Electrosurgical plasma-mediated ablation for application in dermal wound and cartilage debridement

- Biochemical, microbiological and clinical effects

Henrik H. Sönnergren

Department of Dermatology and Venereology
Institute of Clinical Sciences
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2015

Cover illustration: Electrosurgical plasma probe by Morgan Carlsson and Anna Sofia Björk

Electrosurgical plasma-mediated ablation for application in dermal wound and cartilage debridement

henrik.sonnergren@gu.se

ISBN 978-91-628-9390-3

Printed in Gothenburg, Sweden 2015
Kompendiet

To my wife Anna Sofia

Electrosurgical plasma-mediated ablation for application in dermal wound and cartilage debridement

Biochemical, microbiological and clinical effects

Henrik H. Sönnergren

Department of Dermatology and Venereology, Institute of Clinical Sciences
Sahlgrenska Academy at University of Gothenburg
Göteborg, Sweden

ABSTRACT

The state of matter known as plasma has in the latest decades been investigated within different areas of medical treatment. The work presented in this thesis has focused on a specific type of plasma-based electrosurgical treatment modality (Coblation®) and its biochemical, microbiological and clinical effects on treatment of cartilage and of dermal wounds.

Paper I investigated the biochemical effects of plasma ablation exposure of human articular chondrocytes *in vitro*. The plasma ablation induced a well-defined area of immediate cell death, an increased chondrocyte proliferation and up-regulation of cytokines IL-6 and IL-8. Paper II investigated the *in vitro* antimicrobial effect of plasma ablation on *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*. The plasma ablation had a direct microbicidal effect on all strains compared to untreated control and a temperature control. Papers III and IV investigated the bacteria aerosol formation and wound bacteria reduction of debridement using curette, plasma ablation or hydrodebridement in an *ex vivo* porcine wound model inoculated with *S. aureus*. Plasma ablation significantly reduced the wound bacterial load, while curette and hydrodebridement resulted in minor or no reduction. Hydrodebridement gave a significant bacterial spread to the operative environment, while plasma ablation and curette debridement did not. Paper IV also used scanning electron microscopy to detect if there was a bacterial biofilm in the porcine wound model. Paper V investigated the effect of debridement using plasma ablation on ulcer healing, wound bacteria colonization, and complications to the treatment, in a clinical case series of 10 patients with venous ulcers. The

procedure was fast and easy to perform and gave a clean wound bed. The wound area was significantly reduced with a mean of 44 % and 2 of 17 ulcers healed within 8 weeks. The wound bacterial load was reduced by treatment with 1.5 log CFU/ml.

In conclusion, plasma ablation has a direct biochemical effect on chondrocytes indicating an onset of a tissue regeneration response. Plasma ablation can clinically be used for debridement of small ulcers in local anaesthesia. The bactericidal effect seen *in vitro* and *ex vivo* was confirmed clinically, which could be of value for the wound healing process. Further clinical studies should evaluate the plasma ablation method for use in other areas, such as in wound debridement prior to skin transplantation, diabetic foot ulcers, and burns.

Keywords: Ablation techniques, aerosol, antibacterial, arthroscopy, bacterial spread, bactericidal, bipolar radiofrequency, Candida albicans, cartilage, Coblation, debridement, electrosurgery, Escherichia coli, hydrosurgery, plume, Pseudomonas aeruginosa, Staphyloccus aureus

ISBN: 978-91-628-9390-3

SAMMANFATTNING PÅ SVENSKA

Elektrokirurgisk plasma-medierad ablation på sårrengöring och brosk - Biokemiska, mikrobiologiska och kliniska effekter

Plasma är ett av de fyra energitillstånd som materia kan befinna sig i. De senaste åren har plasma-fält utvärderats inom flera olika användningsområden inom medicin. Denna avhandling har undersökt en specifik typ av plasma-baserad elektro-kirurgisk teknik (Coblation®) och dess biokemiska, mikrobiologiska och kliniska effekter på behandling av brosk och hud-sår.

Artikel I undersökte de biokemiska effekterna av plasma ablation på ledbrosk och broskceller i laboratorieförsök. Plasma ablation gav en direkt celldöd närmast plasma-fältet men gav även en ökning av celldelningen och ökning av uttrycket av två olika immunsignalerande substanser.

Artikel II undersökte i laboratorieförsök den bakteriedödande och svampdödande effekten av plasma ablation på stafylokocker och andra bakteriestammar som infekterar sår och på svampen *Candida albicans*. Plasma ablationen hade en starkt bakterie- och svampdödande effekt.

Artikel III och IV undersökte den bakteriedödande effekten i artificiella hudsår i grishud och den bakteriespridande effekten i operationsrummet av sårrengöring med plasma ablation, sårrengöring med kyrettage och med hydrokirurgi. Plasma ablation var den enda metod som effektivt minskade bakteriemängden i såren. Hydrokirurgi visade sig sprida bakterier från såret och ut i operationsrummet.

Artikel V utvärderade effekten av sårrengöring med plasma ablation i en klinisk studie på tio patienter med venösa underbens-sår. Behandlingen med plasma ablation var lätt och snabb att genomföra och minskade bakteriemängden i såren. Under åtta veckors uppföljning minskade såren med i genomsnitt 44 % i storlek och 2 av de 17 behandlade såren läkte helt.

Slutsatserna från studierna är att plasma ablation har en biokemisk effekt på broskceller som kan initiera en regenerationsprocess. Plasma ablation kan användas för sårrengöring av sår och har en bakteriedödande effekt som kan vara till nytta för sårläkningen av svårläkta sår. Framtida studier bör utvärdera plasma ablation inom andra områden såsom behandling före hudtransplantation och rengöring av brännskador.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Lars Enochson, Henrik H. Sönnergren, Vipul I. Mandalia, Anders Lindahl

Bipolar radiofrequency plasma ablation induces proliferation and alters cytokine expression in human articular cartilage chondrocytes

Arthroscopy. 2012 Sep; 28(9): 1275-1282.

II. Henrik H. Sönnergren, Louise Strömbeck, Jan Faergemann

Antimicrobial effects of plasma-mediated bipolar radiofrequency ablation on bacteria and fungi relevant for wound infection

Acta Derm Venereol. 2012 Jan; 92(1): 29-33.

III. Henrik H. Sönnergren, Louise Strömbeck, Frank Aldenborg, Jan Faergemann

Aerosolized spread of bacteria and reduction of bacterial wound contamination with three different methods of surgical wound debridement: a pilot study.

J Hosp Infect. 2013 Oct; 85(2): 112-7.

IV. Henrik H. Sönnergren, Louise Strömbeck, Frank Aldenborg, Bengt R. Johansson, Jan Faergemann

Bacteria aerosol spread and wound bacteria reduction with different methods for wound debridement in an animal model.

Acta Derm Venereol. 2015 Mar; 95(2): 272-7.

V. Henrik H. Sönnergren, Sam Polesie, Louise Strömbeck, Jan Faergemann

Coblation debridement of chronic venous ulcers – A single center, single arm, non-comparative prospective clinical case series

In manuscript.

CONTENT

ABBREVIATIONS

ACI	Autologous chondrocyte implantation
C. albicans	*Candida albicans*
CFU	Colony-forming unit
E. coli	*Escherichia coli*
HMDS	Hexamethyldisilazane
hrs	Hours
IL	Interleukin
LoQ	Limit of quantification
MSD	Mechanical shaver debridement
MRI	Magnetic resonance imaging
MRSA	Methicillin-resistant *S. aureus*
OD	Optical density
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate-buffered saline
Plasma ablation	Plasma-mediated bi-polar radiofrequency ablation
RCT	Randomized controlled trial
S. aureus	*Staphylococcus aureus*
SEM	Scanning electron microscopy
S. pyogenes	*Streptococcus pyogenes*
TC	Thermal control

TNF	Tumour necrosis factor

1 INTRODUCTION

All matter that is observed in everyday life are traditionally classified into three classical states of matter; solid, liquid and gas. However, also a fourth state of matter known as plasma is recognized within physics. Plasmas are actually present all around us, in fluorescent light, neon signs, electric sparks, lightning and in the sun and other stars. A plasma-field can be created by heating a gas or liquid or by subjecting it to a strong electromagnetic field. The term plasma used within physics for recognizing a state is not to be confused with the physiological term plasma, e.g. blood plasma, used in medicine, which is a totally different entity. A state of matter is one of the distinct forms that matter takes on and also other states of matter than the four ones mantioned above are recognized within physics, to occur under extreme temperature conditions such as Bose-Einstein condensates and neutron degenerate matter. Other states of matter are also theoretically believed to be possible, although such aspects of physics expand way beyond the aim of the current thesis.

The state of matter known as plasma has in the latest decades been investigated for usage within different areas of medical treatment for various conditions. The attributes of the plasma that have been investigated and warranted within medical treatment range from biochemical effects on tissue, microbicidal capacity, pro-inflammatory and anti-inflammatory effects, to the ability to dissolve or cut in tissue. In a number of clinical specialties, plasma-based treatment methods have also been evaluated clinically and in some areas been incorporated in every-day clinical practice.

The work presented in this thesis has focused on a specific type of plasma-based electrosurgical treatment modality that has been developed for clinical use and the biochemical, microbiological and clinical effects that this technique have on treatment of cartilage and dermal wounds.

The plasma-based electrosurgical instrument investigated in the thesis is produced under the brand name Coblation®. Within cartilage-treatment, this plasma-based instrument is already used in clinical practice in a number of countries worldwide. In this area, the plasma-instrument is primarily used in orthopaedic arthroscopic surgery, i.e. knee-arthroscopy, to smoothen rough and torn cartilage surfaces. A relatively large amount of preclinical animal data and clinical data have been published on the effects of the treatment. However, the study on effects on cartilage presented in paper I was prompted

by the lack of data on biochemical effects of this treatment modality on human cartilage.

A number of basic-research as well as clinical studies has evaluated different modalities of plasma-based treatment for use in wound treatment. These different plasma-modalities have all in common that they have a bactericidal or general microbicidal effect on wound pathogens and it has also been discussed if they may have positive biochemical effects on the wound bed. However, none of these prior plasma-modalities investigated in wound treatment have had the effect of also debriding and removing devitalized tissue from the wound, which is one of the potential effects of the plasma-modality investigated in this thesis. In papers II through V, different aspects relevant for the use of this specific plasma-based technique in wound treatment are studied.

Paper II evaluated the bactericidal and fungicidal effect of the plasma-technique in *in vitro* planktonic solutions of a number of bacteria species and one fungal species. Papers II-III further evaluated the bactericidal effect of the plasma-technique in comparison to other methods for wound debridement in a porcine *ex vivo* artificial wound model inoculated with the wound pathogen *S. aureus* and also evaluated the risk of bacteria spread during the treatments. Following these basic research studies, paper V was a phase IIa clinical case series study, which evaluated the clinical effect of the plasma-technique for wound debridement.

The introductory sections of the thesis give a theoretical background to the different papers and describe aspects of the cartilage pathology and wound pathology as well as of the electrosurgical plasma-mediated ablation technology.

Henrik H. Sönnergren

Gothenburg, 30th of April 2015

1.1 Cartilage physiology and pathophysiology

Cartilage is a flexible connective tissue that is foremost present in the joints between the bones (articular cartilage), but is also found in the rib cage, the ear, the nose, the bronchial tubes and the intervertebral discs. Cartilage is composed of cells called chondrocytes and collagenous extracellular matrix consisting of proteoglycan and elastin fibres. In general, cartilage is classified in three types, elastic cartilage, hyaline cartilage and fibrocartilage, which differ in composition properties. All types of cartilage though have in common that it physiologically lacks blood vessels and depend on diffusion, which give a low rate of tissue growth and regenerative capacity.(1, 2)

The cartilage of the joints can be injured through acute trauma or from repetitive microtrauma over several years of use. Articular cartilage in the joints are exposed to high mechanical forces during activities in daily life, forces which increase dramatically during sports activities.(1) When arthroscopically treating damaged menisci or anterior cruciate ligaments, defects and cartilage lesions of the articular cartilage are often identified. Different studies have concluded that between 60 to 70 % of patients having arthroscopic knee surgery have concomitant cartilage lesions and full thickness cartilage defects are identified in 4-11 % of knee arthroscopies performed in younger patients.(3-6) It is also the younger individuals who are considered to potentially benefit the most from cartilage repair procedures. The goals of cartilage procedures are the restoration of function, reduction of pain and slowing, or avoidance of the development of osteoarthritis. (1)

In cartilage disease, early osteoarthritis is defined by proliferation of the chondrocytes and altered interleukin (IL) expression.(7) In the disease, a number of different cytokines are involved; however, IL-1β and tumour necrosis factor (TNF, formerly known as TNF-α) are the two most powerful mediators of inflammation.(8-10) HMGB1 is also considered an important mediator of inflammation through induction of cytokine and metalloproteinase expression in osteoarthritis.(11)

1.1.1 The role of cartilage debridement in cartilage injury

Severe cartilage defects, grade III and grade IV according to the Outerbridge classification scale, are often considered for different advanced and invasive treatment options such as autologous chondrocyte implantation (ACI) or microfracture procedures.(1) Loose flaps of cartilage that mechanically

impinge on the joint and cause inflammation of the cartilage surface and smaller grade III defects may though be suitable for debridement using different treatment options to smoothen the cartilage surface and decrease pain and crepitus.(12, 13) The primary alternatives for cartilage debridement consist of using either hand tools, mechanical shaver debridement (MSD) or plasma-mediated bi-polar radiofrequency ablation (plasma ablation) (also termed chondroplasty).(13, 14)

1.2 The skin and wounds and ulcers of the skin

The skin is an organ that functions as a barrier between the body tissues and the external environment. As such, the skin protects the human tissues from external potentially dangerous factors such as microorganisms, chemical substances warm or cold conditions and other potentially hazardous threats. A compromise of the skin integrity is termed a wound. A wound in turn can be of several different kinds and are generally defined as either acute or chronic.

Acute wounds are e.g. abrasions, crush wounds, cuts and surgical wounds that heal spontaneously within an orderly and timely process. Chronic wounds on the other hand are defined as wounds that have failed to proceed through an orderly and timely reparative process to produce anatomic and functional integrity.(15) Chronic wound are often termed ulcers and can be also defined as wounds with a ‘full thickness depth’ and a ‘slow healing tendency’.(16) In some settings the term hard to heal ulcer is used instead of the term chronic ulcer.

Chronic wounds are traditionally classified by aetiology with four major categories: arterial insufficiency ulcers, venous insufficiency ulcers, pressure ulcers and diabetic foot ulcers, but a large proportion of wounds has a mixed aetiology with several underlying causes. Out of these categories, venous ulcers are the largest category accounting for approximately 45-60 %.(16)

With an increasing life expectancy and increasing prevalence of diabetic disease and venous insufficiency the frequency of chronic wounds can be expected to increase.(16-20) Prevalence numbers of ulcers overall range from 1% in the adult population to approximately 3-5% in the population over 65 years of age and it is estimated that approximately 1% of the general population have active or healed venous leg ulcers, contributing to a substantial cost for the society.(16, 21, 22) Chronic wounds also significantly

affect the quality of life, and since it is often a long lasting and recurrent condition it has impact on somatic as well as psychiatric and social aspects for the individual patient.(22)

The treatment approach towards chronic wounds generally consist of a holistic approach which include a treatment approach towards the aetiological basis of the ulcer as well as local wound treatment of the wound bed. Treatment of the underlying aetiology primarily include arterial revascularization surgery for arterial insufficiency ulcers, compression treatment or venous vascular surgery for venous insufficiency ulcers, well-regulated blood glucose control and off-loading with appropriate shoe-wear for diabetic foot ulcers, and pressure-reduction and repositioning schemes for pressure ulcers.(23-26) However, in many cases these treatment modalities are either not suitable for the individual patient or may be insufficient to aid the healing process.

1.2.1 Microbes and infection in wounds

Wound healing is an intricate play demanding good nutritious status as well as recruitment of immune cells. Many clinicians are concerned about the risk of infection in non-healing wounds and wound bacteria-cultures are often obtained from the wound bed even in the absence of clinical signs of infection. However, almost all chronic wounds are colonized with bacteria and the presence of bacteria in a chronic wound does not necessarily indicate that infection has occurred.(27)

Whether or not the presence of bacteria in a wound can inhibit the wound healing process is a matter of debate. Some researchers argue that the presence of bacteria in the absence of clinical signs of wound infection will not lead to impairment of wound healing (28-30), and it has even been suggested that certain low levels of bacteria can actually facilitate wound healing.(31, 32) One example of such a possibly positive effect is that bacteria has in burn wounds been shown to produce proteolytic enzymes such as hyaluronidase, which contribute to wound debridement and stimulate neutrophils to release proteases.(33) There is also no evidence that systemic antibiotic treatment has an effect on healing of venous leg ulcers without clinical signs of infection.(34) However, presence of bacteria in a certain amount or of specific strains has also been argued to possibly delay wound healing.(27, 35) One of the mechanisms suggested is that bacteria form biofilms, which increase the antimicrobial resistance.(36) Schultz *et al.* have categorised bacterial levels in the wound bed as contamination, colonisation, local infection or spreading infection. With this definition contamination and

colonisation are not considered to impair healing while on the other hand critical colonisation and infection are considered to impair the healing process.(37) However, the number of bacteria considered to impede healing of open wounds is controversial, with some studies showing impaired healing if more than 10^5 or 10^6 organisms per gram of tissue can be detected, while other studies have not been able to show such a correlation.(28, 30, 38) A large number of different bacteria strains can be identified in colonized and infected wounds, but the general conception is that aerobic or facultative pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and beta-hemolytic streptococci are the primary causes of delayed healing and infection in both acute and chronic wounds, while anaerobic bacteria are of lesser importance.(35)

Chronic bacteria colonization of the wound can though lead to recurrent wound infection, which often demands different and repeated antibiotic treatments that may contribute to significant side effects. Furthermore, repeated antibiotic treatment amplifies antibiotic resistance, which pose a problem in wound infections as well as in other bacterial infections.(39)

1.2.2 The role of wound bed preparation and debridement in wound treatment

Surgical debridement constitutes an essential role in standard wound care and assists in removing barriers that impairs wound healing.(27, 40) The aim of debridement is to promote wound healing by removing devitalized tissue and reducing the bacterial load that impair the wound healing process.(41) Traditionally, several types of wound debridement techniques have been used in clinical practice including autolytic, enzymatic, biodebridement, mechanical, sharp debridement with curette and surgical debridement as well as debridement with live maggots.(42, 43)

Sharp debridement with a cold steel curette is the most common method for wound debridement, as it only requires a curette, scissor and water for wound cleansing and removal of visible necrotic wound material. This method thus has a low material cost and can often be performed by specially trained nurses in local anaesthesia on an outpatient basis.

In recent years however, a number of alternative methods for wound debridement have with varying success been evaluated, including hydrosurgery (Versajet®), ultrasound and pulsed lavage.(44)

1.2.3 The risk of bacterial spread during wound debridement

In the recent decades there has been an increased focus on nosocomial infections as well as hospital hygiene.(45) There is also a rising concern about perioperative passive and active bacteria aerosol spreading.(46, 47) A recent study has shown that hydrosurgery debridement of wounds induce a significant risk of bacteria aerosol spread (46) and a report by Maragakis *et al*. describe the potential clinical consequences of using wound debridement equipment with inadequate protection against the potential for bacteria transmission and environmental contamination.(47) The report by Maragakis *et al*. describe a hospital outbreak with a multidrug-resistant strain with *Acetinobacter baumannii* caused by the cross infection between patients treated with pulsed lavage wound debridement.

Reducing bacteria aerosol spread is therefore of interest both with regards to decreasing the dispersion of resistant strains to the immediate surroundings, as well as reducing the time to prepare the operation theatre for the next surgery session, and minimizing the risk of cross-infection between patients.

1.3 The electrosurgical plasma-mediated ablation technique

Electrosurgical plasma-mediated ablation is a method for volumetric soft tissue removal established in several surgical fields, such as arthroscopy, spinal surgery, tumour resection, and ear, nose and throat surgery.(48-51) The technique is based on inducing a bipolar radiofrequency current between two electrodes in a conductive medium, such as saline, and thus creating a physical plasma field which is able to break molecular bonds and dissolve tissue at relatively low temperatures.(52, 53) Physical plasma is regarded as a distinct state of matter and is not to be confused with the physiologically well-known blood plasma.

Within arthroscopic surgery the plasma ablation method is used for chondroplasty of type II and III cartilage lesions and meniscal debridement as well as for cutting and coagulating vascular tissue.(13) A recent review concluded that clinical evidence for plasma-mediated chondroplasty shows decreased pain scores postoperatively and increased functional scores in the short-term when compared with mechanical chondroplasty.(54) Laboratory evidence also suggests increased mechanical stability and decreased release of inflammatory mediators with the use of plasma-mediated

chondroplasty.(54) Clinical results have shown plasma-mediated chondroplasty to give better clinical outcomes at up to four years compared to MSD.(13, 51, 55) Two clinical case series have also indicated plasma-mediated chondroplasty to induce a regeneration of the articular cartilage post treatment, with partial or complete filling in more than 50 % of treated lesions.(14, 56)

In preclinical settings, Kaplan *et al.* showed plasma ablation to efficiently ablate and smooth the surface of injured cartilage while maintaining chondrocyte viability and collagen structure in underlying cartilage at time zero.(57) Amiel and colleagues observed, using confocal laser microscopy and a sulfate incorporation assay, that plasma ablation induce a well-defined margin of chondrocyte death while having no significant effect on metabolic activity adjacent to the treatment zone in bovine articular cartilage.(58) Further, Allen *et al.* used similar methods to examine the difference between MSD and plasma ablation in bovine menisci and underlying cartilage. The results showed no difference in cellular viability or metabolic activity of the tibia surface or menisci between groups.(59)

The plasma ablation technique has in tendon and intervertebral disc tissue of different animal models been shown to induce biochemical changes and hyper-cellularity, possibly establishing a tissue healing response of chronically injured tissue.(60-63) In an *in vivo* porcine intervertebral disc model O'Neill and colleagues showed plasma ablation to induce altered cytokine expression.(60) Similar results were also achieved by Rhyu *et al.* on porcine disc cells *in vitro*.(61) In human osteoarthritic cartilage Cook *et al.* showed increased MMP-13 immunoreactivity, less IL-1 release and less nitric oxide release after bipolar radiofrequency treatment.(64)

Within wound treatment the plasma ablation technique has in an *in vivo* porcine acute wound model inoculated with methicillin-resistant *S. aureus* (MRSA) been shown to significantly reduce bacterial counts compared to both sharp debridement and hydrosurgery at up to 21 days after treatment, without any detrimental effects to the wound healing process.(65)

The clinical experience on the use of plasma ablation for wound debridement is limited. A report by Trial *et al.* presented a number of clinical cases where the plasma ablation technique was used for wound debridement of venous leg ulcers, pressure ulcers and burn wounds. Of the 25 cases presented, one plasma ablation debrided and skin grafted venous leg ulcer had heavy postoperative inflammation with a 30% postoperative skin loss, but otherwise no complications were reported. However no data on wound healing and

wound closure was published in this report (66) A recent case report by Yim and colleagues presented a case where plasma ablation had been used for burn excision surgery of deep-dermal and full-thickness burns with immediate reconstruction using split skin grafts. Interestingly, the wounds on the arm and hand debrided with plasma ablation healed with good graft take, while there was extensive graft loss requiring regrafting of the thigh, breast and abdomen which had been debrided with standard methods prior to grafting.(67)

Different plasma ablation probes have been used in the different studies included in this thesis, depending on the study setup and the difference in application and clinical usage between cartilage debridement and wound debridement.

The Paragon T2 Coblation® probe used in study I is specifically designed for and clinically used in arthroscopic cartilage debridement. The Microblator 30 used in study II is actually designed for application in cartilage debridement, but was chosen for this *in vitro* study on antimicrobial effects since no other probe had, at the time when the experiments were conducted, been developed for use in dermal wound debridement. The WoundWand Coblation® probe used in studies III, IV and V is specifically designed for usage in dermal wound debridement and has an integrated saline delivery tube which continually flushes saline over the electrodes and a suction line for evacuation of saline and debrided wound material (Figure 1). All these three different electrosurgical probes are however based on the same electrosurgical plasma ablation technique and are connected to similar electrosurgical generators (Figure 2).

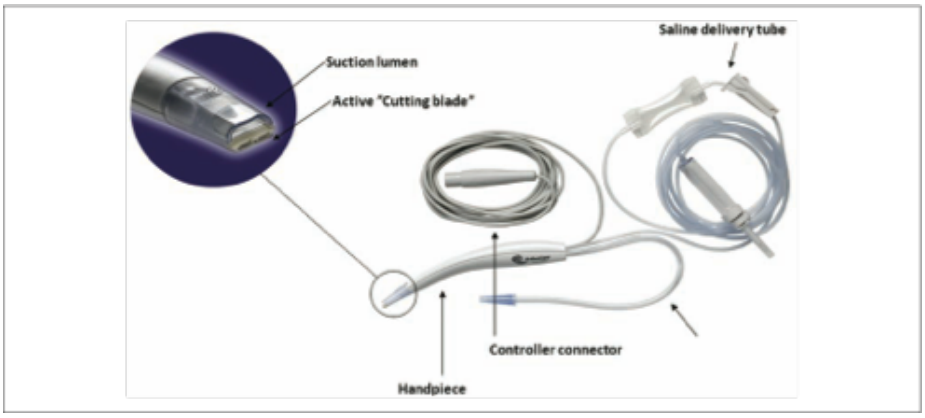

Figure 1. Illustration of the WoundWand Coblation® debridement probe used in studies III, IV and V (Trial et al. Int J Low Extrem Wounds, 2012 Dec;11(4):286-92).

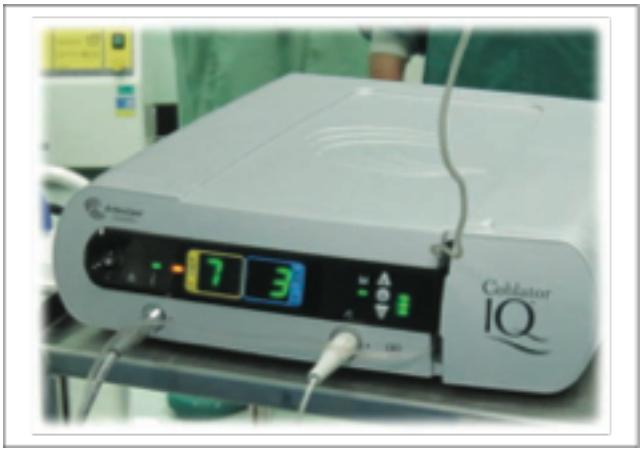

Figure 2. The generator unit to which the plasma ablation debridement probe is attached (Trial et al. Int J Low Extrem Wounds, 2012 Dec;11(4):286-92).

2 AIM

Paper I

To investigate if plasma ablation exposure of human articular chondrocytes *in vitro*;

1) Induce a zone of dead cells in the exposed area,
2) Increase chondrocyte proliferation, and
3) Increase proinflammatory mediator gene expression

compared with untreated control.

Paper II

To investigate;

1) The direct antimicrobial effect of plasma ablation exposure on bacteria and fungi strains relevant to wound infection, and
2) How the parameters of exposure time, temperature increase, and aerobic/ anaerobic growth influence the antimicrobial effect.

Paper III

To investigate;

1) The amount of bacteria aerosol formation, and
2) Wound bacterial load reduction

induced by debridement using either cold steel curette, plasma ablation or hydrodebridement of an *ex vivo* porcine wound model inoculated with *S. aureus*.

Paper IV

To investigate;

1) The reduction of wound bacterial load
2) The amount of bacterial aerosolization

induced by debridement, using either cold steel curette, electrosurgical plasma ablation or hydrodebridement in an *ex vivo* porcine wound model inoculated with *S. aureus*, and

3) To investigate the presence of a bacterial biofilm in the porcine wound model used in the study

Paper V

To investigate the effect of debridement using the electrosurgical plasma ablation Coblation® WoundWand Debridement Device on;

1) The healing of chronic venous ulcers
2) The wound bacteria colonization
3) Potential complications to the treatment

3 MATERIALS AND METHODS

Study I was conducted at the research laboratory of the Department of Clinical Chemistry and Transfusion Medicine at the Sahlgrenska Academy, University of Gothenburg. Studies II, III, IV and the quantitative bacteriological analysis of study V were conducted at the Research Laboratory at the Department of Dermatology, Sahlgrenska University Hospital. Histological specimen analysis in study III and IV were performed at the Department of Pathology, at Sahlgrenska University Hospital. Electron microscopy specimen analyses in study IV were performed at the Department of Medical Biochemistry and Cell Biology at the Sahlgrenska Academy, University of Gothenburg. For study V, the qualitative bacterial assessment was performed at the Department of Clinical Microbiology and the treatment and clinical follow-ups were performed at the Department of Dermatology and Venereology, both at Sahlgrenska University Hospital.

3.1 Paper I

Live/Dead imaging

The effect of the plasma ablation on viability of the cells was investigated in both cartilage and alginate cell cultures. The healthy articular cartilage biopsies were, after informed consent, donated to research from a patient undergoing amputation of the tibia (due to a skeletal tumour without involvement of the cartilage, male, 17 years old) and placed in NaCl 0.9% at surgery. For the cell cultures, human chondrocytes were cultured in alginate gel. Three gel cultures per patient and three cartilage biopsies were exposed to electrosurgical plasma ablation with three gels and three biopsies as control. The samples were put in DMEM-HG (Invitrogen), similar to and with the same ion content as arthroscopic lavage fluid, and the superficial surface of the samples were exposed to electrosurgical plasma ablation with the Paragon T2 ablation probe (Figure 3) connected to a Quantum 2 System generator (ArthroCare, Austin, USA). The probe was inserted in the media, activated at level 6 (equal to 234V ±10%) and moved to the surface of the gel or cartilage sample. The probe tip was then moved by hand once along the sample surface, without touching it, at a through timing estimated velocity of 0.5 mm/second for a total of approximately 3 seconds, where after the probe was turned off and removed from the surface. Paragon T2 was chosen based on that it is commonly used to treat articular cartilage defects. The treatment procedure as it was performed was chosen to simulate the clinical situation

where the plasma ablation usually is performed during arthroscopy of the knee.

Cell viability after exposure was assessed through staining the cells in the samples with the fluorescent dyes fluorescein diacetate for living cells, and propidium iodide for dead cells. Cell viability was determined through comparing amount of cells stained red or green.

Figure 3. The Paragon T2 bipolar radiofrequency plasma ablation probe used in this study is designed with a circular doughnut-shaped tip (Enochson et al. Arthroscopy, 2012 Sep;28(9):1275-82).

Chondrocyte expansion

Surplus chondrocytes from 3 patients (2 male and 1 female, 38-49 years old) undergoing ACI were used in this study. Cartilage biopsies were harvested from minimal load bearing areas on the femoral condyle, chondrocyte cells were extracted and cultured.

Chondrocyte culture in alginate gel

For redifferentiation, the chondrocytes were cultured in alginate gels, as previously described by Rhyu *et al.*(13) After expansion, the chondrocytes were resuspended in 1.2% sodium alginate solution (Sigma, Steinheim, Germany) prepared in phosphate buffered saline at a cell concentration of $4*10^6$ cells/ml. Cell culture inserts (1µm pore size, BD Biosciences, Le Pont De Claix, France) in 12-well culture plates (Corning Inc., Corning, NY, USA) were used to cast each gel and for subsequent cell culture. 575 µL of alginate/cell suspension was added to each insert. 800 µL 102 mmol/L $CaCl_2$ in 0.9% NaCl solution were added to each well around the inserts and the plates were incubated for 2 hours at 37°C/7% CO_2, during which Ca^{2+} diffused through the insert membranes cross-linking the alginate, which formed a 4 mm thick gel inside the inserts. 2 ml of defined culture media (consisting of DMEM-High glucose (PAA Laboratories) supplemented with ITS+ (Life Technologies, Sweden), 5.0 µg/ml linoleic acid (Sigma-Aldrich), 1.0 mg/ml human serum albumin (Equitech-Bio, Tx, USA), 10 ng/ml TGF-beta1 (R&D Systems, UK), 10^{-7} M dexamethasone (Sigma), 14 µg/ml ascorbic acid (Sigma) and 1x Penicillin-Streptomycin (PAA Laboratories)), was added into each well, 1.5 ml in the well around the insert and 0.5 ml on top of the gel inside the insert.(13, 17) The gel constructs were cultured at 37°C/7% CO_2 for 2 weeks. Media was changed twice per week.

Experimental groups

After 2 weeks in culture the inserts were divided into 2 groups, one electrosurgical plasma ablation group and one control group. The groups were further divided into two subgroups each, one subgroup harvested at 3 days and one subgroup harvested at 6 days after exposure as shown by Rhyu *et al.*(61) Each subgroup contained 3 samples. The setup was repeated with cells originating from 3 patients (2 male and 1 female, 38-49 years old). One sample per group was used for live/dead studies according to the procedure described for cartilage samples. Only one sample was used due to the limited amount of cells that could be retrieved from each patient. However, a total of three samples per subgroup were studied, one from each patient. These triplicates showed the same result.

Experimental procedure

For exposure, the Paragon T2 probe was held in the defined chondrogenic medium above the gel, activated at level 6, carefully moved close to the gel surface and continuously moved by hand along the gel surface without touching it, at a through timing estimated speed of 0.5 mm/sec. The probe was moved in 3 straight non-crossing lines over the gel surface and then turned off and removed. The probe was held by hand and the procedure was done with gels and probes immersed in defined culture medium to mimic the clinic arthroscopic procedure. For the control group no exposure was performed. The cell cultures were then cultured for 3 or 6 days.

Harvest and DNA analysis

3 and 6 days after exposure the assigned gels were dissolved. The samples were centrifuged and after removing the dissolved gel, the resulting cell pellets were washed twice with phosphate buffered saline. Cells harvested for qPCR analysis were snap-freezed in liquid nitrogen. Cells harvested for analysis of DNA content were digested with papain solution and mechanically dissolved. The amount of DNA was measured with Hoechst 33258, according to the manufacturer's instructions. The samples were standardized to the non-exposed sample with lowest DNA content.

RNA isolation and analysis

From the Paragon T2 trials RNA was isolated using the RNeasy Mini kit (Qiagen, Solna, Sweden). Removal of residual genomic DNA from all samples was done with DNaseI (Qiagen) according to the manufacturer's protocol. cDNA was prepared from total RNA using HighCapacity cDNA Reverse Transcription Kit. Commercially available human TaqMan Gene expression Assays were used (Applied Biosystems, Foster City, California) (order number in brackets): IL-6, IL-8, IL-1β, TNF, HMGB1, MMP13, collagen IIA and versican. The commercially available assay reagent for Cyclophillin A mRNA labelled with VIC/TAMRA was used as an endogenous control. Samples were analysed in duplicates on a 7900HT instrument (Applied Biosystems, Carlsbad, CA, USA). The relative comparative Ct method was used to analyse the real-time PCR data. Gene expression data are presented in relative units.

Statistic analysis

Statistical analysis of relative DNA-amount and RNA was performed by the non-parametric Mann-Whitney U test using the SPSS software (SPSS Sweden AB, Kista, Sweden). Statistical significance was taken as $P<0.05$.

3.2 Paper II

Microbial experimental setup

All microorganisms were obtained from CCUG (Culture Collection, University of Gothenburg, Sweden). The microorganisms used were *S. aureus* (CCUG 17621), *Streptococcus pyogenes* (CCUG 4207T), *P. aeruginosa* (CCUG 17619), *Escherichia coli* (CCUG 24T) and *Candida albicans* (CCUG 5594). *S. aureus, S. pyogenes, P. aeruginosa* and *E. coli* were maintained on horse blood agar plates at 37° C and *C. albicans* was maintained on Sabouraud's agar (Clinical bacteriology at Sahlgrenska University Hospital, Sweden) at 32° C.

Bacteria and fungi from 24 hrs old cultures were dissolved in 0.9 % saline (pH 7.4) and adjusted to approximately 10^6 cells/ml, as determined by optical density (OD) 2.0 at 550 nm with a DEN-1 McFarland densitometer (Biosan, Riga, Latvia). The suspension was transferred to 96 well microtiter plates (Nunc A/S, Roskilde, Denmark) with 100 µL/well, with every second well and row left empty to avoid thermal effects between samples. The wells were divided into electrosurgical plasma-mediated ablation, thermal control (TC) and untreated (normal) control groups. The two exposure groups were further subdivided into 500, 1000 and 2000 ms exposure time with six samples in each group. The experimental setup was repeated twice for each strain.

Ablation and thermal control equipment

For the exposure, Microblator 30 ICW probes (ArthroCare, Austin, USA), connected to a specifically programmed Quantum generator (ArthroCare), were used. The system can be used in ablation mode where a physical plasma field is generated around the tip of the probe through bi-polar radiofrequency conduction between the probe electrodes applied in a conductive medium, such as saline, or in coagulation mode where the medium is only thermally heated. Both modes use the same electrical waveform, but a certain voltage threshold is required to heat the saline to induce a vapour layer, which in turn enables plasma formation. Voltages for the coagulation mode are below this threshold, and the power delivered only generates thermal increase. In ablation mode, the power delivered generates both plasma and thermal increase.

The generator used was specifically programmed by the producer to allow set activation times of 500, 1000 and 2000 ms and the output voltage for the coagulation mode to provide essentially the same energy and thermal induction per time unit as the ablation mode. Exposure time and mode of activation were controlled via a foot pedal.

Sample exposure

The generator was adjusted to setting 9 (of 1-9), equivalent to 300 V output, and the probes were applied in the wells in ablation mode for the ablation group and coagulation mode for the TC group, for the pre-set exposure times. The probe tips were disinfected in 70 % ethanol and washed in isotonic saline between samples. A new probe was used for each group, and for a maximum of six samples. Plasma formation as confirmed by light emission and bubble formation for ablation samples and a typical fizz in TC samples were monitored to confirm proper probe activation. The normal control samples were left untreated.

Post exposure each sample was serially diluted in 0.9 % saline to 1/100, and plated onto Sabouraud's agar for *C. albicans* and horse blood agar for the bacterial strains. Bacterial plates were incubated at 37°C for 24 hrs and *C. albicans* at 32°C for 48 hrs. All strains were incubated aerobically except *E. coli*, which was incubated in both aerobic and anaerobic conditions using anaerobic jars with AnaeroGen sachets (Oxoid Ltd, Basingstoke, Hampshire, England). The number of colony forming units (CFU) were counted and minimal log reduction and relative reduction (expressed as percentage of absolute amount of CFU reduced by treatment) for ablation exposed groups were calculated, considering a limit of quantification (LoQ) of 1 CFU/group.

Temperature and exposure time measurements

To confirm that the ablation and coagulation modes generated comparable temperature increase a calorimetric trial was performed. Maximum temperature rise of each exposure was measured with a fibre optic temperature sensor (Neoptix Inc., Québec, Canada) at 100 Hz in 100 µL of saline using the same setup as for the microbial trials.

Measurements of the pre-programmed radiofrequency activation times were performed with a DPO4034 Digital Oscilloscope (Tektronix Inc., Oregon, USA), and a P5200 High Voltage Differential Probe (Tektronix), to confirm correct activation times equal between modes. Each measurement was repeated six times.

Statistic analysis

All microbial data were analysed using R version 2.10.1 (The R Foundation for Statistical Computing, Vienna, Austria) using the coin package. The exact permutation form of Wilcoxon-Mann-Whitney's test stratifying for measurement occasion was used for comparison of CFU/ml-values between groups. The values were ranked within each strata. All tests were two-sided and statistical significance was taken at $p < 0.05$.

3.3 Paper III

Sample preparation

Twelve fresh porcine joint specimens were obtained and the skin was disinfected with 70% alcohol. A 75 x 75 mm full thickness artificial dermal wound was created by sharp dissection in each specimen and inoculated with one ml of bacteria suspension of approximately 10^6 CFU/ml of methicillin sensitive *S. aureus* (CCUG 17621, Culture Collection University of Gothenburg, Sweden). The specimens were then incubated in disinfected containers at 37° C for 24 hrs and divided into treatment groups with two specimens in each group.

Six different treatment regimens were used; I) Untreated control wound (positive control), II) Cold steel curette, III) Plasma ablation at default setting (7), IV) Plasma ablation at maximum setting (10), V) Hydrosurgery at default setting (1), and VI) Hydrosurgery at maximum setting (10). Active and passive aerosol sampling was also performed with no biological sample present (negative control).

The default setting of the plasma ablation and hydrosurgery devices is the starting settings recommended by the producers. The maximum settings of the devices have a higher effect with higher bipolar voltage output of the ablation device and higher saline jet flow for the hydrosurgery. The rationale for the maximum settings is to get a more aggressive tissue removal effect.

Sample debridement

The specimens were surgically debrided in a lab fume hood with no airflow. The hood was disinfected by washing all surfaces with 70% ethanol between each debridement. Curette debridement was done by first washing the wound with gauze and water and then debriding using a 7 mm stainless steel curette (Integra Miltex, York, PA, USA), forceps and scissor. Debridement using plasma ablation (Coblation WoundWand®, ArthroCare corp., Austin, USA) and hydrosurgery (Versajet® Exact 14 mm 45° hand piece, Smith & Nephew plc., London, UK) were performed in accordance with the respective Instructions For Use. The plasma ablation probe was connected to a Coblator IQ generator and the suction line was connected to a standard surgical suction unit with a 275 mmHg vacuum pressure. All methods of debridement were performed by doing two debridement passages over each area of the wound bed.

Aerosol bacteria sampling

During and after each debridement bacteria aerosol was measured by active and passive sampling. Active sampling was performed with the bacterial air sampler Sartorius MD8 Airscan (Sartorius Stedim Biotech GmbH, Goettingen, Germany) with the air inlet manifold positioned 0.2 meters from the specimen and a set air throughput of 6.0 m^3/hour. One-minute samples of 100 litres of air were obtained at 0, 5, 15, 30, and 60 minutes post debridement initiation. Passive sampling was performed by placing four 90 mm Ø non-selective horse blood agar plates (Clinical Microbiology, Sahlgrenska University Hospital, Sweden) in the corners of the lab box, approximately 0.5 meters from the wound (Figure 4). The plates were placed out directly prior to each debridement and collected at 60 minutes post debridement initiation. Active and passive aerosol sampling was also performed with no biological sample present (negative control).

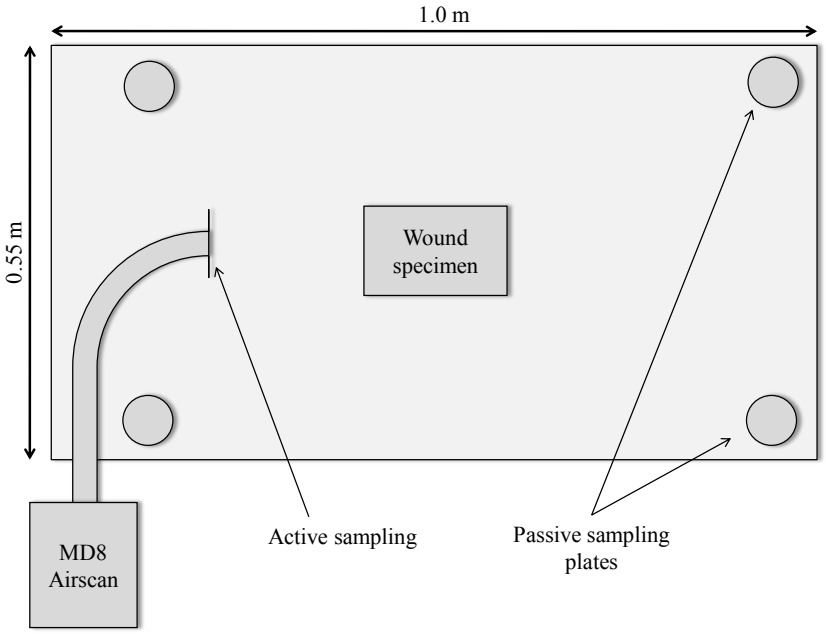

Figure 4. Schematic diagram (plan view) of the study set up illustrating positions of the wound specimen, the Sartorius MD8 Airscan inlet tube and the passive sampling plates (not to scale).

Wound bacteria sampling

To asses wound bacterial load, three quantitative swabs and one cylinder scrub sample were taken from each wound at baseline (pre inoculation), post incubation, and post debridement. Swabs were obtained using the Levine's technique for quantitative culture (68) and immersed in 1 ml of phosphate buffered saline (PBS) and vortex mixed for homogenization. Cylinder scrubs (69, 70) were obtained by placing a 26 mm Ø sterile steel cylinder on the wound surface and adding 1 ml of 0.1 % Triton X in PBS. The area was scrubbed with a sterile glass stirrer for 1 min. and the wash fluid was then obtained by pipetting. The samples were serially diluted and plated on blood agar plates. All agar plates were incubated aerobically at 37°C for 24 hrs and number of colonies was manually counted. Log CFU/m^3 was calculated from the results of the active sampling and Log CFU/dm^2/h was calculated for the passive sampling. Log CFU/ml was calculated for the swab samples and CFU/cm^2 was calculated for cylinder scrub samples. The primary study aim was to detect spread and wound growth of *S. aureus* and thus no anaerobic cultures were performed.

Histology

One (1) 8 mm punch biopsy for histology was taken from each wound at each time point (baseline, post incubation and post debridement) and fixed in neutral buffered 4% formaldehyde solution. The biopsies were dehydrated, embedded in paraffin and 10 µm thick slides from two different levels of each block with approximately 2000 µm between levels were cut out and stained according to a Gram-staining protocol. The histological slides were examined by an experienced pathologist fully blinded to treatment group and sampling time point. The specimens were evaluated for focal clusters and/or defined layers of bacteria and their position at the surface and/or in the deep tissue. The thickest clusters or layers of bacteria and the penetration depth in the tissue were then measured at three different measurement points on each section. This was carried out using a microscope equipped with an eyepiece graticule calibrated against a micrometre slide.

Statistical analysis

For cylinder scrubs and swabs, comparisons were made in the changes of wound bacterial load at post incubation and post debridement for the untreated control wound and each debridement group. For active and passive aerosol samples, comparisons were made between the control wound and negative controls and each debridement group. For cylinder scrubs and active aerosol samples, two sample t-tests were used for comparisons of treatment groups. For swabs and passive aerosol samples, linear mixed effects models were used for comparisons of treatment groups.

The microbiological results were statistically processed with suitable logarithmic transformations for the respective measurement types were used in order to homogenize the variance for the different treatment groups. The significance level was $P \leq 0.05$ and all tests were two-tailed. The histological results were not statistically processed but only qualitatively evaluated.

3.4 Paper IV

This study was largely performed as per paper III. Thirty-two fresh porcine joint specimens were obtained and divided into the six treatment groups into treatment groups with six specimens in each group, with exception of the control wound group, which contained two specimens. Active and passive aerosol sampling was also performed with no biological sample present (negative control). Two quantitative bacterial swabs were taken from each wound at baseline (pre-inoculation), post-incubation, and post-debridement for assessment of wound bacterial load, but no cylinder scrub samples were obtained. One 8 mm punch biopsy for histology was taken from two wounds in each group at each time point. Statistical analysis was performed as per paper III.

Biofilm evaluation

A separate part of the study was performed with scanning electron microscopy (SEM) by a skilled electron-microscopist, for evaluation of biofilm formation, where four porcine leg wounds were prepared, inoculated and incubated as described above. 8 mm punch biopsies for histology were taken from the wounds at baseline and post incubation, and fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2, for 24 h. Samples were then subjected to a triple treatment with osmium tetroxide according to the OTOTO protocol (71) followed by dehydration in ethanol, ending in hexamethyldisilazane (HMDS). The HMDS was allowed to evaporate in a fume hood. The dried tissue blocks were mounted on aluminium stubs and sputter coated with palladium before examination in a Zeiss 982 Gemini scanning electron microscope. Digital images were recorded at a pixel resolution of 1,024 x 1,024. The samples were evaluated for presence of bacteria and bacterial biofilm formation. The biofilm results were not statistically processed but only qualitatively evaluated.

3.5 Paper V

A single centre, single arm, non-comparative prospective clinical case series was performed. The study was approved by the Regional Ethical Review Board at the University of Gothenburg (approval nr 460-12) and was performed in accordance with the Helsinki Declaration of 1964, as revised in 2008. Informed consent was obtained from all patients prior to inclusion in the study. The study was conducted as a Stage 2a prospective development study in accordance with the IDEAL recommendations for Surgical Innovation and Evaluation prepared by the Balliol Collaboration at the University of Oxford, UK.(72-74)

Outcome measures

The study primary outcome measure was decreased wound size including number of wound-closures within the study time frame. The study secondary outcome measures were 1) the efficacy of the debridement in decreasing bacteria-colonization of the ulcer bed, and 2) evaluation of potential complications to the treatment.

Patients with chronic venous leg ulcers were included in the study. Inclusion criteria were; 1) women or men in the age of 50-90 years old with a venous insufficiency ulcer, 2) wound was fibrinous or necrotic and was clinically evaluated to require debridement, 3) wound was no larger than 8 cm in largest diameter, 4) wound had been present for a minimum of 2 months, 5) Ankle Brachial Pressure Index (ABPI) > 0.8.

Exclusion criteria were; 1) insulin treated diabetes mellitus, 2) immunocompromised patient due to disease or medication, 3) patient incapable of understanding the treatment information, 4) on-going treatment with oral antibiotics, 5) surgically inserted pacemaker or other electrical equipment, 6) BMI > 40, 7) patient simultaneously participating in another study.

Venous aetiology of the ulcers was based on clinical diagnosis. Eligible patients who agreed to participate in the study provided informed consent and completed baseline study questionnaires. Baseline patient questionnaires included Visual Analogue Scales (VAS) for wound pain and leg pain for the estimated average for the last seven days. Duration of the wound, previous treatments, previous medical history, and concomitant medication including analgesics, steroids, non-steroidal anti-inflammatory drugs, antibiotics and antimycotics the subject had used in the last seven days were recorded. ABI was measured and wound status was examined.

Procedure

The study used the following equipment: The WoundWand™ probe (ArthroCare, Austin, Tx, USA), which is a handheld sterile single use instrument that is connected to a Coblator IQ controller (ArthroCare, Austin, Tx, USA). The plasma ablation probe used has an integrated saline delivery tube that continually flushes saline over the electrodes and a suction line for evacuation of saline and debrided wound material. The equipment was used in accordance with its Instructions For Use (IFU).

The patient was prepared pre-treatment according to standard procedures. The wound area was anesthetized with local infiltration anaesthesia (lidocaine with epinephrine, 10 mg/ml+5 µg/ml) and/or lidocaine gel as considered appropriate. The Coblator IQ controller was assembled according to the instructions in the Coblator IQ User's Manual and the probe connected to the controller. The probe suction line was connected to a standard surgical suction unit with set vacuum in the range of 150 mmHg to 350 mmHg. The saline delivery line on the probe was connected to a bag of sterile saline. The controller set point and saline flow rate was adjusted as considered appropriate by the operator. All procedures were performed at plasma ablation setting 7 with a NaCl setting of 1-2. Maximum 0.5 l NaCl was used for each procedure. All procedures were performed by the same operator (H.H.S.) whom had good previous experience in preclinical use of the plasma ablation technique and the WoundWand device, and had undergone theoretical and practical education in the clinical use of the technique.

Procedure details were recorded including treatment start and stop time (when probe activated and removed from wound), equipment settings, and comments on device performance/ease of treatment. Immediately prior to debridement, immediately post debridement and at follow-ups, wound status was examined, the wound was digitally photographed (Canon PowerShot G12, Canon Svenska AB, Solna, Sweden) along with a metric scale and wound swabs were obtained. One wound swab was obtained for quantitative bacteria analysis using the Levine's technique for quantitative culture(68) and immersed in 1 ml of PBS and vortex mixed for homogenization. The sample was serially diluted and plated on non-selective blood agar plates (Clinical Microbiology, Sahlgrenska University Hospital, Sweden) which were incubated aerobically at 37°C for 24 hrs. Numbers of colonies were manually counted and Log CFU/ml was calculated. One standard wound swab for qualitative bacteria analysis of bacteria strains was also obtained. After the treatment, in accordance with standard wound care procedures for venous ulcers, the wound edges were rubbed with zinc-paste, the wound was dressed with a standard hydrofibre dressing (Aquacel®, ConvaTec, Bromma, Sweden)

and covered with a Mepore® Pro wound bandage (Mölnlycke Health Care AB, Sweden). The leg was dressed with a low or medium elastic compression bandage as deemed appropriate. The wounds were redressed three times per week during the course of the study with a hydrocolloid dressing or other dressing as considered appropriate depending on the status of the wound.

Follow-up

Follow-up measurements were performed at week 2, 4, 6 and 8 after debridement. Patient questionnaires at follow-up included VAS for leg pain and wound pain, and patient perceived treatment effect on a six grade scale from very dissatisfied to very satisfied. Concomitant medication including analgesics, topical corticosteroids, non-steroidal anti-inflammatory drugs, antibiotics and antimycotics the subject had used were recorded.

Information on complications and adverse events were collected from the baseline visit over the duration of the study. Event, date of onset, severity, duration, and relationship to device was recorded.

Statistical analysis

All data were analysed using R version 3.0.3 (The R Foundation for Statistical Computing, Vienna, Austria) and Photoshop CS4 (Adobe, San Jose, CA, USA). Wound contours were marked on the digital photos using Photoshop and the areas were calculated using R. Wilcoxon's signed rank test was used for pairwise comparisons comparing pre-treatment wound areas and the areas at 2, 4, 6 and 8 weeks post treatment as well as for comparing pre-treatment and post-treatment quantitative bacteria amount. All tests are two-sided and $P<0.05$ was considered as statistically significant. Qualitative bacteria results were not statistically processed.

3.6 Ethical approvals

Paper I

All procedures involving human samples in the study presented in paper I was approved by the Regional Ethical Review Board at the University of Gothenburg.

Paper II, III and IV

For the studies presented in paper II, III and IV, no ethical approval was required since the study did not involve patients, patient data or live animals. Regarding paper III and IV, in accordance with local and national regulations, no ethical application or approval is necessary with regards to

research conducted on organs or body parts originating from already dead animals (Dept. for Animal Welfare and Health, The Swedish Board of Agriculture).

Paper V

The design of the study presented in paper V was reviewed and approved by the Regional Ethical Review Board at the University of Gothenburg. Participants received oral and written information about the study before their written free and informed consent was obtained.

4 RESULTS

4.1 Paper I

Live/Dead imaging

Live/Dead staining of the plasma ablation exposed surfaces showed a well-defined local margin of cell death both in the alginate gels and in the human cartilage biopsies (Figure 5). The cell death reached 150-200 microns deep in both the alginate gels and the biopsies (Figure 5A and B for gels and D and E for biopsies). Underlying tissue seemed viable and undamaged as determined by the staining. The control samples showed no increase in cell death (Figure 5 C for gel and F for biopsy).

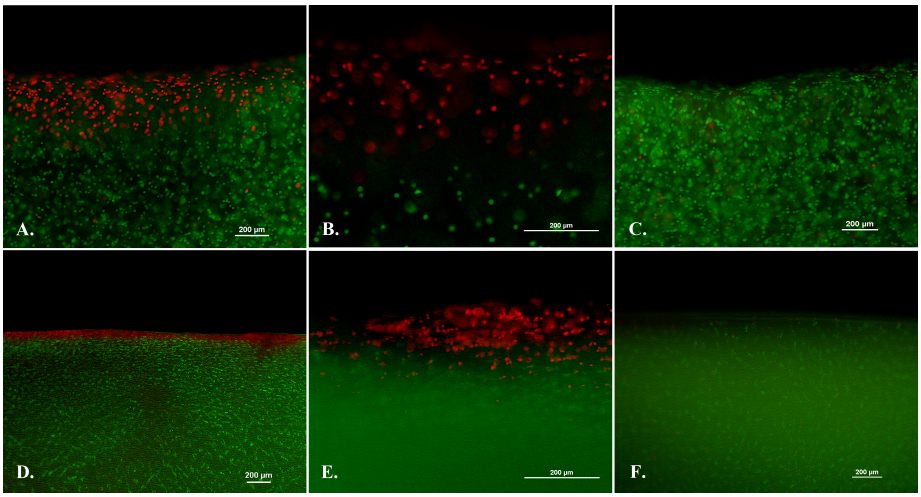

Figure 5. Live/Dead images of human chondrocyte alginate gel cultures and cartilage biopsy specimens exposed to plasma ablation; live cells stained green and dead cells red. (A) Alginate gel culture exposed to Paragon T2 probe showing a defined band of dead cells, 150 to 200 µm wide, close to the exposed area (original magnification X4). (B) Area of gel exposed to Paragon T2 probe (original magnification X10). (C) Non-exposed control gel showing no cell death (original magnification X4). (D) Cartilage biopsy specimen exposed to Paragon T2 probe showing a defined band of dead cells, 150 to 200 µm wide, close to the exposed area (original magnification X2). (E) Area of biopsy specimen exposed to Paragon T2 probe (original magnification X10). (F) Non-exposed control biopsy specimen showing no cell death (original magnification X2) (Enochson et al. Arthroscopy, 2012 Sep;28(9):1275-82).

Cell proliferation

A significant increase in proliferation was discovered in the plasma ablation-exposed group 3 days after exposure, with 51% more relative DNA-amount in the exposed samples compared to the control group ($p < 0.043$). 6 days after exposure the difference was no longer significant, even though the trend was still the same (Figure 6).

Gene expression

The chondrocytes in the gels showed a significant increase in IL-6 expression 3 and 6 days after exposure to plasma ablation compared to the control group. The IL-6 expression at day 3 increased with 70.1 % ($p < 0.020$), and with 56.4 % at day 6 ($p < 0.045$) (Figure 7). The cells also showed a significant increase in expression of IL-8 at 3 days after exposure compared to the control group ($p < 0.048$). There was no significant difference at 6 days after exposure, compared to the control group. IL-8 increased with 31.6 % at day 3 compared to control group (Figure 8).

IL-1β and TNF-α were not detected in the samples, and there was no significant difference between exposed samples and control for HMGB1 (Day 3 increase 7.2 %, $p < 0.45$, day 6 increase 5.8 %, $p < 0.56$). Neither could any effect be detected on the matrix degrading component MMP-13 (Day 3 decrease 3.5 %, $p < 0.76$, day 6 increase 5.1 %, $p < 0.59$) or the matrix components collagen type II (Day 3 increase 8.3 %, $p < 0.33$, day 6 decrease 4.5 %, $p < 0.49$) and versican (Day 3 decrease 6.7 %, $p < 0.12$, day 6 decrease 8.9 %, $p < 0.24$). All compared to control.

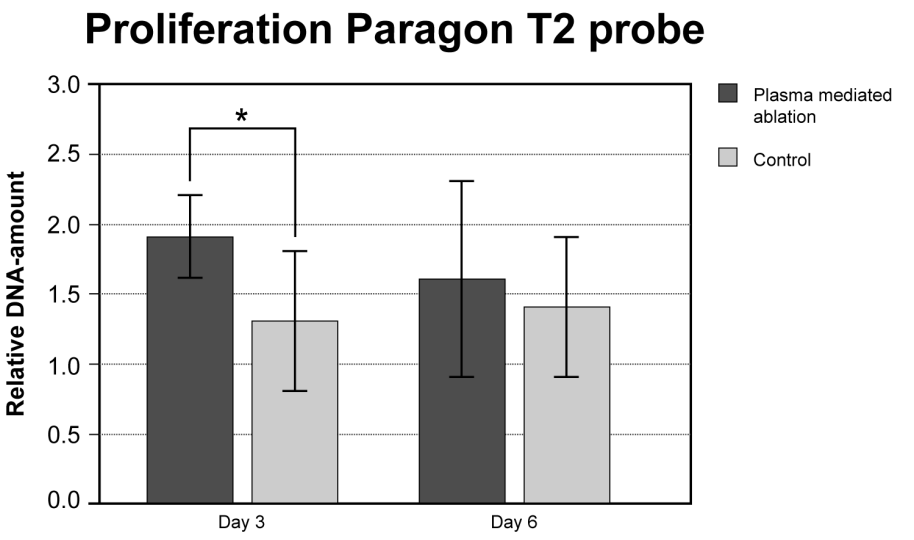

*Figure 6. A significant increase in proliferation with chondrocytes in alginate gel culture was detected 3 days after exposure to plasma ablation with the Paragon T2 probe (*p < 0.043) (Enochson et al. Arthroscopy, 2012 Sep;28(9):1275-82).*

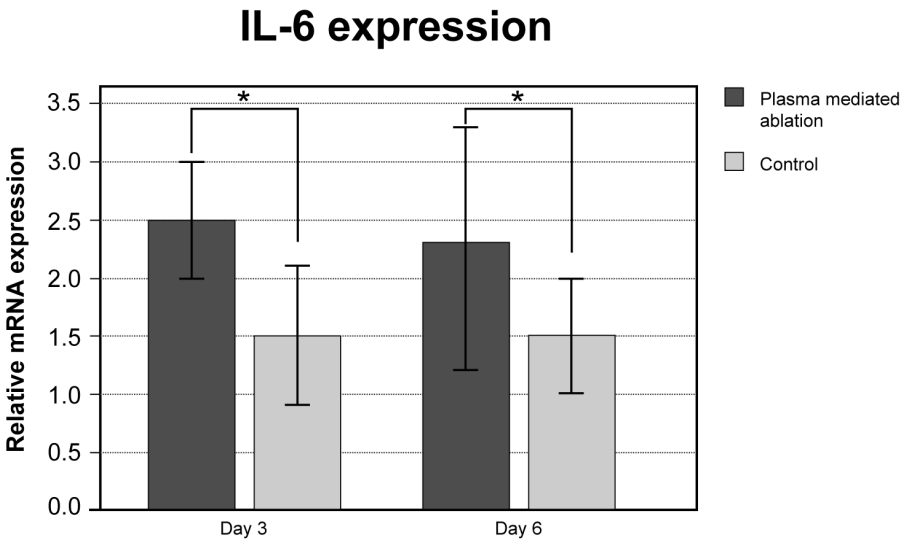

*Figure 7. After exposure to plasma ablation the chondrocytes significantly increased expression of IL-6. The increase was still significant 6 days after exposure (*p < 0.020 at day 3 and p < 0.045 at day 6) (Enochson et al. Arthroscopy, 2012 Sep;28(9):1275-82).*

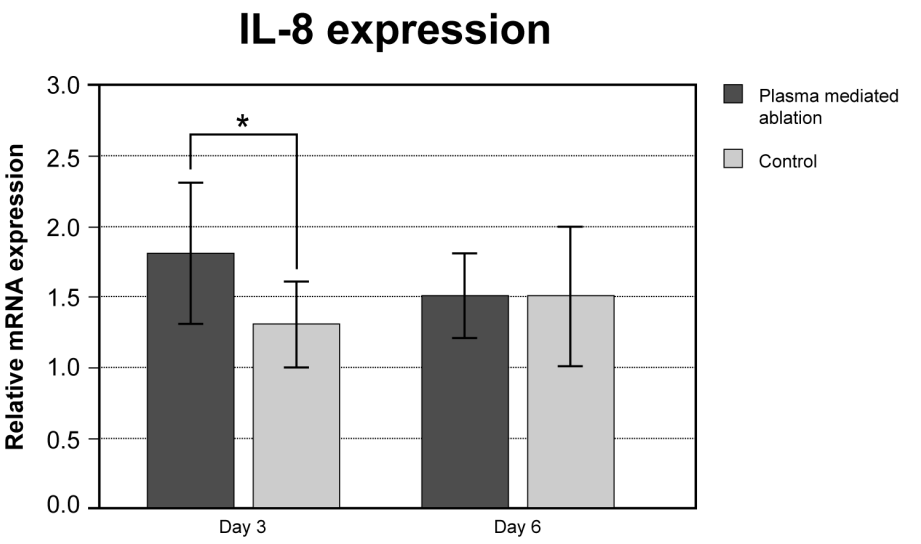

*Figure 8. After exposure to plasma ablation the chondrocytes significantly increased expression of IL-8, the increase was reduced to baseline levels at 6 days after exposure (*p < 0.048) (Enochson et al. Arthroscopy, 2012 Sep;28(9):1275-82).*

4.2 Paper II

The plasma ablation exposure had a direct microbicidal effect on all tested strains. Already at 500 ms plasma ablation there were significant decreases in bacteria/fungi counts compared to both untreated control and 500 ms TC for all strains.

For all strains tested there were significant reductions in CFU/ml for all Plasma ablation groups compared to untreated controls ($p < 0.0001$ for all comparisons). Plasma ablation also significantly reduced CFU/ml compared to each respective TC exposure time for all strains ($p < 0.001$ or less), except for *S. pyogenes* and *C. albicans* where significant differences could be seen only at 500-1000 ms exposure but not at 2000 ms (Figure 9 and Table 1).

The absolute reductions compared to untreated control, considering the LoQ, were for *S. aureus* 99.77 % at 500 ms and 99.98 % at 1000-2000 ms plasma ablation. For *S. pyogenes* the reductions were 99.87 % for all plasma ablation groups. The reductions for aerobically grown *E. coli* were 99.91 % at 500 ms, and 99.99 % at 1000-2000 ms plasma ablation. *E. coli* grown in anaerobic conditions showed reductions of 94.33 % at 500 ms, and 99.98 % at 1000-2000 ms plasma ablation. For *P. aeruginosa* the reductions were 99.52 % at

500 ms, 99.99 % at 1000 ms and 99.97 % at 2000 ms plasma ablation. With *C. albicans* the reductions were 99.73 % at 500 ms, and 99.92 % at 1000-2000 ms plasma ablation. Compared to untreated control mean log reductions reached 4-5 log for all strains and results were consistent between strains (Figure 10).

Results from the exposure time measurements of the Quantum generator showed differences of no more than three ms between set and measured activation times (Table 2). The temperature measurements showed that total temperature increase did not differ significantly ($p > 0.05$) between the two modes of activation used for plasma ablation and TC for any of the exposure times (Figure 11).

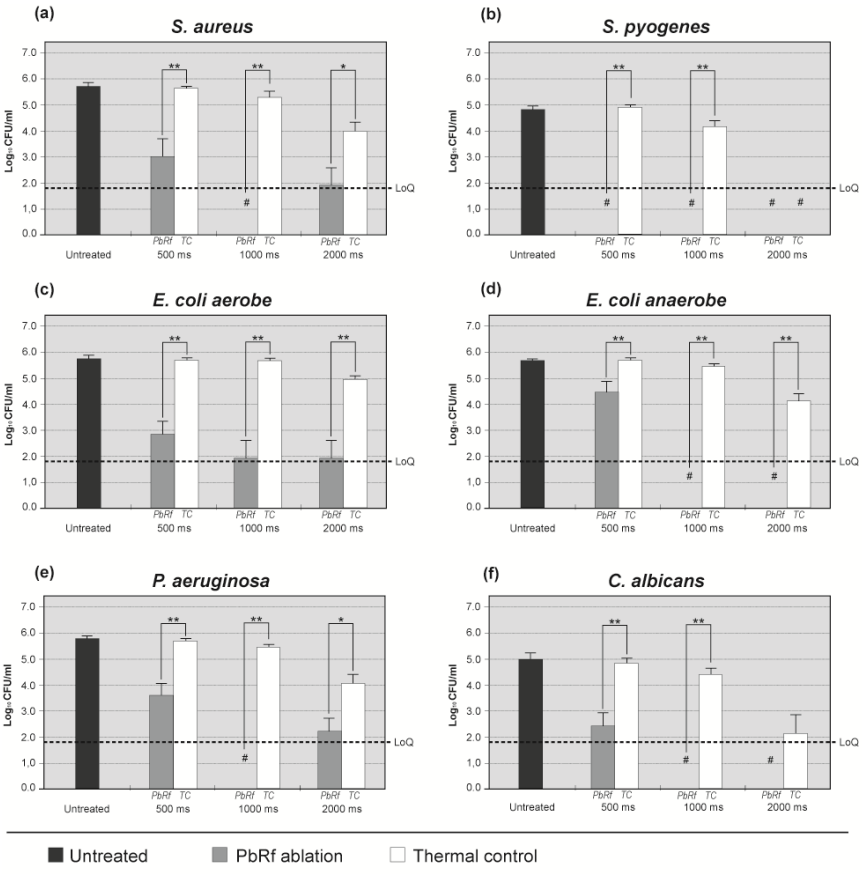

*Figure 9. The charts show the mean amount of CFU/ml + STD on a log scale for the different exposure times and strains. The dotted lines indicate the limit of quantification (LoQ) of 1.92 log CFU/ml for the study setup. In groups indicated with # no CFU were detected. *p < 0.001 and **p < 0.0001. PbRf; Plasma-mediated bipolar radiofrequency (Sönnergren et al. Acta Derm Venereol. 2012 Jan;92(1):29-33).*

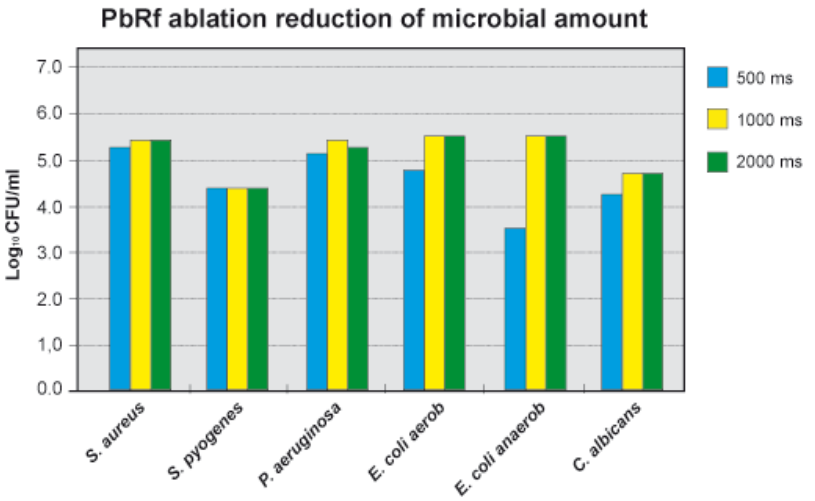

Figure 10. The chart depicts the mean log reduction in microbial amount calculated as the difference between untreated control and each respective plasma ablation exposure time, considering the limit of quantification. PbRf; Plasma-mediated bipolar radiofrequency (Sönnergren et al. Acta Derm Venereol. 2012 Jan;92(1):29-33).

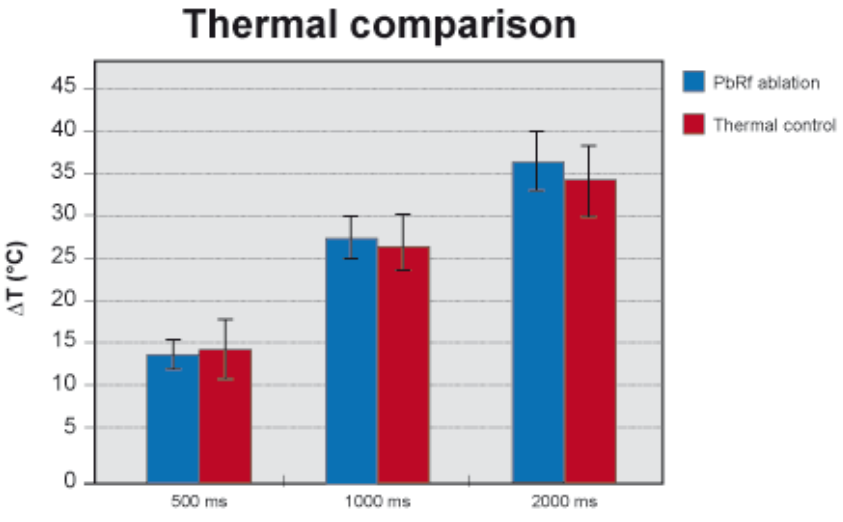

Figure 11. Temperature increase in 100µL of saline with a mean starting temperature of 25°C. Total temperature increase did not differ significantly between the two modes of activation used for plasma-mediated bipolar radiofrequency ablation (PbRf ablation) and thermal control for any of the exposure times.

Table 1. Statistical results as p-values for each comparison

	Thermal control (ms)			Untreated control
	500	1000	2000	
S. aureus				
500 ms Plasma	<0.0001	<0.0001	0.0032	<0.0001
1000 ms Plasma	<0.0001	<0.0001	0.00046	<0.0001
2000 ms Plasma	<0.0001	<0.0001	0.00015	<0.0001
Untreated control	0.00090	0.0044	<0.0001	
S. pyogenes				
500 ms Plasma	<0.0001	<0.0001	1	<0.0001
1000 ms Plasma	<0.0001	<0.0001	1	<0.0001
2000 ms Plasma	<0.0001	<0.0001	1	<0.0001
Untreated control	0.037	<0.0001	<0.0001	
E. coli aerobe				
500 ms Plasma	<0.0001	<0.0001	<0.0001	<0.0001
1000 ms Plasma	<0.0001	<0.0001	<0.0001	<0.0001
2000 ms Plasma	<0.0001	<0.0001	<0.0001	<0.0001
Untreated control	0.0060	<0.0001	<0.0001	
E. coli anaerobe				
500 ms Plasma	<0.0001	<0.0001	0.34	<0.0001
1000 ms Plasma	<0.0001	<0.0001	<0.0001	<0.0001
2000 ms Plasma	<0.0001	<0.0001	<0.0001	<0.0001
Untreated control	0.032	<0.0001	<0.0001	
P. aeruginosa				
500 ms Plasma	<0.0001	<0.0001	0.010	<0.0001
1000 ms Plasma	<0.0001	<0.0001	<0.0001	<0.0001
2000 ms Plasma	<0.0001	<0.0001	0.00085	<0.0001
Untreated control	0.0026	<0.0001	<0.0001	
C. albicans				
500 ms Plasma	<0.0001	<0.0001	0.48	<0.0001
1000 ms Plasma	<0.0001	<0.0001	1	<0.0001
2000 ms Plasma	<0.0001	<0.0001	1	<0.0001
Untreated control	0.0027	<0.0001	<0.0001	

Table 2. Measured exposure times of the Quantum generator

Set time (ms)	Measured time (ms)	
	Plasma ablation mode	Coagulation mode
500	499 ± 1	499 ± 0
1000	1000 ± 1	999 ± 1
2000	2000 ± 0	1997 ± 2

4.3 Paper III

Bacteria aerosol and wound bed bacteria results

The active air sampling (Figure 12) showed significantly higher ($p < 0.01$) bacterial counts for hydrosurgery default and max settings compared to both negative and untreated controls. The bacterial counts for hydrosurgery were initially markedly higher than for other groups, and subsequently went down at 15, 30 and 60 minutes but kept being the highest counts throughout the measurement period. Compared to the negative control, hydrosurgery increased the air bacterial amount with up to 33.000%. Also the passive sampling of bacterial air fallout (Figure 13) was significantly higher ($p < 0.05$) for hydrosurgery default and max settings compared to negative and untreated positive controls.

Curette debridement, plasma ablation default and plasma ablation max groups did not show any significant difference in active or passive sampling compared to the controls.

For the wound bacterial load (Figure 14 and Figure 15) plasma ablation default setting significantly reduced the bacterial counts compared to control wounds as measured by both cylinders scrubs ($p = 0.026$) and swabs ($p = 0.013$). Plasma ablation max significantly reduced the bacterial counts compared to control wounds as measured by swabs ($p < 0.0001$) but not cylinders scrubs. Curette, hydrosurgery default, and hydrosurgery max did not significantly reduce the wound bacteria counts.

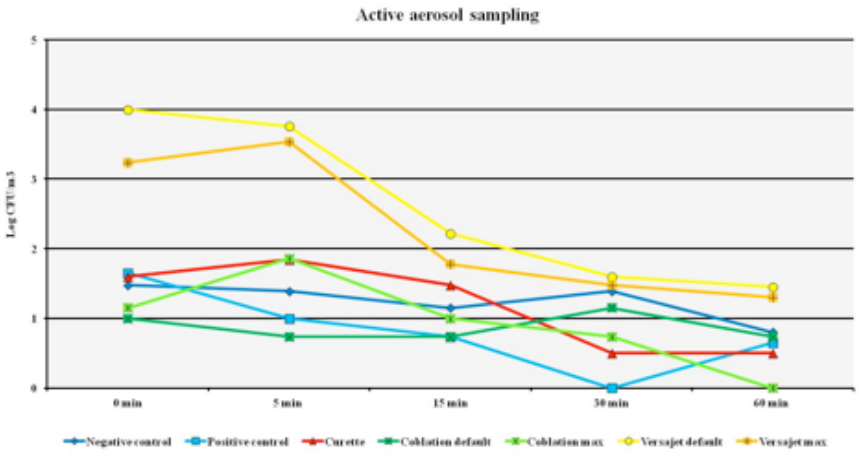

Figure 12. The bacterial amount in the air during and after debridement measured with the Sartorius MD8 Airscan (Sönnergren et al. J Hosp Infect. 2013 Oct;85(2):112-7).

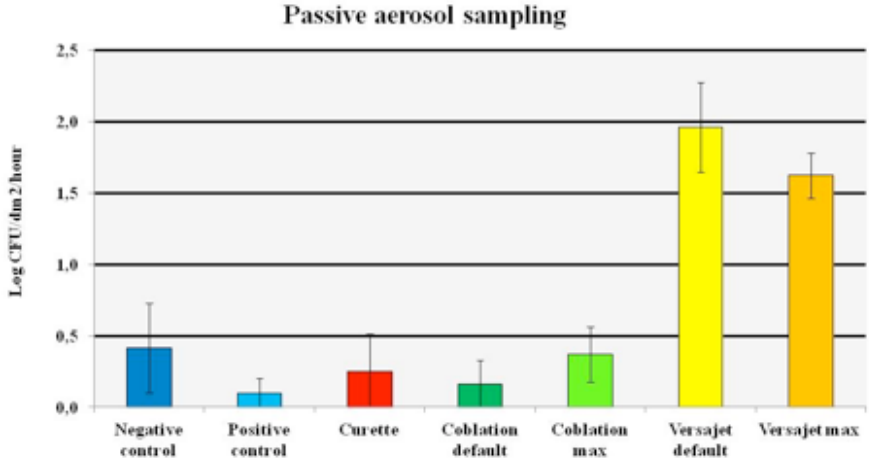

Figure 13. The bacterial fallout on settle plates for the duration of 60 minutes during and after debridement (Sönnergren et al. J Hosp Infect. 2013 Oct;85(2):112-7).

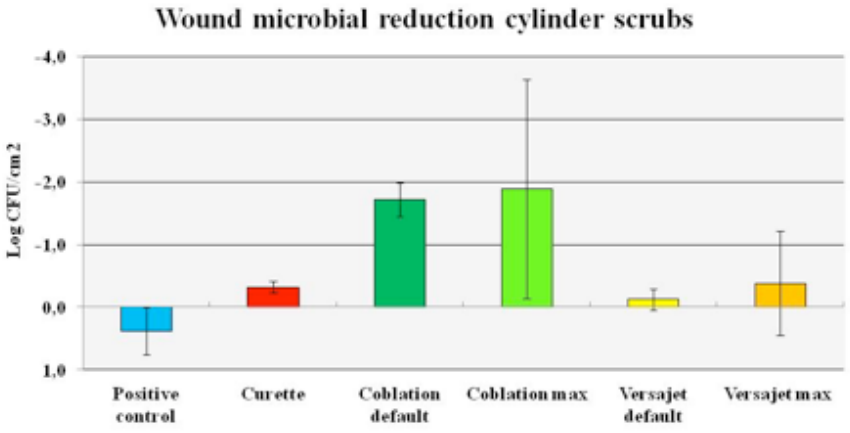

Figure 14. Difference in wound bed bacterial counts at post incubation and post debridement (or respective time point for Positive control) as measured by cylinder scrubs (Sönnergren et al. J Hosp Infect. 2013 Oct;85(2):112-7).

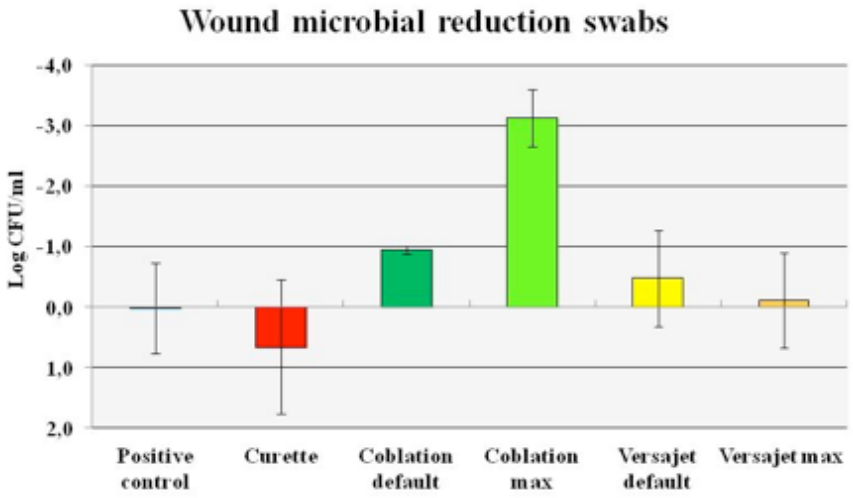

Figure 15. Difference in wound bed bacterial counts at post incubation and post debridement (or respective time point for Positive control) as measured by swabs (Sönnergren et al. J Hosp Infect. 2013 Oct;85(2):112-7).

Histological results

At baseline, before *S. aureus* inoculation, bacteria could not be detected in any of the samples. Post incubation, bacteria were present in all samples in focal clusters and/or diffuse layers, with deep tissue involvement in the majority of samples. At post debridement the bacterial presence was reduced in 100% of plasma ablation default samples and 50% of plasma ablation max samples, and no focal clusters or diffuse layers of bacteria were observed (Figure 16 c-d). In the Control, Curette, hydrosurgery default and hydrosurgery max groups bacteria were still present in all samples in the form of diffuse layers and/or focal clusters (Figure 16 a, b, e, f). Measurements of thickness of bacteria clusters/layers showed reductions in thickness for plasma ablation default and plasma ablation max after debridement. The Control, Curette, hydrosurgery default and hydrosurgery max groups either increased in bacteria cluster/layer thickness or showed no evident difference between post incubation and post debridement.

Plasma ablation default, plasma ablation max and hydrosurgery default showed reduced bacterial penetration depth after debridement. The Control and Curette groups showed increased penetration depth post debridement compared to post incubation.

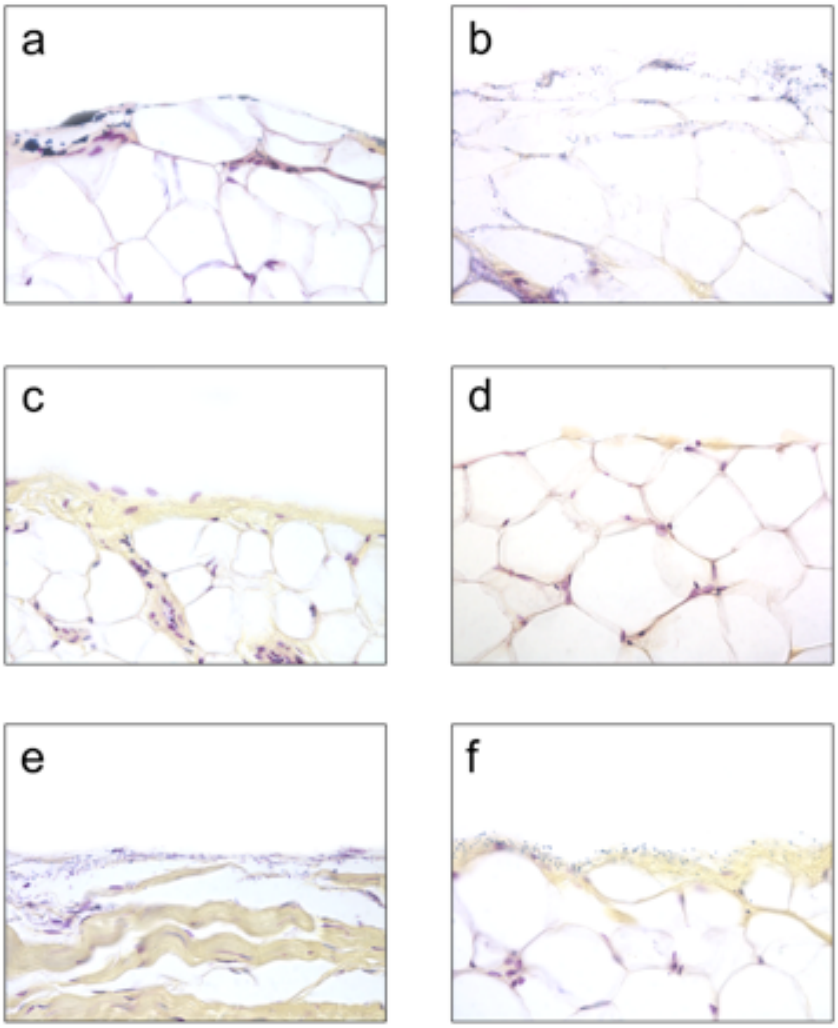

Figure 16. Representative photos of the histological slides, all from post debridement at 520x original magnification. a) Control wound with bacteria in focal clusters and diffuse layers. b) Curette debrided wound with bacteria in diffuse layers. c) Plasma ablation default setting debrided wound with no visible bacteria. d) Plasma ablation max setting debrided wound with only single bacteria present but no focal clusters or diffuse layers. e) Hydrosurgery default setting debrided wound with bacteria in diffuse layers. f) Hydrosurgery max setting debrided wound with bacteria in diffuse layers (Sönnergren et al. J Hosp Infect. 2013 Oct;85(2):112-7).

4.4 Paper IV

Wound bed bacteria and bacteria aerosol results

At baseline, before *S. aureus* inoculation, the wounds had very low bacterial counts with 0.9±1.1 log CFU/ml, compared to 9.8±1.2 log CFU/ml post incubation. For the wound bacterial load after debridement (Figure 17), plasma ablation default and max settings both significantly ($p < 0.0001$) reduced the bacterial counts compared to control wounds as measured by swabs. Hydrosurgery also gave a smaller but significant ($p = 0.04$) reduction in wound bacterial counts at the max setting. Curette and the hydrosurgery default setting did not significantly reduce the wound bacterial counts.

The active air sampling (Figure 18) showed significantly higher bacterial counts for hydrosurgery default ($p < 0.0001$) and max ($p = 0.0003$) settings compared to both negative and untreated controls. The bacterial counts for hydrosurgery were initially markedly higher than for other groups, and subsequently went down at 15, 30 and 60 minutes. However, hydrosurgery default had the highest counts at each time point throughout the measurement period. For measurements at 0 minutes, the majority of both hydrosurgery default and hydrosurgery max bacteria plates were too numerous to count in terms of CFU, and the CFU number was thus assessed to be at least 1000/plate. Compared to the controls, hydrosurgery increased the air bacterial amount with up to at least 20.000 %. Also, the passive sampling of bacterial air fallout (Figure 19) was significantly higher for hydrosurgery default ($p = 0.0002$) and max ($p = 0.002$) settings compared to negative and untreated positive controls.

Curette debridement, plasma ablation default and plasma ablation max groups did not show any significant difference in active or passive sampling compared with the controls.

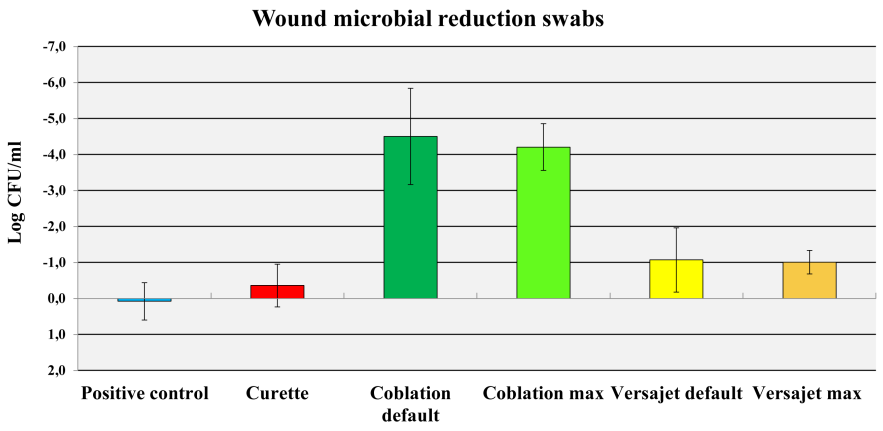

Figure 17. Difference in wound bed bacterial counts post incubation and post debridement as measured by swabs (Sönnergren et al. Acta Derm Venereol. 2015 Mar 9;95(2):272-7).

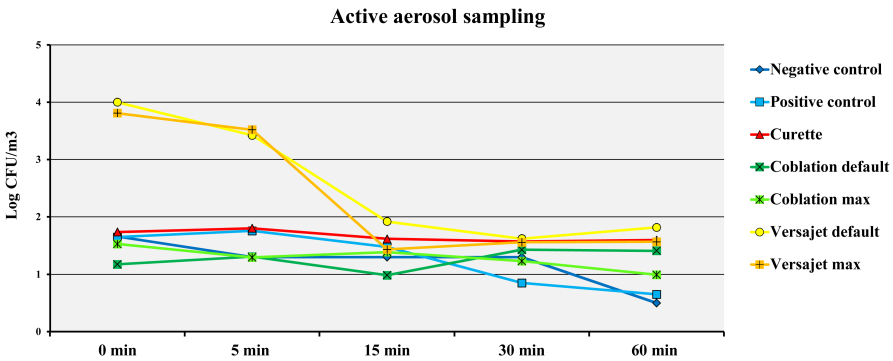

Figure 18. Bacterial amounts in the air during and after debridement as measured with the Sartorius MD8 Airscan (Sönnergren et al. Acta Derm Venereol. 2015 Mar 9;95(2):272-7).

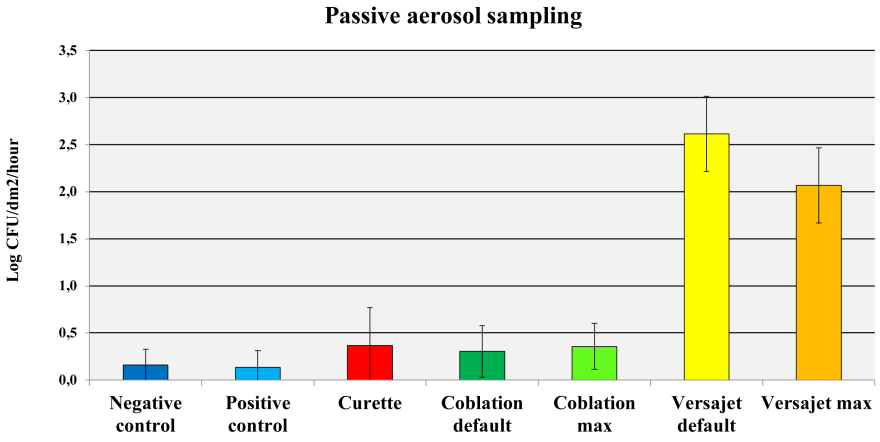

Figure 19. Bacterial fallout on settle plates for a duration of 60 minutes during and after debridement (Sönnergren et al. Acta Derm Venereol. 2015 Mar 9;95(2):272-7).

Histology results

At baseline, before *S. aureus* inoculation, bacteria could not be detected in any of the samples. Post incubation, bacteria was present in all samples in focal clusters and in 58 % of samples also in diffuse layers, with deep tissue involvement in 25 % of the samples. Post debridement, all plasma ablation default samples had focal bacteria clusters but 50 % showed no diffuse bacteria layers. 50 % of plasma ablation max samples had no detectable bacteria (Figure 20 b), and 50 % showed only focal bacteria clusters. No plasma ablation treated samples showed any deep tissue bacteria. In the Curette, hydrosurgery default and hydrosurgery max groups bacteria were still present in all samples in the form of both diffuse layers and focal clusters (Figure 20 a and c). In the Control group, all samples had focal bacteria clusters but 50 % of samples had no diffuse bacteria layers. Measurements of bacterial penetration depth showed either increased bacterial penetration depth or no clear difference between post incubation and post debridement for the Control, Curette, hydrosurgery default and hydrosurgery max groups.

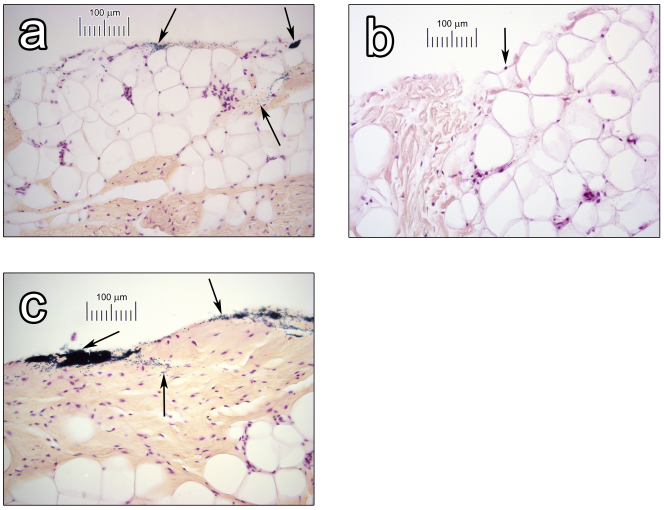

Figure 20. Representative photos of histological slides, all taken using a 20 x objective lens. a) Curette debrided wound with bacteria present in diffuse layers (left arrow), focal clusters (right arrow) and in deep tissue (bottom arrow). b) Plasma ablation max debrided wound with no visible bacteria (only fibroblast cells are visible (arrow)). c) Hydrosurgery (max setting) debrided wound with bacteria present in focal clusters (left arrow), diffuse layers (right arrow) and in deep tissue (bottom arrow) (Sönnergren et al. Acta Derm Venereol. 2015 Mar 9;95(2):272-7).

Biofilm results

The SEM analysis of the wound surface at baseline before bacteria inoculation showed a dense meshwork of collagen fibre bundles partly covered by flattened cell profiles in the low power micrograph (Figure 21 a), the collagen arrangement is enlarged in (Figure 21 b). No biofilm forming bacteria were identified in the baseline samples. The SEM analysis of *S. aureus* inoculated specimens showed a dense growth of both staphylococci-like bacteria and also of coli-like bacteria, morphologically most likely *E. coli,* on the specimen surface. Thus, a mixed population of bacteria was found, forming large aggregates that could reveal curli formation (75) compatible with biofilm development (Figure 21 c).

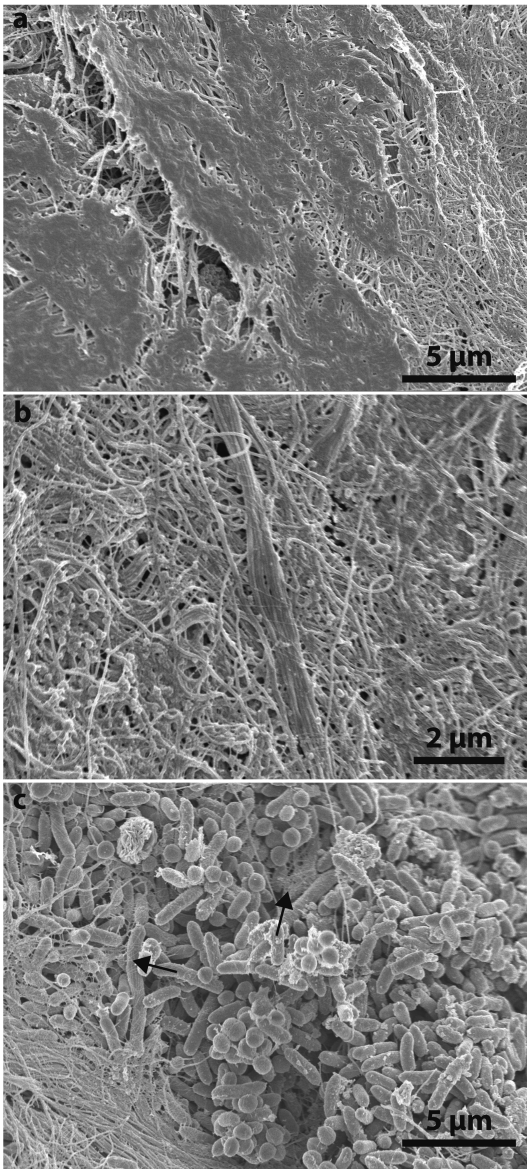

Figure 21. Mount of scanning electron micrographs before (a, b) and after (c) bacteria inoculation. In (a) flattened, slightly elongated cells with an irregular contour cover partly a dense collagenous meshwork which is depicted at a higher magnification of a nude area in (b). A large colony of bacteria cover the wound surface in (c). Both spherical and elongated bacterial cells are identified indicating a mixed growth of added Staphylococci and a contaminating growth of coli-like

species. Arrows indicate regions of biofilm character with fine meshworks of filaments connecting bacteria (visible only after enlargement of digital image file) (Sönnergren et al. Acta Derm Venereol. 2015 Mar 9;95(2):272-7).

4.5 Paper V

Baseline characteristics

89 patients were screened for inclusion in the study. A total of 10 patients, mean age 65 ± 11 years old, with 17 leg ulcers met all inclusion and none of the exclusion criteria and were included in the study. All included patient ulcers were treated according to protocol. One patient missed one follow-up. The patients had some degree of cardiovascular and other comorbidities (Table 3). The current venous ulcers had been present for a mean of 23.3 ± 21.6 months. All patients had undergone a number of prior standard treatments for the ulcer which for all patients included compression therapy with pressure bandages, leg elevation, wound cleansing or debridement and oral antibiotics, and in some cases also included vascular venous surgery, intermittent pneumatic compression therapy, or plastic surgery with skin transplant.

Table 3. Characteristics of the 10 subjects at the time of enrolment

Characteristic		N (%) or Mean ± SD
Male sex		6 (60%)
Age (years)		65 ± 11
Time of wound presence (months)		23.3 ± 21.6
BMI		28.5 ± 6.6
ABPI		1.1 ± 0.2
Medical history		
Dermatological	Contact dermatitis	1
	Eczema	1
Infections (excl. wound infections)	Erysipelas	1
	Hepatitis C	1
	Pneumonia	1
Musculoskeletal	Osteoarthritis	3
	Disc hernia	1
	Fractures	1
	Knee surgery	1
	Lumbago	1
	Spinal surgery	1
Cardiovascular	Atrial fibrillation	2
	CHF	1
	DVT	1
	Hypertension	2
	MI	1
	Stroke	1
	Venous surgery	4
Pulmonary	Asthma	1
Neurological		0
Psychiatric	Depression	3
Allergical	Various antibiotics	4
Haematological		0

ABPI; ankle-brachial pressure index. CHF; chronic heart failure.

DVT; deep vein thrombosis. MI; myocardial infarction.

Procedure details

The procedure time was generally short ranging between 4-15 min. The treatments were for all cases deemed to be easy to perform and gave a result with a clean wound bed with no rests of fibrin or necrotic tissue and a wound bed punctuate bleeding as desired by the operator.

Wound closure and wound size

The wound size was immediately prior to treatment 6.90 ± 7.16 cm^2 and was marginally increased by debridement ($p = 0.50$). Compared to before debridement the wound area was significantly reduced with a mean of 44.1 % to 3.86 ± 5.06 cm^2 at 8 weeks follow-up and 2 of 17 ulcers healed within 8 weeks. The wound area was significantly reduced at each follow up compared to before debridement ($p = 0.0002$ at 2 weeks, $p = 0.03$ at 4 weeks, $p = 0.0001$ at 6 weeks, $p = 0.001$ at 8 weeks) (Figure 22).

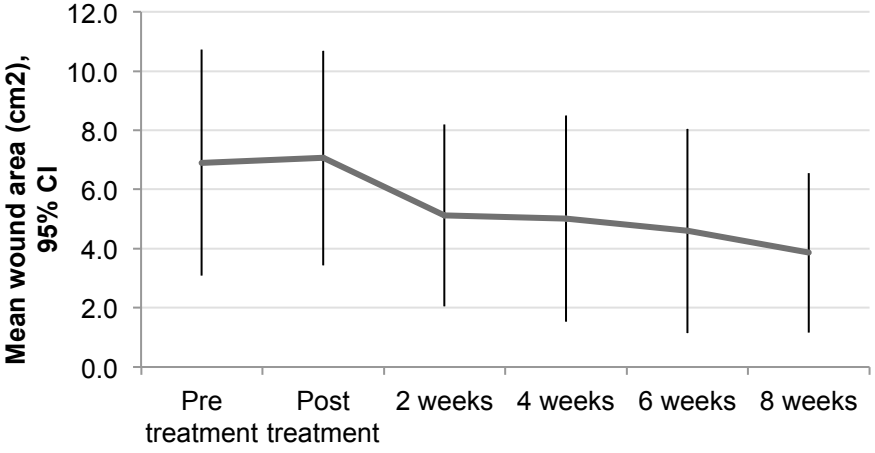

Figure 22. The mean ± 95 % confidence interval wound area before and after treatment and at each follow-up. Pre treatment is the area directly before plasma ablation debridement and post treatment is directly after debridement. The wound area was significantly reduced at each follow up compared to before debridement ($p = 0.0002$ at 2 weeks, $p = 0.03$ at 4 weeks, $p = 0.0001$ at 6 weeks, $p = 0.001$ at 8 weeks).

Wound bed bacteria results

The wound quantitative bacteria load was immediately prior to treatment 5.4 ± 1.1 log CFU/ml and was significantly reduced with 1.5 log CFU/ml ($p = 0.0002$). However, at follow-up measurements, the wound bacteria load had risen again (Figure 23). One patient had no bacteria growth in the quantitative or qualitative cultures before or after treatment and at the majority of follow-ups and was thus excluded from the quantitative bacteria analysis. The qualitative bacteria measurements showed that wounds were colonized with a number of different bacteria species both prior to treatment and at follow-ups; the most common species being *S. aureus*, *P. aeruginosa* and *Enterobacter spp.* No multi-resistant strains were detected in the wound cultures. The numbers of bacteria species were immediately post treatment reduced in 5 of 17 wounds, however no systematic and lasting reduction in bacteria species could be seen at follow-up (Table 4).

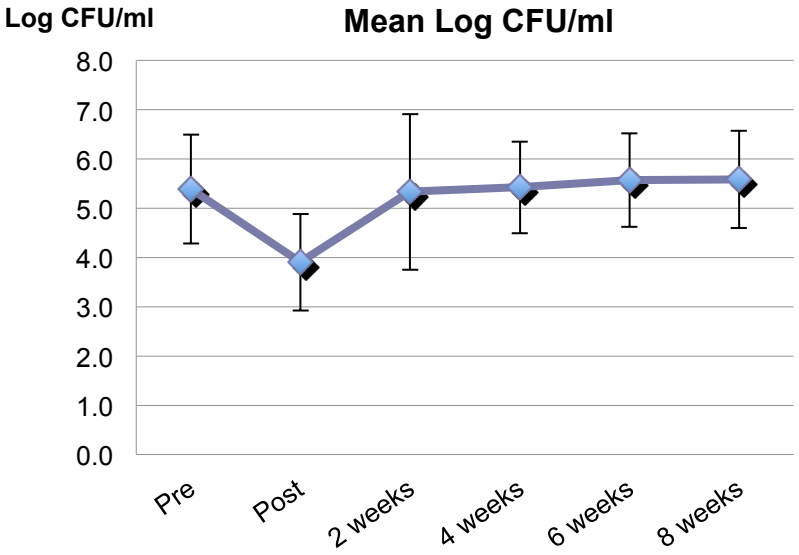

Figure 23. The mean ± SD quantitative bacteria amount before and after treatment and at each follow-up. Pre treatment is the bacteria amount directly before plasma ablation debridement and post treatment is directly after debridement. The wound quantitative bacteria load was significantly reduced with 1.5 log CFU/ml ($p = 0.0002$) after debridement.

Table 4. Microbiological qualitative data for the 17 plasma ablation treated venous wounds at each follow-up

Patient (wound)	Pre treatment	Post treatment	2 weeks	4 weeks	6 weeks	8 weeks
1	1;2;3	1;2;3	1;2;3	1;3;5	1;2;3	1;2;3
2	1;6;7	1	1;6;8;9	1;6;8	1;6;9	1;6;9
3(1)	1;2;4;6;11	1;2;4;6;11	1;2;4;12	1;2;4;11;13	1;2:11:13	1;4;11
3(2)	1;4;6;11	1;4;6;11	1;4;11	1;2;11;13	1;2;4;13	1;2;4;9;11
4(1)	4;9;14;15	14;15	4;14;15;16	3;4;6;8;9; 14;15	1;4;14;15	4;14;15; 16;19
4(2)	4;9;14;15	4;9;14;15	4;12;14;16	3;4;6;8;9; 14;15	1;14;15	12;14;15; 16;18
4(3)	4;9;14;15	4;9;14;15	4;14;15	3;4;14;15	4;14;15	1;14;15; 16;17
4(4)	4;9;14;15	4;9;14;15	4;14;15;16	4;8;9;14;15	4;14;15	14;15;16
4(5)	9;15	4;8;9;14;15	4;14;15;16	H	H	H
5	1	1	1	1;10;16	1;9	H
6	1;14	14	1;14;20	1;14;20	1;14	14
7	1;4;14	1;4;14	1;4	1;14	1	4;6;9;14
8(1)	6;9;15;21	6;9;11;21; 22	3;10;11;21	3;6;10;11	NA	3;6;15;16
8(2)	6;9;11;15	6;11;15	3;10;11;21	3;6;10;11	NA	3;6;11;15; 16
8(3)	6;9;15;21	6;9;15;21	3;10;11;21	3;6;10;11	NA	1;3;6;11; 15;16
9	8	8	6;8	8	1;8	1;8
10	0	0	0	0	1	1

Bacteria no

1=*Staphylococcus aureus*	9=*Enterococci spp.*	17=*Escherichia hermannii*
2=*Streptococcus Group B*	10=*Alpha-hemolytic streptococcus*	18=*Brevibacterium casei*
3=*Escherichia coli*	11=*Morganella morganii*	19=*Mixed gram-negative rods*
4=*Pseudomonas aeruginosa*	12=*Klebsiella spp.*	20=*Streptococcus Group G*
5=*Streptococcus Group C*	13=*Mixed anaerobe spp.*	21=*Stenotrophomonas maltophilia*
6=*Coagulase-negative staphylococcus*	14=*Proteus mirabilis*	22=*Pseudomonas stutzeri*
7=*Mixed gram-positive spp.*	15=*Enterobacter cloacae*	
8= *Coryneform bacilli* (‘diphtheroids’)	16=*Enterococcus faecalis*	

H; Healed. NA; Data not available.

Wound and leg pain

The patients generally had some wound related pain and some general leg pain at baseline which both had a decreasing trend during the course of the study (Figure 24). The patient use of pain medication for wound pain was slightly decreased during the study. At baseline 6 patients had non-prescription analgesics, 2 had prescription non-narcotics, and 6 had narcotic pain medication, with a mean of 4.7 tablets/day. At final follow-up 5 patients had non-prescription analgesics, 3 had prescription non-narcotics, and 4 had narcotic pain medication, with a mean of 3.4 tablets/day.

The patients were generally satisfied with the treatment effect with 80 % very satisfied and 20 % satisfied.

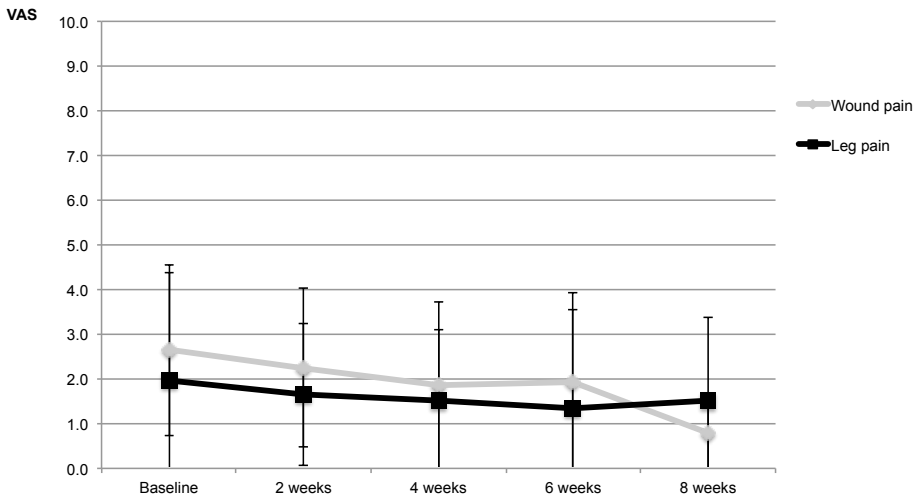

Figure 24. Mean visual analogue scale (VAS) for wound related pain and general leg pain before treatment and at each follow-up. Baseline VAS was recorded prior to the plasma ablation debridement. No major increase or decrease in patient-perceived pain could be detected though there was a trend towards decrease in both wound pain and leg pain during follow-up.

Adverse events

One device related adverse event in the form of increased wound pain during the first days after the procedure occurred during the study. However, this

increase in pain resolved without additional pain medication. One adverse event which was deemed to be non-device related had occurred in the form of a new foot wound on the heel of the foot on the opposite side of the treated leg at 8 weeks follow-up. No other adverse events or procedure related complications occurred. No wound infection signs were noted at follow-ups and no patient required systemic antibiotics for the treated wound during the course of the study.

5 DISCUSSION

5.1 Paper I

This is to our knowledge one of the first studies showing the biochemical effects of plasma ablation on human articular chondrocytes *in vitro*. In agreement with the study hypothesis, there was an increase in proliferation after exposure, which indicates that the plasma ablation has a direct biological effect on differentiated chondrocytes in alginate culture. The proliferation effect seemed to be transient, indicating that these effects are short term.

A previous *in vitro* study on the effect of plasma ablation on animal tissues was not been able to detect an increase in DNA-amount as an indicator of cellular proliferation.(61) In that study porcine intervertebral disc cells from young animals were used, and cultured in expansion media which likely would make an effect on cell proliferation more difficult to detect. In the current study, human articular cartilage chondrocytes were used which were differentiated in a chondrogenic media for 2 weeks before exposure, which is known to shift the cells from a dedifferentiated proliferative state to a differentiated and less proliferative state more similar to the *in vivo* situation.(76) Therefore, the current study could more easily detect alterations in the DNA-amount, and also better represent the human *in vivo* situation and the possible effect which plasma ablation has on human articular cartilage.

In the gel cultures and cartilage biopsies exposed to plasma ablation a well-defined band of dead cells was observed. These results are also in consistency with a previous study showing plasma ablation to induce a well defined margin of chondrocyte death in bovine cartilage.(58) The plasma ablation exposure thus induces a trauma resulting in an immediate cell death, but at a later stage induce a proliferative response. It has been shown in several tissues that trauma induces a compensatory proliferation as part of a regenerative tissue response.(77-79) Compensatory proliferation in cartilage is, however, poorly studied, but it seems intuitive that the observed proliferative effect in the current study might be induced as a compensatory proliferation. Cartilage tissue is known to have a limited capacity of self renewal,(12) and the triggered cell proliferation thus might support and improve a regenerative process which would have a positive effect on damaged cartilage after a cartilage trauma or in the early phase of cartilage degenerative disease.

Alterations in interleukin expression were detected after exposure. IL-1β and TNF were not affected, whilst both IL-6 and IL-8 were up-regulated. IL-6 has been reported both to increase and decrease chondrocyte proliferation,(80) but both IL-6 and IL-8 have been shown to induce mesenchymal stem cell proliferation in a dose dependent manner.(81) The up-regulation of these interleukins might also be the reason for the increase in proliferation.

The specific reason for the detected increase in IL expression was not investigated further in the current study. However, two likely reasons are either a primary effect of the plasma field, or secondary to the cell death induced by the plasma field. Inducing cell death in chondrocytes has been shown to increase the release of several interleukins.(80, 81)

The plasma field that is formed during ablation has been shown to generate reactive oxygen species, such as the hydroxyl radical and nitric oxide, and radicals have been shown to induce production of both IL-6 and IL-8 in human chondrocytes.(53, 82-84)

Within inflammatory diseases in general and in cartilage disease such as early osteoarthritis specifically, cell proliferation and altered interleukin expression is considered as a part of the inflammatory response.(7, 8) However, in the inflammatory process, IL-1β and TNF are the two most powerful mediators of inflammation (8-10) and in the current study; these interleukins were not up-regulated. This strengthens the theory that both IL-6 and IL-8 might in the current context serve as mediators of a regenerative response and that the response of the cells to the plasma exposure is anabolic and regenerative. The up-regulation of interleukins and the proliferatory response induced by the plasma ablation may thus indicate that the cells have reacted in an anabolic fashion to the exposure.

A weakness with the current study is that a cell culture never perfectly mimics the *in vivo* situation, and as with all *in vitro* studies conclusions for the clinical situation are limited. To be able to transfer the results to the *in vivo* situation, further studies are required. Another limiting factor is that only mRNA expression and not protein production was investigated. To better mimic the arthroscopic situation, a fluid flow over the exposed surface would have been preferable. The mechanical integrity of the gels did however not allow for the fluid flow in this setup. Further, it has been discussed if mounting the probe in a mechanical jig is better than applying it by free-hand as done in the current study.(58) That the current study was done using the probe at free-hand may have given inconsistencies in the results and make the setup less repeatable. However, it has been concluded that a free-hand

application better mimic clinical use and also gives a low result variability.(58)

5.2 Paper II

The results of the current study show that plasma ablation has a direct local microbicidal effect on the tested bacteria and fungi strains *in vitro* at exposure times of between 0.5 to 2 seconds.

The four tested bacteria strains are among the most frequently found in both infected and non-infected wounds and *S. aureus* is considered to be the most important pathogen.(85) Different *Candida* species have been concluded to be the most common fungus found in diabetic feet and *C. albicans* has by Hansson *et al.* been identified as the most frequently found fungus in several clinical leg ulcer studies.(28, 86-88) The choice to use 10^6 CFU/ml in this study was based on the established concept that a level of more than 10^5 or 10^6 CFU per ml or gram of tissue has been characterized as a clinically relevant colonisation of the wound bed and considered a key factor in wounds that fail to heal.(38, 68, 85, 89, 90)

Based on the comparison of the plasma ablation and TC mode of the device with regards to thermal increase, it can be concluded that the TC was a relevant thermal and energy control. For all strains the 500 ms plasma ablation was significantly more microbicidal than both 500 and 1000 ms TC. It can thus be concluded that the antimicrobial effect of the plasma ablation is not only thermal or due to the energy input in the suspension *per see* but instead associated with the plasma and its characteristics. At the longest TC exposure time a clear reduction in CFU/ml was seen for all strains as would be expected with the thermal increase in a small amount of fluid. It is well established that the different strains utilized are sensitive to short exposures of temperatures in the range of around 52-70°C.(91-95)

The hydroxyl radicals known to be produced by the plasma ablation (53, 84) do not only effect cells and tissues with induction of a cytokine response as discussed above, but are also well recognised to have direct bactericidal and fungicidal effects.(96, 97) There are also a number of other medical devices and techniques that are based on different forms of physical plasmas, primarily gas plasmas, which have been investigated in recent years. These techniques have been suggested for use in e.g. medical equipment sterilisation but also for use in wound care, and have been shown to directly destruct the bacterial cell wall. This could thus also be hypothesised as the potential mechanism of action of the plasma ablation.(97-101)

In the current study, both gram negative and gram positive bacteria as well as fungi were tested and in general the same microbicidal effect of the plasma ablation was seen. As a very similar decrease in CFU was seen for aerobically and anaerobically grown *E. coli,* the effect also seems to be independent of bacterial aerobic/anaerobic growth. The microbicidal effect could thus from these set of experiments be said to be general.

In the results section above and in the publication of Paper II it has been noted that the maximum microbial reduction achieved for the different strains was between 4.5 to 5.6 log. It should however be noted that these calculations are based on a LoQ of 1 CFU for all plates in one study group, since no serial dilution with plating of each dilution was performed in the study set up. In standard bacteriological experiments such a serial dilution with plating of each dilution is usually performed which give a more precise quantification of the bactericidal effect. In the current study only the 1/100 dilution was plated and presented, due to lack of resources. The precise estimate of the microbicidal effect for each strain in the study should thus be looked upon with this in consideration.

At comparing the results from the different studies in the thesis it should be considered that different versions of the plasma ablation probes have been used for the different studies. The probe used for the experiments in paper II creates a plasma field at the tip of the probe. It has been estimated that plasma probes similar to the one used in the current study create a plasma field about 100 µm thick around the active electrode.(84) This would also concur with the results of paper I showing a cartilage cell death of approximately 150-200 µm deep into the tissue. From the results we can thus only conclude that the microbicidal effect is local within the direct vicinity of the probe tip within the microtiter well. The absolute distance of the effect can therefor not be determined by the current results.

5.3 Paper III-IV

Papers III and IV were in major parts conducted as a pilot study setup and a final full study setup however published in separate publications, and the results are thus best discussed in parallel.

These two studies found that wound debridement with plasma ablation reduced the wound bacterial load in the porcine *ex vivo* wound model with 1-3 log CFU in paper III and with in the range of 4 log CFU in paper IV, while curette and hydrosurgery debridement resulted in minor or no wound bacteria reduction. Hydrosurgery gave rise to a significant bacterial spread from the

wound to the operative environment, while plasma ablation and curette debridement did not. These results are concurrent with the results of Bowling *et al*. which evaluated the effect of hydrosurgery on bacterial spread in an operative room setup.(46)

In comparison to paper II as discussed above, the two studies of paper III and IV were performed with quantification of bacteria CFU with serial dilution with plating of each dilution to better precisely estimate the quantitative bactericidal effect and compare between treatment methods. There was however a quantitative difference in wound bacteria reduction seen between the two studies in paper III and IV, which is notable since in all major aspects of the study setups were the same. One possible explanation to the difference in results could be that the wound debridement procedure was more effectively performed in the second study due to increased practical experience of the device operator (H.H.S.), another possible explanation is that the difference is simply due to statistical variation since the pilot study was based on a very small sample size. In either case, the results from paper IV should be deemed to be more reliable based on the larger sample size.

The current studies thus confirm the bactericidal effect of plasma ablation and a similar 4-5 log reduction of *S. aureus* in an *ex vivo* porcine wound biofilm model as was seen in the *in vitro* planktonic bacteria solutions of paper II. A likely reason for this effect is thus that plasma ablation has a direct bactericidal effect, as shown in paper II, rather than merely removing the bacteria from the wound bed as would be the aim of hydrosurgery and curette debridement. This is also the likely reason for the bacteria aerosol spread seen with the hydrosurgery device and considering to the increasing problem with antibiotic resistant bacteria strains in chronic wounds and wound infections, these results may have important clinical implications for safe and effective wound treatment. A report by Maragakis *et al*. describes the potential consequences of using wound debridement equipment with inadequate protection against the potential for bacteria transmission and environmental contamination.(47) The report describe a hospital outbreak with a multidrug-resistant strain with *Acetinobacter baumannii* caused by the cross infection between patients treated with pulsed lavage wound debridement. Pulsed lavage can be considered to be a similar method to the hydrosurgery as both methods utilizes a jet of irrigated saline under direct pressure as a method for wound cleansing.

The SEM results confirm the formation of a biofilm after inoculation with *S. aureus* and incubation for 24 h. The SEM also showed the presence of coliform bacteria. This is not unexpected since the wound surfaces are not

entirely sterile at baseline, and additional bacterial growth of these contaminating strains may have taken place during incubation. This is also compatible with clinical wound colonization since clinical studies have shown that chronic wounds are in most cases colonized with multiple bacterial strains, including *S. aureus* and coli-form bacteria (28, 102-104).

The studies have some limitations. Due to local laboratory regulations, a MRSA strain, or other multi-resistant strain, could not be used in the experiments. However, there is no reason why the results should differ between methicillin-sensitive and methicillin-resistant strains for the surgical debridement methods tested. Further, the studies in paper III and IV were done in an *ex vivo* wound model and that wound healing as well as wound closure time could not be investigated *in vivo*.

5.4 Paper V

Based on the results of papers I- IV, it was postulated that the plasma ablation method may have positive clinical effects on debridement of wounds and ulcers.

The clinical case series in paper V is to our knowledge the first prospective study on the use of plasma ablation for wound debridement. The study results show a significant reduction in wound bacteria after debridement, even though this reduction is not as large as shown in the *in vitro* and *ex vivo* results of papers II-IV and in the *in vivo* porcine wound model used by Nusbaum *et al*.(65)

The role of bacteria in chronic leg ulcers has long been under discussion. Control of wound bacteria and minimizing the risk of infection is generally considered a key component in standard wound bed preparation.(37) However, the number of bacteria considered to impede healing of open wounds is controversial, with some studies showing impaired healing if more than 10^5 organisms per gram of tissue can be detected, while other studies have not been able to show such a correlation.(28, 30, 38) There is also no evidence that systemic antibiotic treatment has an effect on healing of venous leg ulcers without clinical signs of infection.(34) The potential importance for the wound healing process of the significantly bactericidal effect of the plasma ablation debridement method shown in the current study is thus not clear and would need to be further evaluated.

The rate of full wound closure was rather low in the current study in comparison to other clinical venous ulcer studies,(105) even though the

wounds decreased considerably in size during follow-up. Possible reasons for the relatively low rate of full wound closure could be that the included wounds had a very long duration, a number of previous treatments had been tried without success, and that the current study had a relatively short follow-up time. Also, no control group was used in the current study and it is generally acknowledged that healing rates for the patient group do vary largely between different clinical studies and between clinical settings. Considering the lack of control group, the current study thus cannot conclude if the plasma ablation debridement method has the ability to further aid the wound healing process.

6 CONCLUSION

Paper I shows that exposure to plasma ablation induces a well-defined area of immediate cell death and a short term increased proliferation with human articular chondrocytes *in vitro*. The exposure also alters cytokine expression for the same period causing up-regulation of IL-6 and IL-8. In conclusion this indicates that the cells react in an anabolic fashion to the exposure indicating a possible onset of a tissue regeneration response.

Paper II demonstrates that plasma ablation has a general microbicidal effect on bacteria and fungi common in wound infection. Thus, with the combination of its known ability to effectively remove tissue and the microbicidal capacity it is a promising technique for use in surgical areas such as chronic wound treatment.

Paper III and IV shows that plasma ablation is a promising wound debridement method, which effectively reduces the wound bed bacterial load without the risk of bacterial aerosol spread. Hydrosurgery on the other hand gave a statistically and clinically significant aerosol spread of bacteria. These results emphasize that extra care and protective means should be used when utilizing hydrosurgery debridement for infected and bacteria-colonized wounds, especially for wounds potentially contaminated with MRSA or other multiresistant strains. These precautions may for example need to include extra disinfection procedures of the operating room after a debridement procedure, to only use the device in operating rooms with high efficacy ventilation systems and to avoid consecutive debridement procedures of open wounds in the same operating room. The same precaution does not seem to be needed when using curette or plasma ablation for debridement. Paper IV further shows that a bacterial biofilm was formed in the porcine skin wound model, but the effect of debridement on the biofilm was not further evaluated.

Paper V shows that the plasma ablation method can be used for debridement of small chronic ulcers and can be performed with local anaesthesia on an outpatient basis. The study show that the wound bed bacterial load significantly reduced after treatment with plasma ablation. This should in general be of value for the wound healing process, even though the clinical significance of the finding is yet to be concluded. The ulcers investigated in this study tended to progress well towards healing after treatment with plasma ablation. Further clinical studies should evaluate the plasma ablation method for use in other areas than venous ulcers, such as in wound debridement prior to skin transplantation, debridement of bacteria colonized

or infected diabetic foot ulcers, and debridement of burns. However, to fully conclude the effect of plasma ablation treatment on wound healing, the treatment would need to be compared to standard of care including standard curette debridement in a randomized controlled trial (RCT).

7 FUTURE PERSPECTIVES

This thesis has shown that the plasma ablation technique has a pro-proliferative effect on chondrocytes *in vitro*. The plasma ablation technique is currently already in clinical use for cartilage repair and has been evaluated in RCTs with regards to the effect on knee pain. A possible cartilage regenerative effect has however not been fully clinically investigated in comparative clinical trials. It would thus be of great interest to investigate in a RCT, e.g. with MRI of the knee joint as outcome measure, if plasma ablation debridement has the capability induce a clinical regenerative effect on damaged cartilage.

This thesis has further investigated a number of preclinical effects of plasma ablation wound debridement and performed a first clinical evaluation of the application in venous ulcers. Before considering clinical routine implementation of plasma ablation treatment for venous ulcers or other wounds, the effects of plasma ablation on wound healing rate as well as potential adverse effects and complications to treatment need to be investigated further in comparative trials, preferably in a RCT setup.

A potential future clinical application for plasma ablation wound debridement could be as debridement method prior to split-thickness skin graft transplantation of venous ulcers with e.g. punch-grafts or pinch-grafts. A clean wound bed is essential prior to implantation of skin grafts and the bactericidal and debriding effect of plasma ablation may thus pose a clinical advantage compared to standard curette debridement and other currently existing techniques. This potential application should therefore be clinically evaluated. The plasma ablation debridement technique may also have a great potential in other types of wounds, e.g. in burn wounds that undergo debridement prior to skin grafting.

ACKNOWLEDGEMENT

I would like to thank:

Jan Faergemann, my supervisor, for your scientific skills and your encouragement and support.

Olle Larkö, my co-supervisor, for encouragement and for always keeping the spirits up with humorous stories about dermatology and the academia.

Louise Strömbeck and **Conny Eriksson** for being fantastic co-workers in the lab.

Sam Polesie, for being a great scientific co-worker.

Martin Gillstedt, for excellent statistical assistance.

Frank Aldenborg for excellent histological evaluations in projects III and IV.

Bengt R. Johansson and **Kanita Cukur** for excellent electron microscopy work in project IV.

Elisabeth Liljensten, for excellent advisory and scientific guidance during your time at ArthroCare.

All past and current **co-workers at Hudkliniken, Sahlgrenska** – you are the reason why it has been great place to work.

My parents **Christina** and **Håkan**, my sister **Anna** and my brothers **Oscar** and **Edwin**, for supporting me even when you are not exactly sure of what I am doing.

My wife **Anna Sofia**, for always believing in me and supporting me and for being the most loving and wonderful wife a man can have.

Disclosures and Grant Acknowledgements

Projects I-IV were fully or in part financially supported by ArthroCare Corporation.

Project V was supported by a limited economic research grant from the foundation Mary von Sydows, född Wijk, donation fund, and by a research grant from ArthroCare corporation including and limited to support of the WoundWands and lending of the CIQ generator used in the study.

I, Henrik H. Sönnergren, have during Projects I-IV had a consultancy agreement with ArthroCare Corporation. I have during Project V and at the time of writing this thesis not had any financial relation to ArthroCare corporation or any of the other producers or manufacturers related to the products and techniques investigated in this thesis.

REFERENCES

1. *Gomoll AH, Minas T. The quality of healing: articular cartilage. Wound Repair Regen. 2014;22 Suppl 1:30-8.*
2. *Mariani E, Pulsatelli L, Facchini A. Signaling pathways in cartilage repair. International journal of molecular sciences. 2014;15(5):8667-98.*
3. *Aroen A, Loken S, Heir S, Alvik E, Ekeland A, Granlund OG, et al. Articular cartilage lesions in 993 consecutive knee arthroscopies. Am J Sports Med. 2004;32(1):211-5.*
4. *Curl WW, Krome J, Gordon ES, Rushing J, Smith BP, Poehling GG. Cartilage injuries: a review of 31,516 knee arthroscopies. Arthroscopy. 1997;13(4):456-60.*
5. *Hjelle K, Solheim E, Strand T, Muri R, Brittberg M. Articular cartilage defects in 1,000 knee arthroscopies. Arthroscopy. 2002;18(7):730-4.*
6. *Widuchowski W, Widuchowski J, Trzaska T. Articular cartilage defects: study of 25,124 knee arthroscopies. Knee. 2007;14(3):177-82.*
7. *Goldring MB, Goldring SR. Osteoarthritis. J Cell Physiol. 2007;213(3):626-34.*
8. *Goldring MB, Otero M, Tsuchimochi K, Ijiri K, Li Y. Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism. Ann Rheum Dis. 2008;67 Suppl 3:iii75-82.*
9. *Kobayashi M, Squires GR, Mousa A, Tanzer M, Zukor DJ, Antoniou J, et al. Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. Arthritis Rheum. 2005;52(1):128-35.*
10. *Lubberts E, van den Berg WB. Cytokines in the pathogenesis of rheumatoid arthritis and collagen-induced arthritis. Adv Exp Med Biol. 2003;520:194-202.*
11. *Andersson U, Harris HE. The role of HMGB1 in the pathogenesis of rheumatic disease. Biochim Biophys Acta.1799(1-2):141-8.*
12. *Shah MR, Kaplan KM, Meislin RJ, Bosco JA, 3rd. Articular cartilage restoration of the knee. Bull NYU Hosp Jt Dis. 2007;65(1):51-60.*
13. *Spahn G, Klinger HM, Muckley T, Hofmann GO. Four-year results from a randomized controlled study of knee chondroplasty with concomitant medial meniscectomy: mechanical debridement versus radiofrequency chondroplasty. Arthroscopy. 2010;26(9 Suppl):S73-80.*
14. *Voloshin I, Morse KR, Allred CD, Bissell SA, Maloney MD, DeHaven KE. Arthroscopic evaluation of radiofrequency chondroplasty of the knee. Am J Sports Med. 2007;35(10):1702-7.*

15. *Robson MC, Barbul A. Guidelines for the best care of chronic wounds. Wound Repair Regen. 2006;14(6):647-8.*

16. *Mekkes JR, Loots MA, Van Der Wal AC, Bos JD. Causes, investigation and treatment of leg ulceration. Br J Dermatol. 2003;148(3):388-401.*

17. *Abbade LP, Lastoria S. Venous ulcer: epidemiology, physiopathology, diagnosis and treatment. Int J Dermatol. 2005;44(6):449-56.*

18. *Abraham TM, Pencina KM, Pencina MJ, Fox CS. Trends in Diabetes Incidence: The Framingham Heart Study. Diabetes Care. 2014.*

19. *Beebe-Dimmer JL, Pfeifer JR, Engle JS, Schottenfeld D. The epidemiology of chronic venous insufficiency and varicose veins. Ann Epidemiol. 2005;15(3):175-84.*

20. *Arai H, Ouchi Y, Yokode M, Ito H, Uematsu H, Eto F, et al. Toward the realization of a better aged society: messages from gerontology and geriatrics. Geriatrics & gerontology international. 2012;12(1):16-22.*

21. *Evans CJ, Fowkes FG, Ruckley CV, Lee AJ. Prevalence of varicose veins and chronic venous insufficiency in men and women in the general population: Edinburgh Vein Study. J Epidemiol Community Health. 1999;53(3):149-53.*

22. *Bergan JJ, Schmid-Schonbein GW, Smith PD, Nicolaides AN, Boisseau MR, Eklof B. Chronic venous disease. N Engl J Med. 2006;355(5):488-98.*

23. *Steed DL, Attinger C, Colaizzi T, Crossland M, Franz M, Harkless L, et al. Guidelines for the treatment of diabetic ulcers. Wound Repair Regen. 2006;14(6):680-92.*

24. *Whitney J, Phillips L, Aslam R, Barbul A, Gottrup F, Gould L, et al. Guidelines for the treatment of pressure ulcers. Wound Repair Regen. 2006;14(6):663-79.*

25. *Robson MC, Cooper DM, Aslam R, Gould LJ, Harding KG, Margolis DJ, et al. Guidelines for the treatment of venous ulcers. Wound Repair Regen. 2006;14(6):649-62.*

26. *Hopf HW, Ueno C, Aslam R, Burnand K, Fife C, Grant L, et al. Guidelines for the treatment of arterial insufficiency ulcers. Wound Repair Regen. 2006;14(6):693-710.*

27. *Schultz GS, Sibbald RG, Falanga V, Ayello EA, Dowsett C, Harding K, et al. Wound bed preparation: a systematic approach to wound management. Wound Repair Regen. 2003;11 Suppl 1:S1-28.*

28. *Hansson C, Hoborn J, Moller A, Swanbeck G. The microbial flora in venous leg ulcers without clinical signs of infection. Repeated culture using a validated standardised microbiological technique. Acta Derm Venereol. 1995;75(1):24-30.*

29. *Kerstein MD. The scientific basis of healing. Adv Wound Care. 1997;10(3):30-6.*

30. *Dow G, Browne A, Sibbald RG. Infection in chronic wounds: controversies in diagnosis and treatment. Ostomy Wound Manage. 1999;45(8):23-7, 9-40; quiz 1-2.*

31. *De Haan BB, Ellis H, Wilks M. The role of infection on wound healing. Surg Gynecol Obstet. 1974;138(5):693-700.*

32. *Pollack SV. The wound healing process. Clin Dermatol. 1984;2(3):8-16.*

33. *Stone LL, Dalton HP, Haynes BW. Bacterial debridement of the burn eschar: the in vivo activity of selected organisms. J Surg Res. 1980;29(1):83-92.*

34. *O'Meara S, Al-Kurdi D, Ologun Y, Ovington LG, Martyn-St James M, Richardson R. Antibiotics and antiseptics for venous leg ulcers. The Cochrane database of systematic reviews. 2014;1:CD003557.*

35. *Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev. 2001;14(2):244-69.*

36. *Davis SC, Ricotti C, Cazzaniga A, Welsh E, Eaglstein WH, Mertz PM. Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. Wound Repair Regen. 2008;16(1):23-9.*

37. *Schultz GS, Barillo DJ, Mozingo DW, Chin GA, Wound Bed Advisory Board M. Wound bed preparation and a brief history of TIME. International wound journal. 2004;1(1):19-32.*

38. *Robson MC, Lea CE, Dalton JB, Heggers JP. Quantitative bacteriology and delayed wound closure. Surg Forum. 1968;19:501-2.*

39. *O'Meara S, Al-Kurdi D, Ologun Y, Ovington LG. Antibiotics and antiseptics for venous leg ulcers. The Cochrane database of systematic reviews. (1):CD003557.*

40. *Falabella AF. Debridement and wound bed preparation. Dermatol Ther. 2006;19(6):317-25.*

41. *Attinger CE, Janis JE, Steinberg J, Schwartz J, Al-Attar A, Couch K. Clinical approach to wounds: debridement and wound bed preparation including the use of dressings and wound-healing adjuvants. Plast Reconstr Surg. 2006;117(7 Suppl):72S-109S.*

42. *Madhok BM, Vowden K, Vowden P. New techniques for wound debridement. International wound journal. 2013;10(3):247-51.*

43. *Wolff H, Hansson C. Larval therapy--an effective method of ulcer debridement. Clin Exp Dermatol. 2003;28(2):134-7.*

44. *Hess CL, Howard MA, Attinger CE. A review of mechanical adjuncts in wound healing: hydrotherapy, ultrasound, negative pressure therapy, hyperbaric oxygen, and electrostimulation. Ann Plast Surg. 2003;51(2):210-8.*

45. *Wenzel RP. The Lowbury Lecture. The economics of nosocomial infections. J Hosp Infect. 1995;31(2):79-87.*

46. *Bowling FL, Stickings DS, Edwards-Jones V, Armstrong DG, Boulton AJ. Hydrodebridement of wounds: effectiveness in reducing wound bacterial contamination and potential for air bacterial contamination. J Foot Ankle Res. 2009;2:13.*

47. *Maragakis LL, Cosgrove SE, Song X, Kim D, Rosenbaum P, Ciesla N, et al. An outbreak of multidrug-resistant Acinetobacter baumannii associated with pulsatile lavage wound treatment. JAMA. 2004;292(24):3006-11.*

48. *Gerszten PC, Monaco EA, 3rd. Complete percutaneous treatment of vertebral body tumors causing spinal canal compromise using a transpedicular cavitation, cement augmentation, and radiosurgical technique. Neurosurg Focus. 2009;27(6):E9.*

49. *Gerszten PC, Smuck M, Rathmell JP, Simopoulos TT, Bhagia SM, Mocek CK, et al. Plasma disc decompression compared with fluoroscopy-guided transforaminal epidural steroid injections for symptomatic contained lumbar disc herniation: a prospective, randomized, controlled trial. J Neurosurg Spine. 2010;12(4):357-71.*

50. *Shah UK, Dunham B. Coblation for tonsillectomy: an evidence-based review. ORL J Otorhinolaryngol Relat Spec. 2007;69(6):349-57.*

51. *Spahn G, Kahl E, Muckley T, Hofmann GO, Klinger HM. Arthroscopic knee chondroplasty using a bipolar radiofrequency-based device compared to mechanical shaver: results of a prospective, randomized, controlled study. Knee Surg Sports Traumatol Arthrosc. 2008;16(6):565-73.*

52. *Stalder KR, McMillen DF, Woloszko J. Electrosurgical Plasmas. J Phys D: Appl Phys. 2005;38:1728-38.*

53. *Stalder KR, Woloszko J, Brown IG, Smith CD. Repetitive plasma discharges in saline solutions. Applied Physics Letters. 2001;79(27):4503-5.*

54. *Kosy JD, Schranz PJ, Toms AD, Eyres KS, Mandalia VI. The use of radiofrequency energy for arthroscopic chondroplasty in the knee. Arthroscopy. 2011;27(5):695-703.*

55. *Owens BD, Stickles BJ, Balikian P, Busconi BD. Prospective analysis of radiofrequency versus mechanical debridement of isolated patellar chondral lesions. Arthroscopy. 2002;18(2):151-5.*

56. *Voloshin I, DeHaven KE, Steadman JR. Second-look arthroscopic observations after radiofrequency treatment of partial thickness articular cartilage defects in human knees: report of four cases. J Knee Surg. 2005;18(2):116-22.*

57. *Kaplan L, Uribe JW. The acute effects of radiofrequency energy in articular cartilage: an in vitro study. Arthroscopy. 2000;16(1):2-5.*

58. *Amiel D, Ball ST, Tasto JP. Chondrocyte viability and metabolic activity after treatment of bovine articular cartilage with bipolar radiofrequency: an in vitro study. Arthroscopy. 2004;20(5):503-10.*

59. *Allen RT, Tasto JP, Cummings J, Robertson CM, Amiel D. Meniscal debridement with an arthroscopic radiofrequency wand versus an arthroscopic shaver: comparative effects on menisci and underlying articular cartilage. Arthroscopy. 2006;22(4):385-93.*

60. *O'Neill CW, Liu JJ, Leibenberg E, Hu SS, Deviren V, Tay BK, et al. Percutaneous plasma decompression alters cytokine expression in injured porcine intervertebral discs. Spine J. 2004;4(1):88-98.*

61. *Rhyu KW, Walsh AJ, O'Neill CW, Bradford DS, Lotz JC. The short-term effects of electrosurgical ablation on proinflammatory mediator production by intervertebral disc cells in tissue culture. Spine J. 2007;7(4):451-8.*

62. *Takahashi N, Tasto JP, Ritter M, Ochiai N, Ohtori S, Moriya H, et al. Pain relief through an antinociceptive effect after radiofrequency application. Am J Sports Med. 2007;35(5):805-10.*

63. *Tasto JP, Cummings J, Medlock V, Harwood F, Hardesty R, Amiel D. The tendon treatment center: new horizons in the treatment of tendinosis. Arthroscopy. 2003;19 Suppl 1:213-23.*

64. *Cook JL, Kuroki K, Kenter K, Marberry K, Brawner T, Geiger T, et al. Bipolar and monopolar radiofrequency treatment of osteoarthritic knee articular cartilage: acute and temporal effects on cartilage compressive stiffness, permeability, cell synthesis, and extracellular matrix composition. J Knee Surg. 2004;17(2):99-108.*

65. *Nusbaum AG, Gil J, Rippy MK, Warne B, Valdes J, Claro A, et al. Effective method to remove wound bacteria: comparison of various debridement modalities in an in vivo porcine model. J Surg Res. 2012;176(2):701-7.*

66. *Trial C, Brancati A, Marnet O, Teot L. Coblation technology for surgical wound debridement: principle, experimental data, and technical data. Int J Low Extrem Wounds. 2012;11(4):286-92.*

67. *Yim GH, Ahmad Z, Jeffery SL. The WoundWand and Its Novel Use in Burn Excision Surgery. Eplasty. 2015;15:e1.*

68. *Levine NS, Lindberg RB, Mason AD, Jr., Pruitt BA, Jr. The quantitative swab culture and smear: A quick, simple method for determining the number of viable aerobic bacteria on open wounds. J Trauma. 1976;16(2):89-94.*

69. *Williamson P, Kligman AM. A new method for the quantitative investigation of cutaneous bacteria. J Invest Dermatol. 1965;45(6):498-503.*

70. *Hambraeus A, Hoborn J, Whyte W. Skin sampling-validation of a pad method and comparison with commonly used methods. J Hosp Infect. 1990;16(1):19-27.*

71. *Friedman PL, Ellisman MH. Enhanced visualization of peripheral nerve and sensory receptors in the scanning electron microscope using cryofracture and osmium-thiocarbohydrazide-osmium impregnation. J Neurocytol. 1981;10(1):111-31.*

72. *Barkun JS, Aronson JK, Feldman LS, Maddern GJ, Strasberg SM, Altman DG, et al. Evaluation and stages of surgical innovations. Lancet. 2009;374(9695):1089-96.*

73. *Ergina PL, Cook JA, Blazeby JM, Boutron I, Clavien PA, Reeves BC, et al. Challenges in evaluating surgical innovation. Lancet. 2009;374(9695):1097-104.*

74. *McCulloch P, Altman DG, Campbell WB, Flum DR, Glasziou P, Marshall JC, et al. No surgical innovation without evaluation: the IDEAL recommendations. Lancet. 2009;374(9695):1105-12.*

75. *Romling U, Bian Z, Hammar M, Sierralta WD, Normark S. Curli fibers are highly conserved between Salmonella typhimurium and Escherichia coli with respect to operon structure and regulation. J Bacteriol. 1998;180(3):722-31.*

76. *Tallheden T, Dennis JE, Lennon DP, Sjogren-Jansson E, Caplan AI, Lindahl A. Phenotypic plasticity of human articular chondrocytes. J Bone Joint Surg Am. 2003;85-A Suppl 2:93-100.*

77. *Fan Y, Bergmann A. Apoptosis-induced compensatory proliferation. The Cell is dead. Long live the Cell! Trends Cell Biol. 2008;18(10):467-73.*

78. *Ruifrok AC, Mason KA, Lozano G, Thames HD. Spatial and temporal patterns of expression of epidermal growth factor, transforming growth factor alpha and transforming growth factor beta 1-3 and their receptors in mouse jejunum after radiation treatment. Radiat Res. 1997;147(1):1-12.*

79. *Sakurai T, He G, Matsuzawa A, Yu GY, Maeda S, Hardiman G, et al. Hepatocyte necrosis induced by oxidative stress and IL-1 alpha release mediate carcinogen-induced compensatory proliferation and liver tumorigenesis. Cancer Cell. 2008;14(2):156-65.*

80. *Guerne PA, Carson DA, Lotz M. IL-6 production by human articular chondrocytes. Modulation of its synthesis by cytokines, growth factors, and hormones in vitro. J Immunol. 1990;144(2):499-505.*

81. *Li D, Wang GY, Dong BH, Zhang YC, Wang YX, Sun BC. Biological characteristics of human placental mesenchymal stem cells and their proliferative response to various cytokines. Cells Tissues Organs. 2007;186(3):169-79.*

82. *Campo GM, Avenoso A, Campo S, D'Ascola A, Traina P, Sama D, et al. Purified human plasma glycosaminoglycans reduced NF-kappaB*

activation, pro-inflammatory cytokine production and apoptosis in LPS-treated chondrocytes. Innate Immun. 2008;14(4):233-46.

83. Villarete LH, Remick DG. Nitric oxide regulation of interleukin-8 gene expression. Shock. 1997;7(1):29-35.

84. Woloszko J, Stalder KR, Brown IG. Plasma characteristics of repetitively-pulsed electrical discharges in saline solutions used for surgical procedures. IEEE transactions on plasma science 2002;30(3):1376-83.

85. Bowler PG. Wound pathophysiology, infection and therapeutic options. Ann Med. 2002;34(6):419-27.

86. Mlinaric-Missoni E, Kalenic S, Vazic-Babic V. Species distribution and frequency of isolation of yeasts and dermatophytes from toe webs of diabetic patients. Acta dermatovenerologica Croatica : ADC / Hrvatsko dermatolosko drustvo. 2005;13(2):85-92.

87. Hansson C, Jekler J, Swanbeck G. Candida albicans infections in leg ulcers and surrounding skin after the use of ointment impregnated stockings. Acta Derm Venereol. 1985;65(5):424-7.

88. Hansson C, Faergemann J, Swanbeck G. Fungal infections occurring under bandages in leg ulcer patients. Acta Derm Venereol. 1987;67(4):341-5.

89. Raahave D, Friis-Møller A, Bjerre-Jepsen K, Thiis-Knudsen J, Rasmussen LB. The infective dose of aerobic and anaerobic bacteria in postoperative wound sepsis. Archives of surgery (Chicago, Ill : 1960). 1986;121(8):924-9.

90. Robson MC, Heggers JP. Delayed wound closure based on bacterial counts. J Surg Oncol. 1970;2(4):379-83.

91. Arguelles JC. Thermotolerance and trehalose accumulation induced by heat shock in yeast cells of Candida albicans. FEMS Microbiol Lett. 1997;146(1):65-71.

92. Rodriguez-Calleja JM, Cebrian G, Condon S, Manas P. Variation in resistance of natural isolates of Staphylococcus aureus to heat, pulsed electric field and ultrasound under pressure. J Appl Microbiol. 2006;100(5):1054-62.

93. Sorqvist S. Heat resistance in liquids of Enterococcus spp., Listeria spp., Escherichia coli, Yersinia enterocolitica, Salmonella spp. and Campylobacter spp. Acta Vet Scand. 2003;44(1-2):1-19.

94. Spinks AT, Dunstan RH, Harrison T, Coombes P, Kuczera G. Thermal inactivation of water-borne pathogenic and indicator bacteria at sub-boiling temperatures. Water Res. 2006;40(6):1326-32.

95. Tsuji A, Kaneko Y, Takahashi K, Ogawa M, Goto S. The effects of temperature and pH on the growth of eight enteric and nine glucose non-fermenting species of gram-negative rods. Microbiol Immunol. 1982;26(1):15-24.

96. *Iwasawa A, Saito K, Mokudai T, Kohno M, Ozawa T, Niwano Y. Fungicidal action of hydroxyl radicals generated by ultrasound in water. J Clin Biochem Nutr. 2009;45(2):214-8.*

97. *Moisan M, Barbeau J, Moreau S, Pelletier J, Tabrizian M, Yahia LH. Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms. Int J Pharm. 2001;226(1-2):1-21.*

98. *Joaquin JC, Kwan C, Abramzon N, Vandervoort K, Brelles-Marino G. Is gas-discharge plasma a new solution to the old problem of biofilm inactivation? Microbiology. 2009;155(Pt 3):724-32.*

99. *Matthes R, Bekeschus S, Bender C, Koban I, Hubner NO, Kramer A. Pilot-study on the influence of carrier gas and plasma application (open resp. delimited) modifications on physical plasma and its antimicrobial effect against Pseudomonas aeruginosa and Staphylococcus aureus. GMS Krankenhaushygiene interdisziplinar. 2012;7(1):Doc02.*

100. *Ermolaeva SA, Varfolomeev AF, Chernukha MY, Yurov DS, Vasiliev MM, Kaminskaya AA, et al. Bactericidal effects of non-thermal argon plasma in vitro, in biofilms and in the animal model of infected wounds. J Med Microbiol. 2011;60(Pt 1):75-83.*

101. *Isbary G, Heinlin J, Shimizu T, Zimmermann JL, Morfill G, Schmidt HU, et al. Successful and safe use of 2 min cold atmospheric argon plasma in chronic wounds: results of a randomized controlled trial. Br J Dermatol. 2012;167(2):404-10.*

102. *Gjodsbol K, Christensen JJ, Karlsmark T, Jorgensen B, Klein BM, Krogfelt KA. Multiple bacterial species reside in chronic wounds: a longitudinal study. International wound journal. 2006;3(3):225-31.*

103. *Schmidt K, Debus ES, St J, Ziegler U, Thiede A. Bacterial population of chronic crural ulcers: is there a difference between the diabetic, the venous, and the arterial ulcer? Vasa. 2000;29(1):62-70.*

104. *Dowd SE, Sun Y, Secor PR, Rhoads DD, Wolcott BM, James GA, et al. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. BMC Microbiol. 2008;8:43.*

105. *O'Meara S, Cullum N, Nelson EA, Dumville JC. Compression for venous leg ulcers. The Cochrane database of systematic reviews. 2012;11:CD000265.*