great debate in the scientific literature (Cha and Falanga, 2007, Lee et al., 2009, Kim et al., 2007, Maharlooei et al., 2011, Lee et al., 2011b). Stem cells have the ability to renew themselves as well as differentiate into specialized cell types and their role has been demonstrated in tissue regeneration (Rodrigues et al., 2011, Sterodimas et al., 2010). While we know that the introduction of stem cells can improve wound healing, it is currently not clearly understood by what mechanism this occurs. It is also known that they enhance healing and there have been several published animal studies that attest to that (Maharlooei et al., 2011, Levi et al., 2011, Cherubino et al., 2011, Wu et al., 2007b). However, commonly these studies utilize an intravenous route for the mechanism of stem cell delivery, and often the stem cells are bone marrow derived. Stem cells can be isolated from adipose tissue. The stem cells derived from adipose tissue (adipocyte derived stem cells or ASC) are ideal to

be studied because these cells are able to differentiate into cells of various lineages. In addition fat is abundant throughout the human body and therefore, it is also relatively easy to harvest large quantities of these cells when compared to bone marrow.

If in fact ASC can facilitate and promote wound healing, then what is the ideal manner to transport them to the target tissue? Currently the favored mechanisms are intravenous or subcutaneous injection. However, rather than look at systemic delivery we believe a more focused local application would be more desirable. While this could be achieved with a subcutaneous injection beneath the wound, in some cases, this may be particularly challenging because of poor skin quality/depth of wound and/or due to the extensive nature of the wound and its location. Topical application of the ASC could be a good alternative.

Permeability of the skin to transdermal applications is dependent on a number of factors; in particular the *stratum corneum* and the drug composition play vital roles. In order to assist penetration of the *stratum corneum* a fractional laser can be used as it has been shown to facilitate transdermal delivery of drugs both *in vitro* and *in vivo* (Lee et al., 2001, Haedersdal et al., 2010, Gomez et al., 2011).

The aim of this study is to determine if ASC can be delivered topically into the skin following pretreatment with a fractional laser. If viable stem cells can be delivered topically, then this will be a more patient friendly approach than subcutaneous injection. I believe that a fractionated ablative erbium laser can be used to create channels in the skin through which the stem cells can be delivered into the dermis/subcutaneous tissue.

6.2 Method:

Method and materials:

Ethics approval for the study was sought and granted by the IACUC board at UT Southwestern (appendix 3). Two adult female domestic pigs (pig A and pig B) were acclimatized for one week prior to use in the pilot study. The animals were kept nil by mouth the night prior to surgery. Under general anesthesia the skin of the belly of each pig was shaved.

For pig A: Four areas (3cm x 3cm) were marked on the abdomen of the animal; area 1 was the control, area 2, 3 and 4 were pretreated with laser. All four areas had topical labeled stem cells applied. Pig A was kept under anesthesia for 4 hours then euthanized.

For pig B: three areas (3cm x 3cm) were marked on the abdomen of the animal; area 1 was the control and areas 2 and 3 were pretreated with laser. Area 2 had stem cells applied topically as before and area 3 had stem cells injected subcutaneously for comparison. Pig B was allowed to recover and then euthanized at 48 hours.

Laser type and setting:

Erbium-doped yittrium aluminium garnet (Er:YAG) laser (Profile, Sciton) was used for this study. The settings were 1000μm depth, at 22% density, which is the equivalent of 375 J/cm^2. A depth of 1000 microns was arbitrarily chosen, as we were sure that at this setting the columns would extend into the deep dermis. This would allow us better ease of seeing the channels on histology, and subsequent immunohistochemistry. A 1cm^2 spot size was used to deliver the laser energy.

Preparation of the porcine adipocyte derived stem cells:

Allograft adipocyte stem cells were extracted from pigs that had undergone prior surgery and according to Björntorp protocol and Lequex *et al*.(Lequeux et al., 2011, Bjorntorp et al., 1978). The adipose tissue was minced, digested with collagenase (0,120U/ml, Roche) at 37°C for 30 min and was subjected to constant shaking. Digestion was stopped by adding Dulbecco's Modified Eagle's Medium (DMEM with glutamax (Gibco, Invitrogen, Carlsbad, CA) containing 10 % fetal calf serum (FCS, HyClone, Logan, USA). Floating adipocytes were discarded and cells from the stomal vascular fraction (SVF) were pelleted, rinsed with media, centrifuged (1000 rpm) and incubated in an erythrocyte lysis buffer for 10 min at 37°C. The cell suspension was centrifuged and cells were counted using trypan blue and seeded at a density of 2 $x10^4$ cells/cm^2 and cultured in preadipocytes expansion medium containing DMEM, HAM-F12 L-Glutamine (Gibco, Invitrogen, Carlsbad, CA), 10 % FCS (HyClone, Logan, USA), 10 ng/ml basic Fibroblast growth Factor (Sigma-Aldrich, St Louis, MO, USA) and antibiotics (20 µg/ml of gentamycin and 100 UI/ml of penicillin). The medium was changed every 3 days until 60% confluence was reached at 37°C, 5% CO_2 and 95% air humidity.

Bromodeoxyuridine (BrdU) stem cell labeling of the porcine ASC:

BrdU is a thymine analogue that competes with thymidine and is substituted into DNA during the S-phase of the cell cycle. BrdU can therefore, be used to label stem cells and to track the DNA as the cells divide and replicate. Once the ASC reached 60% confluence they were labeled with BrdU. To prepare the solution, 7.65 mg of BrdU (Invitrogen, Carlsbad, CA) was dissolved in 2.5 ml PBS. The solution was then sterilized by filtration

using a 70 µm cell filter. The solution was then further diluted with expansion medium containing fibroblast growth factor, to a 10 µm concentration. The medium was then added to the culture flasks and the cells proliferate for 48 hours. The action of the BrdU was stopped by removal of the medium. The cells were washed three times with PBS and trypsinized ready to use for topical application.

Trypsinisation of the cells:

After 48 hours incubation with BrdU, the culture flasks were washed twice with PBS. 4mls of trypsin containing 0.01% EDTA (Invitrogen, Carlsbad, CA) was added to each flask. After incubation at 37°C for 5 minutes the cells were detached and the action of the enzyme was stopped with DMEM medium. The cells were then centrifuged at 1200rpm for 5 minutes, the supernatant removed and the cell pellet resuspended in 5mls DMEM. The cells suspension was subject to a further centrifugation at 1200rpm for 5 mins, the supernatant again discarded and the cell pellet resuspended in DMEM medium. The cells were counted with a haemocytometer (Fisher Scientific, Waltham, MA) prior to use.

BrDU Immunohistochemistry:

Porcine ASC on cover glass slips from passage 1 were fixed with 2% paraformaldehyde in PBS for 5 min, followed by incubation in 2N HCl for 10 min at 37°C for DNA denaturation. Cells were washed twice with PBS, incubated with Triton 0.5% for 10 min and washed again. For non-specific antibody reactions, ASC were blocked with 1.5% normal horse serum (Vector, Burlingame, USA) in PBS and then incubated with mouse

anti-BrdU antibody (Sigma-Aldrich, St Louis, MO) with 0.1% bovine serum albumin (Sigma-Aldrich, St Louis, MO) in PBS for 16-18 h at 25°C. Cells were washed, incubated with anti-mouse-Ig-biotinylated (Vector, Burlingame, USA) for 30 min at 25°C, washed again and incubated with X streptavidin for 30 min at 25°C. After washing with PBS, cells were counterstained with propidium iodide (Invitrogen, Carlsbad, CA). The coverslips were then mounted on slides using vectashield (Vector, Burlingame, USA) and viewed under the fluorescent microscope (Figure 1).

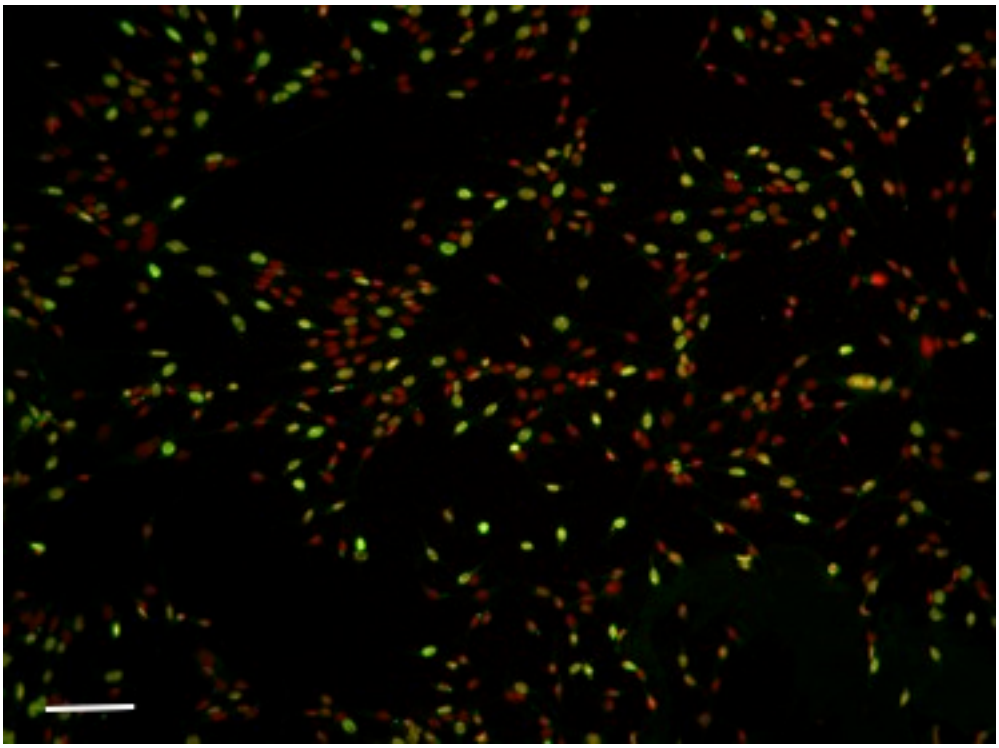


Figure 1: BrdU labeled porcine adipocyte derived stem cells *in vitro* counterstained with propidium iodide

Topical application of labeled cells:

For pig A, following pretreatment to areas 2, 3 and 4, the BrdU labeled stem cells were applied directly to the skin on all four marked areas and then covered with an occlusive dressing for four hours with the animal under anesthesia. Approximately 20 million cells in 1.5mls of medium were applied topically to each of the 4 marked areas. At four hours the dressings were removed from the skin. The areas were cleansed with normal saline to remove any residual fluid. Areas 1-4 were excised *in toto* and submitted for immunohistochemistry and H&E staining. The animal was then euthanized.

For pig B following pretreatment to areas 2 the BrdU labeled stem cells were applied topically directly to the skin areas 1-2 and then covered with an occlusive dressing. Approximately 20 million cells in 1.5mls of medium were applied topically to each of the 2 marked areas and the dressings were left in place for 48 hours. For area 3, approximately 20 million cells in 1.5mls of medium were injected subcutaneously under the laser-pretreated area. Again the area was dressed with an occlusive dressing. The animal was allowed to recover from anesthesia. At 48 hours the animal was then euthanized and areas 1-3 were excised *in toto* and submitted for immunohistochemistry and H&E staining.

Tissue analysis:

Each biopsy specimen was fixed in 10% buffered formaldehyde solution at pH7.4 for at least 48 hours, embedded into paraffin wax and cut into 5μ sections and stained with immunohistochemistry counterstains and haematoxylin and eosin standard staining.

Tissue immunohistochemistry:

The 5µ paraffin embedded sections were deparaffinised, then incubated in 2N HCl for 60 min at 37°C for DNA denaturation. The acid was neutralized using 0.1% borate acid and the sections were then washed in PBS, incubated with Triton 0.5% for 10min and washed again with PBS. The counterstains were then applied as for the cells (see above).

Labeled stem cell counts:

Three independent blinded observers analyzed two 5µm sections per laser treated area. The counts were performed under fluorescent microscopy. All visible fluorescent stem cells were counted. Averages across the observers were taken. To calculate total number of stem cells found in the 3x3cm tissue the following formula was used:

Number of cells x 30000 (length of specimen in µm)/5 (thickness of section) = total number of cells in specimen

6.2 Results:

Four-hour study:

No positively labeled cells were found in the epidermis or dermis of the control group.

Positively stained BrdU cells were found within the microchannels that had been created by the laser (Figure 2). They were found in different distributions within the microchannels, some suspended within the channel, some adherent to the walls of the

channels, and some at the base of the channel (Figure 3). No positively labeled stem cells were seen outside of the channel.

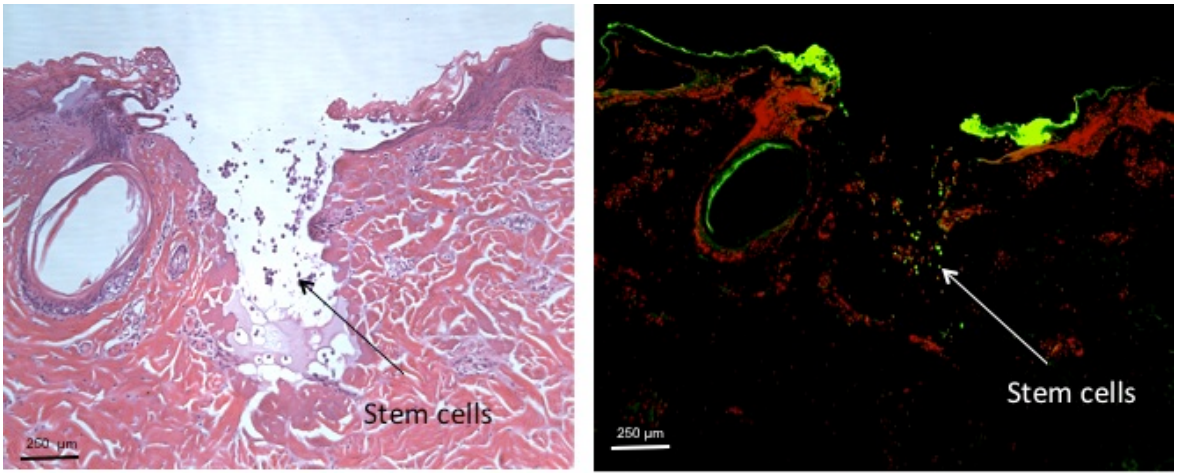


Figure 2: H&E (left) and immunohistochemically stained (right) fluorescent microscopy demonstrating BrdU labeled stem cells within the laser channel

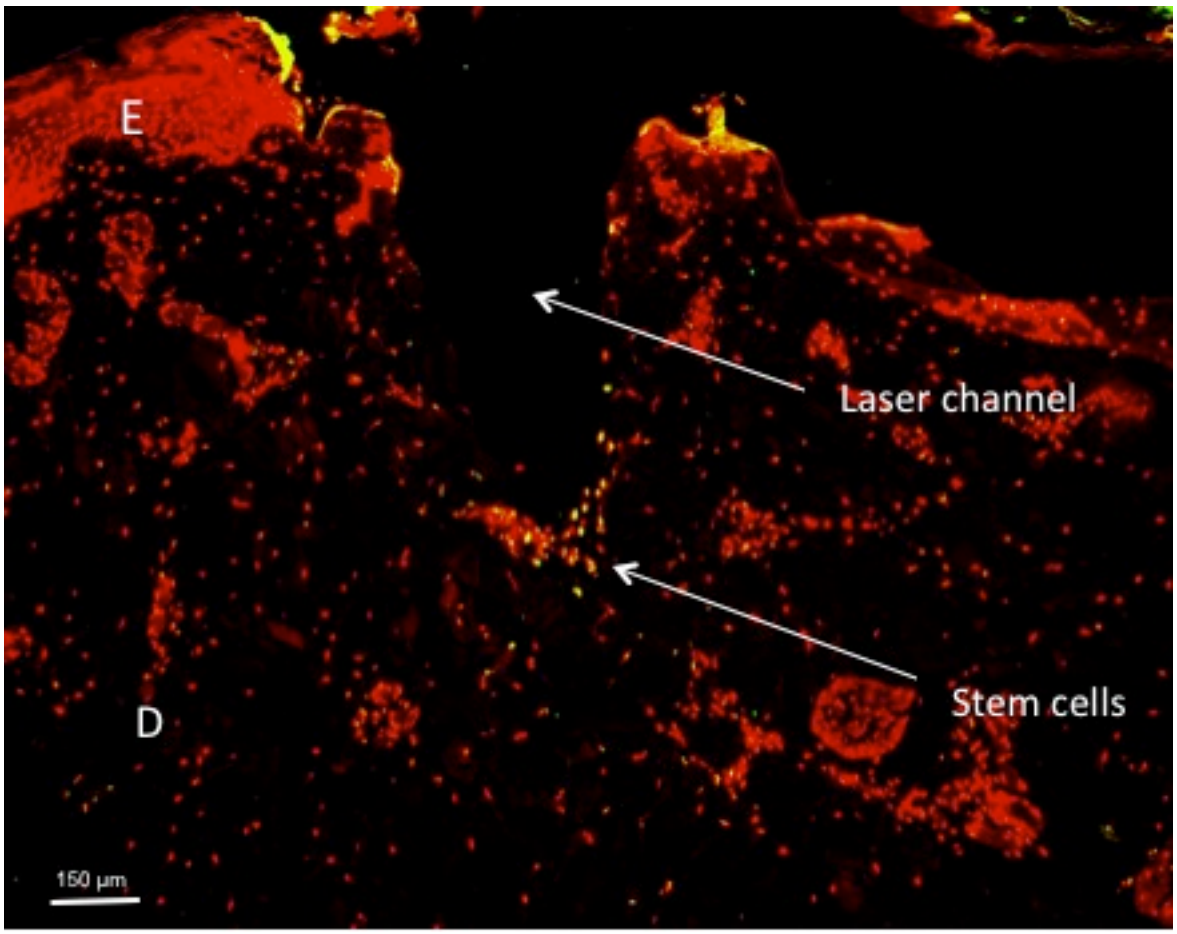


Figure 3: BrdU labeled stem cells (yellow) at base of laser channel. E - epidermis D-Dermis

Total cell counts are summarized in Table 1. The average counts for the three areas were 2.18 x 10^6, 2.27 x10^6 and 2.74 x 10^6 respectively (p=0.751). This corresponds to 10.9%, 11.4%, and 13.7% of the 20 million cells that were applied.

Table 1: Total stem cell counts for the 4-hour study

Area on pig A*	Observer Cell counts** ($x10^6$)			Average total cell count ($x10^6$)
	1	2	3	
2a	2.08	3.80	2.25	
2b	1.33	1.94	1.67	2.18
3a	3.37	6.42	2.92	
3b	1.51	1.93	0.98	2.27
4a	3.93	3.99	3.55	
4b	2.15	2.44	1.97	2.74

*areas on pig – area 1 control, areas 2, 3 and 4 laser pretreatment

*each column represents a separate observer

Forty-eight hour study:

No positively labeled cells were found in the epidermis or dermis of the control group. For the injected labeled ASC there was a dense collection of cells found in discrete areas in the subcutaneous tissue. The cells were too dense to count (Figure 4).

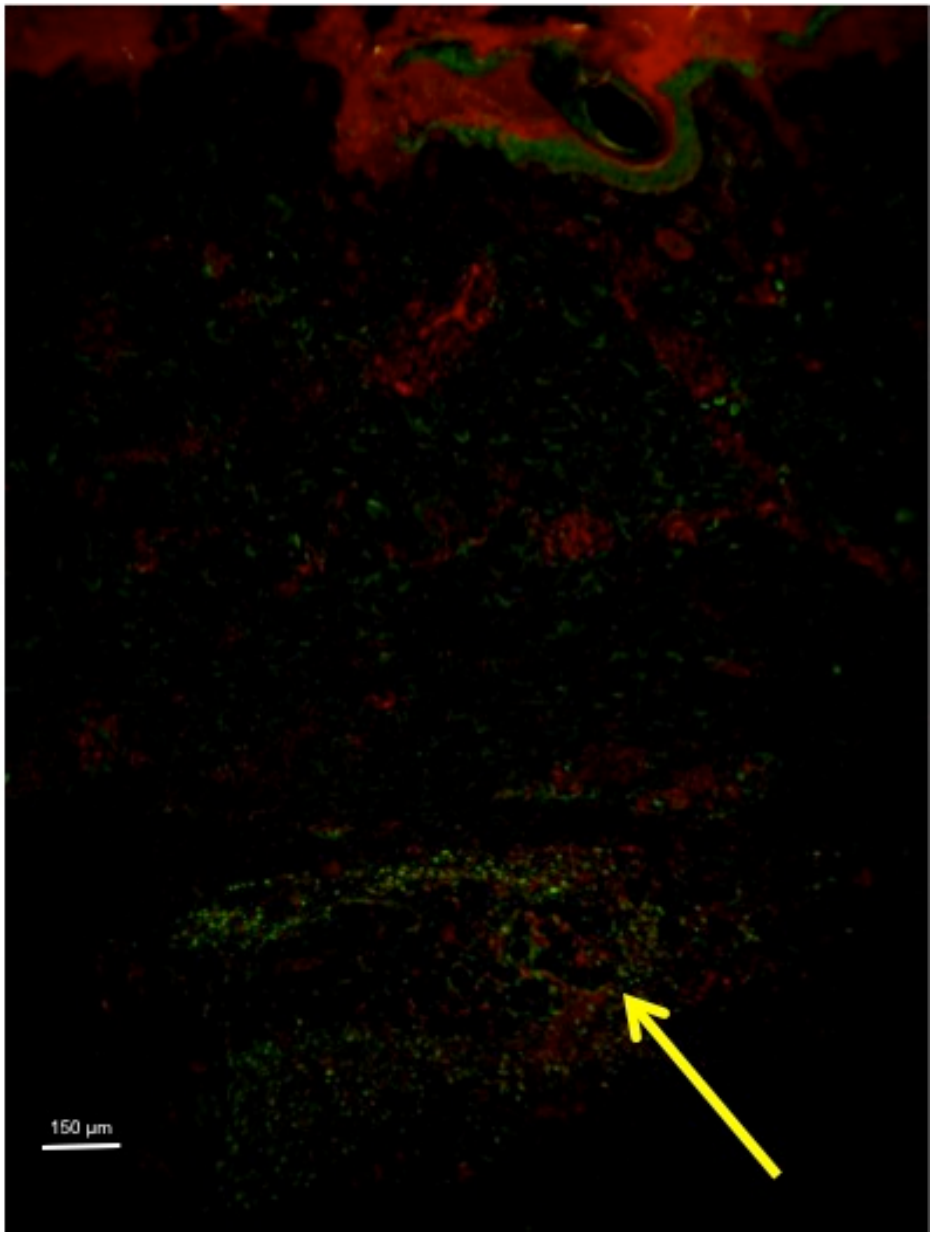


Figure 4: BrdU immunochistochemistry demonstrating dense collection of positively stained stem cells that were injected subcutaneously. The cells were too numerous to count.

Few positively stained BrdU cells were found within the tissue (Figure 5). H&E staining demonstrated that at 48 hours the microchannels had re-epithelialised (Figure 5).

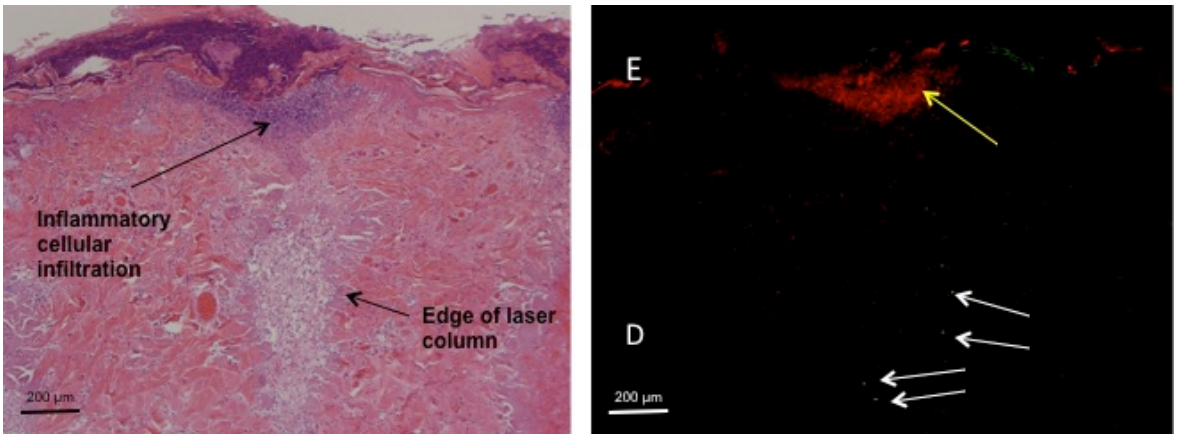


Figure 5: H&E (left) note large inflammatory cellular infiltration and re-epithelialisation of the laser channel. BrdU immunohistochemistry (right), note few scanty BrdU labeled cells (white arrows) towards the base of the channel

Therefore the BrdU labeled cells were found to be within the channels in the dermis of the skin. The cell numbers appeared to be much reduced at 48 hours compared to 4 hours. No positively labeled stem cells were found outside of the channel. Again the cells were found along the wall of the channel with some cells found at the base. Total cell counts are recorded in Table 2. The average counts for the two areas were 0.73 $x10^6$ and 1.47$x10^6$ respectively (p=0.025). This represents 3.64% and 7.34% respectively of the 20 million cells applied, fewer cells than were seen in the 4-hour study.

Table 2: Total stem cell counts for the 48-hour study

Area on pig B*	Observer Cell counts** ($x10^6$)			Average total cell count ($x10^6$)
	1	2	3	
2a	109	138	117	0.73
2b	193	276	265	1.47

*areas on pig – area 1 control, areas 2 laser pretreatment, area 3 injected.

**each column represents a separate observer

6.4 Discussion:

Adipocyte derived stem cell (ASC) therapy is an area of great interest currently in the literature. This is in part related to the fact that fat is an abundant and therefore renewable source from which to harvest stem cells. ASC are pluripotent, having the ability to differentiate into numerous cell lines including adipose tissue, nerve, bone and cartilage. Many of the animal studies to date have focused on intravenous or local injection of stem cells in order to exert their effects on distant targets and have shown success in acceleration of wound healing (Williams and Hare, 2011, Kuo et al., 2011, Zografou et al., 2011, Kim et al., 2011, Tseng et al., 2008, Falanga et al., 2007, Wu et al., 2010). Other methods have included fat grafts augmented with the stromal vascular fraction (SVF) from collagenased fat. This technique was first described by Yoshimura *et al.* and was termed

cell assisted lipotransfer (CAL) (Yoshimura et al., 2008). This has been demonstrated to have had a beneficial effect on radiation injuries (Rigotti et al., 2007) and longevity of the fat grafts themselves (Ko et al., 2011). There has been considerable interest in the role of stem cells in skin rejuvenation in particular improvement in skin wrinkles in the literature. A paper by Mojallal *et al.* describes anecdotal improvement of skin texture following fat grafting to the face in fat grafts, which they investigated with an athymic rat model (Mojallal et al., 2009). They found that the animal had a thickened dermis when grafted with increased synthesis of collage I fibers and concluded that this was due to the presence of mesenchymal stem cells in the adipose tissue. Park *et al.* again demonstrated improvement in skin wrinkles of a micro pig injected with autologous adipocyte derived stem cells (Park et al., 2008). Histology from this study again showed an increase in dermal thickness and collagen expression. However to date these methods of stem cell delivery are fairly invasive and require some form of intravenous or subcutaneous injection.

The aim of this pilot study was to ascertain if ASC could be delivered via the transdermal route into the dermal layer of the skin following creation of microchannels with the laser. This study showed evidence that approximately 10% of topically applied cells at 4 hours were still present and 5% present at 48 hours. It is important to note that the cells remained within the microchannels but were not seen within the surrounding tissue.

There may be several reasons why the cell count decreased from the 4-hour study to the 48-hour study. The study was performed on the abdominal skin, which once the animal is awake and ambulating is subjected to gravitational forces. Additionally, at higher fluences the microchannels can sometimes become partially occluded with the normal inflammatory

exudate that accompanies a laser injury potentially preventing the cells from entering the channel further. Cell death due to lack of nutritional support as the media evaporated could also account for a decrease in cell count from the 4-hour to the 48-hour point. Finally, the number of histological samples taken may limit our cell count.

Previous animal studies from this group have shown that the microchannels remain patent for up to 90 minutes depending on the fluences used (data not shown). From this study we were able to demonstrate that the pigskin re-epithelializes within 48 hours of laser treatment, at the fluence used (375 J/cm^2), with a strong acute inflammatory infiltrate both within and outside of the channel.

Injecting the cells was definitely superior in terms of cell number compared to topical application, but this in itself poses some interesting questions. It is still unclear *how many* stem cells *over what area* are required to exert an effect. Further investigation needs to be performed in this area, as an injection can be unpleasant for patients. If they are already undergoing a laser treatment for example for facial rejuvenation, then a topical application of stem cells would be more appropriate and patient friendly than a subcutaneous or intradermal injection. Intra-dermal injections may be less precise than a predetermined laser depth unless some sort of imaging is used at the same time.

Limitations/Future directions:

As it has been previously mentioned there were some limitations to this study. The ASC used in this study were not autologous but allogenic and this may have had an effect on cell viability *in vivo* as the immune system of the animal would identify these cells as foreign bodies. The use of autologous ASC would be preferable, and would be

recommended for further investigations. They were not used here because it would have meant a two-stage procedure, first for adipose tissue harvest and the second for ASC application and given this was a proof of principle study. For further studies, for example, investigating the effect of ASC on wound healing, the immunological ramifications of autologous versus allogenic cells must definitely be considered.

The variations in distribution of the stem cells suggest that there is good penetrance of the ASC, however they may have been disrupted during the process of removing the dressing and tissue staining. A method which allows continuous delivery of the stem cells over time such as an impregnated scaffold (e.g. acellular dermal matrix), or fibrin glue applied topically would be more preferable than applying the cells freely in solution. Autologous platelet rich plasma applied topically following fractionated laser resurfacing has already been demonstrated to improve wound healing (Na et al., 2011, Lee et al., 2011a). In addition a small study of four patients injected intradermally with autologous platelet rich fibrin matrix (PRFM) performed by Sclafani *et al.* suggested that dermal collagenesis, angiogensis and adipogenesis were all induced by the PRFM (Sclafani and McCormick, 2011). It may well be that combining PRFM with ASC may lead to an additive stimulatory effect, which could have ramifications not only within wound healing but possibly within the aesthetic realm for skin rejuvenation therapies.

In addition while we were able to demonstrate the presence of cells in the channels at 48 hours, there was no evidence of migration into the tissues. A longer time point would be necessary to see if this occurs. The BrdU label being a nuclear stain becomes diluted as the cell divides. This makes it difficult to use this stain to track the cells after a short period of time. Lequeux *et al.* found that the BrdU signal detectable declines after 2 weeks

in their *in vitro* study, although is present for up to 30 days (Lequeux et al., 2011). There is also some debate as to whether the BrDU label persists even after the cell is dead before it is phagocystosed. In future studies other methods of stem cell tracking may be more efficient, such as fluorescence-activated cell sorting (FACS) or RNA evaluation. These methods are more objective and do not rely on counts by the human eye which can be confounded by clerical error.

This study only looked at the physical location of the labeled stem cells, it did not investigate what effect they exerted by being present. Further investigations could be undertaken to see if the stem cells secrete cytokines or other factors, which are implicated in wound healing. It would also be of interest to note whether the stem cells had an effect on the amount of inflammatory infiltrate present, utilizing a method, which more adequately quantifies it.

The potential benefit for this application could range from wound healing implications to rejuvenation procedures. The laser could be used to 'reactivate' a chronic dormant wound and the stem cells applied topically to stimulate angiogenesis and other pro-healing growth factors found in acute wounds such as PDGF and VEGF. It may be possible that a gel formulation containing stem cells could be applied following the laser procedure. This would accelerate healing and shorten downtime, which can be considerable, for example, following facial resurfacing. Alternatively, depths of injury could be set by the laser allowing for a more precise deliver of ASCs to a specified target within the skin.

6.5 Conclusion:

This pilot study has demonstrated that it is possible to deliver adipocyte-derived stem cells topically following pretreatment with an ablative laser. Further in depth studies need to be conducted to determine whether those stem cells are viable and if so what their mode of action will be once delivered into the skin. In addition, one needs to investigate enhancing delivery of cells perhaps by seeding them first on an acellular dermal matrix or plasma rich fibrin matrix in order to increase the number of cells found within the dermis. This study is the first step of a pathway, which potentially could lead to enhancing wound healing by the introduction of adipocyte stem cells.

CHAPTER 7 - An *in vivo* histopathological comparison of single and double-pulsed modes of a fractionated CO_2 Laser

7.1 Introduction:

For many years, full ablative laser treatments, especially with the carbon dioxide (CO_2) laser were considered the gold standard for resurfacing treatments due to the strong dermal remodeling and collagen contracture leading to marked clinical improvements. However these 'aggressive' treatments led to adverse events such as prolonged erythema, pigmentary changes and in some cases scarring (Papadavid and Katsambas, 2003, Hunzeker et al., 2009, Duke et al., 1998, Dover et al., 2000, Railan and Kilmer, 2005). The significance of the advent of fractional laser therapy was the reduction in down time for the patient following their procedure. Reduced erythema, weeping, and recovery times were the benefits of this change in mode of laser energy delivery (Neaman et al., 2010, Hunzeker et al., 2009). Those benefits in combination with the concomitant reduction in altered pigmentation, and scarring has seen the popularity of fractional laser treatments increase steadily.

The histological injury of fractional laser devices was first described by Rox Anderson et al (Manstein et al., 2004). Their work demonstrated that the clinical benefits seen with fractional devices was in part due to the microscopic treatment zones (MTZ) resulting from thermal injury to the skin. These zones are bridged by normal tissue and therefore, re-epithelialization is much quicker which shortens the recovery time. Basic science research studies published in humans looking at patterns of laser injury are usually limited to either

ex vivo tissue or abdominal or preauricular tissue just prior to excision. However skin on the abdomen differs from skin on the face and our group has shown that the laser injury from the CO_2 fractional laser also differs on the abdomen from the face (Bailey et al., 2011a). This study builds on that work by comparing the acute histopathological response of human facial and abdominal skin across several more clinically relevant treatment settings utilizing the fractional ablative CO_2 laser system, using the subjects from our previous study as a historic control.

The goals in this study were to better characterize histologically the acute response of human facial skin to differing fractional ablative laser treatments as compared to abdominal skin. In particular to compare higher single pulse mode of energy delivery and also to examine the thermal injury caused by double pulsing.

7.2 Methods:

Twenty-seven healthy subjects were consented and screened in accordance with Title 45 Code of Federal Regulations, Part 46, Protection of Human Subjects (45 CFR part 46) for participation in this UT Southwestern Medical Center Institutional Review Board (IRB) study (appendix 4+5). Fifteen of those subjects were the historic control from Bailey *et al.* (2011). The remaining twelve subjects were split into two groups. The subjects recruited via posters that were placed throughout the UT Southwestern campus and were from a broad range of age groups, sex and Fitzpatrick type.

Laser procedure:

One $1cm^2$ area on the face—right mid-pupillary line, against the hairline—and one $1cm^2$ area on the abdomen—1-2 cm infraumbilical—were identified for evaluation. Only skin areas free of scar or other dermatologic abnormality were chosen; any hair in the selected site was shaved before treatment. The selected sites were first anesthetized with BLT (20% benzocaine - 6% lidocaine -4% tetracaine) triple topical anesthetic cream then treated with a Lumenis CO_2 laser Deep FX scanner. Six (6) subjects were treated with laser energy of 15 mJ, double-pulse mode (lag time <1ms), while six (6) were treated with a fluence of 30 mJ, single-pulse mode, at 300Hz repetition rate, single pass. All subjects received the same treatment at both sites. Immediately after treatment, treated sites were further anesthetized with an injection of 1% lidocaine with epinephrine. One 3 mm punch biopsy was taken at each site for histological analysis and the wounds were closed with absorbable suture.

Tissue processing and histological analysis:

Skin biopsy specimens were fixed in 10% neutral buffered formalin on shaker for 30 hours. The specimens were then rinsed in a 70% ethanol solution, embedded in paraffin, sectioned, mounted on poly-L-lysine slides, and stained with haemotoxylin and eosin staining. Additional sections were stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining according to Farkas *et al* (Farkas et al., 2010a). TUNEL is an immunohistochemical stain which, allows visualization of terminally injured and apoptotic nuclei.

All slides were examined under light and fluorescent microscope. The microscopic thermal zones (MTZ) depths were measured in triplicate using QCapture Pro 6.0 software (Q Imaging, BC, Canada). The measurements were averaged to yield one value per MTZ, and the depths of multiple MTZs per sample were averaged to finally yield a single average MTZ depth per sample.

For the purposes of this study, the MTZ depth was defined as the distance from the surface of the epidermis to the deepest extent of thermal injury (be that ablation or coagulation) in a single column of laser-induced thermal injury. Due to the potential of obtaining an oblique section of an MTZ, measurements of MTZs that were felt to not represent a full column of injury were discarded from analysis. MTZ widths were calculated based on the furthest lateral TUNEL florescence either side of the ablated column.

Statistical analysis:

All data was entered in to an Excel (Microsoft Corp, Redmond, VA) spreadsheet and statistical analysis was performed using Student's t test. Results are expressed as a mean with standard deviations (SD), significance $p<0.05$.

7.3 Results:

12 subjects were included in this study and were split into two groups. These subjects were then compared against the historic control group from Bailey *et al.* (Bailey et al.,

2011a). The subjects' demographics are summarized in Table 1. There were no adverse effects as a result of the treatment.

Table 1: Patient demographics

	15mj Single pulse	15mj Double pulse	30mj single pulse
No of patients	15	6	6
Average Age	37.4 (Range 23-73)	36 (Range 23-56)	31 (Range 26-46)
Sex	F-11 M-4	F – 3 M – 3	F -2 M- 4
Fitzpatrick type	III-6 IV-4 V- 4 VI- 1	II - 1 III – 4 IV - 1	II – 1 III – 5

Dose Response (Table 2, Figure 1):

A summary of the average MTZ depths at each site and laser setting is presented in Table 2. From the historic control we showed that single pulsing at 15 mj yielded average MTZ depths in the abdomen of 582 µm (range 344-776 µm) and in the face of 416 µm (range 315-530 µm). When increasing this energy to single pulsing at 30mj this yielded average MTZ depths of 1345 µm (range 998-1772 µm) in the abdomen and 854 µm (range 682-1015 µm) in the face. The increase in MTZ depths was significantly different in both the abdomen ($p< 0.0001$) and facial skin ($p=0.05$).

Table 2: Average MTZ depths at each respective laser setting and treatment site

Treatment	MTZ depth (µm)		
	Abdomen	***Face***	***P value***
15 mJ Single pulsed	582 µm (344-776 µm)	416 µm (315-530 µm)	<0.01
15 mJ Double pulsed	822 µm (648-992 µm)	881 µm (772-992 µm)	0.53
30 mJ Single pulsed	1345 µm (998-1772 µm)	854 µm (682-1015 µm)	<0.01

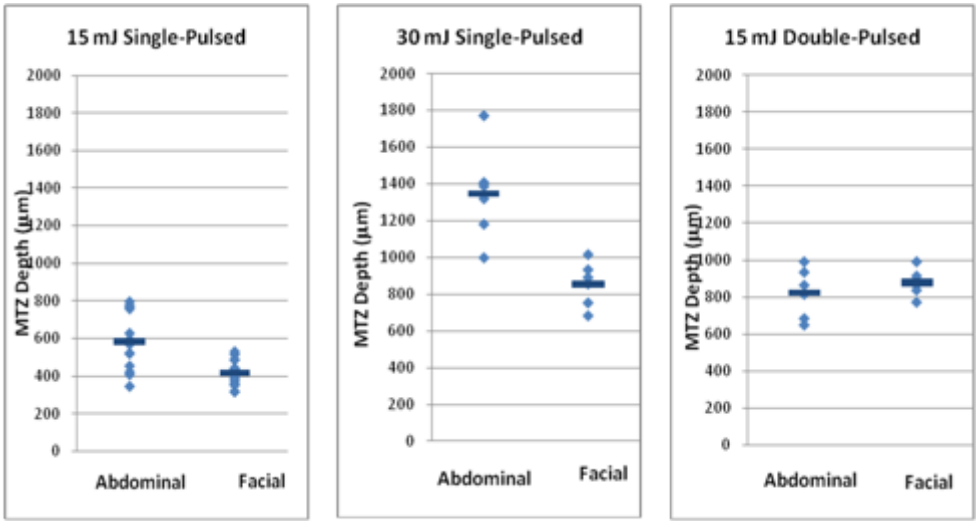


Figure 1: Comparison of the MTZ depths at each laser setting by treatment site (abdominal vs. facial). N=5 for each group. Solid bar denotes average MTZ depth for that group.

Double pulsing at 15 mj yielded mean MTZ depths of 822 µm (range 648-992 µm) in the abdomen and 881 µm (range 772-992 µm) in facial skin. There was no significant difference in MTZ depths when comparing abdominal skin to facial skin (p=0.53). However when comparing single pulsing to double pulsing at 15mj there were significant increase in MTZ depths both for the abdomen (p=0.002) and for facial skin (p=0.001).

When comparing 15 mJ double-pulsed to 30 mJ single-pulsed, this yielded a significant increase in the MTZ depths in abdominal skin (means 822µm compared with 1345µm, $p < 0.01$), but there was no significant difference in the facial MTZ depths (means 881µm compared with 854 µm, p= 0.69). Figure 2 demonstrates the TUNEL stain for facial skin from one subject in each of the three different laser-setting groups.

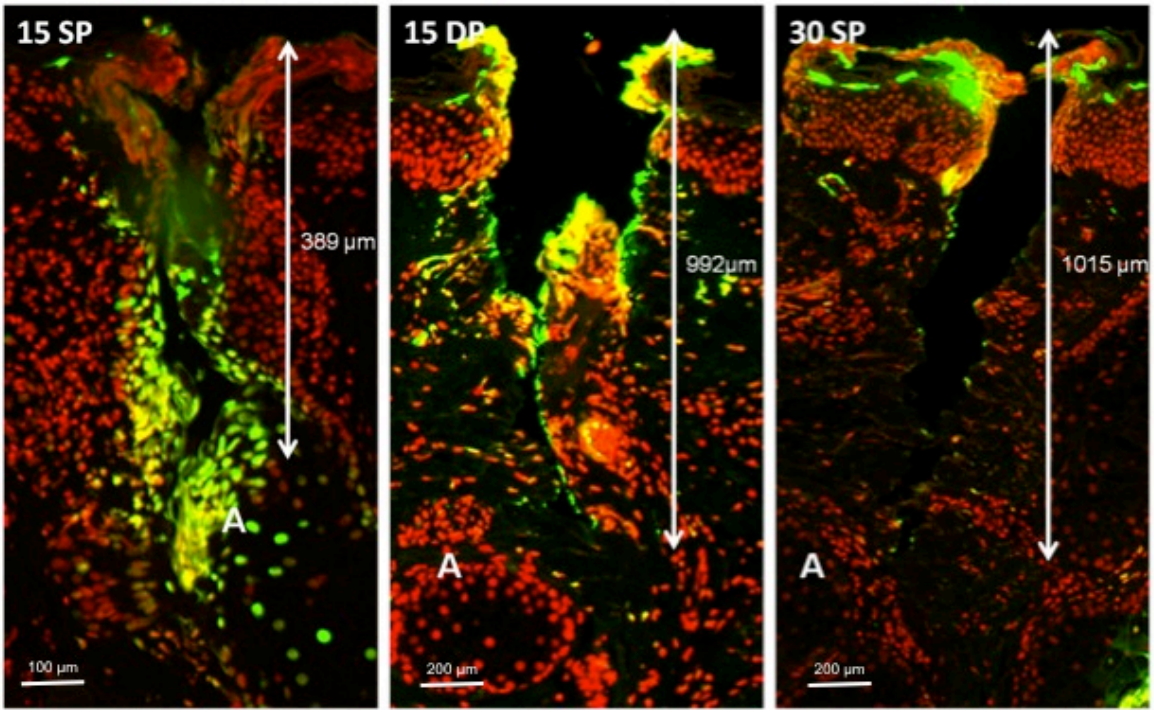


Figure 2: TUNEL stain (magnification x10) comparing of the MTZ depths at each laser setting in facial skin. TUNEL positive nuclei fluoresce green whereas *all* nuclei fluoresce red, so when the images are superimposed, yellow represents the damaged nuclei. Note that there is a significant increase in MTX (*p*=0.001) from 15mJ single pulse (SP) to 15mJ double pulse (DP) in facial. There was no significant difference in MTZ (*p*=0.69) when comparing 15mJ DP to 30 mJ SP in facial skin. 'A' represents glands within the facial tissue.

MTZ widths in facial skin– single pulse versus double pulse (table 3)

There were significant differences between the MTZ widths when comparing all three groups ($p<0.0001$). The mean width for the MTZ for single pulse at 15mj was 311.6 µm (range 203– 373 µm) and this was significantly smaller ($p=0.0002$) than the mean width for double pulse at 15 mj which was 493.1 µm (range 363-606 µm). When comparing the mean width for single pulse at 30mj (534 µm, range 337–669 µm) to double pulse at 15mJ there was no significant difference ($p=0.502$). When comparing 15mJ single pulse (mean

311.6 µm) to 30mJ single pulse (mean 534.3µm) there is a significant difference (p< 0.0001). Figure 3 highlights the differences between the three groups.

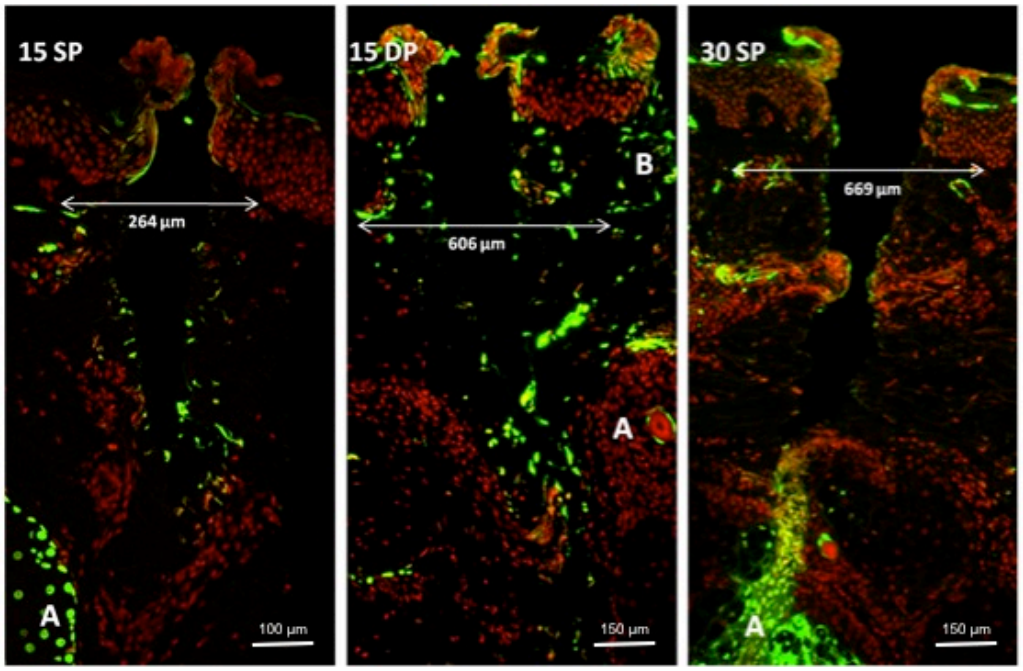


Figure 3: TUNEL stain comparing the MTZ widths at each laser setting in facial skin. TUNEL positive nuclei fluoresce green whereas *all* nuclei fluoresce red, so when the images are superimposed, yellow represents the damaged nuclei. Note that there is a significant increase (p=0.0002) in MTZ width when comparing 15mJ single pulse (SP) to 15mJ double pulse (DP) in facial skin. 'A' represents glands within the facial tissue.

Table 3*:* Average MTZ widths at each respective laser setting for facial skin

Abdominal versus facial skin response (attenuation factor):

Treatment	MTZ width for facial skin
15 mJ Single pulsed	311.6 µm (203– 373 µm
15 mJ Double pulsed	493.1 µm (363-606 µm)
30 mJ Single pulsed	534 µm (337–669 µm)

It was previously reported that there was a significant decrease in MTZ depth in facial skin compared to abdominal skin treated with 15 mJ single-pulsed of around 30%. This was in part due to absorbance of energy from the laser beam by appendages in facial skin (Figure 4), this trend was also observed in 30 mJ single-pulsed treatments ($p<0.01$). However, in 15 mJ double-pulsed treatments, there was no significant difference between MTZ depths in abdominal and facial skin ($p=0.53$).

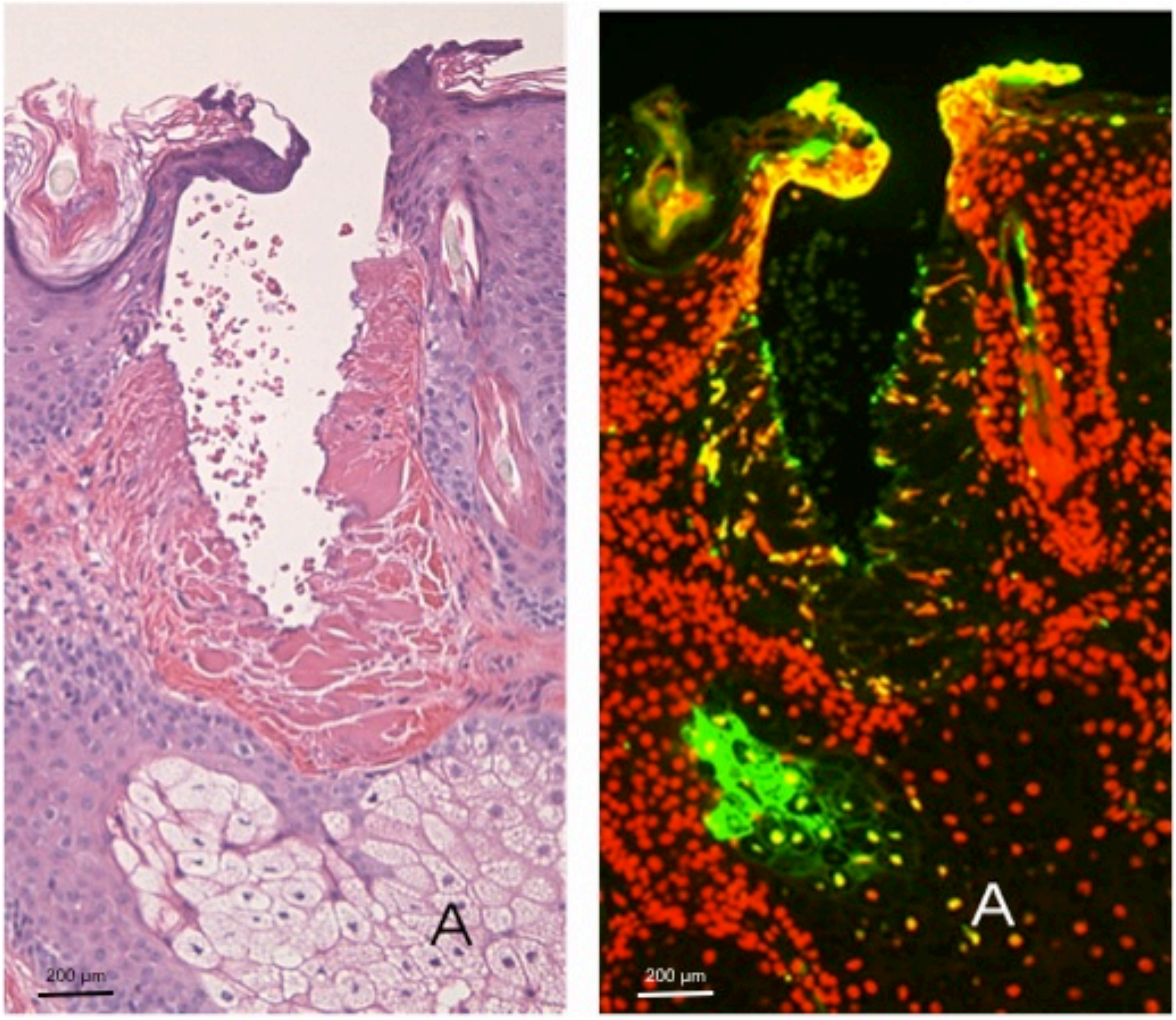


Figure 4: H&E and TUNEL image demonstrating termination of the laser energy by the presence of glandular structures (A) in facial skin. TUNEL positive nuclei fluoresce green whereas *all* nuclei fluoresce red, so when the images are superimposed, yellow represents the damaged nuclei. Note the gland itself absorbs some energy.

Statistical analysis of the calculated attenuation factors of facial MTZ depths compared to abdominal MTZ depths yielded no significant difference between the attenuation factors calculated for the 15 mJ single-pulsed and 30 mJ single-pulsed treatments (p = 0.21). Thus, a single average attenuation factor of 0.70 or around 30% (which is similar to that

found by Bailey *et al.*) was determined for both laser fluences. This factor was used to derive a predicted facial MTZ depth from each measured abdominal MTZ depth from the 15 mJ and 30 mJ single-pulsed treatments; there was no significant difference between the predicted facial MTZ depths and the actual facial MTZ depths ($p = 0.44$).

7.4 Discussion:

Non surgical cosmetic treatments are on the increase and laser resurfacing remains a popular choice by patients for a variety of indications ranging from removal of sun damaged skin, wrinkles/fine lines, melasma and acne scarring (Hunzeker et al., 2009, Railan and Kilmer, 2005, Papadavid and Katsambas, 2003, Duke et al., 1998, Dover et al., 2000). The advent of fractional devices afforded shorted downtimes, with reduced erythema, weeping/crusting, and faster re-epithelialization (Rokhsar and Fitzpatrick, 2005). In addition, these procedures can be performed under local anesthesia with or without conscious sedation. However, many practitioners have reported less favorable results than when they have used full ablative devices (Brightman et al., 2009, Alexiades-Armenakas et al., 2008). Often laser practitioners, both with full surface ablative and fractional ablative lasers, increase fluence settings or increase the number of passes in efforts to improve their clinical results (Burkhardt and Maw, 1997, Tanzi and Alster, 2003). This study has shown that double pulsing at lower fluences would provide increased amounts of thermal damage to the dermis as evidenced by the increased MTZ width and depth. This, therefore, leads to an expectation that the neo-collagen synthesis and therefore clinical outcome possibly could approach the levels more comparable with traditional, full ablative resurfacing. The

question remains at what *depth* and to what extent does dermal injury have to occur in order to maximize collagen synthesis/remodeling in order to achieve maximal cosmetic benefit? Width of injury may be as important as or more important than depth when evaluating increased neo-collagen synthesis and ultimately improved outcomes for facial rejuvenation.

Mode of energy delivery

Double pulsing offers a different modality of the delivery of laser energy. It causes two discrete pulses of laser energy to be delivered to the same treatment area within quick succession, at less than 1ms between the pulses in the device used in this study. Using the theory of selective photothermolysis the thermal heat does not have sufficient time to dissipate following the first pulse before the second is delivered. The net effect therefore, is greater coagulation of the tissue. It was expected that double pulse treatments at energy of 15 mJ would deliver exactly twice the energy as a single-pulse 15 mJ treatment and the same energy as a single-pulsed 30 mJ treatment. However, these settings were found to be not necessarily equivalent in terms of acute histopathological response, suggesting that the thermal effects were actually more attenuated than expected with double pulsing. The question therefore is, does double pulsing cause an increase in depth or the width of the MTZ and how does this correlate with clinical outcomes? Our study has shown that double pulsing at 15mj generated greater lateral thermal damage than single pulsing at 15mj but similar MTZ widths were found when single pulsing at 30mj. This suggests that double pulsing at a fixed energy increases both the depth and width at which dermal injury

occurs, which in turn will also reduce the amount of unaffected normal tissue between MTZs. Clinically this may correlate to improved outcomes, as the amount of collagen synthesis is related to the amount of dermal injury. Full surface ablative treatments were thought to have better clinical results than fractional treatments, but the drawback is the increased downtime and potential side effects (Tanzi and Alster, 2003, Alster and Lupton, 2000, Metelitsa and Alster, 2010, Alexiades-Armenakas et al., 2008, Tierney et al., 2011). It may well be that double pulsing would improve the clinical outcome while keeping the downtown shorter than traditional full ablative treatments, because the lateral thermal injury is still bridged by areas of unaffected tissue (albeit with narrower bridges than with a single pulse treatment at the same energy level). A clinical study would need to be carried out comparing double pulse clinical outcomes with single pulse clinical outcomes in order to address this question.

Microscopic differences between skin on the face and abdomen

The significant differences in the patterns of laser injury observed in abdominal and facial skin subjected to single-pulsed fractional ablative treatments at both 15 mJ and 30 mJ may be explained largely by the histological differences between skins from these regions. The tissue response to CO_2 laser treatments is largely dependent on the water content of the skin. The highest water content is in the epidermis and papillary dermis, followed by reticular dermis (Rokhsar et al., 2005). Furthermore, the epidermis has a largely intracellular water content compared to the dermis which is mainly extracellular (Riggs et al., 2007). Therefore, ablation will reduce as the energy travels from epidermis to dermis,

but not necessarily coagulation. This is evidenced histologically by the V shaped columns seen with H&E stains (Figure 5). The dermal layer of the skin on the face contains a higher density of pilo-sebaceous units and greater vascularity than abdominal skin may largely account for the attenuating effect of facial skin compared to abdominal skin. Blood vessels in the path of the laser energy were observed to have absorbed some of the laser energy along its path, and they showed evidence of laser energy encountering hair follicles and sebaceous glands and being absorbed within these structures, or terminated by the appendage directly in its path. With double pulsing there was less observed absorption of energy around structures such as blood vessels, and greater absorption of energy in glandular structures. However with single pulsing the ablative column was terminated by a glandular structure, whilst with double pulsing the ablative column would continue beyond.

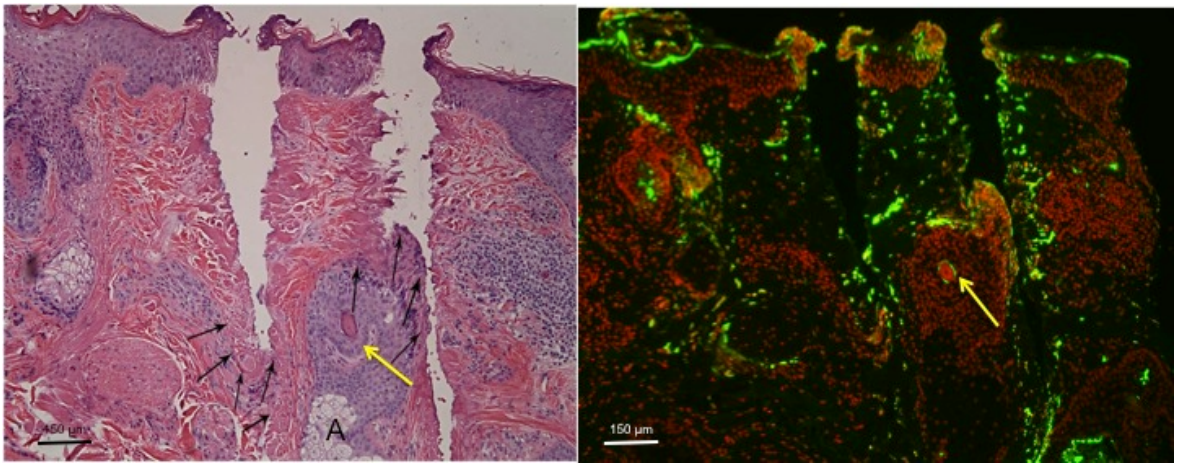


Figure 5: H&E and TUNEL image demonstrating 'v' shaped column within the skin. TUNEL positive nuclei fluoresce green whereas *all* nuclei fluoresce red, so when the images are superimposed, yellow represents the damaged nuclei. Arrows demonstrate divergence around appendages such as sebaceous glands (A) and blood vessels (yellow arrows).

Abdominal skin model versus facial skin

This study confirms what was previously reported by Bailey *et al.,* that an attenuation factor could be used to predict based on the depth of injury on an abdominal model what the depth of injury would be on facial skin. On average with single pulsing MTZs in the abdomen are 30% greater than in the face. There are non-invasive instruments that can be used for measuring facial skin depth such as the Derma Scan C 20Mhz ultrasound device. We can then use this information to predict how deep the laser injury would be in the face. Thus giving the clinician a better grasp of what laser setting corresponds to depth of injury, in particular within the dermis. This should in turn improve clinical outcomes for laser treatments.

Histopathological injury and neo-collagen synthesis

Studies, which examine the *in vivo* histopathological changes produced by the laser, tend to be in non-visible areas such as the abdomen, forearm or buttocks. Resurfacing treatments tend to be on the face. Our group has already demonstrated that there are significant differences between skin on the face and skin on the abdomen and this was clearly shown again with this current study. A study by Orringer *et al.* looked at collagen synthesis (Orringer et al., 2004). In comparison the same group looked at a non ablative laser on the forearm and buttock which, interestingly showed that stimulation of collagen synthesis varied from subject to subject and was not consistent (Orringer et al., 2005). They concluded that this could explain why some patients are ‘non responders’, but acknowledged that the effect of multiple passes had not been ascertained.

The Deep FX laser system used in this study provides an additional modification to the mode of fractional ablative laser energy delivery. This system sets the laser microbeam distribution at a constant pitch of 250 microns and pulse width of 250 microseconds, allowing potentially very high fluences to be delivered to relatively small skin areas, and at pulse widths significantly below the thermal relaxation time of the dermis (200-600ms in the skin) (Riggs et al., 2007). Double pulsing delivers two discrete energy pulses less than 1ms apart, and therefore, the tissue does not have time to dissipate heat between two pulses. The net effect as demonstrated by this study is increased thermal damage both laterally and in depth for a given energy. This maximizes both the depth of ablation and the ablation-to-coagulation ratio of the zones of injury, producing the characteristic injury patterns of deep, narrow columns of ablation surrounded by relatively wider zones of coagulation. It is feasible that this injury pattern yields more predictable results with less down time than increasing single pulse fluences.

Future directions:

This study provides good data as to changes that we can expect at a microscopic level in the skin for given settings of the CO_2 laser. Combining the laser physics and the histopathological injury pattern may be useful for predicting how to best achieve a good clinical response. With current techniques multiple passes of the laser are required in order for the treatment to be effective. This has to be balanced with the risk of pigmentation changes and scarring as a result of aggressive treatment. Further, a study by Fitzpatrick *et al.* looked at the effect of pulse stacking with a high energy CO_2 laser (Ultrapulse), and he

concluded that multiple stacks lead to increased thermal necrosis and less ablation i.e. a less ‘precise wound’ with a concomitant increase in risk of adverse effects (Fitzpatrick et al., 1999). Broad crater like ablation of the epidermis/dermis was evidence of this. It may be that a double pulse mode allows a more precise delivery of thermal ablation and thus more targeted collagen denaturation. This would be the subject of future histopathological work to compare single and multiple passes in a double pulse mode at several energy settings. It may well be that fewer passes in a double pulse mode at a lower fluence delivers the energy required to stimulate collagen synthesis without the adverse effects. The next step would be to devise a clinical trial that would test out this hypothesis.

7.5 Conclusions:

This study has demonstrated that there are distinct differences between skin of the face and abdomen for a given fluence and the mode of delivery with the Deep FX CO_2 laser. These differences can be in part attributed to the increased density of skin appendages in the face compared to the abdomen. For a given energy setting (15mJ) the histopathological injury in the face and abdomen is significantly greater when delivered in a double pulse fashion compared to single pulse. This could have implications for clinical outcomes, and a clinical trial would be the next step in correlating microscopic injury patterns with efficacy of clinical treatments.

CHAPTER 8: Comparison of five commonly available lidocaine containing topical anesthetics and their effect on serum levels of lidocaine and its metabolite monoethylglycinexylidide (MEGX)

8.1 Introduction:

Topical anesthetics are used for a wealth of procedures in a variety of disciplines. They come in several different forms, many of which are available over the counter (OTC) without the need for prescription. There have been some documented serious adverse events and even fatalities after the application of topical lidocaine (Marra et al., 2006, Hahn et al., 2004, Curtis et al., 2009, Bursell et al., 2009). Serious adverse events are not limited to one preparation and are often accompanied by unsupervised application of the drug by the patient. The OTC products tend to have vague instructions as to how much to apply, over what area, and with what frequency they should be used. It is therefore imperative that the systemic absorption profiles of these drugs be properly investigated.

It has always been assumed that these topical anesthetic delivery systems are safe. There are several studies in the literature attesting to this when used in areas such as the arms, leg or abdomen (Friedman et al., 2001, Friedman et al., 1999). However, recently our group has shown that there is great inter-individual differences in the manner that lidocaine is absorbed and metabolized for just one of the OTC products that contain lidocaine (Oni et al., 2010). Unfortunately there are numerous lidocaine-containing topical anesthetic products that are commonly used on the face for a number of different procedures. The skin on the face is different than the skin elsewhere in the body in that it has a greater

vascular supply and a stronger density of glandular structures (Bailey et al., 2011a). To date, there is a paucity of data looking at the safety of these OTC products when applied to the face.

This study evaluates five commonly used topical anesthetics, three of which are available OTC and two that are compounded (Table 1). We compare the absorption of a fixed amount of each of the drugs when applied to the face, by measuring the serum levels of lidocaine and its active metabolite monoethylglycinexylidide (MEGX). In this way we hope to provide more information on the safety profile of the use of these drugs on facial skin.

Table 1: Comparison between the five different local anesthetics

Drug	Composition	Recommended doses	Metabolized	Patient instruction
LMX 4®	4% lidocaine Liposomal carrier	Apply externally to the affected area up to 3 to 4 times a day	Liver	For use on adults and children 2 years and older Children under 2 years – ask a doctor. May be applied under occlusive dressing
Generic form of EMLA™	2.5% lidocaine and 2.5% prilocaine eutectic mixture (oil in water)	See data sheet [33]– instructions given depending on clinical scenario	Liver	See data sheet[33] – instructions given depending on clinical scenario
Topicaine®	4% lidocaine. Amide. Translucent microemulsion gel	Adults and children >2yrs. Apply external to affected area up to 3-4times per day	Liver	Apply a moderately thick layer to the affected area (approximately 1/8 inch thick). Allow time for numbness to develop. Best results obtained 20 minutes to 1 hour post-application
LET	4% lidocaine, 1:2000 epinephrine, 0.5% tetracaine Gel, Methylcellulose base	N/A	Liver (lidocaine) Plasma (tetracaine)	Apply under direction of prescribing health professional
BLT	20% Benzocaine, 6% lidocaine, 4% tetracaine Emollient base	N/A	Liver (lidocaine) Plasma (benzocaine, tetracaine)	Apply under direction of prescribing health professional

8.2 Method:

Twenty five (25) healthy subjects were consented and screened in accordance with Title 45 Code of Federal Regulations, Part 46, Protection of Human Subjects (45 CFR part 46) for participation in this UT Southwestern Medical Center Institutional Review Board (IRB) study (appendix 6+7). Each enrolled participant was subject to a patch test for drug sensitivity before inclusion into the next phase of the study. The patch test consisted of a 1cm application of each of the study drugs to the forearm with an occlusive dressing for ten minutes. Any subject with adverse skin reactions such as erythema or welting were excluded at this point.

The subjects were then randomly assigned to one of five groups, with an N=5 for each group:

Group A: 30g Topicaine® (active ingredient 4% lidocaine, Esba Laboratories, Jupiter, FL) – non prescription, available over the counter

Group B: 30g eutectic mixture 2.5% lidocaine, 2.5 % prilocaine (generic form of EMLA™, High tech Pharmaceutical, Amityville, NY) - non-prescription, available over the counter

Group C: 30g LMX 4® (active ingredient 4% lidocaine, Ferndale Laboratories, Ferndale, MI) - non prescription, available over the counter

Group D: 30g LET (4% lidocaine, 1:2000 epinephrine and 0.5% tetracaine) – prescription required, compounded in pharmacy

Group E: 30g BLT (20% benzocaine, 6% lidocaine, and 4% tetracaine) - prescription required, compounded in pharmacy

Each subject in received 30g of the assigned drug applied to the face and neck area (Figure 1). This was then covered immediately with an occlusive dressing and left for 60 minutes (Figure 2). Vital signs (ECG, blood pressure, heart rate and oxygen saturations) were measured for the duration of the 60 minutes that the study drug was applied to the face/neck. At 60 minutes the study drug was removed and the subjects were asked to cleanse their face with Cetaphil® (Galderma Laboratories, Fort Worth, Texas, USA) skin cleanser to ensure all traces of topical preparation were removed. Blood samples were taken via an intravenous catheter that was left in place for the duration of the study. Time intervals of the blood samples were 90, 120, 150, 240 and 480 minutes. Any adverse events relating to the procedure were recorded.

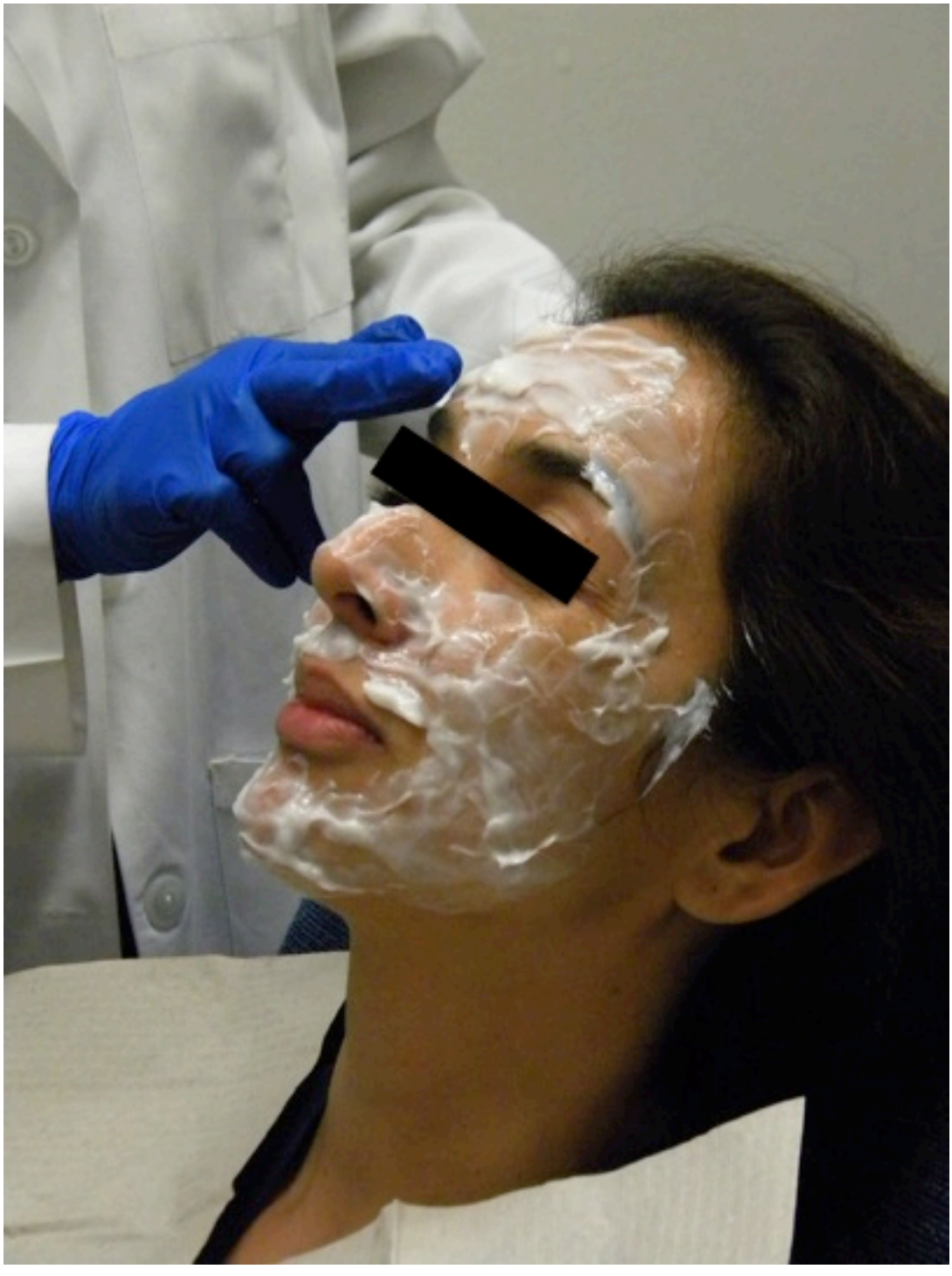


Figure 1: Topical lidocaine containing cream is applied to the face and neck (image obtained with patient consent).

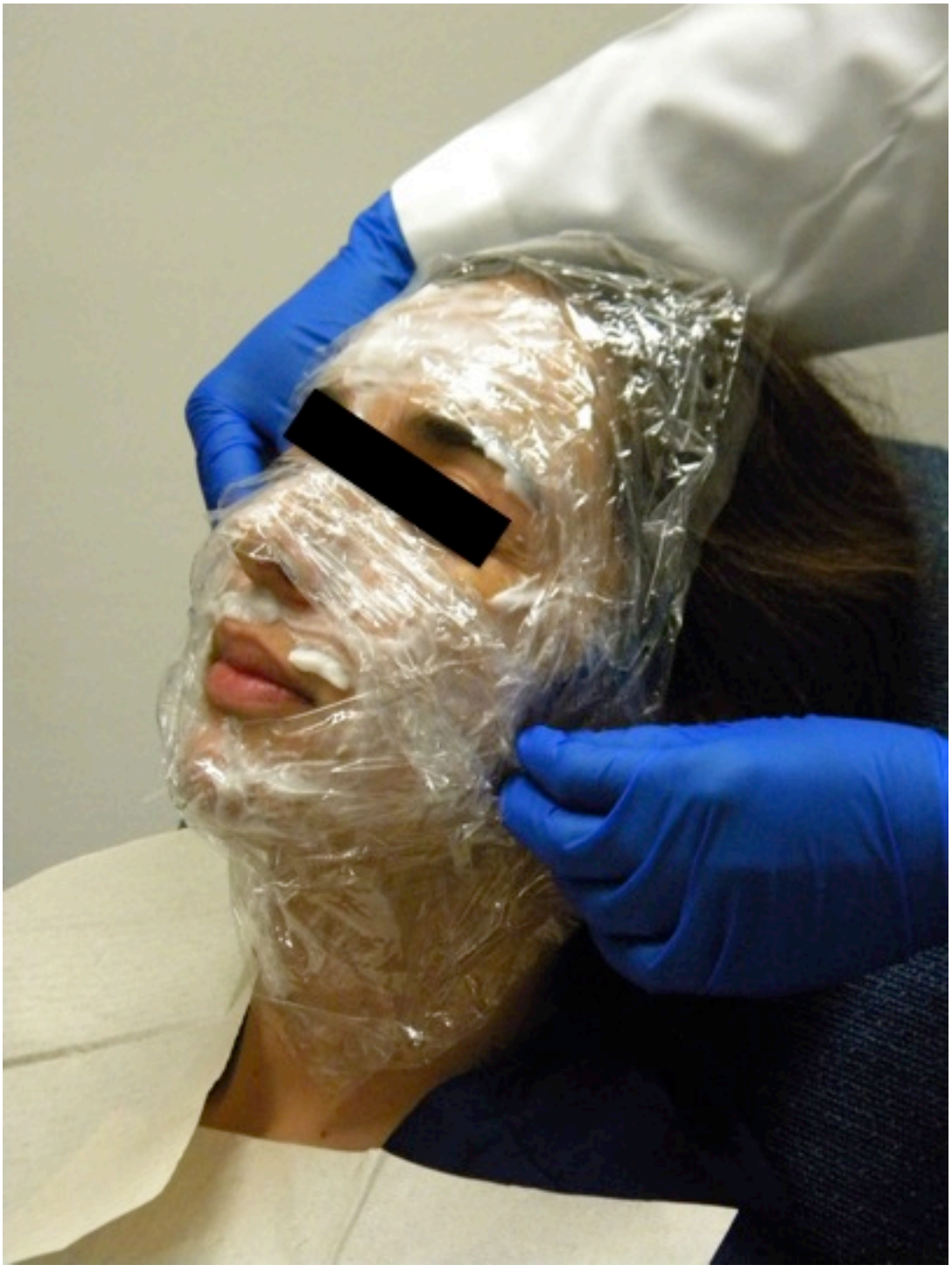


Figure 2: An occlusive dressing is placed over the face for 60 minutes duration while the subject is cardiac monitored (imaged obtained with patient consent).

Blood sample procurement and analysis:

Whole blood samples (approximately 15ml of blood) were collected in ethylenediaminetetraacetic acid (EDTA) containing vials and allowed to clot. The blood samples were then centrifuged (3000 rpm for 10 minutes at 4°C) and the serum stored at -80°C until analyzed. The Department of Clinical Chemistry, George-August University (Goettingen, Germany) analyzed lidocaine and MEGX in serum using a previously described technique (Streit et al., 2001).

Statistical analysis:

The serum concentration-time courses of lidocaine and MEGX were characterized using Microsoft Excel (Microsoft Corp., Redmond, Wash.). A repeated measures ANOVA test for non-parametric data was used to analyze differences between the groups. Associations with $p<0.05$ were considered statistically significant.

8.3 Results:

All 25 subjects completed the study. There were 17 females and 8 males included in the study with an average age of 26 years (range 22-62) and an average weight of 70.9kg (range 46.4kg to 96.4kg). All patients described numbness of the face and neck after 60 minutes, although time to anesthesia and depth of anesthesia were not formally assessed. The subject demographics are shown in table 2.

Table 2: Patient demographics

No of patients	25
Age	26 years (range 22-62)
Sex	Male – 8 Female - 17
Weight (kg)	70.9 (range 46.4- 96.2)

Drug absorption:

The over the counter preparations had the highest serum lidocaine and MEGX levels. Topicaine® had the greatest serum levels of lidocaine absorption (0.808µg/ml) for an individual, followed by generic EMLA™ (0.72 µg/ml), LMX 4 (0.44 µg/ml), BLT (0.17µg/ml), and lastly LET (0.13µg/ml). On average Topicaine® had the highest serum lidocaine and MEGX levels, 0.438µg/ml and 0.0678µg/ml respectively.

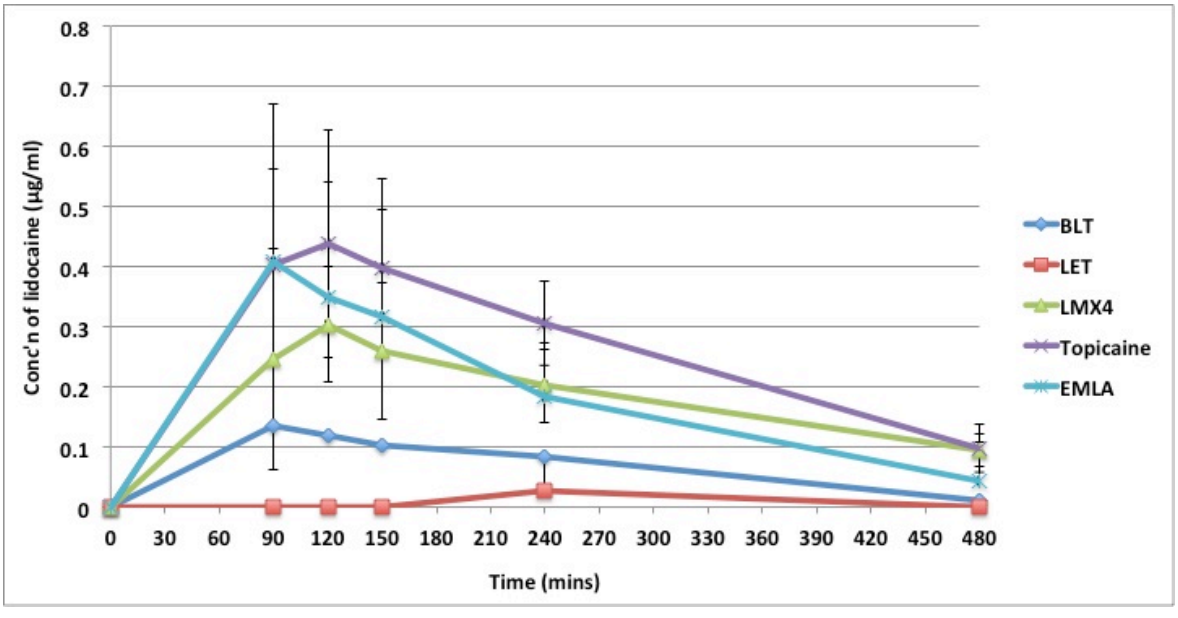


Figure 3: Comparison of all 5 groups - Serum lidocaine. There is a significant difference in the serum levels of lidocaine between the different groups (*p*=0.0002)

There were significant differences across the time points between the serum levels of lidocaine (*p*=0.0002) and MEGX (*p*=0.0045) when comparing all five groups (Figure 3 and 4). LET had the least absorption of all five study drugs, in fact 4 out of 5 patients in this group had no detectable levels of lidocaine or MEGX in their serum.

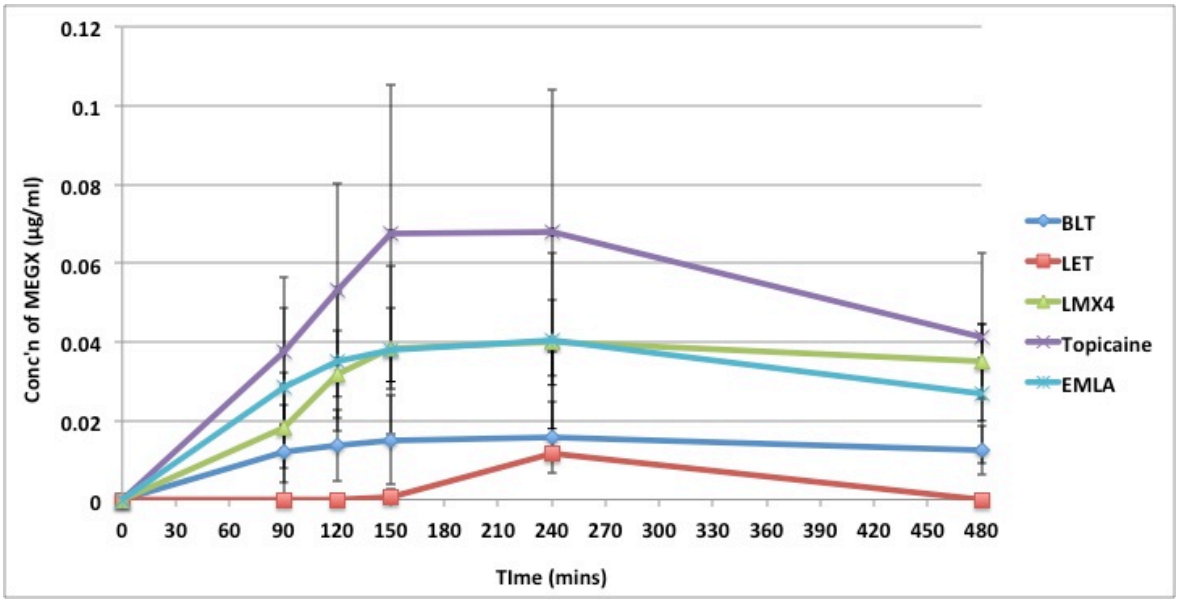


Figure 4: Comparison of all 5 groups - Serum MEGX. There is a significant difference in the serum levels of MEGX between the different groups (*p*=0.0002)

When averaged across each group, for all study drugs there were still detectable levels of lidocaine and MEGX in the serum of participants at 8 hours.

Table 3 summarizes the different groups and the average peak serum levels of lidocaine and MEGX.

Table 3: Average and Peak combined serum levels for the five different topical anesthetic preparations

Drug	*Peak levels (µg/mL)	Average levels (µg/mL)	†Time (min)
Topicaine® (4% lidocaine)	0.808	0.4996	90/120
Generic EMLA™ (2%lidocaine +2% prilocaine)	0.779	0.4384	90/90
LMX-4® (4% lidocaine)	0.477	0.3359	150/120
BLT (20% benzocaine, 6% lidocaine, 4% tetracaine)	0.178	0.1482	90/90
LET (4% lidocaine, 1:2000 epinephrine, 0.5% tetracaine)	0.188	0.0376	240/240

***individual within the group**

†first reading is peak time for individual, second reading is peak time for the group average

Inter-individual variation within the groups:

Inter-individual variation was shown in each group except for LET. Topicaine® ($p<$ 0.0001), generic EMLA™ ($p<$ 0.0001), LMX-4® ($p<$ 0.0001), and BLT ($p=<$ 0.0001) all showed significant differences between the serum levels of MEGX and lidocaine combined in their respective groups.

Differences between 4% lidocaine containing preparations (Figure 5):

Topicaine® had the greatest serum absorption (0.491µg/ml) followed by LMX-4® (0.336 µg/ml) and then LET (0.038 µg/ml). The difference between these three groups was statistically significant (p=0.0439).

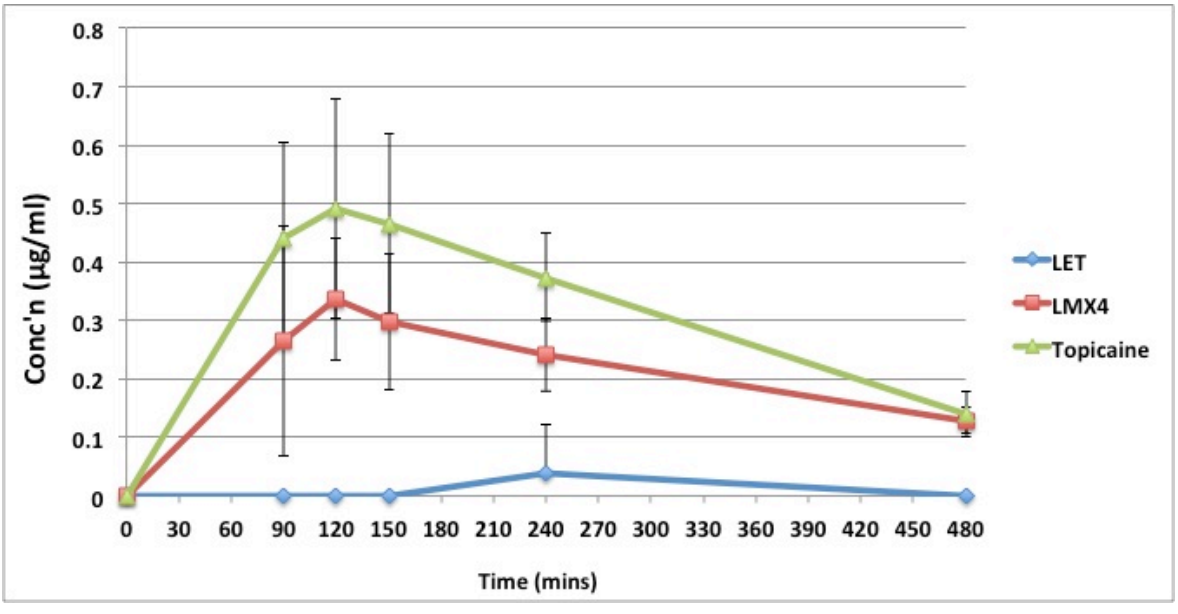


Figure 5: Combined serum lidocaine and MEGX levels for the different 4% lidocaine containing preparations. There is a significant difference between the groups (*p*=0.0439)

Differences between preparations containing differing lidocaine concentrations (Figure 6):

The 2.5% lidocaine containing preparation had greater absorption with a peak combined serum level of 0.4384 µg/ml compared with 0.276 µg/ml for the 4% containing preparations and 0.1482 µg/ml for the 6% lidocaine containing preparation, which had the least absorption. The differences between the different lidocaine concentrations were statistically significant (*p*=0.0016).

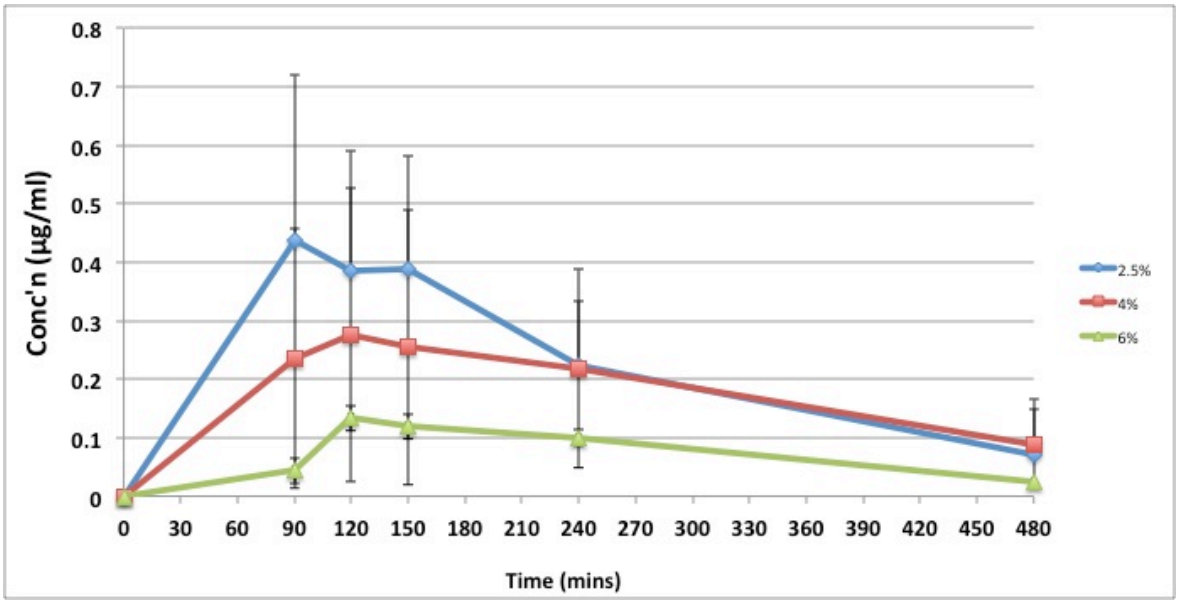


Figure 6: Differences between preparations containing differing lidocaine concentrations. There is significant difference between the combined serum concentrations of lidocaine and MEGX among the groups (*p*=0.0016)

Adverse skin reactions:

Two participants developed minor adverse reactions of erythema within the first 4 hours, with scaling and prolonged erythema at 48 hours, 1 in the Topicaine® (4% lidocaine) group and 1 in the 2.5% lidocaine group (EMLA™). All skin reactions resolved by 7 days. One subject (Fitzpatrick V) in the Topicaine® group developed a more severe skin reaction resulted in post inflammatory hyperpigmentation over the cheeks and dorsum of the nose (Figure 7). This resolved with conservative care.

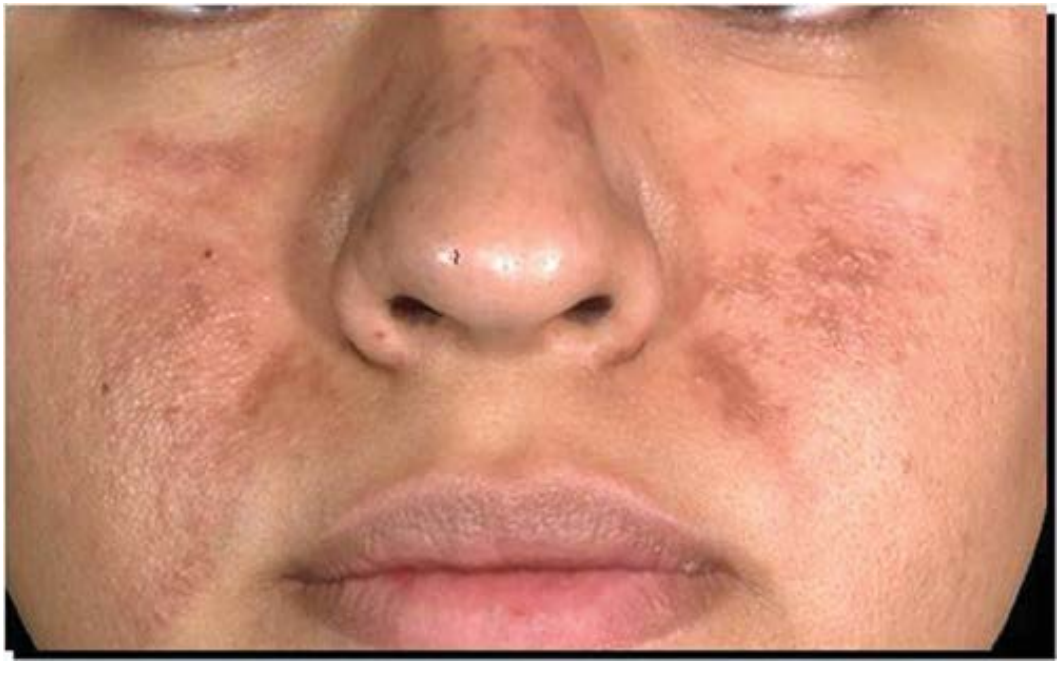


Figure 7: Hyperpigmentation following an inflammatory reaction to Topicaine® topical anesthetic (image obtained with patient consent).

8.4 Discussion:

The ideal topical anesthetic would have high efficacy with minimal systemic absorption. They are used for a variety of applications ranging from venipuncture in children (Lander et al., 2006) to skin graft harvesting (Goodacre et al., 1988, Ohlsen et al., 1985), to laser treatments such as facial rejuvenation and hair removal in adult (Yun et al., 2002, Friedman et al., 1999, Guardiano and Norwood, 2005). They are commonly available and some can be purchased over the counter without the need for a prescription. While these topical lidocaine-containing compounds are considered safe, they have been poorly studied. There is some systemic absorption of lidocaine and in some rare cases this has led to severe cardiac and neurological side effects and in extreme cases, death (Marra et al.,

2006, Rincon et al., 2000, Hahn et al., 2004). This study has quantified the systemic absorption of 5 different lidocaine containing topical anesthetics, three of which are available without prescription, and has shown with the exception of LET that all these drugs have considerable systemic absorption.

Local anesthetics (LAs) can be split into two classes; amides (e.g. lidocaine, prilocaine) and esters (e.g. benzocaine and tetracaine). The basic chemical structure of local anesthetics consists of a lipophilic group and intermediate bond (which is either an ester or an amide) and a hydrophilic group. All local anesthetics are weak bases, inflammation causes an acidic environment which causes more of the LA to be ionized leading to a slower onset of action. Amides are metabolized in the liver by the p450 cytochrome pathway more slowly than esters that are metabolized in the plasma by cholinesterases. (Lui and Chow, 2010). Both are excreted through the kidney. Therefore because they are metabolized more slowly, amides can be more toxic than esters, e.g. lidocaine (amide) has a relative potency of 4, whereas tetracaine (ester) has a relative potency of 10 (Ruetsch et al., 2001). Prilocaine is less toxic than lidocaine for a given dose because it has less vasodilatory effect and is metabolized faster, doses of >8mg/kg can lead to toxic effects (Carruthers et al., 2010, Ruetsch et al., 2001). LAs bound to proteins have a longer duration of action and those in the non-ionized form lead to a more rapid onset of action. The higher the pH the longer acting the LA; for example, lidocaine (pH7.6-7.8) has a faster onset than tetracaine (pH 8.1-8.9). Both amide and ester type LAs work by binding to receptors on Na+ channels reducing the uptake of NA+ ions into the nerve, thus preventing depolarization and therefore, conduction of the nerve impulse.

Many clinicians use a combination of local anesthetics; indeed three of the five drugs that were used in this study contain more than one type of local anesthetic. The effect of the

different anesthetics is additive. For each drug there is a safe limit in mg/kg that should be administered to the patient and this should always be adhered to.

Over the counter topical anesthetics have greater absorption than prescription anesthetics

This study has shown that the over the counter (OTC) products, which did not require prescription, had greater levels of lidocaine in the bloodstream than the prescription preparations. The literature relating to lidocaine toxicity is largely centered on its intravenous use for cardiac arrhythmias. Clinical symptoms of lidocaine toxicity include light headedness, paraesthesia, nausea and vomiting and this can progress to seizures, arrhythmias and cardio-respiratory depression (Kaweski, 2008, Bursell et al., 2009, Curtis et al., 2009, Brosh-Nissimov et al., 2004). Toxic levels of lidocaine are said to occur with serum levels above 5µg/ml, with CNS disturbance such as seizures occurring at levels greater that 8µg/ml (Becker and Reed, 2006). While the observed levels did not exceed 1 µg/ml (the lower limit of therapeutic intravenous lidocaine therapy for arrhythmias) it is still a sobering thought that, for example, one individual with Topicaine® applied to the face had serum levels in excess of 0.8 µg/ml. The doses that were used in this study and the application of occlusive dressings were all within the manufacturer guidelines.

Allergy rarely occurs with amides, but can occur with esters such as benzocaine because when metabolized it forms p-aminobenzoic acid (PABA) which has been associated with allergic reactions (Eggleston and Lush, 1996). In addition benzocaine can cause methemoglobinemia, as can lidocaine and prilocaine (Rincon et al., 2000), which can be

potentially fatal particularly in the pediatric population (Logan and Gordon, 2005, Dahshan and Donovan, 2006).

All adverse skin effects were seen with the OTC drugs. Interestingly some studies have shown that Topicaine® and EMLA™ can be used under occlusion with 'mild and transient' adverse events (Friedman et al., 2001) These studies were performed on the legs and forearms, and not on the face, as with our study. Other studies support our findings particularly with EMLA™, that it can cause minor skin irritations (Huang and Vidimos, 2000). The composition of Topicaine® contains alcohol; a hypersensitivity to this could be the cause of the more severe post inflammatory hyperpigmentation reaction that one patient experienced. It has already been shown that the prilocaine component of generic topical anesthetic which resembles the same composition as EMLA™ can cause contact sensitivity and we had one adverse skin reaction in this group (Friedman et al., 2001). For these reasons this study highlights the need for caution when patients use these drugs unsupervised. Even when a test patch is offered, as in this study, skin reactions may not be evident until the drug is actually applied to the face.

Inter individual variability

This study compared 5 different lidocaine containing topical anesthetics and demonstrated that absorption of the drug through the facial skin varies from individual to individual. Our group has previously been demonstrated this with topical anesthetic, and liposuction studies (Oni et al., 2010, Kenkel et al., 2004). It has also been shown in other studies looking at the use of local anesthetics in breast augmentation. (Rygnestad et al., 1999). The significance of this is that one cannot predict the amount of drug a patient will absorb.

Lidocaine is metabolized in the liver via the p450 cytochrome pathway and its breakdown products including MEGX are excreted via the kidney. As a result patients who have liver or kidney problems will have a reduced capacity for lidocaine metabolism and therefore will have greater circulating levels of the drug. In addition, patients who have broken areas on the skin prior to drug application will also have greater absorption of the drug, as the barrier to drug absorption is the *stratum corneum* (the outermost layer of the skin).

Drug delivery vehicle influences drug absorption

This study has highlighted the effect of the drug delivery vehicle on the absorption through the skin. We had three different products all of which contained 4% lidocaine, and all three had differing absorption profiles. One of the drugs had an alcohol-based composition, the second was a liposomal drug delivery system and the third was an emollient-based product. Alcohols act as skin penetration enhancers, by removing lipid from the *stratum corneum* and therefore increasing its permeability (Lachenmeier, 2008). Liposomes facilitate drug penetration through the skin because they have a lipid bilayer construct that encapsulate the drug and emollients are lipid based (de Leeuw et al., 2009). In both these cases the lipophilic nature of the drug delivery system enhances penetration through the *stratum corneum* (Gillet et al., 2011). What was interesting to note is that the 2.5% lidocaine-containing drug had the greatest absorption compared to the 4% and 6% lidocaine-containing drugs. This is because the drug exists in a eutectic mixture with 2.5% prilocaine. The significance of this is that the active ingredients (i.e. lidocaine and prilocaine) exist as an oil in water mixture with a lower melting point of 18°C. This means that at room temperature lidocaine and prilocaine exist as a liquid rather than a solid and

the absorption is therefore enhanced. This is further facilitated by the addition of an occlusive dressing. LET had the least absorption of all 5 drugs, with only 1 participant in this group having any detectable levels in the blood stream. This is probably attributable to the fact that this drug contains epinephrine, which is a known vasoconstrictor. The benefit of this is that the drug is confined to the epidermis/dermis, and is not typically absorbed systemically.

Compounded drugs:

Two of the local anesthetic mixtures that were used were compounded in pharmacy. Recently the FDA has issued warnings to five compounding companies with regard to their practice of mass production of compounded products. Compounded products should be prescribed specifically for the patient and should not therefore, be mass-produced. The reason for this is that compounded drugs are not FDA approved and often have higher concentrations of active ingredients than OTC products. These drugs should be used under the care of health care professional, which give the patient clear instructions on how to use the compounds. Fatalities related to compounded products have been secondary to unsupervised use, as they are not packaged with the usual patient inserts/written instructions such as with OTC local anesthetics.

Recommendations and future directions

This study and previous studies by this group have demonstrated that while topical anesthetics are safe, there can be considerable systemic absorption of lidocaine containing

topical anesthetics. In particular, it is difficult to predict those patients that may be 'sensitive' to topical applications, resulting in high circulating serum levels of lidocaine. We would therefore, advise caution particularly with OTC lidocaine preparations which we have shown to have the greatest systemic absorptions.

This study has focused on lidocaine and its metabolites, because it has well documented guidelines for toxicity and the serum drug levels at which symptoms occur. It is also the most potent of the active drugs in the topical anesthetics. Esters such as benzocaine and tetracaine are less well researched in the literature, but have less potency than amides due to their method of metabolism (Lui and Chow, 2010). What can be said is that where there are combinations of drugs they will have an additive effect. For future studies levels of benzocaine, tetracaine and prilocaine could be measured in the serum to give an indication of their relative contribution.

Occlusive dressings enhance penetration of the drug, and the length of time over which the occlusive dressing is applied will influence drug absorption. Added to this, participants in this study for all drugs still had detectable levels of lidocaine and MEGX in their serum at 8 hours which has ramifications for repeat applications. Ideally, these drugs should be used under the supervision of a healthcare professional, so that patients do not apply large amounts of the drug to large surface areas under occlusive dressings, all of which result in enhanced absorption. While these drugs will come with instructions on maximum recommended surface areas for application, maximum dose of drug to be applied as well as the need for occlusion, it is still prudent for the healthcare professional to recognize warning signs of lidocaine toxicity. Systemic drug absorption following topical application will be influenced by several factors, but again this study demonstrates that it also is dependent on individual patient physiology. Facial skin would most likely have an

increased absorption profile to skin, for example, on the leg or abdomen. Further studies would need to be carried out to compare these areas, before recommendations can be made on specific drugs and their doses.

It is well known that disruption of the *stratum corneum* leads to enhanced drug absorption. There are several clinical studies which show that disruption of the *stratum corneum* by laser pretreatment, followed by topical application of anesthetic can lead to a faster onset of anesthesia (Koh et al., 2007, Baron et al., 2003, Yun et al., 2002). Our group has already conducted preclinical studies in an animal model which show that laser pretreatment leads to increased systemic absorption of topically applied lidocaine (Oni et al., 2012b). The next step is to look at the safety profile of laser pretreatment as a method to facilitate faster anesthesia in a clinical model, for procedures such as laser facial resurfacing.

8.5 Conclusion:

Topical anesthetics are safe however systemic absorption in some individuals can reach unpredictably high levels. The type of drug delivery system, occlusive dressings as well as patient factors such as liver function can affect systemic absorption, metabolism and excretion of lidocaine. Over the counter products have been shown in this study to have the greatest level of systemic absorption, and can persist in the blood stream at 8 hours post application. Therefore, we would recommend that topical anesthetics are used under the supervision of a health care professional to avoid adverse toxic effects and in rare cases death.

CHAPTER 9: Pre treatment with a laser – a safe and effective method of analgesia for facial rejuvenation

9.1 Introduction:

Non surgical cosmetic surgery procedures such as botox, fillers and laser treatments are on the increase (Oni et al., 2011). Often these non-surgical procedures require either a topical anesthetic or injectable anesthetic to ensure adequate analgesia during the procedure. Injectable forms of anesthesia although efficacious are uncomfortable for the patient during administration. Laser pretreatment has been previously studied in the context of improving efficacy of analgesia of topical lidocaine prior to procedures such as venipuncture and facial resurfacing (Baron et al., 2003, Koh et al., 2007, Shapiro et al., 2002). These studies have been subjective based on pain perceptions. The aim of this study therefore is twofold; firstly to ascertain whether laser assisted transdermal delivery is safe and more effective than topical anesthetics alone and secondly to examine whether we can enhance the anesthetic ability of topical anesthetics to help diminish the need for local infiltration and nerve blocks.

Pre clinical studies by our group and others have demonstrated that laser pretreatment at low energies enhances absorption of topical lidocaine (Oni et al., 2012b). This study demonstrated that at very low energy settings pre treatment with the laser causes increased systemic absorption of lidocaine in a porcine model. The serum levels of lidocaine and its metabolite are however well below the levels that would be considered toxic. Physiologically, lidocaine is broken down into monoethylglycinexylidide (MEG-X), an active metabolite that has a similar toxicity. Lidocaine typically has a half-life of

approximately 2 hours in individuals with normal hepatic function. We have seen in previous studies performed by this group that there is significant inter-person variability with regard to lidocaine metabolism (Oni et al., 2010).

It is presumed that serum lidocaine levels will rise and the risk of lidocaine toxicity will increase in individuals who undergo treatments leading to the disruption of normal physiological barriers to dermal absorption, although this has not been proven. Lasers can be used to ablate the *stratum corneum* of the skin and therefore enhance absorption of topically applied drugs. There are no documented studies, which look at the serum concentrations of lidocaine and its metabolite MEGX in this context. Our study aims to look at the serum levels of lidocaine and MEGX after the application of topical lidocaine following pretreatment of the skin with an ablative laser.

9.2 Method:

Ten patients were consented and screened in accordance with Title 45 Code of Federal Regulations, Part 46, Protection of Human Subjects (45 CFR part 46) for participation in this UT Southwestern Medical Center Institutional Review Board (IRB) study (appendix 7+8). The patients were recruited from flyers that were posted on the UT Southwestern campus. Prior to treatment all patients had photographs taken. The inclusion/exclusion criteria are summarized in table 1. Patients were randomly assigned to either group A or group B.

Table 1: Inclusion/Exclusion criteria:

Inclusion	Exclusion
All races, genders and ethnicities	Pregnancy
Age - 18 and 89 years	Allergy to topical anesthetics
Seeking consultation for laser correction of fine lines, melasma, and acne scarring	History of heart arrhythmia, liver dysfunction, congestive heart failure, hypertension, chronic renal insufficiency, chronic renal failure, anxiety disorder, psychosis or seizure
Wish to undergo full facial rejuvenation	Had microderm abrasion, severe sunburn, chemical peel, laser treatment, or other severe abrasions to the face/neck in the past 45 days
	Severe acne or subjects being treated with acutane.
	Had any disease causing abrasion or sloughing of skin of treatment area in the past 45 days.

At time 0 all patients had 15g of BLT (20% benzocaine, 6% lidocaine, and 4% tetracaine) triple anesthetic cream applied to their face for 20 minutes with no occlusion. The topical anesthetic was then removed and the first blood draw made.

First superficial laser treatment:

Patients in group A had a superficial pretreatment with the full ablative erbium-doped yittrium aluminium garnet (Er:YAG) laser (Sciton Profile 2940nm) to the face. Laser

settings were 15 microns (2.8 J/cm^2). Patients in group B had a superficial pretreatment with the fractionated carbon dioxide (CO_2) laser (Ultrapulse, Lumenis) 15mJ (1.1 J/cm^2) 300 mHz (4.5W), density 2, column spot size 1.3mm.

Immediately after, a further 15g of BLT was reapplied to the face and left without occlusion for another 20mins.

Second deeper laser treatment:

After removal of the second application of topical anesthetic the second laser treatment commenced. For group A patients the Er:YAG laser settings for the deeper treatment was 100 microns (25 J/cm^2) on the full ablative setting. For group B patients the fractionated CO_2 laser (Ultrapulse, Lumenis) setting for the deeper treatment was 50 mJ (3.8J/cm^2) 300mHz (15W), density 3, column spot size 1.3mm.

At the patient's request analgesia will be supplemented with 1% lidocaine (plain) in the form of nerve blocks. This will be recorded –these patients will still have their bloods taken. In total patients will have no more than 2mg/kg lidocaine administered by any route.

Blood draws:

Blood serum samples (approximately 1 tablespoon or 7 mL) were taken at 20 (immediately after the topical anesthesia is removed), 60, 90, 120, 180 and 240

minutes after application. These samples were taken from an intravenous line left in place for the course of the procedure.

Patient pain score questionnaire:

Patients were required to complete a short questionnaire rating the pain experience prior to their discharge (Figure 1).

ID_____

We would like you to rate your experience today, please answer the following questions.

1. Rate your experience during the first laser treatment (tick appropriate box):

Numerical Pain Rating Scale	0	1	2	3	4	5	6	7	8	9	10
Verbal Descriptor	NO PAIN		MILD PAIN		MODERATE PAIN		MODERATE PAIN		SEVERE PAIN		WORST PAIN POSSIBLE
Wong-Baker FACES Pain Scale											
Activity Tolerance Scale	NO PAIN		CAN BE IGNORED		INTERFERES WITH TASKS		INTERFERES WITH CONCENTRATION		INTERFERES WITH BASIC NEEDS		BEDREST REQUIRED

2. Rate your experience after the anesthetic was removed and the second laser treatment began

Numerical Pain Rating Scale	0	1	2	3	4	5	6	7	8	9	10
Verbal Descriptor	NO PAIN		MILD PAIN		MODERATE PAIN		MODERATE PAIN		SEVERE PAIN		WORST PAIN POSSIBLE
Wong-Baker FACES Pain Scale											
Activity Tolerance Scale	NO PAIN		CAN BE IGNORED		INTERFERES WITH TASKS		INTERFERES WITH CONCENTRATION		INTERFERES WITH BASIC NEEDS		BEDREST REQUIRED

3. Rate your experience half way through the second laser treatment

Numerical Pain Rating Scale	0	1	2	3	4	5	6	7	8	9	10
Verbal Descriptor	NO PAIN		MILD PAIN		MODERATE PAIN		MODERATE PAIN		SEVERE PAIN		WORST PAIN POSSIBLE
Wong-Baker FACES Pain Scale											
Activity Tolerance Scale	NO PAIN		CAN BE IGNORED		INTERFERES WITH TASKS		INTERFERES WITH CONCENTRATION		INTERFERES WITH BASIC NEEDS		BEDREST REQUIRED

4. Rate your experience at the end of your treatment

Numerical Pain Rating Scale	0	1	2	3	4	5	6	7	8	9	10
Verbal Descriptor	NO PAIN		MILD PAIN		MODERATE PAIN		MODERATE PAIN		SEVERE PAIN		WORST PAIN POSSIBLE
Wong-Baker FACES Pain Scale											
Activity Tolerance Scale	NO PAIN		CAN BE IGNORED		INTERFERES WITH TASKS		INTERFERES WITH CONCENTRATION		INTERFERES WITH BASIC NEEDS		BEDREST REQUIRED

Would you have this laser treatment done again with this pain relief method? Yes/No

Figure 1: Patient questionnaire to assess pain scores during the treatment.

Blood sample procurement and analysis:

Whole blood samples were collected in serum separator tubes (BD Vacutainer, Franklin Lakes, NJ), and allowed to clot. The blood samples were then centrifuged (3000 rpm for 10 minutes at 4°C), the serum aliquoted into eppendorf tubes in duplicate and stored at -80°C until analyzed. The samples were sent to the Department of Clinical Chemistry, George-August University (Goettingen, Germany) analyzed lidocaine and MEGX in serum using a previously described technique (Streit et al., 2001).

Statistical analysis:

The serum concentration-time courses of lidocaine and MEGX were characterized using Microsoft Excel (Microsoft Corp., Redmond, Wash.). A repeated measures ANOVA test for non-parametric data was used to analyze differences between the groups. Associations with $p<0.05$ were considered statistically significant.

9.3 Results:

All ten patients completed the study with no adverse events relating to the topical anesthesia.

Pain scores (Table 2):

Group A:

For group A patients the average pain score for the first pass was 2.8 (range 1-6). For the second pass the average pain score at the beginning was 1.4 (range 0-4), during the

treatment rose to 3.0 (range 1-4) and by the end of the treatment reached a peak of 4.6 (range 2-7). All patients stated that they would have repeat treatments with this method of analgesia again.

Group B:

For group B patients the average pain score for the first pass was 3.0 (range 2-4). For the second pass the average pain score at the beginning was 3.4 (range 3-4), during the treatment rose to 3.6 (range 2-6) and by the end of the treatment it reached a peak of 4.0 (range 2-6). All patients stated that they would have repeat treatments with this method of analgesia again.

Table 2: Average pain scores taken during laser treatments (*denotes statistical significance)

Laser pass	Group A	Group B	P value
1st	**2.8**	**3.0**	**0.436**
2nd – beginning	**1.4**	**3.4**	**0.045***
2nd – middle	**3.0**	**3.6**	**0.213**
2nd – end	**4.6**	**4.0**	**0.323**

Comparing pain scores between group A and group B (Table 2):

All patients in both groups complained of the most discomfort either around the lips or in the pre-auricular region. For the first pass pain scores were equivocal between the two groups (p=0.436). At the start of the second pass the pain scores were significantly higher in group B compared to group A (p=0.045). However, there was no statistical difference in pain scores between the groups during the middle of the second pass (p=0213) and by the end (p=0.323).

Blood serum lidocaine and MEGX levels:

For all patients in all groups the serum levels of lidocaine and MEGX did not reach toxic levels (serum concentrations of greater than 5 μg/ml).

Group A (Figure 2):

For group A patients the maximum combined serum levels of lidocaine and MEGX in an individual patient was 0.935 μg/ml and this occurred at 90 minutes. For all patients in this group peak serum levels occurred between 60-90 minutes and started to decline thereafter. The average peak reading was 0.61 μg/ml, which occurred at 90 minutes. There was significant inter-individual variation amongst the patients within the group (p< 0.0001).

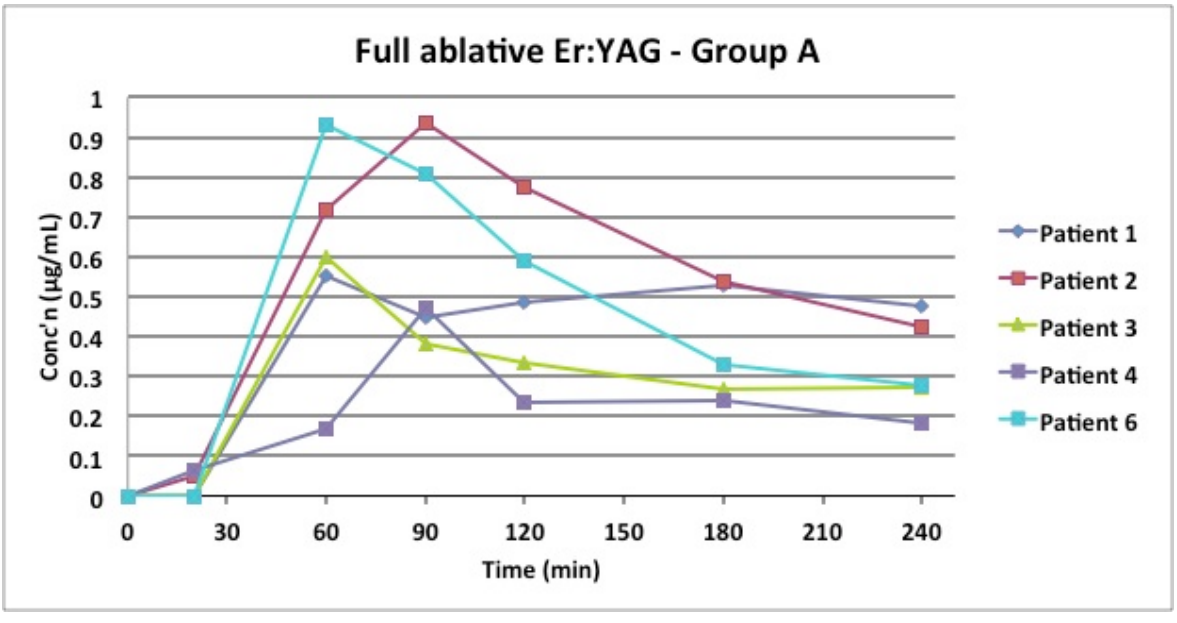


Figure 2: Group A – serum lidocaine and MEGX combined levels over a 4-hour period. There was a significant difference in levels between the subjects in the group (*p*<0.0001). The peak reading for a single individual was 0.935 µg/ml.

Group B (Figure 3):

For group B patients the maximum combined serum levels of lidocaine and MEGX in an individual patient was 0.533 µg/ml and this occurred at 90 minutes. For all patients in this group peak serum levels occurred between 90-120mins and thereafter started to decline thereafter. The average peak reading was 0.315 µg/ml, which occurred at 120 minutes. There was significant inter-individual variation amongst the patients within the group (*p*=< 0.0001).

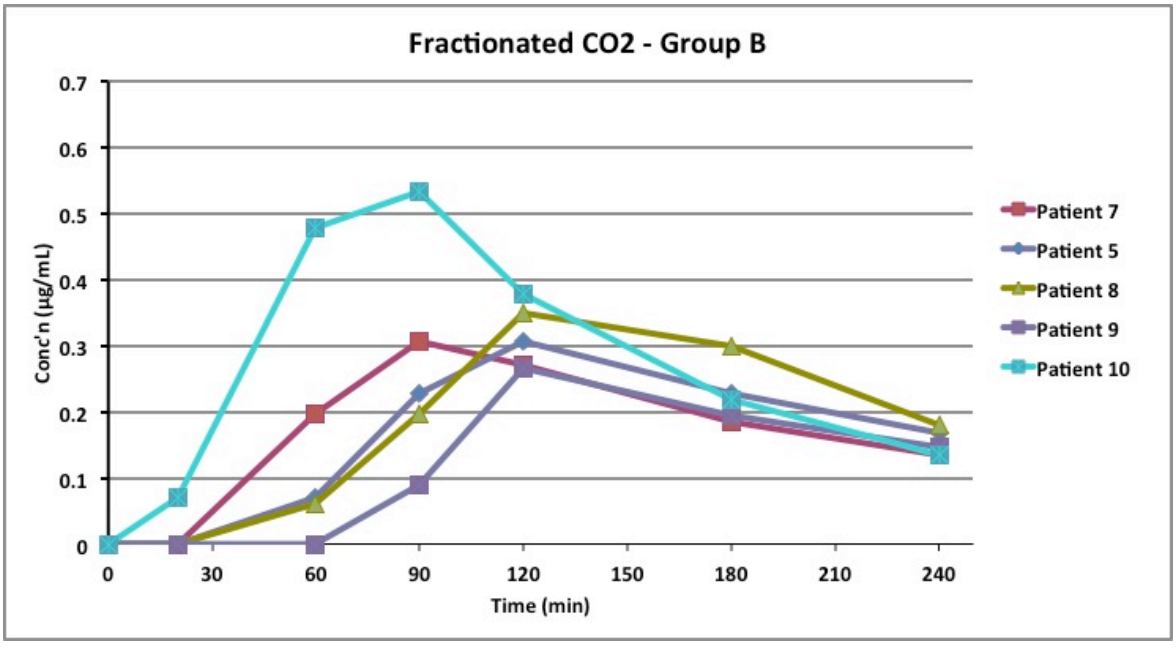


Figure 3: Group B – serum lidocaine and MEGX combined levels over a 4-hour period. There was a significant difference in levels between the subjects in the group ($p<0.0001$). The peak reading for a single individual was 0533 µg/ml.

Comparing group A and group B:

When comparing the two groups over the whole time course, group A patients, on average had higher serum levels of lidocaine and MEGX and this was statistically significant ($p=0.0253$). On average the serum levels of combined lidocaine and MEGX rose and fell quicker in group A, compared to group B.

Correlating pain scores to combined serum levels of lidocaine/MEGX:

For group A patients, there was a rapid increase in serum levels of lidocaine/MEGX by 60-90 minutes. These patients had the lowest pain scores at the start of the second pass. This correlates to rapid absorption of lidocaine and its metabolism. Hence the increase in pain

scores by the end of the second pass. In contrast group B patients saw a slow increase in serum lidocaine/MEGX levels with overall lower serum levels, but also had a fairly consistent pain score throughout both first and second pass laser treatments compared to group A. This correlates to slower absorption, and therefore, metabolism of lidocaine.

Adverse events:

No patient developed any complications with regard to the topical anesthesia. One patient in group B (fractionated laser group), developed post treatment hyperpigmentation. This was despite being commenced on hydroquninones pretreatment to reduce her risk. Post laser treatment she had a one-month course of hydroquinone cream and the hyperpigmentation resolved completely.

9.4 Discussion:

Topical anesthetics are used to perform countless procedures across a number of medical disciplines. Non-surgical cosmetic/rejuvenation procedures such as laser resurfacing of the face are becomingly increasingly popular. For laser resurfacing, topical anesthesia is often used, either with conscious sedation or with nerve blocks. This is the first study to our knowledge, which looks at the use of lidocaine containing topical anesthesia as the sole means of anesthesia for laser resurfacing. In addition it has demonstrated enhanced analgesic efficacy by the elimination of the superficial epidermis and correlates this to the serum levels of lidocaine. Previous studies from our group have demonstrated the histopathological injury that fractionated devices can cause in human skin (Oni et al., 2012c, Bailey et al., 2011a, Farkas et al., 2010a, Farkas et al., 2010b, Farkas et al., 2009, Farkas et al., 2008).

The aim of this study was to investigate whether the principles of laser assisted transdermal drug delivery could be used to provide complete anesthesia for facial rejuvenation without the need for supplementary nerve blocks. There are several articles in the literature, which attest to the use of laser-assistance to enhance topical anesthesia (Baron et al., 2003, Singer et al., 2006, Singer et al., 2005) for example, for venipuncture and intramuscular injections (Shapiro et al., 2002). However there is little, which examines the role of laser-assisted anesthesia for facial rejuvenation and/or correlates this to serum levels of the drug. Previous work performed by our group has demonstrated that the depth of fractional ablation used influences the absorption of lidocaine and subsequent metabolite levels. Animal pre clinical studies performed in our laboratories investigated the role of the fractionated laser (Er:YAG) to assist transdermal delivery of drugs, using lidocaine as the study drug (Oni et al., 2012b). This study found that as the depth of laser treatment increased so too did the serum levels of lidocaine. In addition, I have also investigated the safety of topical anesthetics applied to the face (Oni et al., 2010). All the prior clinical studies by this group regarding lidocaine containing topical anesthetics demonstrated considerable inter-individual variability in the way lidocaine is metabolized.

Yun *et al.* compared laser assisted topical anesthesia and topical anesthesia alone for facial rejuvenation (Yun et al., 2002). One half of the subject's face was pre treated with laser, and then the whole face was covered with EMLA for 60 minutes. Patients then underwent two passes of treatment with the ablative Er:YAG laser. They found that they had to give supplemental nerve blocks, both during the first and the second pass of treatment. However, they concluded that laser pretreatment reduced the need for supplemental nerve blocks. In our study no patient requested supplemental analgesia.

Kilmer *et al.* used a supplemental protocol in which patients had topical EMLA applied to their face, 30g for 90 minutes with occlusion, prior to oral analgesia, sedatives, and intramuscular analgesia being given (Kilmer et al., 2003). Then another 30g of EMLA was applied for 45-60 minutes before the actual laser treatment. Of the 200 patients treated with this protocol, 5% required supplemental nerve blocks. Most patients had between 2-3 laser passes on the face, and 1 laser pass to the neck. No evidence of lidocaine toxicity was recorded in any of their patients, but serum levels were not carried out.

Carruthers *et al.* investigated the serum levels of lidocaine applied to the face, neck and chest after intense pulsed light (IPL) treatment (Carruthers et al., 2010). Their study drug contained 15% lidocaine and 5% prilocaine (less than 10g in all cases). They found low levels of serum lidocaine and no evidence of lidocaine toxicity in any of their patients. IPL is a broad spectrum focused treatment and does not disrupt the *stratum corneum* in the way that a mono wavelength laser would. Its ability in increasing transdermal drug delivery is most likely through warming of the skin, as opposed to perforation of the *stratum corneum*.

This clinical study looks not only at the analgesic effect of laser assisted drug delivery, but more importantly the safety profile. It also illustrates the differences between ablative and fractionated lasers and their effect on transdermal drug delivery. Patients who had full ablative treatments with removal of all of the *stratum corneum* had significantly higher levels of lidocaine/MEGX in their blood stream compared to those who had a fractionated treatment and therefore only partial removal of the *stratum corneum*. The highest serum level seen in this study for any one patient was 0.935 μg/ml. To put this into context the lower therapeutic limit for intravenous lidocaine treatment of cardiac arrhythmias is 1.0

μg/ml. The toxic levels for lidocaine are 5.0 μg/ml and therefore, the levels seen in this study are significantly below this threshold, and no patient had any adverse events.

Duration of analgesia:

The patients who had the full ablative treatments demonstrated significantly lower pains scores and therefore better anesthesia at the start of the second pass of treatment than the patients in the fractionated laser group. However, by the end of the second treatment the pain scores were equivocal. This suggests that in the full ablative group the lidocaine is quickly absorbed through the skin and metabolized compared to the fractional group. This was reflected in the faster rise in serum levels in the full ablative group compared to the fractionated group. All patients stated that they would have their laser treatment performed with this method of anesthesia again. The pain scores suggest that the fractionated laser group had more consistent anesthesia from the first to the second pass, suggesting that drug is retained in the dermis over a longer period of time. This may well be affected by changing the treatment density of the fractionated laser. The serum levels of lidocaine/MEGX in this group started to fall after 120 minutes. Compare this to the ablative group where the pain scores were very low at the start of the second pass but had risen by the end. This suggests that the drug is not being retained within the dermis. For these patients the serum levels started to fall after 90 minutes, but reached significantly higher levels than the fractionated group. Extrapolating this trend it could be concluded that multiple pass treatments using this topical lidocaine regime may provide sufficient analgesia for fractionated and ablative laser treatments, but reapplication of the topical anesthesia would probably be required between passes. The amount and timing of doses would need further investigation.

Efficacy of topical lidocaine enhanced by laser treatments:

There are few studies that, look at the use of topical anesthesia as the sole method of analgesia for laser treatments, and even fewer that, include serum levels of lidocaine/MEGX to assess systemic toxicity. Alster *et al.* compared the analgesic effect of EMLA™ to S-Caine peel (which is no longer manufactured) when a 4x4 cm area on the cheek was treated with the CO_2 fractionated laser (Alster and Lupton, 2002). They used assessors to rate the patient's discomfort rather than asking the patient directly and found that 85% of patients had moderate pain (pain score average 5.25) on the EMLA™ side. Our study treats the whole face, and asks the patient to rate the experience. Marra *et al.* described the case of a woman with a BMI of 17 who developed systemic toxicity following fractional laser treatment to the face (Marra et al., 2006). In this instance 30% lidocaine was applied topically to the face and neck and left for 60 minutes before treatment began. She then underwent multiple pass treatment (8 on the face), and developed symptoms of toxicity with a serum level of 1.5 μg/ml 60 minutes after the onset of symptoms. There have also been reported lidocaine toxicity arising from application of topical lidocaine applied copiously to the lower limbs and wrapped in occlusive dressings prior to hair laser treatments (Brosh-Nissimov et al., 2004). Several lessons are to be learnt with cases such as these. Our study has demonstrated, that peak serum levels of lidocaine and its metabolite occur between 60-90 minutes after treatment depending on the laser used. In addition one would expect a greater absorption when 30% of the topically applied substance is lidocaine. Also the drug was left on for 60 minutes before treatment. The half-life of lidocaine is 90-120 minutes and therefore, it is not surprising that this patient developed toxic symptoms, especially as they had laser treatments which, depending on the laser, we know will perforate or obliterate the *stratum corneum* in its

entirety. This would then lead to absorption of any remaining lidocaine in the skin into the bloodstream. Increasing temperature also facilitates drug diffusion and the laser treatment generates heat within the skin, as does the occlusive dressing. The cumulative effect therefore of *stratum corneum* disruption and tissue heating caused by the laser would lead to increasing absorption of the topical lidocaine.

Effect of laser device:

In this study the fractionated CO_2 laser device was used. Previous studies by our group and by many others in pre-clinical animal models have used the fractionated Er:YAG laser to facilitate transdermal drug delivery (Oni et al., 2012b). Farkas et al demonstrated that the fractionated CO_2 device causes more thermal injury than the fractionated Er:YAG (Farkas et al., 2010b, Farkas et al., 2010a). It may well be that the amount of drug absorption would be affected by the differences in thermal injury between the devices. We have already alluded to this, with the ablative Er:YAG device facilitating greater drug absorption than the fractionated CO_2 device. Therefore one could extrapolate that the fractionated Er:YAG device would cause less drug absorption than the fractionated CO_2 device, because less thermal injury occurs initially. It may well be that if the topical drug was applied after a delay the channels generated would become sealed off with exudate/blood and generally tissue edema thus preventing any further drug absorption. This would be more marked with the fractionated CO_2 laser because it generates more thermal injury than the fractionated Er:YAG laser. Therefore, *timing* of drug application may be important in the amount of drug absorption.

In addition, the treatment density of the fractionated device could be examined. In preclinical studies on a porcine model conducted by our group reducing the treatment density of the fractionated Er:YAG laser led to less lidocaine being absorbed into the animal's blood stream (data not published). A further study would have to be conducted to test this hypothesis.

Limitations and future directions:

In this study only serum levels of lidocaine and its metabolite MEGX were measured. However BLT contains 20% benzocaine as well as 4% tetracaine. Both of these drugs also have potential toxicity. The toxicity profile of lidocaine is fairly well investigated because of the work done in its role as an intravenous treatment for cardiac arrhythmias. However, the literature examining safety profile of these two drugs is extremely limited, but it would have been interesting to measure the serum levels of these drugs to note if they rise in a similar fashion to lidocaine.

Despite all patients stating that they would have their laser treatment with this method of anesthesia again, it is unclear whether they would have tolerated more than one pass at the deeper fluence. The amount of topical anesthesia applied was based on our previous study in which 30g of BLT was applied to the face with occlusion for one hour. While there were no controls, this present study demonstrated on average a three-fold rise in serum levels with full ablative treatments compared to no laser at all (Figure 4), based on historic data by this group (Oni et al., 2012a).

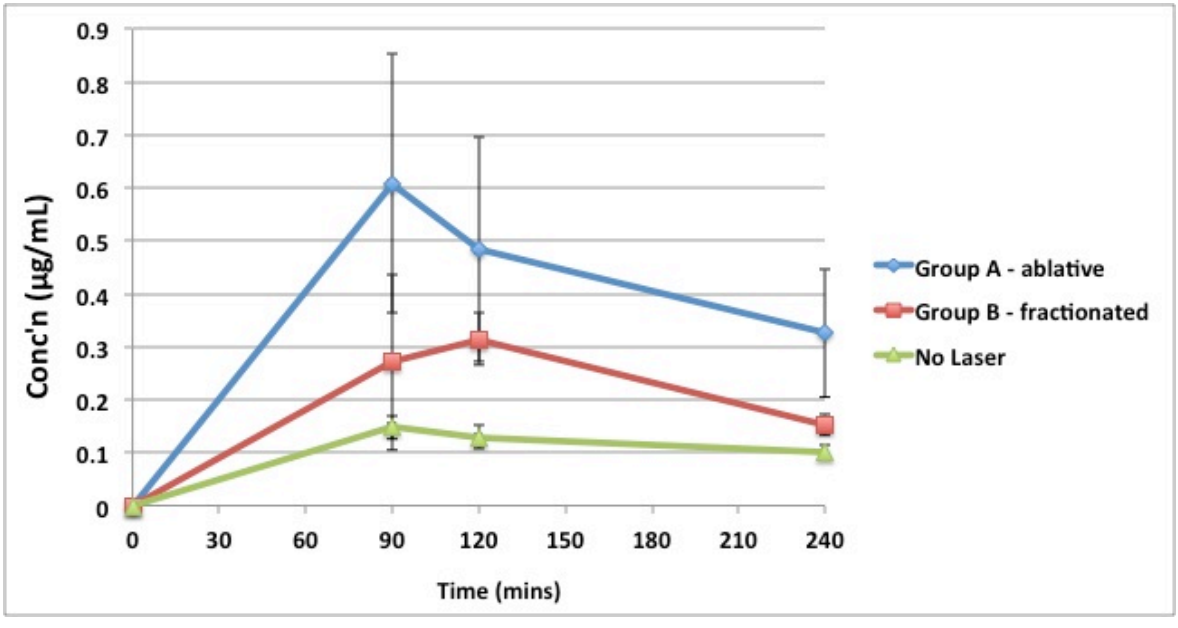


Figure 4: Historic data comparison, note that there is an increase in drug absorption with laser pretreatment compared to no laser treatment at all.

This suggests several areas for further investigation. Firstly, in this study a time of 20 minutes was arbitrarily chosen as sufficient to achieve anesthesia. There is a possibility that if the time were shortened there would be a different effect be on serum levels of lidocaine/MEGX and pain scores. Secondly, one could compare the amount of BLT applied with serum levels and patient pain scores. The benefit of such as study would be that perhaps a lower dose of BLT (and therefore, serum lidocaine/MEGX levels) could be used with similar anesthetic effect. Thirdly, if the dose of BLT is reduced could it be reapplied at this lower dose e.g. 5g between each laser pass, thus facilitating multipass laser treatments? An accumulative effect on the rise in serum lidocaine/MEGX levels would be expected and this study would allow titration of serum levels against multipass laser treatments. Fourthly, previous studies by this group demonstrated that preparation of drug has an effect on serum levels of lidocaine/MEGX when applied to the face with occlusion. Much of the literature surrounding laser assisted topical analgesia uses EMLA

as the study drug, most probably because it is one of few FDA approved topical anesthesias (Sobanko et al., 2012). A study looking at the effect on differing drug absorption when used in combination with laser would be useful as there are a number of different topical lidocaine preparations available over the counter as well as by prescription. It may well be that one specific preparation demonstrates better efficacy and safety than the others when used for laser treatments. Certainly from previous studies performed by this group LET topical anesthesia has a low systemic absorption compared to its counterparts (Oni et al., 2012a). This formulation may persist for longer at the dermal level because it includes epinephrine, and therefore, would have a longer analgesic effect. This would need further investigation.

Lastly, the influence of surface area treated in relationship not only to the laser treatment and the amount of drug applied has not been fully examined. In this study the whole face was treated. However, laser treatments for specific cosmetic units, e.g. the periorbital region for wrinkles, would require less amount of topical anesthetic applied to a smaller area, and thus the amount of drug absorbed would be expected to be much less. This would render this method of analgesia less of a toxicity risk.

9.5 Conclusion:

This study is the first step in understanding the relationship between lidocaine containing topical anesthetics and their interaction with laser treatments. It has demonstrated that there are differences in the level of drug absorption depending on the type of laser used. Ablative lasers significantly increase the amount of topical lidocaine absorbed compared to fractionated lasers. It also highlights that patient's ability to metabolize lidocaine is highly individual. It is therefore not surprising that there are instances of toxicity and even death

with seemingly innocuous topical applications of the drug. Topical anesthetics however, can be a good form of anesthesia for laser treatments thus removing the need for nerve blocks, conscious sedation or general anesthetics. Therefore, further work needs to be done to define the safety parameters for this method of analgesia.

CHAPTER 10: Discussion – summary of key findings and future clinical directions

10.1 Summary of key findings

The use of lasers to assist transdermal delivery of drugs has been demonstrated in numerous publications using *in vitro* systems. Little published work however has been done to move this work from the bench into an *in vivo* model. The aim of this thesis was to look at the feasibility of laser assisted drug delivery for clinically translational applications.

The experiments demonstrate several progressive steps:

1. Microchannels can be produced by fractionated laser systems within the skin that can be manipulated in terms of depth and collateral thermal injury. This was corroborated with a preclinical model using the erbium-doped yittrium aluminium garnet (Er:YAG) laser and a human model using the carbon dioxide (CO_2) laser. The laser parameters such as energy, pulse width, number of pulses, spot size, and wavelength all play a critical role in determining the resultant effect on the target. Additionally, skin appendages including glands, hair, and vasculature may alter the efficacy of the laser. Clinically, the anatomic region chosen for the proposed transdermal drug application must also consider these parameters.

2. Laser generated microchannels possess the capability of enhancing drug delivery as demonstrated by topically applied lidocaine being detected systemically in the porcine model used. Although limited to one drug in this thesis, this does however pose an interesting question. The aim of the laser assistance may be to deliver

drugs systemically, but there may be instances in which local drug administration is desirable. For example, in a chronic wound where topical application is insufficient to achieve adequate penetration of the drug just below the wound surface. The benefit of utilizing laser technology is that one can manipulate the depths at which the laser microchannels are generated. For example, if drug delivery is required to target the epidermis/superficial dermis, then the laser can be set to depths of around 200-400µm. Accordingly if the reticular dermis/subcutaneous tissues are the targets then depths of 1000µm+ may be required. It should be noted is that the more superficial the laser depths, the shorter the recovery time in terms of re-epithelialisation. Conversely, the deeper the microchannel, the greater the thermal injury and therefore, the longer the down time.

3. The potential for the delivery of other modalities such as stem cells has not been fully elucidated and further research is warranted. The studies conducted shows deposition of stem cells within microchannels, but migration of those cells was not clearly demonstrated.

Having conducted this investigative work, the question therefore, is one of clinical application and areas in which this technology can be utilized, which is discussed below.

10.2 Feasibility in clinical practice:

There are now a variety of lasers used every day in clinical practice. Many of these are used by aestheticians and nurse practitioners for a number of different applications ranging from hair removal to facials. However, the stronger Er:YAG and CO_2 lasers tend to be

used more by physicians because of their more extensive profile. These types of lasers as has been demonstrated in this thesis can be fractionated and thus cause discrete thermal injury via microchannels into the epidermis and dermis. Both Er:YAG and CO_2 lasers can be used as fractionated devices with the degree of tissue injury being controlled by adjustments in fluence and density. With the Er:YAG laser for instance, injury to a depth of just 25µm (12.5j/cm2) was sufficient to affect the lidocaine absorption profile in the porcine model. At a clinical level a treatment at this fluence may not even require topical anesthetic depending on the pain threshold of the patient. A superficial treatment such as this will not require much in the way of "downtime" for the patient. Much of the clinical work performed in the literature has been in the context of improving the pediatric experience for venipuncture. For example Koh *et al.* in 2007, performed a study in which they used the Er:YAG laser (Epiture Easytouch ™) to pretreat the anticubtal fossa before venepuncture (Koh et al., 2007). This device 'painlessly removes the *stratum corneum*' prior to application of the topical anesthetic. They found, that there was no statistical difference between the lower fluence of 2.0J/cm^2 and the higher fluence of 3.5J/$cm^{2.}$ However, there were more adverse events in the higher fluence group but not significantly so. Singer *et al.* found similar results in 2006. Their study demonstrated in a randomized control trial that prior treatment with the Er:YAG laser at a fluence of 3.5j/cm^2 followed by application of the topical anesthetic, resulted in significantly reduced pain of cannulation both in adults and children (Singer et al., 2006). These studies illustrate that pretreatment with the Er:YAG laser at very low fluences is painless and can assist the action of topical anesthetics for pain control.

10.3 Clinical applications beyond topical anesthesia:

The uses of laser pretreatment could be far reaching clinically. The theory is that because the laser opens up channels within the skin, it will broaden the type of drug (hydrophilic or hydrophobic) or size of molecule that could be delivered into the skin.

i. Chronic wounds:

Chronic wounds pose a particular challenge to healthcare professionals and significant morbidity to patients. Chronic wounds can be difficult to manage and very slow to heal, if they heal at all. Any advances in this area can make huge differences to patient quality of life and the economics involved in the care of these chronic patients. There are three phases to wound healing; the immediate inflammatory phase, followed by the proliferative phase and finally the remodelling phase. Chronic wounds are those which fail to progress through these steps and are trapped in an inflammatory uncoordinated cycle of wound healing, sometimes, in part due to bacterial colonization. There is also down regulation of many cytokines such as TGF-β, VEGF and PDGF. In normal acute wound healing these cytokines are all upregulated and coordinate the recruitment of the necessary cells to facilitate wound healing such as fibroblasts.

Platelet derived growth factor (PDGF) stimulates fibroblasts to produce collagenases for connective tissue remodeling, which has a role in the laying down of the extracellular matrix for wound remodelling. In addition it stimulates other connective tissue matrix components such as glycosaminoglycans and proteoglycans. PDGF is also important in angiogenesis. It is released not only by platelets, but also by macrophages, vascular endothelial cells, fibroblasts, epithelial cells and vascular smooth muscle cells.

Bercaplermin gel is a recombinant platelet derived growth factor commercially available as a gel (Regranex 0.01% gel, Ortho-McNeil Pharmaceutical Inc., Raritan, NJ). It was approved in 1998 to induce soft tissue granulation in the treatment of non-ischaemic diabetic neuropathic ulcers (Senet et al., 2011, Papanas and Maltezos, 2008). However results have not been as impressive as was anticipated. A potential further study could involve the use of a preclinical model to look at the effect of PDGF on wound healing and whether this could have been enhanced with pretreatment with the fractional laser. This could be done with the use of a series of full thickness wounds in one animal such as the pig. Each group of wounds would be subject to a different clinical condition for example; no treatment (control), laser treatment only, PDGF gel only, and combination laser and PDGF gel. In this way it could be ascertained firstly which modalities enhanced wound healing and whether they work synergistically.

One of the chapters within this thesis looked at delivering stem cells to the dermis using the microchannels generated by the fractionated laser. Bone marrow and adipocyte derived stem cells and other mesenchymal stem cells are currently at the fore of many regenerative techniques. There is tremendous interest in the use of stem cells to solve many of the challenges in medicine. To date, there are several published works, which attest to the benefit of intravenous/systemic stem cell therapies on wound healing in animal models (Levi et al., 2011, Yang et al., 2012, Curley et al., 2012, Fu and Li, 2009). However, there are few that explore topical application. Luo *et al.* in 2010 used mesenchymal stem cells isolated from umbilical cords of the mouse to demonstrate increased wound healing when applied topically to a wound (Luo et al., 2010). They suggested that the mechanism for this was through differentiation of the stem cells into keratinocytes. Wu *et al.* in 2007, injected fluorescent labeled bone marrow derived stem

cells around the periphery of splinted wounds and also applied topically to the wound bed in a mouse model (Wu et al., 2007a). They found that wound healing was increased with the presence of stem cells and that this was due to differentiation of the cells into dermal keratinocytes as well as enhancing angiogenesis (although not directly found in the vasculature).

It may well be that combining low level light lasers which have been shown to increase vascularity to the irradiated area (Costa et al., 2010, Melo et al., 2011) in combination with fractionated lasers which can be used to deliver the stem cells can lead to improvement in chronic wounds that have failed to heal using other methods. Further work needs to be done in this area as many of the studies are either in animal models or non-randomized poorly controlled trials of low level evidence.

In addition there is controversy over the use of stem cells in the human. Concerns remain around purity of cells lines, methods of extraction and potential for tumorigenic differentiation (Yu et al., 2008, Locke et al., 2011, Gir et al., 2012).

ii. Skin cancer treatments:

There has already been progress made in this department. Investigational work has been published regarding transdermal drug delivery to treat precancerous and cancerous skin lesions. Photodynamic therapy (PDT) is a form of treatment for precancerous skin lesions such as actinic keratoses (AK) and Bowen's disease. It has also been explored for use in the treatment of non-melanomatous skin cancers. It works by combining a photosensitizer topical chemotherapy agent that can be activated by a light source. Sources of light may include blue light, pulse dye, diode, and intense pulse light among others. This treatment though effective is limited to depths of 2mm (Choudhary et al., 2011). At present there

have been some clinical trials whereby the lesion is first flattened using the Er:YAG laser as an ablative tool (Smucler and Vlk, 2008). However subsequently preclinical studies have shown that the fractional Er:YAG laser can increase the depth of penetration of the photosensitizer, both *in vitro* and with *in vivo* animal models (Shen et al., 2006, Lee et al., 2010, Forster et al., 2010, Haak et al., 2012b, Haedersdal et al., 2011). As yet there have been no human clinical trials, but laser pretreatment may be an effective method of improving the success rate of this treatment for more nodular lesions. Although Togsverd-Bo *et al* .in 2012 have shown that after curettage of actinic keratoses, PDT is more effective when combined with a fractional CO_2 laser (Togsverd-Bo et al., 2012).

iii. Fertility:

The painless laser epidermal system (P.L.E.A.S.E, Pantech Biosolutions, Liechtenstein) has been licensed for use in Europe. This again is a variation of the Er:YAG laser technology and has been reported to be painless to the patient. Yu *et al.* in 2010, using this laser system in a porcine model have demonstrated histologically the presence of what they term ‘micropores’ (Yu et al., 2010). In the same study they showed that this system facilitated the transdermal delivery of prednisone, a corticosteroid, and that the amount of drug delivered increased with increasing fluence. The same group demonstrated the same phenomena with lidocaine (Bachhav et al., 2010) and diclofenac (Bachhav et al., 2011). Zech *et al.* in 2011, utilizing the same laser-microporation system for the delivery of infertility drugs (Zech et al., 2011). In their case report they combined a hormone patch containing follicle stimulating hormone (FSH). FSH is a large molecular weight hydrophilic drug, which ordinarily would not be able to pass through the *stratum corneum*. However, they were able to demonstrate the transdermal passage of this drug and the patient successfully became pregnant.

10.4 Limitations and Future directions:

While laser pretreatment seems to be a promising modality for facilitating transdermal drug delivery, there are some potential drawbacks to its use. The most obvious of which would be the initial cost and the training involved for personnel. Er:YAG and CO_2 lasers can cost upward of £50,000 depending on the make and model. In addition there needs to be a laser safety office in charge of ensuring that the correct maintenance and proper training is delivered. The room in which the laser is used also needs to be adapted to ensure that the laser beam does not reflect off shiny surfaces and all windows are covered. This therefore represents a big investment for the clinic/hospital setting in which the laser will be used.

The purpose of the fractionated laser is to perforate the *stratum corneum*; however this may mean damage to the underlying epidermis. Epidermal disruption is the main contributor to many of the side effects that are found with fractional lasers, ranging from erythema, oedema, blistering, and rarely infections. In addition, some patients have developed pigmentary changes, and thus caution is advised when attempting to treat darker Fitzpatrick IV to VI skins. While many of the adverse effects are reversible, pigmentary changes can sometimes be permanent.

Other issues to bear in mind are the anatomic location and frequency over which the pretreatment can be given. From work done in this thesis and from the research department, it is clear that there are differences between appendages in human facial skin and human abdominal skin (Bailey et al., 2011a, Bailey et al., 2011b). How this affects absorption of drugs has not been elucidated. There may well be variation in the time taken for diffusion and concentration of drug passing transdermally depending on the anatomic

location. It may also be that patients would tolerate laser pretreatment more comfortably on the abdomen more than they would on their upper arm. Further work would have to be done to investigate further the effect of anatomic location.

Another issue to consider is the effect of the cutaneous injury of skin healing from fractional laser treatments. From human studies conducted, superficial fractional laser treatments may take anywhere from 7-21 days for the skin to re-epithelialized. If, for example, this pretreatment is to be used in combination with a topical patch that has to be changed every 72 hours, then it may be necessary for the anatomic locations to be rotated on the patient to ensure that the adverse effects are minimized and the skin has a chance to fully heal in between laser pretreatments.

This thesis has focused on topical lidocaine because it is a commonly used topical anesthetic with a proven safety profile, minimal side effects and available over the counter. There are however, numerous other drugs and treatments which may benefit from delivery in this manner, some of which have already been tested either using *in vitro* models or *in vivo* but only in animal models. Prior published human studies have been related to enhancing the effect of topical anaesthetics, but have not ascertained safety profiles in terms of systemic absorption, only efficacy in terms of patient questionnaires. We have demonstrated both efficacy and systemic absorption in humans pretreated with both fractional and full ablative laser systems. With regards to wound healing models, ethics approval was sought and granted in a porcine model to explore the use of laser to enhance wound healing with or without the addition of stem cells and platelet derived growth factor. This work would be the next logical step to distinguish the effect of lasers on wounds healing.

10.5 Conclusion:

The body of work contained within this thesis demonstrates a journey from the laboratory bench into a clinical forum. Using animal models this thesis demonstrates that fractionated laser through its discrete injury to the *stratum corneum* generates microchannels through which drugs and stem cells could be delivered transdermally. Within the thesis it has also been demonstrated that the same microchannel injury can be replicated in the human skin. The microchannels generated within the skin significantly increased the amount of topically applied lidocaine absorbed, thus proving that these microchannels can be used for transdermal drug delivery. The future uses for this modality remains to be proven, but the studies performed within this thesis, and published in the literature as discussed, suggest that this technology holds promise across a wealth of medical disciplines and therefore merit further investigations.

Appendix 1: IACUC ethics approval for animal study

UT SOUTHWESTERN MEDICAL CENTER

Institutional Animal Care and Use Committee

Date: April 12, 2010

APN	2010-0011
Approval Date	3/26/2010
Expiration Date	3 / 26 / 2011

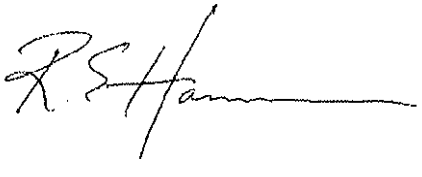
RE: Do Fractional Lasers Assisted Transdermal Delivery of Molecules? Implications for Transdermal Drug Delivery

Dear Dr. Kenkel, Jeffrey:

The Institutional Animal Care and Use Committee (1ACUC) reviewed your animal use protocol 2010-0011 at a convened meeting with a quorum on 2/12/2010. The committee voted to APPROVE the proposed use of Pigs as submitted, effective on the approval date listed above.

Please note, this letter documents approval of animal use only. Any proposed use of hazardous biological material or chemicals with your animals may not commence until you have obtained approval of a safety plan from the Biological and Chemical Safety Advisory Committee and have discussed the implementation of that plan with the ARC manager in your animal use facility. Similarly, the use of radioactive materials or radiation-producing devices with animals must first be authorized by Environmental Health and Safety (EH&S) and discussed with the ARC manager in your animal use facility. Additional training for study personnel and animal care staff may also be required.

Sincerely,



Robert E. Hammer, PhD
Chair, Institutional Animal Care and Use Committee (IACUC)

5323 Harry Hines Blvd. / Dallas, Texas 75390-9107 / 214-645-6423 FAX 214-645-6426

Appendix 2: IACUC ethics approval for animal study

UT SOUTHWESTERN MEDICAL CENTER

Institutional Animal Care and Use Committee

Date: October 6, 2010

APN	2010-0162
Approval Date	9/30/2010
Expiration Date	9/30/2011

RE: Do Fractional Lasers Assisted Transdermal Delivery of Molecules?- Implications for Transdermal Drug Delivery

Dear Dr. Kenkel, Jeffrey:

The Institutional Animal Care and Use Committee (IACUC) reviewed your animal use protocol 2010-0162 at a convened meeting with a quorum on 9/10/2010. The committee voted to APPROVE the proposed use of Pigs as submitted, effective on the approval date listed above.

Please note, this letter documents approval of animal use only. Any proposed use of hazardous biological material or chemicals with your animals may not commence until you have obtained approval of a safety plan from the Biological and Chemical Safety Advisory Committee and have discussed the implementation of that plan with the ARC manager in your animal use facility. Similarly, the use of radioactive materials or radiation-producing devices with animals must first be authorized by Environmental Health and Safety (EH&S) and discussed with the ARC manager in your animal use facility. Additional training for study personnel and animal care staff may also be required.

Sincerely,

Robert E. Hammer, PhD
Chair, Institutional Animal Care and Use Committee (IACUC)

5323 Harry Hines Blvd. / Dallas, Texas 75390-9107 / 214-645-6423 FAX 214-645-6426

Appendix 3: IACUC ethics approval for animal study

UT SOUTHWESTERN MEDICAL CENTER

Institutional Animal Care
and Use Committee

UT Southwestern Medical School
UT Southwestern Graduate School
of Biomedical Sciences
UT Southwestern Allied Health Sciences School

Date: June 24, 2010

APN	2010-0096
Approval Date	6/21/2010
Expiration Date	6/21/2011

RE: Do BrdU Labeled Adipocyte Stem Cells Survive in Vivo When Applied Topically to the Skin Compared to Subdermal Injection Following Laser Pretreatment in the Porcine Model
Departmental

Dear Dr. Kenkle, Jeffrey:

The Institutional Animal Care and Use Committee (IACUC) reviewed your animal use protocol 2010-0096 at a convened meeting with a quorum on 6/11/2010. The committee voted to APPROVE the proposed use of Pigs as submitted, effective on the approval date listed above.

Please note, this letter documents approval of animal use only. Any proposed use of hazardous biological material or chemicals with your animals may not commence until you have obtained approval of a safety plan from the Biological and Chemical Safety Advisory Committee and have discussed the implementation of that plan with the ARC manager in your animal use facility. Similarly, the use of radioactive materials or radiation-producing devices with animals must first be authorized by Environmental Health and Safety (EH&S) and discussed with the ARC manager in your animal use facility. Additional training for study personnel and animal care staff may also be required.

Sincerely,

Robert L. Hammer, PhD
Chair, Institutional Animal Care and Use Committee (I

NOTE: It is your responsibility to schedule a meeting with Julie Muszynski in the Animal Resources Center and Patrick Thobe in Environmental Health and Safety at least 30 days prior to beginning work with hazardous materials in the animal facility.

5323 Harry Hines Blvd. / Dallas, Texas 75390-9107 / 214-645-6423 FAX 214-645-6426
www.utsouthwestern.edu

Appendix 4: IRB ethics approval

THE UNIVERSITY OF TEXAS
SOUTHWESTERN MEDICAL CENTER
AT DALLAS

Institutional Review Board

October 1, 2009

To: Jeffrey Kenkel, MD
c/o Angela Smith
Plastic Surgery - 9132

From: George Buchanan, MD
Institutional Review Board 2 Chairperson
IRB - 8843

DATE: October 1, 2009

RE: **Final Approval of the Protocol/ Project Summary, NR1, Consent Form, Spanish Short Consent Form, HIPAA Authorization, Spanish HIPAA Authorization, and HIPAA Waiver**
IRB Number: 082009-039
Title: A Histological comparison of ablative laser treatment in Human Facial and Abdominal Skin.

Having met the conditions as set forth by the IRB at the September 14, 2009 meeting, your research protocol is now approved for a period of 12 months. This approval period will begin October 1, 2009 and last until September 13, 2010. If the research continues beyond twelve months, you must apply for updated approval of the protocol and informed consent document(s) one month before the date of expiration noted above.

Please Carefully Read Important Compliance Information Below:

Your approved number of evaluable subjects is 15. If during the course of your study you feel that you need to change this number, you must submit a completed MOD Form applying for prospective approval to do so.

All subjects must sign a copy of the attached IRB-approved and stamped consent form(s) and HIPAA Authorization, if applicable, before undergoing any study procedures, including screening procedures that would not otherwise be performed for a patient/subject's medical condition in a non-research context.

The above referenced study is not approved to enroll non-English speaking subjects until the principal investigator receives written acknowledgment from the IRB of the proposed translated consent form and HIPAA Authorization. DHHS regulations permit oral presentation of informed consent information in conjunction with a short form written consent document (stating that the elements of consent have been presented orally) and a written summary of what is presented orally. A witness to the oral presentation is required, and the subject must be given copies of the short form document and the summary.

When this procedure is used with subjects who do not speak or read English, (1) the oral

Southwestern Medical School 5323 Harry Hines Boulevard / Dallas, Texas 75390-8843 / (214)648-3060

presentation and the short form written document should be in a language understandable to the subject; (2) the IRB-approved English language informed consent document may serve as the summary; and (3) the witness should be fluent in both English and the language of the subject.

At the time of consent, (1) the short form document should be signed by the subject (or the subject's legally authorized representative); (2) the summary (i.e., the English language informed consent document) should be signed by the person obtaining consent as authorized under the protocol; and (3) the short form document and the summary should be signed by the witness. When the person obtaining consent is assisted by a translator, the translator may serve as the witness.

For research involving therapeutic or prophylactic interventions or invasive diagnostic procedures, a bilingual translator must be continuously available to facilitate communications between research personnel and a subject. If a bilingual translator will not always be available, it may be unsafe for an otherwise eligible candidate to participate in the research if that person does not speak and read English.

Important Note: You must use a photocopy of the attached IRB-stamped consent form(s). Use of a copy of any consent form on which the IRB- stamped approval and expiration dates are replaced by typescript or handwriting is prohibited.

A photocopy of the signed consent form(s) and HIPAA Authorization should be given to each participant. The copy of the consent form(s) bearing original signatures should be kept with other records of this research for at least five years past the completion of the study. For research involving treatment or invasive procedures, a photocopy of the signed consent form(s) should be on file in a subject's medical record.

The Department of Health and Human Services (DHHS) regulations for the protection of human subjects require that informed consent information be presented in a language understandable to the subject(s), and, in most situations, that informed consent be documented in writing.

Where informed consent is documented, the written consent document(s) should embody, in language understandable to the subject, all of the elements necessary for legally effective informed consent. Potential subjects who do not speak or read English should be presented with a consent document written in a language understandable to them. The Office for Human Research Protections (OHRP) strongly encourages the use of this procedure whenever possible.

In the future, should you require a change or need to modify the research, including the informed consent document(s) and HIPAA Authorization, per federal regulation you must obtain prospective review and approval of the Institutional Review Board. For any change to the research, prior review and approval before implementing such changes is mandatory except when prompt implementation is necessary to eliminate apparent immediate hazard to a subject.

Approval by the appropriate authority at a collaborating facility or performance site is required before subjects may be enrolled on this study.

If you have any questions related to this approval or IRB policies and procedures, you may telephone Vicki McNamara at 214-648-8430.

Attachment(s):NR1
Project Summary
Consent Form
Spanish Short Form
HIPAA Authorization Form
Spanish HIPAA Authorization Form
HIPAA Waiver

GB/vm

Appendix 5: Patient consent form

The University of Texas Southwestern Medical Center at Dallas

CONSENT TO PARTICIPATE IN RESEARCH

Title of Research: A Histological comparison of ablative laser treatment in Human Facial and Abdominal Skin.

Funding Agency/Sponsor: UT Southwestern Department of Plastic Surgery

Study Doctors:
Dr. Jeffrey Kenkel
Dr. Steven Bailey
Dr. Spencer Brown
Dr. Rod Rohrich

Research Personnel:
Angela Smith
Heather Caine
Debby Noble
John Hoopman, CMLSO

You may call these study doctors or research personnel during regular office hours and at other times at 214-645-3112.

Instructions:
Please read this consent form carefully and take your time making a decision about whether to participate. As the researchers discuss this consent form with you, please ask him/her to explain any words or information that you do not clearly understand. The purpose of the study, risks, inconveniences, discomforts, and other important information about the study are listed below. If you decide to participate, you will be given a copy of this form to keep.

Why is this study being done?

This study is being done to compare laser injury of facial and abdominal tissue. The Investigators would like to compare the changes using tissue samples from the face and the abdomen after laser treatment. The Investigators hope that this will make laser treatments safer for all patients.

- Physical exam and medical history;
- Photographs of your face and abdomen; and
- Demographic information (age, sex, ethnic origin)
- Urine Pregnancy Test.

Procedures and Evaluations during the Research

Visit 1 (- Up to 2 hours -May be combined with Screening):

- Face and Abdomen will be photographed.
- Dermal Scanner Measurements on your face and abdomen, a hand piece will be placed on your face and abdomen using ultrasound to measure thickness of your skin. This measurement procedure is painless.
- BTC2000 measurements on your face and abdomen, a hand piece will be placed on your face and abdomen using a light suction that will measure how elastic your skin is. This measurement procedure is painless.
- Sebumeter measurements on your face and abdomen, a hand piece will be placed on your face and abdomen that measures oil production and pH [acidity] concentration of your skin. This measurement procedure is painless.
- Chromometer measurements on your face and abdomen, a hand piece will be placed on your face and abdomen that measures color. This measurement procedure is painless.
- Laser Treatment of your face and abdomen will be approximately 1cm X 1cm. The location of the treatment area will be located either on the forehead, cheek, nasolabial fold, upper and lower lip, nasal tip and chin. You will be numbed with numbing cream prior to treatment. During treatment you will feel slight "prickly" sensation similar to a snap of a rubberband.

3mm punch biopsy from the laser treated area on your face and abdomen (a total of 2 biopsies), a small amount of tissue will be removed for analysis. Prior to the procedure you will receive a Lidocaine injection at the site. Lidocaine is used to numb the area. During this injection you will feel the prick of the needle and a slight sting of the lidocaine. The biopsy will be taken from the selected sites soon after laser treatment.

Visit 2 (Follow-up – Up to 15 minutes):

- You will come into the clinic to have your biopsy site assessed to ensure that it is healing properly.

The skin measurements in this study are designed for research, not for medical purposes. These devices will be used to compare facial and abdominal skin in order to assess differences after laser treatment. They are not useful for finding problems or diseases. Even though the researchers are not looking at your skin measurements to find or treat a medical problem, you will be told if they notice something unusual. You and your regular doctor can decide together whether to follow up with more tests or

treatment. Because the skin measurements done in this study are not for medical purposes, the research results will not be sent to you or to your regular doctor. The researchers will record and use your Social Security Number (SSN) in order to process your payment. You do not have to give this information to the researchers; however, it may result in not being paid for participating in this study. This information will remain confidential unless you give your permission to share it with others or if we are required by law to release it.

How long can I expect to be in this study?
You can expect to be in this study for up to 2 weeks, or until after your follow-up visit.

You can choose to stop participating for any reason at any time. However, if you decide to stop participating in the study, we encourage you to tell the researchers. You may be asked if you are willing to complete some study termination tests.

What are the risks of the study?
Loss of Confidentiality
Any time information is collected; there is a potential risk of loss of confidentiality. Every effort will be made to keep your information confidential; however, this cannot be guaranteed.

Laser Treatment

Less than 2% of patients who have received laser treatment have experienced the following complications: Blistering, Scarring, Hypo-pigmentation or Hyper-pigmentation. Some redness and slight swelling is common and may be present in the area that has been treated for up to 48 hours. For some patient's recovery time will be less while other patients will have prolonged redness. Most patients describe laser treatment as feeling "prickly," or like the light snap of a rubber band against the skin. Afterwards, it might feel like mild sunburn. Each patient has his or her own level of skin sensitivity and pain threshold; different areas on the body are also more sensitive than others. There are no potential risks of interaction between the laser and medications that you may be taking. In the unusual circumstance where a blister occurs following treatment, you will be treated medically with a topical antibiotic until the blister has successfully epithelialized (new skin has formed - typically occurs in 24-48 hours). If a scar occurs, it will be treated with pressure, massage, and observation. On rare occasion, steroid ointment or injections may be required.

Risks associated with injectable anesthetic:
Lidocaine may cause nausea, drowsiness, mental/mood changes, ringing in the ears, dizziness, vision changes, tremors, numbness, headache or backache. Other serious but unlikely side effects include fever, fast or slow pulse, trouble breathing, seizures, or chest pain.

Risks associated with the biopsy:
Risks associated with the biopsy include infection, bleeding, bruising at the biopsy site, pain from the numbing injection and appearance of a small scar at the biopsy site. The risk of infection will be minimized by proper wound care.

Risks associated with triple anesthetic cream:
The risks associated with the use of Triple Anesthetic cream are temporary swelling, itching and formation of a rash.

Risks associated with the photographs:
The risks associated with photographs may be loss of confidentiality. Individually identifying information will be removed from photographs.

Risks associated with devices:
There are no risks identified with the use of the Dermal Scanner Ultrasound, BTC 2000, Sebumeter or chromometer measurements.

Other Risks
There may possibly be other side effects that are unknown at this time. If you are concerned about other, unknown side effects, please discuss this with the researchers.

Psychological Stress
Some of the questions we will ask you as part of this study may make you feel uncomfortable. You may refuse to answer any of the questions, take a break or stop your participation in this study at any time.

There are no known risks to the fetus associated with the light from the Short Pulse Fractional CO2 system.

How will risks be minimized or prevented?
In order to minimize the risk of eye injury in this study, you must wear protective goggles at all times during the treatments. If you have any serious pain, discomfort or adverse effects during or after the treatment, treatment may be discontinued or a different approach may be taken. You should follow any instructions given to you by the study doctor, such as wearing sunscreen and proper care and cleansing of the treated areas. You should avoid sun exposure to the treated area for up to 30 days to avoid permanent skin color changes. Also, you should avoid over-the-counter drugs, like aspirin, or prescription drugs like steroids and/or Accutane.

An independent reviewer will also monitor the study data and safety of the study to ensure accuracy. Data will be monitored by a co-research coordinator approximately every 6 to 8 weeks.

What will my responsibilities be during the study?

IRB File Number: 082009-039
Approval Period Begins: OCT 01 2009
Approval Period Ends: SEP 13 2010

While you are part of this study, the researchers will follow you closely to determine whether there are problems that need medical care. It is your responsibility to do the following:

- Ask questions about anything you do not understand.
- Keep your appointments.
- Follow the researchers' instructions.
- Let the researchers know if your telephone number or address changes.
- Tell the researchers before you take any new medication, even if it is prescribed by another doctor for a different medical problem or something purchased over the counter.
- Tell your regular doctor about your participation in this study.
- Report to the researchers any injury or illnesses while you are on study even if you do not think it is related.

If I agree to take part in this research study, will I be told of any new risks that may be found during the course of the study?
Yes. You will be told if any new information becomes available during the study that could cause you to change your mind about continuing to participate or that is important to your health or safety.

What should I do if I think I am having problems?
If you have unusual symptoms, pain, or any other problems while you are in the study, you should report them to the researchers right away. Telephone numbers where they can be reached are listed on the first page of this consent form.

If you have a sudden, serious problem, like difficulty breathing or severe pain, go to the nearest hospital emergency room, or call 911 (or the correct emergency telephone number in your area). Tell emergency personnel about any medications you are taking, including any medications you are taking for this study.

What are the possible benefits of this study?
If you agree to take part in this study, there will not be direct benefits to you. The researchers cannot guarantee that you will benefit from participation in this research.

We hope the information learned from this study will benefit others who would like laser treatment in the future. Information gained from this research could lead to better care.

What options are available if I decide not to take part in this research study?
This is not a treatment study.

Will I be paid if I take part in this research study?
You will receive compensation of $50 for your participation in this study.

IRB File Number: 082009-039
Approval Period Begins: OCT 01 2009
Approval Period Ends: SEP 13 2010

There are no funds available to pay for parking expenses, transportation to and from the research center, lost time away from work and other activities, lost wages, or child care expenses.

Your Social Security Number (SSN) will be given to The University of Texas Southwestern Medical Center in order to process your payment as required by law. This information will remain confidential unless you give your permission to share it with others, or if we are required by law to release it.

If you are an employee of UT Southwestern, your payment will be added to your regular paycheck and income tax will be deducted.

UT Southwestern, as a State agency, will not be able to make any payments to you for your participation in this research if the State Comptroller has issued a "hold" on all State payments to you. Such a "hold" could result from your failure to make child support payments or pay student loans, etc. If this happens, UT Southwestern will be able to pay you for your taking part in this research 1) after you have made the outstanding payments and 2) the State Comptroller has issued a release of the "hold."

Will my insurance provider or I be charged for the costs of any part of this research study?
No. Neither you, nor your insurance provider, will be charged for anything done only for this research study (i.e., the Screening Procedures, Experimental Procedures, or Monitoring/Follow-up Procedures described above).

What will happen if I am harmed as a result of taking part in this study?
It is important that you report any illness or injury to the research team listed at the top of this form immediately.

Compensation for an injury resulting from your participation in this research is not available from the University of Texas Southwestern Medical Center at Dallas.

You retain your legal rights during your participation in this research

Can I stop taking part in this research study?
Yes. If you decide to participate and later change your mind, you are free to stop taking part in the research study at any time.

If you decide to stop taking part in this research study, it will not affect your relationship with the UT Southwestern staff or doctors. Whether you participate or not will have no effect on your legal rights or the quality of your health care.

If you are a medical student, fellow, faculty, or staff at the Medical Center, your status will not be affected in any way.

Your doctor is a research investigator in this study. He is interested in both your medical care and the conduct of this research study. At any time, you may discuss your care with another doctor who is not part of this research study. You do not have to take part in any research study offered by your doctor.

If I agree to take part in this research study, can I be removed from the study without my consent?

Yes. The researchers may decide to take you off this study if:

- The researchers believe that participation in the research is no longer safe for you.
- The researchers believe that other treatment may be more helpful.
- The sponsor cancels the research.
- You are unable to keep appointments or to follow the researcher's instructions.

Will my information be kept confidential?

Information about you that is collected for this research study will remain confidential unless you give your permission to share it with others, or if we are required by law to release it. You should know that certain organizations that may look at and/or copy your medical records for research, quality assurance, and data analysis include:

- Representatives of the Food and Drug Administration (FDA). The FDA may oversee the Research Project to confirm compliance with laws and regulations. The FDA may photocopy your health information to verify information ; and
- The UT Southwestern Institutional Review Board.

In addition to this consent form, you will be asked to sign an "Authorization for Use and Disclosure of Protected Health Information." This authorization will give more details about how your information will be used for this research study, and who may see and/or get copies of your information.

Your photographs will be presented in a manner that does not reveal your identity (your eyes will be covered).

Are there procedures I should follow after stopping participation in this research?

Yes. If you, the researchers, or the sponsor stops your participation in the research, you may be asked to do the following:

- Let the researchers know immediately that you wish to withdraw from the research.
- Return to the research center for tests that may be needed for your safety.

Whom do I call if I have questions or problems?

IRB File Number: 082009-039
Approval Period Begins: OCT 01 2009
Approval Period Ends: SEP 13 2010

For questions about the study, contact Dr. Jeffrey Kenkel at 214-645-3112 during regular business hours and after hours and on weekends and holidays.

For questions about your rights as a research participant, contact the UT Southwestern Institutional Review Board (IRB) Office at 214-648-3060.

I understand that if I refuse photographs or biopsies I will not be able to participate in the study because they are a vital endpoint of the researcher's data.

Photograph Release:
I voluntarily agree to the taking and use of photographs of the evaluated area by Dr. Kenkel for UT Southwestern, Department of Plastic Surgery, which will be the owner of the copyright. Any photographs taken during the study will become the property of UT Southwestern, Department of Plastic Surgery. The photographs may be used as part of a scientific publication.

The photographs will be presented in a manner that does not reveal my identity (my eyes will be covered).

Photographs will be taken at Visit 1.

In addition to the above uses, I agree to the following uses of photographs (as indicated by writing my initials below):

_____For Research Purposes

_____For Educational Purposes

_____For Informational Purposes

I hereby release Dr. Kenkel and UT Southwestern, Department of Plastic Surgery from any claim or cause of action arising out of the publication or distribution of the photographs when used for the purposes indicated above.

SIGNATURES:

YOU WILL BE GIVEN A COPY OF THIS CONSENT FORM TO KEEP.

Your signature below certifies the following:

IRB File Number: 082009-039
Approval Period Begins: OCT 01 2009
Approval Period Ends: SEP 13 2010

- You have read (or been read) the information provided above.
- You have received answers to all of your questions and have been told who to call if you have any more questions.
- You have freely decided to participate in this research.
- You understand that you are not giving up any of your legal rights.

_____

Participant's Name (printed)

_____ _____

Participant's Signature Date

_____

Name of person obtaining consent (printed)

_____ _____

Signature of person obtaining consent Date

If applicable:

INTERPRETER STATEMENT:

I have interpreted this consent form into a language understandable to the participant and the participant has agreed to participate as indicated by their signature on the associated short form.

_____

Name of Interpreter (printed)

_____ _____

Signature of Interpreter Date

IRB File Number: 082009-039
Approval Period Begins: OCT 01 2009
Approval Period Ends: SEP 13 2010

- You have read (or been read) the information provided above.
- You have received answers to all of your questions and have been told who to call if you have any more questions.
- You have freely decided to participate in this research.
- You understand that you are not giving up any of your legal rights.

_____

Participant's Name (printed)

_____ _____

Participant's Signature Date

_____

Name of person obtaining consent (printed)

_____ _____

Signature of person obtaining consent Date

If applicable:

INTERPRETER STATEMENT:

I have interpreted this consent form into a language understandable to the participant and the participant has agreed to participate as indicated by their signature on the associated short form.

_____

Name of Interpreter (printed)

_____ _____

Signature of Interpreter Date

IRB File Number: 082009-039
Approval Period Begins: OCT 01 2009
Approval Period Ends: SEP 13 2010

Appendix 6: IRB ethics approval

UT SOUTHWESTERN MEDICAL CENTER eIRB System

From: George Buchanan

Institutional Review Board Chairperson
IRB - 8843

To: Jeffrey Kenkel , Deborah Noble , Andrea Selmon

Date: March 3, 2011

Re: Study Approval

IRB Number: STU 122010-083

Title: Serum levels of lidocaine and MEGX: A randomized comparison of five commonly available topical anesthetics after application to the face.

Documents: Protocol, Consent Form, HIPAA Authorization Form and Other Study-Related Documents

The UT Southwestern Institutional Review Board (IRB) reviewed the above-referenced research study at a convened meeting of the full board on February 28, 2011. Having met all applicable requirements, the research study is approved. The approval period for this research study begins on February 28, 2011 and lasts until January 23, 2012.

The research study cannot continue beyond the approval period without continuing review and approval by the IRB. In order to avoid a lapse in IRB approval, the Principal Investigator must apply for continuing review of the protocol and related documents before the expiration date. A reminder will be sent to you approximately 90 days prior to expiration of study approval.

The approved number of subjects to be enrolled is 30. The IRB considers a subject to be enrolled once s/he signs a Consent Form. If, additional subjects are needed, you first must obtain permission from the IRB to increase the sample size.

If you have any questions related to this approval letter or about IRB policies and procedures, please telephone the IRB office at 214-648-3060.

Appendix 7: Patient Consent form

The University of Texas Southwestern Medical Center at Dallas Parkland Health & Hospital System Children's Medical Center Texas Scottish Rite Hospital for Children Presbyterian Hospital of Dallas

CONSENT TO PARTICIPATE IN RESEARCH

Title of Research: Serum levels of lidocaine and MEGX: A randomized comparison of five commonly available topical anesthetics after application to the face.

Funding Agency/Sponsor: Aesthetic Surgery Education and Research Foundation (ASERF) grant

Study Doctors: Jeffrey Kenkel, MD Georgette Oni, MD Jayne Coleman, MD

Research Personnel: Spencer Brown, Ph.D Debby Noble Angela Smith

You may call these study doctors or research personnel at 214-645-3112.

Instructions:
Please read this consent form carefully and take your time making a decision about whether to participate. As the researchers discuss this consent form with you, please ask him/her to explain any words or information that you do not clearly understand. The purpose of the study, risks, inconveniences, discomforts, and other important information about the study are listed below. If you decide to participate, you will be given a copy of this form to keep.

Why is this study being done?
This study is being done to look at the effects of dosage, absorption time, and occlusion (covering) for five formulations of the over the counter topical anesthetic lidocaine. We want to measure the amount of lidocaine in your bloodstream over time and use this information to develop dosing instructions for physicians.

Why is this considered research?
This is a research study because there is a lack of information about the proper clinical dose of these anesthetic creams and we believe this could present an unseen risk to patients.

The following definitions may help you understand this study:

☐ Randomization means you will be placed by chance (like drawing straws) in one of the study groups ☐

from your personal doctor if you choose not to participate in this research. ☐

Researchers means the study doctor and research personnel at the University of Texas Southwestern Medical Center at Dallas and its affiliated hospitals.

Why am I being asked to take part in this research study?

You are being asked to take part in this study because you are a healthy adult.

Do I have to take part in this research study?"

No. You have the right to choose whether you want to take part in this research study. If you decide to participate and later change your mind, you are free to stop participation at any time.

If you decide not to take part in this research study it will not change your legal rights or the quality of health care that you receive at this center.

How many people will take part in this study?

About 30 people will take part in this study at UT Southwestern.

What is involved in the study?

If you volunteer to take part in this research study, you will be asked to sign this consent form and will have the following tests and procedures. Screening Visit To help decide if you qualify to be in this study, the researchers may ask you questions about your health, including medications you take and any surgical procedures you have had.

☐ Informed Consent (reading and signing this document)

☐ Complete all screening procedures: medical history, vital signs (temperature, pulse, respirations, blood pressure), height, weight, physical exam, current medications, allergies

☐ Urine pregnancy test for females of childbearing potential

☐ The surface area of your face will be measured prior to the experiment, utilizing 3D photography which is painless. Your confidentiality in the facial photographs will be protected to the greatest extent possible by masking of the eyes or use of pixilation (digital 'fuzzing'), as appropriate

☐ Allergy patch tests for each of the five topical lidocaine preparations will be performed at this visit.

This visit will last about 30 minutes. Group Assignment If the researchers believe you can take part in this study, you will be assigned randomly (like drawing straws) to receive one of the five creams containing lidocaine that will be applied during the study. You have a 1 in 5 chance of receiving one of the five creams described below.

The group you will be in is decided by a computer program. Neither you nor the researchers will be allowed to choose which group you are assigned to.

Study Medication/Intervention Twenty-five (25) subjects will be randomly assigned into one of the experimental groups by the research coordinator.

☐ Group 1: LMX-4 (4% lidocaine, Ferndale laboratories), will be applied to the face and neck for 1 hour. ☐
AstraZeneca), will be applied to the face and neck for 1 hour. ☐ Gr
Topicaine (4% lidocaine, ESBA Laboratories), will be applied to the face and neck for 1 hour. ☐ Grou
epinephrine), will be applied to the face and neck for 1 hour. ☐
(6% lidocaine, 20% Benzocaine, 4% tetracaine), will be applied to the face and neck for 1 hour.

Procedures and Evaluations during the Research

Study Visit (up to 4 weeks after Screening)
a thin layer over your face and neck. [
over your face and neck.

☐ Blood serum samples (approximately 1 tablespoon each time) will be taken from a vein in your arm at 90 minutes (30 minutes after the topical anesthesia is removed), 2 hours, 2 and ½ hours, 4 hours and 8 hours after application. We will insert an intravenous line in your arm that will be left in place for the course of the study.

A total of 5 tablespoons of blood will be taken.

This visit will last about 10 hours.

The blood tests in this study are designed for research, not for medical purposes. They are not useful for finding problems or diseases. Because the blood tests done in this study are not for medical purposes, the research results will not be sent to you or to your regular doctor.

Procedures for storing of extra or left over samples
Your blood samples will be sent to The Department of Clinical Chemistry, George-August University (Goettingen, Germany) to be analyzed. Samples will be labeled with a study identification number with no personal identifying information. Your blood samples will be destroyed after testing is completed.

How long can I expect to be in this study?
You will make two (2) visits over the course of 4 weeks. Your participation in this research will end when you complete your study visit.

You can choose to stop participating for any reason at any time. However, if you decide to stop participating in the study, we encourage you to tell the researchers. You may be asked if you are willing to complete some study termination tests.

What are the risks of the study?

Study Procedure/Intervention Because of your participation in this study, you are at risk for the following side effects. You should discuss these with the researchers and your regular health care provider.

There is a minimal risk of lidocaine toxicity (poisoning) due to the application of topical lidocaine.

Common minor side-effects (up to 5%) include localized skin reactions (redness, itching, rash), anxiety, agitation, psychosis (seeing/hearing things that aren't there), tinnitus (ringing in the ears), tingling around the mouth, metallic taste, dysgeusia (altered sense of taste), light-headedness, nausea, diplopia (double vision), nystagmus (involuntary eye movements), slurred speech, hallucinations, and muscle tremors.

Rare (less than 1%), but serious side effects include rare instances of seizures, palpitations (rapid heartbeats), arrhythmia (irregular heartbeats), cardiovascular collapse, methemoglobinemia (a disorder of the red blood cells), hypotension (low blood pressure) and in some rare instances death.

Loss of Confidentiality Any time information is collected; there is a potential risk for loss of confidentiality. Every effort will be made to keep your information confidential; however, this cannot be guaranteed.

Risks to Embryo, Fetus or Breast-fed Infant **Females:** If you are part of this study while pregnant or breast-feeding an infant, it is possible that you may expose the unborn child or infant to risks. For that reason, pregnant and breast-feeding females cannot participate in the study. If you can become pregnant, a blood pregnancy test will be done (using 1 teaspoon of blood drawn from a vein by needle-stick), and it must be negative before you participate in this study. If you take part in this study and you are sexually active, you and any person that you have sex with must use medically-acceptable birth control (contraceptives) during the study. Medically-acceptable birth control (contraceptives) includes:

(1) surgical sterilization (such as hysterectomy or "tubes tied"),

(2) approved hormonal contraceptives (such as birth control pills, patch or ring; Depo-Provera, Depo-Lupron, Implanon),

(3) barrier methods (such as condom or diaphragm) used with a spermicide (a substance that kills sperm), or

(4) an intrauterine device (IUD). If you do become pregnant during this study, you must tell the researchers immediately.

Intravenous Catheter (for Blood Draws) Risks associated with insertion of the catheter and drawing blood from your arm include minimal discomfort and/or bruising. Infection, excess bleeding, clotting, and/or fainting also are possible, although unlikely.

You will have five (5) tablespoons of blood collected because you are in this research study.

Other Risks There may possibly be other side effects that are unknown at this time. If you are concerned about other, unknown side effects, please discuss this with the researchers.

How will risks be minimized or prevented?

You will be carefully screened to be sure that participation in this research is safe for you. An anesthesiologist (Dr. Jayne Coleman) will be present for the course of the study to monitor and treat any adverse reactions that could occur during the study.

Dr. Kenkel, Dr. Brown and Dr. Oni will review the study and monitor for your safety.

What will my responsibilities be during the study?

While you are part of this study, the researchers will follow you closely to determine whether there are problems that need medical care. It is your responsibility to do the following:

☐ Ask questions about anything you do not understand.
☐ Keep your appointments.

- ☐ Follow the researchers' instructions.
- ☐ Let the researchers know if your telephone number or address changes.
- ☐ Tell the researchers before you take any new medication, even if it is prescribed by another doctor for a different medical problem or something purchased over the counter.
- ☐ Tell your regular doctor about your participation in this study.
- ☐ Report to the researchers any injury or illnesses while you are on study even if you do not think it is related.

If I agree to take part in this research study, will I be told of any new risks that may be found during the course of the study?
Yes. You will be told if any new information becomes available during the study that could cause you to change your mind about continuing to participate or that is important to your health or safety.

What should I do if I think I am having problems?
If you have unusual symptoms, pain, or any other problems while you are in the study, you should report them to the researchers right away. Telephone numbers where they can be reached are listed on the first page of this consent form.

If you have a sudden, serious problem, like difficulty breathing or severe pain, go to the nearest hospital emergency room, or call 911 (or the correct emergency telephone number in your area). Tell emergency personnel about any medications you are taking, including any medications you are taking for this study.

What are the possible benefits of this study?
If you agree to take part in this study, there will not be direct benefits to you.

We hope the information learned from this study will provide useful safety information for the clinical usage of topical lidocaine for patients in the future.

What options are available if I decide not to take part in this research study?
This is not a treatment study. You do not have to be part of it to get treatment for your condition.

Will I be paid if I take part in this research study?
Yes.

You will be paid $130.00 at the end of the study. If you stop taking part in this study or are withdrawn by the research team, you will receive payment for only the visits you have completed. For example, if you complete the screening visit, you will be paid $30.00.

There are no funds available to pay for parking expenses, transportation to and from the research center, lost time away from work and other activities, lost wages, or child care expenses.

Your Social Security Number (SSN) will be given to The University of Texas Southwestern Medical Center in order to process your payment as required by law. This information will remain confidential unless you give your permission to share it with others, or if we are required by law to release it.

If you are an employee of UT Southwestern, your payment will be added to your regular paycheck and income tax will be deducted.

UT Southwestern, as a State agency, will not be able to make any payments to you for your participation in this research if the State Comptroller has issued a "hold" on all State payments to you. Such a "hold" could result from your failure to make child support payments or pay student loans, etc. If this happens, UT Southwestern will be able to pay you for your taking part in this research 1) after you have made the outstanding payments and 2) the State Comptroller has issued a release of the "hold."

Will my insurance provider or I be charged for the costs of any part of this research study?
No. Neither you, nor your insurance provider, will be charged for anything done only for this research study (i.e., the Screening Procedures, Study Procedures described above).

What will happen if I am harmed as a result of taking part in this study?
It is important that you report any illness or injury to the research team listed at the top of this form immediately.

Compensation for an injury resulting from your participation in this research is not available from the University of Texas Southwestern Medical Center at Dallas.

You retain your legal rights during your participation in this research

Can I stop taking part in this research study?
Yes. If you decide to participate and later change your mind, you are free to stop taking part in the research study at any time.

If you decide to stop taking part in this research study, it will not affect your relationship with the UT Southwestern staff or doctors. Whether you participate or not will have no effect on your legal rights or the quality of your health care.

If you are a medical student, fellow, faculty, or staff at the Medical Center, your status will not be affected in any way.

Your doctor is a research investigator in this study. S/he is interested in both your medical care and the conduct of this research study. At any time, you may discuss your care with another doctor who is not part of this research study. You do not have to take part in any research study offered by your doctor.

If I agree to take part in this research study, can I be removed from the study without my consent?

Yes. The researchers may decide to take you off this study if:

- ☐ You withdraw your consent
- ☐ You are unable to stay for the full 10 hours on the treatment day
- ☐ The researchers believe that participation in the research is no longer safe for you.
- ☐ The sponsor or the FDA stops the research for the safety of the participants.
- ☐ The sponsor cancels the research.
- ☐ You are unable to keep appointments or to follow the researcher's instructions.

Will my information be kept confidential?

Information about you that is collected for this research study will remain confidential unless you give your permission to share it with others, or if we are required by law to release it. You should know that certain organizations that may look at and/or copy your medical records for research, quality assurance, and data analysis include:

- ☐Representatives of the Food and Drug Administration (FDA). The FDA may oversee the Research Project to confirm compliance with laws and regulations. The FDA may photocopy your health information to verify information submitted to the FDA by the Sponsor.
- Representatives of government agencies, like the U.S. Food and Drug Administration (FDA), involved in keeping research safe for people; and
- The UT Southwestern Institutional Review Board.

In addition to this consent form, you will be asked to sign an "Authorization for Use and Disclosure of Protected Health Information." This authorization will give more details about how your information will be used for this research study, and who may see and/or get copies of your information.

Are there procedures I should follow after stopping participation in this research?

Yes. If you, the researchers, or the sponsor stops your participation in the research, you may be asked to do the following:

☐ Discuss your future medical care, if any, with the researchers and/or your personal doctor.

Whom do I call if I have questions or problems?
For questions about the study, contact Dr. Jeffrey Kenkel at 214-645- 3112 during regular business hours and after hours and on weekends and holidays.

For questions about your rights as a research participant, contact the UT Southwestern Institutional Review Board (IRB) Office at 214-648-3060.

SIGNATURES:

YOU WILL BE GIVEN A COPY OF THIS CONSENT FORM TO KEEP.

Your signature below certifies the following:

You have read (or been read) the information provided above.
You have received answers to all of your questions and have been told who to call if you have any more questions.
You have freely decided to participate in this research.
You understand that you are not giving up any of your legal rights.

Participant's Name (printed)

Participant's Signature Date

Name of person obtaining consent (printed)

Signature of person obtaining consent Date

☐ Let the researchers know immediately that you wish to withdraw from the research.

Appendix 8: IRB ethics approval

UT SOUTHWESTERN MEDICAL CENTER eIRB System

From: George Buchanan
Scott Roberts

Institutional Review Board Chairperson
IRB - 8843

To: Jeffrey Kenkel , Deborah Noble , Deborah Noble

Date: June 10, 2011

Re: Study Approval

IRB Number: STU 032011-058

Title: Is pre treatment with fractional laser on serum levels of lidocaine and MEGX following topically applied 4% lidocaine a safe and effective method of analgesia for facial rejuvenation.

Documents: Protocol, Consent Form, HIPAA Authorization Form and Other Study-Related Documents

The UT Southwestern Institutional Review Board (IRB) reviewed the above-referenced research study at a convened meeting of the full board on March 28, 2011. Having met all applicable requirements, the research study is approved. The approval period for this research study begins on June 10, 2011 and lasts until March 26, 2012.

The research study cannot continue beyond the approval period without continuing review and approval by the IRB. In order to avoid a lapse in IRB approval, the Principal Investigator must apply for continuing review of the protocol and related documents before the expiration date. A reminder will be sent to you approximately 90 days prior to expiration of study approval.

The approved number of subjects to be enrolled is 15. The IRB considers a subject to be enrolled once s/he signs a Consent Form. If, additional subjects are needed, you first must obtain permission from the IRB to increase the sample size.

If you have any questions related to this approval letter or about IRB policies and procedures, please telephone the IRB office at 214-648-3060.

Appendix 9: Patient consent form

The University of Texas Southwestern Medical Center at Dallas

Children's Medical Center

CONSENT TO PARTICIPATE IN RESEARCH

Title of Research: **Is pre treatment with fractional laser on serum levels of lidocaine and MEGX following topically applied 4% lidocaine a safe and effective method of analgesia for facial rejuvenation.**

Funding Agency/Sponsor: UTSW

Study Doctors: Dr. Jeffrey Kenkel

Research Personnel: Georgette Oni

Jennifer White

Debby Noble

You may call these study doctors or research personnel during regular office hours and at other times at 214-645-3112.

Instructions:

Please read this consent form carefully and take your time making a decision about whether to participate. As the researchers discuss this consent form with you, please ask him/her to explain any words or information that you do not clearly understand. The purpose of the study, risks, inconveniences, discomforts, and other important information about the study are listed below. If you decide to participate, you will be given a copy of this form to keep.

Why is this study being done?

This study is being done to see if pretreatment with the laser prior to topical anesthetic application improves the pain relief for your facial rejuvenation procedure. We obtain blood serum levels (the amount of a certain substance in the blood) from patients following the application of topical lidocaine (a numbing product applied to the skin). The serum levels (amount in the blood) of lidocaine and its metabolic products (the products that result when it is broken down by the body) will then be used to calculate the maximum amount of lidocaine allowed to be applied in clinical settings (in doctor's offices) before the risk of lidocaine toxicity (lidocaine poisoning).

Why am I being asked to take part in this research study?

You are being asked to take part in this study because you are a healthy individual between the ages of 18-65 who wants to have a facial rejuvenation procedure eg to remove fine lines or dark patches on the skin.

How many people will take part in this study?

Approximately 14 people will participate in this study at UT Southwestern Medical Center at Dallas.

What is involved in the study?

If you agree to be in this study, you will be asked to sign this consent form and will have the following tests and procedures.

Procedures and Evaluations during the Research:

You will have the following tests and/or evaluations:

Visit 1:

- *You will fill out a short form about your medical history.*
- *Females will undergo a pregnancy test.*
- *You will come to the clinic and a photograph will be taken of you to calculate your facial surface area*

Visit 2:

- *You will have topical anesthetic cream applied to your face for 20 minutes.*
- *Then after a light treatment with the laser you will have topical anesthetic cream applied to your face again and left on for a maximum of 20 mins*
- *Blood levels will be drawn through an IV 6 times over 4 hours, starting after the first topical anesthetic is removed*
- *The process will take approximately 5 hours*
- *You will then be asked to complete a questionnaire.*

How long can I expect to be in this study?

You can expect to be in this study until the end of your second visit, approximately 8 weeks.

You can choose to stop participating for any reason at any time. However, if you decide to stop participating in the study, we encourage you to tell the researchers.

What are the risks of the study?

Study Intervention

Because of your participation in this study, you are at risk for the following minimal side effects:

Neurotaxic (nerve) effects: anxiety, agitation, altered mental state, tinnitus (ringing in the ears), perioral tingling (tingling around the eyes), metallic taste, dysgeusia (less sense of taste), light-headedness, nausea, diplopia (double vision), nystagmus (rapid eye movement), slurred speech, hallucinations, shaking of your muscles that you cannot control, and seizures.

Cardiotaxic (heart) effects: palpitations (irregular heart beating), arrhythmia (disturbance of heart rhythm), cardiovascular collapse (collapse of the heart and blood vessels), methemoglobinemia (not enough oxygen getting to body tissues), hypotension (low blood pressure).

Laser treatments: erythema (redness of the face), hypo or hyper pigmentation (light or dark areas on the skin), serous exudates (weeping from the treated areas), crusting, pain, and herpes simplex infection (if you have had it in the past).

Loss of Confidentiality

Any time information is collected; there is a potential risk of loss of confidentiality. Every effort will be made to keep your information confidential; however, this cannot be guaranteed.

Health Risks

The following health risks are all rare, occurring in (<1%) of individuals

Other Risks

There may possibly be other side effects that are unknown at this time. If you are concerned about other, unknown side effects, please discuss this with the researchers.

What are the possible benefits of this study?

The direct benefit for participation in this research study is that you may not require an injectionto be able to undergo their laser procedure. To the wider population the results of this study may provide a better understanding of the way lidocaine behaves once it is absorbed by the body following laser treatment. In addition if adequate pain relief can be demonstrated to be provided using this method it would remove the need for anesthetic injections not only for laser procedures, but also to wider applications such as skin grafts.

What options are available if I decide not to take part in this research study?

This is not a treatment study. You are not being treated for any medical condition therefore do not need treatment outside of this study. You therefore, you do not have to participate.

Will I be paid if I take part in this research study?

Yes. You will be compensated with your laser treatment for free plus $20 towards travel costs following your inclusion in the study and the completion of the experimental procedure outlined above.

Will my insurance provider or I be charged for the costs of any part of this research study?

No. Neither you, nor your insurance provider, will be charged for anything done only for this research study

What will happen if I am harmed as a result of taking part in this study?

It is important that you report any illness or injury to the research team listed at the top of this form immediately.

Compensation for an injury resulting from your participation in this research is not available from the University of Texas Southwestern Medical Center at Dallas.

You retain your legal rights during your participation in this research

Can I stop taking part in this research study?

Yes. If you decide to participate and later change your mind, you are free to stop taking part in the research study at any time.

If you decide to stop taking part in this research study, it will not affect your relationship with the UT Southwestern staff or doctors. Whether you participate or not will have no effect on your legal rights or the quality of your health care.

If you are a medical student, fellow, faculty, or staff at the Medical Center, your status will not be affected in any way.

Your doctor is a research investigator in this study. He is interested in both your medical care and the conduct of this research study. At any time, you may discuss your care with another doctor who is not part of this research study. You do not have to take part in any research study offered by your doctor.

Will my information be kept confidential?

Information about you that is collected for this research study will remain confidential unless you give your permission to share it with others, or if we are required by law to release it. You should know that certain organizations that may look at and/or copy your medical records for research, quality assurance, and data analysis include:

- Representatives of government agencies, like the Food and Drug Administration (FDA), involved in keeping research safe for people, and
- The UT Southwestern Institutional Review Board.

In addition to this consent form, you will be asked to sign an "Authorization for Use and Disclosure of Protected Health Information." This authorization will give more details about how your information will be used for this research study, and who may see and/or get copies of your information.

Whom do I call if I have questions or problems?

For questions about the study, contact Dr. Jeffrey Kenkel at 214-645-3112 during regular business hours, after hours and on weekends and holidays.

For questions about your rights as a research participant, contact the UT Southwestern Institutional Review Board (IRB) Office at 214-648-3060.

SIGNATURES:

YOU WILL BE GIVEN A COPY OF THIS CONSENT FORM TO KEEP.

Your signature below certifies the following:

- You have read (or been read) the information provided above.
- You have received answers to all of your questions and have been told who to call if you have any more questions.
- You have freely decided to participate in this research.
- You understand that you are not giving up any of your legal rights.

_____

Participant's Name (printed)

_____ _____

Participant's Signature Date

_____

Name of person obtaining consent (printed)

_____ _____

Signature of person obtaining consent Date

Interpreter Statement:

I have interpreted this consent form into a language understandable to the participant and the participant has agreed to participate as indicated by their signature above.

_____

Name of Interpreter (printed)

_____ _____

Signature of Interpreter Date

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