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Destruction of in situ oral biofilms formed on titanium surfaces by cold atmospheric plasma

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Dedication

To my beautiful homeland watered with the blood of innocents

To all martyrs of the word, the right and the free

To every freeman who holds an olive branch

To every child that carries the hope in their thin hearts

To every woman who lost a husband, a son or a brother for the sake of freedom

The sun will shine again and the warmth will return to Syria

To my family,

For their love, patience, encouragement, support and prayers

Contents

List of Abbreviations

AA:	Air-abrasion
CAP:	Cold atmospheric plasma
CC:	Clean control
CHX:	Chlorhexidine
DL:	Diode laser
CLSM:	Confocal laser scanning microscopy
DNA:	Deoxyribonucleic acid
FM:	Fluorescence Microscope
GC:	Gas control
Ma:	Machined
MP:	Mean power
MS:	Microstructured
RNA:	Ribonucleic acid
SEM:	Scanning electron microscope
Ti:	Titanium
UC:	Untreated control
V:	Volunteer

1. Summary

1.1. English summary

The decontamination of implant surfaces represents the basic procedure in the management of peri-implant diseases, but it still remains a challenge. This study is aimed to evaluate the degradation of oral biofilms grown *in situ* on machined (Ma) and microstructured (MS) titanium (Ti) discs through cold atmospheric plasma (CAP).

Material and Methods

As many as 400 Ti discs[*1] were exposed to the oral cavities of five healthy human volunteers for 72 h. The Ti discs were divided randomly between the following treatment groups: Untreated biofilms as negative control, four groups treated with CAP (which varied in mean power, treatment duration, and/or the gas mixture), and treated biofilms as positive controls (gas, diode laser, air-abrasion, chlorhexidine 60 s and chlorhexidine 200 s). The viability, quantity, and morphology of control and the treated biofilms were determined by live/dead staining, inoculation onto blood agar, quantification of the total protein content, as well as by scanning electron microscopy.

Results

All the volunteers showed similar colonization patterns of *Streptococcus mitis, Streptococcus gordonii, Streptococcus constellatus, Actinomyces odontolyticus, Actinomyces viscosus, Fusobacterium nucleatum, Viellonella parvula, Eikenella corrodens, and Capnocytophaga* on the Ma and MS Ti discs. No differences in composition, regarding the colonization of the investigated bacteria, were observed between the biofilms accumulated on the Ma and MS Ti surfaces. The personal factor and the roughness of the Ti discs played a large role in determining the amount of the accumulated biofilms.

All plasma parameters exhibited great abilities to inhibit bacterial growth on blood agar and resulted in a significant decrease in the total amount of biofilms and the average percentage of viable bacteria, compared with the untreated control. The treatment of oral biofilms with cold atmospheric plasma led to the destruction of the structure of biofilms; it reduced the biofilms

[*1] 200 Ma and 200 MS Ti discs, d = 5 mm, h: 1 mm.

by the amount as well as by the thickness. Although the superficial bacteria were reduced, a complete removal of biofilms was not achieved. There was a correlation between an increase in the effectiveness of CAP and an increase in the mean power and/or the treatment duration. The antiseptic efficacy of CAP was comparable with diode laser and chlorhexidine.

No method of diode laser, air-abrasion, and chlorhexidine has been able to completely remove the biofilms or has achieved complete inhibition of bacterial growth on blood agar. The treatment with air-abrasion, however, exhibited the best ability to eliminate the oral biofilms.

The SEM analysis of plasma-treated specimens failed to reveal any micromorphologically detectable changes of the machined or microstructured titanium surfaces. The highest measured temperature on the Ti surfaces reached approximately 52 °C. An increase in the measured temperature correlated with the surface roughness, mean power, and the amount of biofilms present on the titanium surfaces.

Conclusion

The present experimental investigation showed that cold atmospheric plasma has a distinct antibacterial efficacy and it is suitable for disintegrating the matured *in situ* oral biofilms formed on machined and microstructured titanium surfaces without causing any thermal or mechanical damages. Comparison with other techniques showed that cold atmospheric plasma could be compared to diode laser and chlorhexidine 0.2%.

1.2. Deutsche Zusammenfassung

Biofilme sind Ursache der oralen Erkrankungen periimplantäre Mukositis und Peri-implantitis. Die Therapie dieser Erkrankungen stellt eine Herausforderung dar. Das aktuelle Therapiekonzept beruht auf der Dekontamination von Implantatoberflächen, die in der zahnmedizinischen Praxis jedoch nur unvollkommen möglich ist. Die vorgelegte Arbeit hatte das Ziel, orale Biofilme auf maschinierten (Ma) und mikrostrukturierten (MS) Titanoberflächen unter Verwendung kalten atmosphärischen Plasmas zu beseitigen.

Materialien und Methoden

Vierhundert Titanprüfkörper[*1] wurden über 72 h in den Mundhöhlen von fünf gesunden Probanden zur Gewinnung oraler Biofilme exponiert. Die biofilmtragenden Prüfkörper wurden randomisiert Test- und Kontrollgruppen zugeordnet, bei denen die Oberflächen mit unterschiedlichen physikalisch und chemisch antimikrobiell wirksamen Maßnahmen behandelt wurden. In den Testgruppen wurden die Biofilme mit physikalischen Plasmen unterschiedlicher Intensität und Komposition behandelt.

Bei der Auswahl der Behandlungsparameter wurde auf biologische Kompatibilität hinsichtlich der resultierenden Temperaturen geachtet. Die Biofilme der Kontrollgruppen wurden gegenüber Diodenlaser, abrasivem Glycinpulver-Wasser-Gemisch oder Chlorhexidin exponiert. Zusätzlich dienten unbehandelte Biofilme und Prüfkörper ohne Biofilme als Negativkontrollen.

Nach der Behandlung wurden die Biofilme morphologisch, quantitativ und hinsichtlich ihrer Vitalität im Rasterelektronenmikroskop, mittels Bestimmung der zurückgebliebenen Proteinmenge sowie fluoreszenz-vitalmikroskopisch und mikrobiologisch untersucht.

Ergebnisse

Es waren bei allen Probanden in den Biofilmen auf maschinierten und mikrostrukturierten Titanprüfkörpern die folgenden Mikroorganismen nachweisbar: *Streptococcus mitis, Streptococcus gordonii, Streptococcus constellatus, Actinomyces odontolyticus, Actinomyces viscosus, Fusobacterium nucleatum, Viellonella parvula, Eikenella corrodens and Capnocytophaga*.

[*1] 200 Ma and 200 MS Ti discs, d = 5mm, h: 1 mm.

Die Behandlung oraler Biofilme mit kaltem atmosphärischem Plasma führte zur Zerstörung der Biofilmstruktur, Biofilmmenge und Biofilmvitalität wurden im Vergleich mit den unbehandelten Kontrollen statistisch signifikant reduziert. Erhöhte Plasma Bestrahlungsintensität führte zu höherer Reduktion der Biofilmmenge und –vitalität. Eine komplette Entfernung von Biofilmen mit ausschließlicher Plasmabehandlung konnte jedoch nicht beobachtet werden. Allerdings zeigte auch keine der verwendeten Kontrollmethoden die komplette Biofilmentfernung.

Plasma- und Laserbehandlung von Biofilmen zeigten vergleichbare Ergebnisse hinsichtlich residualer Biofilmmenge und –vitalität. Die Nutzung eines abrasiven Pulver-Wasser-Gemischs führte zur stärksten Reduktion der Biofilmquantität, der verbleibende Biofilm war jedoch vital.

Weder auf maschiniertem noch auf mikrostrukturiertem Titan konnten morphologische Oberflächenalterationen nach Plasmabehandlung festgestellt werden. Die höchste gemessene Oberflächentemperatur auf Titan erreichte im Zentrum des Plasmajets 52 °C. Ein Aufheizen der Prüfkörper wurde nicht beobachtet.

Zusammenfassung

Im Vergleich mit klinisch etablierten Methoden der Oberflächendekontamination zeigte die Behandlung von oralen 72 h-Biofilmen vergleichbare Ergebnisse hinsichtlich Reduktion und Desinfektion. Die Oberflächenstruktur der Titanprüfkörper wurde dabei nicht alteriert.

2. Introduction

Over the past four decades, dental implantology has devised new solutions and therapy choices in dental medicine; in particular, the need to support the functional and cosmetic requirements of patients without harming sound tissues adjacent to the edentulous area.

In recent years, rigorous research has contributed immensely to the improvement of the biological acceptance and osseointegration of dental implants. Branemark *et al*. (1977) exhibited the possibility of long-term biological acceptance of dental implants through their 10-year follow-up study "Osseointegrated implants in the treatment of the edentulous jaw". This study provided a positive concept and opened the way for other clinical research which afterward contributed to a variety of discussions and demonstrations of the long-term biological acceptance of titanium implants (Adell *et al*., 1981; Ekelund & Lindquist, 2003; Pjetursson *et al*., 2005; Rasmusson *et al.,* 2005; Lekholm *et al.,* 2006; Jemt & Johansson, 2006; Astrand *et al*., 2008). The installation of oral implants has become a routine procedure to reconstruct partially and fully edentulous individuals.

After the installation of an implant into the oral cavity, the already resident microorganisms begin to colonize the implant surfaces (Pontoriero *et al*., 1994; Zitzmann *et al*., 2000; Lindhe & Meyle, 2008). Biofilms are generated by microbial communities developing on the interfaces between solid surfaces and biological fluids. Besides microorganisms, biofilms consist of a matrix of exopolysaccharides, proteins, and nucleic acids (Hannig & Hannig, 2009; Marsh, 2006). A lot of diseases in the oral cavity, such as caries, stomatitis, periodontal, and peri-implant diseases, are associated with biofilms (Renvert *et al*., 2009). Experimental and clinical studies demonstrated that peri-implant and periodontal tissues react similarly to oral biofilms (Berglundh *et al*., 1992; Ericsson *et al*., 1992; Pontoriero *et al*., 1994; Lindhe & Meyle, 2008). Also, the removal of biological contamination from dental and implant surfaces is one of the most important factors affecting the healing of periodontal and peri-implant tissues (Mombelli *et al.,* 1992).

Therefore, this study highlights the importance of developing new techniques to clean and disinfect dental implant surfaces and help to improve the biological properties without having any negative effects on the implant surfaces.

2.1. Physical Plasma

Definition

In physical sciences, plasma is a term which refers to the fourth state of matter. In medicine and biology, plasma is known as the non-cellular fluid component of blood (Compton, 1930). Physical plasma jet is defined as ionized local gas flows containing a collection of positively and negatively charged ions, free electrons, and activated neutral species which act collectively (Kieft *et al*., 2005).

Plasma can be classified into two types, thermal and non-thermal, based on the differences in characteristics. Thermal plasma is the state of fully ionized gas, characterized by a high gas temperature and an approximate equality between the gas and electron temperature, and it can be generated under atmospheric pressure. The energy content of this plasma is high enough to break any chemical bond; therefore, this type of plasma can be excluded from most areas of organic chemistry as well as from the field of pharmaceutical science. In contrast, cold plasma is mostly characterized by a low gas temperature and a high electron temperature, and it is easily generated by electric discharges under reduced pressure (Fridman *et al*., 2008).

In all plasmas supported by electric field, electrons receive the external energy faster than the heavier ions and have the opportunity to heat up to thousands of degrees before their environment heats up. In non-thermal plasma, the cooling of ions and uncharged molecules is more effective than energy transfer from electrons, and the gas remains at a low temperature. For this reason, non-thermal plasma is also known as non-equilibrium plasma. In thermal plasma, on the other hand, the energy flux from electrons to heavy particles equilibrates the energy flux from heavy particles to the environment only when the temperature of heavy particles becomes almost equal to the electron temperature (Kieft *et al*., 2005). Sometimes, even small differences in the temperature of the heavier species can play a substantial role. This is particularly important when various plasma chemical processes are considered. It is certainly important when plasma is used to treat heat-sensitive objects.

The effectiveness of plasma

The efficacy of cold atmospheric plasma is manifested through two relevant therapeutic effects: physical and biological (Laroussi, 2002; Kieft *et al*., 2005; Vleugels *et al*., 2005; Manner, 2008; Dobrynin *et al*., 2009). The physical effects, attained through heating of tissues, can induce different effects; they depend not only on the reached temperature of plasma, but also on the rate and duration of heating (Manner, 2008). For human tissues, in general, no irreversible cellular damage occurs at temperatures below 40 °C. The phenomena occurring for temperatures between 40 °C and 50 °C are summarized under the term "hyperthermia". Changes at cellular membranes and molecular structures set in, and the interstitial fluid may increase due to water secretion by the cells (oedema formation). Depending on the duration of heating, those changes may still be reversible; but after several minutes, the tissue may begin to die. At higher temperatures, the cells are irreversibly damaged only after a few seconds. Between 60 °C and 80 °C, the denaturation of internal proteins occurs first. This process is called coagulation and leads to cell death (necrosis). Also, the intercellular proteins then coagulate and transform into gelatin. In addition, the cell membranes are destroyed since they contain a large proportion of proteins. At 100 °C, the evaporation of the cellular liquid sets in. Additional heat is now converted into the energy of evaporation. The vapor pressure leads to mechanical destruction of the tissue. Depending on the rate of the heat dissipation, the evaporation either slowly leads to desiccation or violently leads to vaporization of the tissue. Only after the evaporation of the entire liquid, the temperature can rise beyond 100 °C and the remaining material may burn and thereby induce carbonization (Zenker, 2008; Table 1).

Table 1: Overview of the effects of heating on tissues (modified after Zenker 2008)

Temperature in tissue	Effect
< 40 °C	None
40-50 °C	Hyperthermia: changes in cellular membrane and internal molecular structures, oedema formation. Time-dependent cell death (necrosis) and devitalisation
60-80 °C	Denaturation of the internal proteins (coagulation). Destruction of cell membranes. Coagulation of the extracellular collagen. Devitalisation
100 °C	Evaporation off cell liquid. Desiccation/Vaporization (depending on heating power)
> 100 °C	Carbonization

The second therapeutic effect of plasma is biological; it gets through reactive species such as oxygen and nitric radicals. The short-living radicals like O and OH are the most important sterilizing agents in atmospheric pressure plasma which have more powerful disinfectants and stronger oxidation mechanism than ozone (Laroussi, 2002; Kieft *et al*., 2005). Furthermore, ultraviolet irradiation, ions, and electrons are also emitted with the plasma discharges which play an important role in enhancing the physical and mechanical effectiveness (Laroussi & Leipold, 2004; Vleugels *et al*., 2005). Lastly, the mechanisms of interaction of reactive plasma components with living organisms depend on the way the plasma had been generated and delivered, as well as on the organism to which it had been applied (Shimizu *et al*., 2008; Stoffels *et al*., 2006).

Cold atmospheric plasma in human medicine

One of the important medical applications of plasma therapy is tissue coagulation through the monopolar electrosurgical technique that uses argon discharges at atmospheric pressure. The major application fields are hemostasis, tissue devitalization, and tissue reduction (Hoffmann *et al*., 2012; Manner, 2008; Tsiamoulos *et al*., 2012). Argon plasma offers a new tonsillectomy method which significantly reduces blood loss, surgical time and post-operative pain, and help to avoid the general problems associated with electrosurgery (Bergler *et al*., 2001). Argon plasma coagulation is effective and safe in the management of gastrointestinal vascular lesions, bleeding of the large intestine, and hemorrhagic cystitis (Kwan *et al*., 2006; Quinlan *et al*., 1992; Villavicencio *et al*., 2002).

Devitalization and shrinkage are often used for the treatment of tumors and obstructing tissues. In the case of early stage tumors, the desired effect is to kill the cancerous tissues which can then be removed mechanically, whenever necessary. If high power is applied, the tissue may be carbonized or even vaporized. Examples of devitalization and tissue reduction by argon plasma are the treatment of overlarge nasal conches, the minimally invasive treatment of tumors, and metastases in the respiratory or gastrointestinal tract (Bergler *et al*., 2001). In skin surgery, argon atmospheric plasma is used to treat warts, actinic keratosis, and hemangioma (Brand *et al*., 1998).

Several *in vivo* and animal studies have demonstrated that plasma discharges are able to accelerate tissue granulation and heal wounds (Zhiliaev *et al*., 1998). The use of plasma flows at the time of surgical treatment of purulent wounds reduces pain during the post-operative

period and considerably improves the result of the treatment (Khrupkin *et al*., 1998). Furthermore, experimental studies suggest that NO-containing gaseous flows are useful in the treatment of eye wounds and they speed up the healing of cornea erosion and penetrating corneal wounds (Gundarova *et al*., 2001; Chesnokova & Gundorova, 2003).

The antibacterial effect of non-thermal plasma has been demonstrated by numerous studies for a variety of microorganisms (Laroussi, 2002; Lee *et al*., 2006; Vleugels *et al*., 2005; Yu & Hsieh, 2006; Goree *et al*., 2006; Sladek *et al*., 2007; Hübner *et al*., 2010; Rupf *et al*., 2010; Rupf *et al*., 2011; Gerspach *et al*., 2011; Idlibi et al., 2013). However, some studies have shown that bacterial DNAs are not completely destroyed by cold plasma (Venezia *et al*., 2008; Kim & Kim, 2006). Plasma can also be used to render hydrophilic properties to surfaces (Duan *et al*., 2007; Duske *et al*., 2012).

Cold atmospheric plasma in dentistry

The removal of carious dentin through the plasma technique has been suggested as an alternative to the conventional method of drilling (Stoffels *et al*., 2002; Sladek *et al*., 2004). Ritts *et al*. (2010) found that the plasma treatment of the peripheral dentin surface increases the interfacial bonding strength.

The methods to decontaminate and condition intraoral surfaces are of great interest in the field of dentistry. Only a few papers have dealt with the application of plasma jets for the removal and inhibition of *in situ* oral biofilms. The *in vitro* disinfection of *Streptococcus mutans* grown on agar plates and in oral biofilms has been demonstrated (Goree *et al*., 2006; Sladek *et al*., 2007; Rupf *et al*., 2010). Hauser-Gerspach *et al*. (2011) investigated the anti-microbial efficacy of gaseous ozone on *Streptococcus sanguinis* and *Porphyromonas gingivalis* adhered to various titanium and zirconia surfaces. Hübner *et al*. (2010) compared the efficacy of chlorhexidine digluconate (0.1%) with that of polihexanide and tissue-tolerable plasma against *Pseudomonas aeruginosa*. The tissue-tolerable plasma led to significant reduction of colony-forming units and it was shown to be effective against the *Pseudomonas aeruginosa* biofilms on polystyrene and silicone materials. The microbicidal effect of plasma was comparable to that of chlorhexidine digluconate (0.1%).

2.2. Peri-implant diseases

Definition

The term ‘‘peri-implantitis’’ was first introduced in the late 1980s. It describes the pathological changes in soft and hard tissues around a functioning osseointegrated dental implant (Mombelli *et al*., 1987). Peri-implant diseases, in the consensus report of the first European Workshop on Periodontology, were divided into a reversible inflammatory reaction when the inflammation remains confined to soft tissues, and an irreversible inflammatory reaction when the inflammation extends to hard tissues and leads to the loss of the supporting bone around the implant (Albrektsson *et al*., 1994). In the consensus report of the sixth European Workshop on Periodontology, the reversible and irreversible form was modified to peri-implant mucositis and peri-implantitis, respectively (Lindhe & Meyle, 2008).

Prevalence

Roos-Jansåker *et al*. (2006) reported in a cross-sectional study (216 patients, 987 implants, 9 to 14 years as functional time) that peri-implant mucositis was diagnosed in 79% of the patients and 50% of the implants, while peri-implantitis was diagnosed in 55-77% of the patients and 43% of the implants. Fransson *et al*. (2008) examined 3,413 Branemark implants; of them, 12% showed bone loss extended to the apical third of the implant, while clinical examination revealed that 94% of the affected implants exhibited bleeding on probing.

Diagnosis

Peri-implant mucositis may be identified clinically by redness, swelling, and bleeding on probing. In peri-implantitis, the mucosal lesion is associated with the loss of the supporting marginal bone and increasing of the peri-implant pocket (Lindhe & Meyle, 2008).

Peri-implant probing: The presence of bleeding on gentle probing (0.25 N) is a useful parameter to diagnose mucosal inflammation. Healthy peri-implant tissues showed absence of bleeding on probing, while bleeding on probing occurred in 67% of the patients in the case of peri-implant mucositis and 91% of the patients in the case of peri-implantitis (Lang et al., 1994). Other studies have showed an association between the increase of probing depth and loss of the hard tissue around the implant (Lang *et al*., 1993 and 1994; Schou *et al*., 1993; Zitzmann, 2000; Heitz-Mayfield, 2008).

Radiological evaluation: Radiographic techniques, including intra-oral radiography by using long cone, paralleling techniques, and panoramic tomography have been widely used to monitor marginal bone levels at implants and diagnose interproximal bone loss (Kullman *et al*., 2007). While a panoramic tomograph allows the entire implant to be visualized, its limitations, such as image resolution and distortion, are also well known. Further limitations of conventional radiography include the inability to monitor facial and lingual/palatal bone levels, low sensitivity in the detection of early bone changes, and the underestimation of bone loss (Brägger *et al*., 1988; Akesson *et al*., 1993; De Smet *et al*., 2002; Heitz-Mayfield, 2008). Recently, multi-slice computer tomography and cone beam volume imaging have been used in implant dentistry to the advantage that osseous structures can be represented in three planes, true to scale, and without overlay or distortion (Mengel *et al*., 2006; Heitz-Mayfield, 2008).

Implant mobility: The mobility of an implant refers to complete lack of osseointegration and the implant should be removed (Heitz-Mayfield, 2008; Fransson *et al*., 2008).

Suppuration: The presence of purulent exudates is a result of infection, and it may be associated with progressive bone loss and an increase of the peri-implant pocket depth (Roos-Jansåker *et al*., 2006; Fransson *et al*., 2008).

Aetiology

The causative factors of peri-implant diseases are generally divided into two major categories: biomechanical and biological (Tonetti, 1999; Klinge *et al*., 2005; Quirynen *et al*., 2006). The biomechanical factors are associated with functional loads exceeding the implant bone interface due to overloading conditions, such as bruising, clenching, and fracture. The biological failures are associated with microbial plaque accumulation and bacterial infections, as well as sensory disturbances and fibrointegration due to surgical trauma (Tonetti, 1999; Esposito *et al*., 1999; Quirynen, 2002; Klinge *et al*., 2005; Teughels *et al*., 2006).

The biological failures may further be divided into early and late failures. The early failures are associated with dental implant infections as a result of contaminated surgical placement or impaired host healing (Esposito *et al*., 1998 and 1999; Snauwaert *et al*., 2000; Quirynen, 2002; Norowski & Bumgardner, 2009). Dental implant infections generally occur after more than one year of the implant placement and are termed as the plaque-induced peri-implantitis (Tonetti, 1999; Quirynen, 2002; Norowski & Bumgardner, 2009).

Risk factors of peri-implant disease

Heitz-Mayfield (2008), in a cross-sectional analysis, investigated the following factors: 1) Considerable factors associated with peri-implant diseases: poor oral hygiene, history of periodontitis, and smoking; 2) Factors that have limited evidence, such as diabetes, poor metabolic control, and alcohol consumption; and 3) Factors with conflicting and limited evidence, such as implant surface properties and genetic traits.

Patients with healthy oral hygiene show lower plaque scores and less bleeding on probing (Lindquist *et al*., 1997; Ferreira *et al*., 2006). Patients with a history of periodontitis show an increased risk for peri-implant diseases and peri-implant marginal bone loss, compared with healthy periodontal subjects (Hardt *et al*., 2002; Karoussis *et al*., 2003; Schou *et al*., 2006; Karoussis *et al*., 2007; Quirynen *et al*., 2007). Several studies have referred to an association between smoking and development of peri-implant diseases (Haas *et al*., 1996; Lindquist *et al*., 1996; Lindquist *et al*., 1997; Carlsson *et al*., 2000; Feloutzis *et al*., 2003; Attard & Zarb, 2002; McDermott *et al*., 2003; Karoussis *et al*., 2004; Peñarrocha *et al*., 2004; Wennström *et al*., 2004; Galindo-Moreno *et al*., 2005; Nitzan *et al*., 2005; Schwartz-Arad *et al*., 2005; Strietzel *et al*., 2007). Lindquist *et al.* (1997) indicated that smokers with poor oral hygiene suffer three times more marginal bone loss than non-smokers.

The presence of diabetes has been statistically associated with greater risks for peri-implantitis, especially in subjects with poor metabolic control (Ferreira *et al*., 2006). Good oral hygiene is very difficult to achieve around dental restorations without the protection of a band of keratinized gingival tissue. A higher plaque accumulation, bleeding tendencies on lingual sites, and larger soft-tissue recession on buccal sites were found when the width of keratinized mucosa on the peri-implant soft-tissue was lesser than 2 mm (Schrott *et al*., 2009).

Plaque biofilm as causative factor in the development of peri-implant disease

The maturation of plaque biofilms on dental and implant surfaces played a major role in the development of gingivitis and peri-implant mucositis (Pontoriero *et al*., 1994). No significant differences of clinical and microbiological measures were found between the experimentally induced peri-implant mucositis and gingivitis (Löe *et al.,* 1967; Pontoriero *et al*., 1994).

Biofilm formation on dental surfaces

The formation of plaque biofilms on dental surfaces is a multiple-stage process that begins minutes after tooth eruption or cleaning (Gibbons & van Houte, 1973). The enamel gets covered by salivary pellicle to form an acellular proteinaceous film on the tooth surface. Salivary pellicles are proteins and glycoproteins that selectively bind to tooth surfaces, dental implants, restorations, and dental prostheses. The salivary pellicles act as an adhesive binding to tooth surfaces, and on the other hand, promote the adhesion and coaggregation of oral bacteria (Hannig, 1997; Marsh, 2004).

Oral bacteria begin to colonize on tooth surfaces within zero to four hours of pellicle formation. After formation of the pellicle layer, there begins the weak, long-range physicochemical interactions (reversible adhesion) between the pellicle on the tooth surface and the cell surface of the oral bacteria, thus encouraging the bacteria to invert their previous relation and approach the tooth surface. The weak, long-range interactions transform into stronger short-range attachments (irreversible adhesion) within the approach of bacteria to the pellicle layer (Marsh, 2006).

Owing to the fact that many oral microbial species have multiple adhesion types on their cell surfaces, they can participate in an excess of interactions simultaneously with other microbes and the host surface molecules (Marsh, 2006). Later, other cells adhere to the primary layer of bacteria (co-aggregation or co-adhesion) to form multilayered cell clusters in the polymer matrix. During the next four to 24 h, the attached bacteria multiply and grow to form distinct micro-colonies. Such confluent growth of micro-colonies results in the formation of plaque biofilms which increase in complexity over time. The bacterial cells get nutrition from the intermicrobial polymer matrix of salivary origin in the supragingival plaque, as well as from gingival crevicular fluid and crevicular exudation origin in the subgingival plaque (Marsh, 2006).

A large proportion of the initial colonizers are *Streptococci* (*S. sanguis, S. oralis,* and *S. mitis*). In one to 14 days, the *Streptococcus*-dominated plaque changes to a plaque dominated by *Actinomyces* species. In two to four weeks, the bacterial species become more diverse with high levels of gram-negative anaerobic filamentous species (Marsh, 2006).

Biofilm formation on implant surfaces

After implantation, bacteria move from saliva, oral tissues, and periodontal pockets, in the case of periodontitis, to colonize the implant surfaces (Takanashi *et al*., 2004; Quirynen *et al*., 2006; Heuer *et al*., 2007). The colonization of implant surfaces starts at irregular supragingival areas, and then, spreads down toward the base. The surface irregularities protect colonizing bacteria from natural removal forces, e.g. salivary fluid flow and oral hygiene measures. The implant-abutment interface may also provide a protected site for bacterial accumulation and contribute to the peri-implant inflammatory reactions (Piattelli *et al*., 1995; Quirynen, 2002; Broggini *et al*., 2006).

The sequence of biofilm formation on implant surfaces seems to be similar to that of dental surfaces. The periopathogenic species of natural dentition and dental implants are essentially the same, though there may be some differences in relative numbers and species present (Mombelli, 1993; Silverstein *et al*., 1994; Meffert, 1996; Tanner *et al*., 1997). The methods of Gingival crevicular fluid sampling and DNA probing have shown similarities to biofilm microflora on titanium implants and tooth surfaces (Leonhardt *et al*., 2002). The exposure of different implant materials *in situ* to the oral environment showed that *Streptococci* were the predominant initial colonizing microbes after four hours and that the anaerobes increased within 48 h (Nakazato *et al*., 1989; Rams *et al*., 1991; Leonhardt *et al*., 2002; Fürst *et al*., 2007). The early colonizers prepare a favorable environment for late colonizers that require more demanding growth conditions (Mombelli *et al*., 1995).

The putative periodontal pathogens such as *Porphyromonas*, *Prevotella*, *Capnocytophaga* and *Fusobacterium* species are also considered as the causative microbes for peri-implant infections (Mombelli & Lang, 1994; Gerber *et al*., 2006). These pathogens produce endotoxins such as collagenase, hyaluronidase, and chondroitin sulphates which elicit an inflammatory response, resulting in the loss of supporting bone and tissues around the implant.

Healthy and infected implants show differences in the composition of the associated microbiota (Mombelli *et al*., 1987 and 1993; Silverstein *et al*., 1994; Quirynen, 2002; Heydenrijk *et al*., 2002). Successful implants are reported to be populated with the gram-positive coccoid cells, very few rods, a low ratio of anaerobe/aerobes, and a low number of gram-negative anaerobes (George *et al*., 1994; Quirynen, 2002). Infected and failing implants show greater proportions of the gram-negative anaerobe rods, motile rods, fusiform

bacteria, and spirochetes, including large numbers of *Fusobacterium* ssp, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*, *Peptostreptococcus micros*, *Campylobacter rectus*, *Capnocytophaga spp*, and *Porphyromonas gingivalis* (Mombelli *et al*., 1987; Sbordone *et al*., 1995; Leonhardt *et al*., 1999; Romeo & Ghisolfi, 2004).

Effect of roughness on biofilm formation

Bacterial adhesion and early biofilm formation are significantly influenced by the nanoscale morphological features and modifications of implant surfaces (Al-Ahmad *et al*., 2010; Badihi Hauslich *et al*., 2011; Singh *et al*., 2011).

The roughness of implant surfaces is associated with higher incidences of peri-implant diseases, especially in the periodontally compromised patient (Wu-Yuan *et al*., 1995; Quirynen *et al*., 2007). In a scanning electron microscopy study, which was performed to evaluate the attachment of oral bacteria on titanium discs with different surface morphologies (smooth, grooved, and rough), the highest degree of bacterial attachment was observed on rough titanium surfaces, while smooth surfaces showed poor attachment (Rimondini *et al*., 1997). The initial biofilm formation and composition were affected by the microtopography and hydrophilicity of the surface (Almaguer-Flores *et al*., 2011).

The initial bacterial adhesion is primarily influenced by the roughness factor, while the influence of surface-free energy seems to be of only minor importance (Bürgers *et al*., 2010). Al-Ahmad *et al*. (2010) evaluated the biofilm formation on titanium and zirconia implants with different roughness degrees *in vivo* (machined titanium, modified titanium, modified zirconia, machined alumina-toughened zirconia, sandblasted alumina-toughened zirconia, and machined zirconia); no significant differences were observed in biofilm compositions on the different implant surfaces; the biofilm composition present on the different implant materials seems to be similar to that on tooth enamel. The chemical composition of the implant material do not have any influence on bacterial behavior once the biofilm has matured, and the influence of the surface roughness on the adhesion of microorganisms is compensated by the proceeding maturation of the oral biofilm.

Treatment of peri-implant diseases

Non-surgical treatment

Mechanical non-surgical therapy and oral hygiene could be effective in the treatment of peri-implant mucositis lesions and the adjunctive use of anti-microbial mouth rinses enhances the outcome of mechanical therapy (Renvert *et al*., 2008 and 2009; Lindhe & Meyle, 2008; Thöne-Mühling *et al*., 2010; Heitz-Mayfield *et al*., 2011).

The sub-mucosal debridement of implant surfaces, with increasing peri-implant depth (> 5 mm), showed improvement of bleeding score, while a complete removal of all adhering microorganisms and peri-implant pockets had not been observed (Lindhe & Meyle, 2008; Renvert *et al*., 2009; Heitz-Mayfield *et al*., 2011). Antibiotics, antiseptics, and laser treatments have been proposed to improve the non-surgical treatment of peri-implantitis (Schwarz *et al*., 2005; Sculean *et al*., 2005; Schwarz *et al*., 2006; Kotsovilis *et al*., 2008; Jan Lindhe & Meyle, 2008; Renvert *et al*., 2008; Renvert *et al*., 2009; Máximo *et al*., 2009).

Surgical treatment

The surgical treatment of peri-implant lesions aims to facilitate the elimination of all granulation tissues from the defect area, debridement and decontamination of implant surfaces as well as to render the surface conductive to bone regeneration and reosseointegration (Claffey *et al*., 2008; Lindhe & Meyle, 2008). Schwarz *et al*. (2006) compared the outcome of open and closed debridement of implant surfaces by using different decontamination techniques (Er:YAG laser, an ultrasonic device, and plastic curettes with local application of metronidazole gel). Regardless of the used decontamination technique, the open debridement of implant surfaces improved the outcome of treatment, in comparison with closed debridement. Mariano *et al*. (2011) compared the results of multiple treatments of peri-implant diseases. They concluded that surgical therapy was more effective than non-surgical approaches in the treatment of peri-implantitis. Regenerative procedures, such as bone graft techniques, with or without guided tissue regeneration resulted in various degrees of success (Schwarz *et al*., 2006a; 2006b & 2006c; Lindhe & Meyle, 2008; Mariano *et al*., 2011). The resective surgical therapy with modification of the topography of the implant surface (removal of the roughness and exposed implant threads) seems to have a positive influence on the survival of oral implants affected by peri-implantitis (Romeo *et al*., 2005 & 2007).

Decontamination of implant surfaces

The treatment strategy of peri-implant diseases appears to be largely based on the same procedures that are used to treat periodontal diseases. The mechanical debridement and the surface disinfection represent the basic tenets of these procedures.

Surface complications of dental implants, such as the screw-shaped designs and surface roughness modifications, hinder effective cleaning and biofilm removal from the infected implant surfaces. Furthermore, insufficient removal of biological contamination from implant surfaces is considered as one of the most important factors which may prevent healing and lead to a failure of the conservative and regeneration therapy. Various methods have been evaluated, e.g. manual debridement with plastic or titan curets, rotating brush with pumice, air powder abrasion, ultrasonic, laser therapy, and application of topical medication such as citric acid, chlorhexidine irrigation, peroxide treatment, among others; but none of them was found to be the gold standard (Claffey *et al*., 2008; Lindhe & Meyle, 2008).

The metal scaler, curettes, and ultrasonic tips led to damages on the titanium surfaces. The remaining plaque and calculus did not differ significantly after the treatment with carbon, plastic, and metallic scaler tips (Kawashima *et al*., 2007). Also, the therapy with the vector ultrasonic system damages the following implant surfaces: sand-blasted and acid-etched, titanium plasma-sprayed, machine-polished, and hydroxyapatite-coated; debris of carbon fibers were visible on implant surfaces (Schwarz *et al*., 2003). Air-abrasion displayed high efficacy with regard to biofilm removal (Dennison *et al*., 1994).

The irradiation with 980-nm gallium aluminum laser (diode laser) showed reduction of bacteria on the machined, sand-blasted, and acid-etched titanium surfaces contaminated with *Enterococcus faecalis* and *Porphyromonas gingivalis* (Gonçalves *et al*., 2010). Diode laser succeeded in reducing the amount of 10 days *in situ* oral biofilm formed on titanium surfaces (Sennhenn-Kirchner *et al*., 2007). The irradiation with diode laser effectively reduced the viability of the adhered *Streptococcus sanguinis* and *Porphyromonas gingivalis* (Hauser-Gerspach *et al*., 2010). Several studies have suggested that diode laser (980 nm) does not damage titanium surfaces (Romanos *et al*., 2000; Theodoro *et al*., 2003; Castro *et al*., 2007; Stubinger *et al*., 2010; Gonçalves *et al*., 2010), but may produce a temperature increase above the critical threshold (Geminiani *et al*., 2012).

Er:YAG laser was more effective than the ultrasonic system and plastic curettes to remove supragingival early plaque biofilms grown on sand-blasted and acid-etched titanium implants (Schwarz *et al*., 2005). The Er:Yag laser application showed cleaning effectiveness on different implant surfaces contaminated by *Porphyromonas gingivalis* (Quaranta *et al*., 2009). Stubinger *et al*. (2010) reported that the use of Er: YAG laser for implant surface irradiation is safe only with the settings no higher than 300 mJ/10 Hz. The irradiation of implant surfaces with Er:YAG lasers may produce a temperature increase above the critical threshold (10 °C) after 10 s of continuous irradiation (Geminiani *et al*., 2011).

Gosau *et al*. (2010) compared the effect of six different peri-implantitis disinfection methods (sodium hypochlorite, hydrogen peroxide 3%, Chlorhexidine gluconate 0.2%, plax, listerine, citric acid 40%) on *in vivo* human oral biofilm for one minute. Sodium hypochlorite, hydrogen peroxide, chlorhexidine, and listerine showed a significant bactericidal effect against the adhering bacteria. The highest amounts of dead bacteria in proportion to all the adhering bacteria were found for sodium hypochlorite, chlorhexidine, and listerine without any statistical differences among them. Ntrouka *et al*. (2011), in a systematic review, reported that citric acid is the chemotherapeutic agent with the highest potential to remove biofilms from contaminated titanium surfaces *in vitro*, although it does not achieve complete removal.

2.3. Aims of the study

No single method was found to be superior in decontaminating dental implant surfaces and no investigations were available to evaluate cold atmospheric plasma as a technique to remove and disinfect *in situ* oral biofilms on the titanium implant surfaces. On the basis of these considerations, the aims of the present research are:

1. Investigating the influence of cold atmospheric plasma on three-day oral biofilms formed on machined and microstructure titanium surfaces.

2. Comparing the effects of cold atmospheric plasma with diode laser, air-abrasion, and chlorhexidine 0.2%.

3. Testing the effects of cold atmospheric plasma on machined and microstructured titanium surfaces (the surface structure and the surface temperature).

3. Material and Methods

3.1. Specimens

The used specimens are machined (Ma) and microstructured (MS)[*1] titanium (Ti) discs 5 mm diameter and 1 mm thickness (Friadent Germany). Numbers and distribution of groups, specimens and treatment parameters are described in Tables (2-a) and (2-b).

Table 2-a: The total numbers and distribution of Ma and MS Ti discs

Ma	MS		Analysing methods	Aim
5	5	72 h. biofilm	ParoCheck	Identify the bacterial species
5	5	72 h. biofilm	Confocal laser microscope	Measurement of biofilm thickness
200	200	72 h. biofilm	10 subgroups (Table 2-b)	Plasma treatment and controls
10	10	clean	Cold atmospheric plasma	Studying the surface alterations

Table 2-b: Description of groups, specimen's numbers and treatment parameters

Groups	Ti (n)	V (n)	Time (s)	MP	Gas	d
Untreated control (UC)	20 Ma + 20 MS	5	------	-------	-------	------
Gas control (GC)	20 Ma + 20 MS	5	196.25	-------	He 2.0 slm	2 mm
Cold atmospheric plasma pa. 1 (CAP1)	20 Ma + 20 MS	5	196.25	5 W	He 2.0 slm	2 mm
Cold atmospheric plasma pa. 2 (CAP2)	20 Ma + 20 MS	5	196.25	3 W	He 2.0 slm	2 mm
Cold atmospheric plasma pa. 3 (CAP3)	20 Ma + 20 MS	5	49.06	5 W	He 2.0 slm	2 mm
Cold atmospheric plasma pa. 4 (CAP4)	20 Ma + 20 MS	5	49.06	5 W	He 2.0 slm + O_2 5 sccm	2 mm
Diode laser (DL)	20 Ma + 20 MS	5	60	2.5 W	-------	1 mm
Air-abrasion (AA)	20 Ma + 20 MS	5	60	-------	-------	5 mm
Chlorhexidin pa. 1 (CHX1)	20 Ma + 20 MS	5	60	-------	-------	0 mm
Chlorhexidin pa. 2 (CHX2)	20 Ma + 20 MS	5	200	-------	-------	0 mm

Ti (n): number of titanium discs; V (n): number of volunteers; MP: Mean power; d: distance; slm: standard litres per minute; sccm: standard cm per minute

[*1] Sand blasted and acid etched, Ti grade 2, mean roughness (Ra): 1.96 µm, Vickers hardness: 150, tension resistance: 470 N/mm², E-module: 110 Gpa.

3.2. *In situ* biofilm formation

As many as 420 Ti discs (210 Ma and 210 MS) were equally divided between five healthy volunteers (aged 20–32 years; three female and two male; Table 2-b). Every 10 Ti discs were fixed with silicon impression material[*1] at the buccal site of the molar and premolar teeth on the custom-made maxillary splints (Fig. 1). After 72 h of exposure to the oral cavity, the specimens were rinsed for 10 s in a sterile saline solution (0.9%), and then, were checked by stereo light microscopy before further processing of the samples.

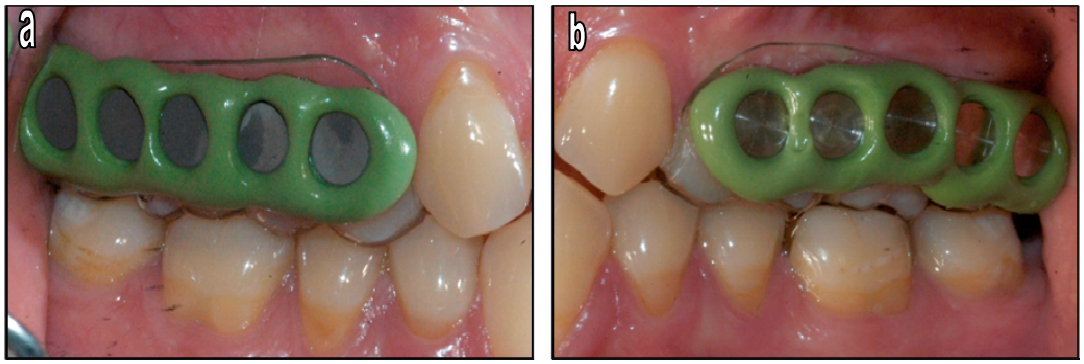

Fig. 1: Individual removable custom-made maxillary splints. The Ti discs were fixed with silicon impression material at the buccal sites of molar and premolar teeth (a: MS and b: Ma)

3.3. Study population

The study protocol was approved by the Ethical Committee of the Saarland Medical Association (Vote No. 39/09) and written consents were obtained from all the participants.

The inclusion criteria for volunteers were: good general health, non-smokers, no antibiotic treatment in the last six months, no signs of inflammation of periodontal tissues, and a good level of oral hygiene[*2]. The volunteers adapted their regimes of oral hygiene to create reproducible amounts of viable biofilms; they stopped using mouth rinses two weeks prior to starting the study. They did not use toothpaste and did not brush the Ti surfaces.

[*1] President light body, Coltene, Switzerland.

[*2] Plaque index < 1 (Loe, 1967): The plaque index was measured at 6 sites (3 buccal and 3 lingual) of each teeth 0 = No plaque. 1 = A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque is seen only after passing the probe along the tooth surface. 2 = Moderate accumulation of soft deposit within the gingival margin can be seen by the naked eye. 3 = Abundance of soft matter within the gingival pocket and / or the tooth and gingival margin.

3.4. Devices

Cold atmospheric plasma (CAP)

An experimental plasma source[*1] was mounted on a CNC 3-axes linear stage motion system[*2] to ensure the reproducible time, distance, and treatment parameters (Rupf et al., 2010). The pulse width of the microwave was adjusted at 5 µs and 250 W, which resulted in a plasma jet width of ~0.5 mm and a length of 5 mm.

The treatment was carried out under ambient pressure at a distance of 2 mm between plasma jet nozzle and sample surface, and in a meander-like scanning mode line by line with a line speed of 1 mm/s or 4 mm/s and a distance between the lines of 0.1 mm (Rupf *et al.,* 2011). The basic process gas flow was adjusted to 2 standard liters per minute (slm) helium for all the four CAP parameters. For CAP4, oxygen was admixed 5 standard cm per minute (sccm)[*3].

The evaluated CAP variables were the mean power (3 or 5 W), the line speed of scanning (CAP1 and CAP2: 1mm s^{-1} or CAP3 and CAP4: 4mm s^{-1}), and the process gas (an O_2 admixture).

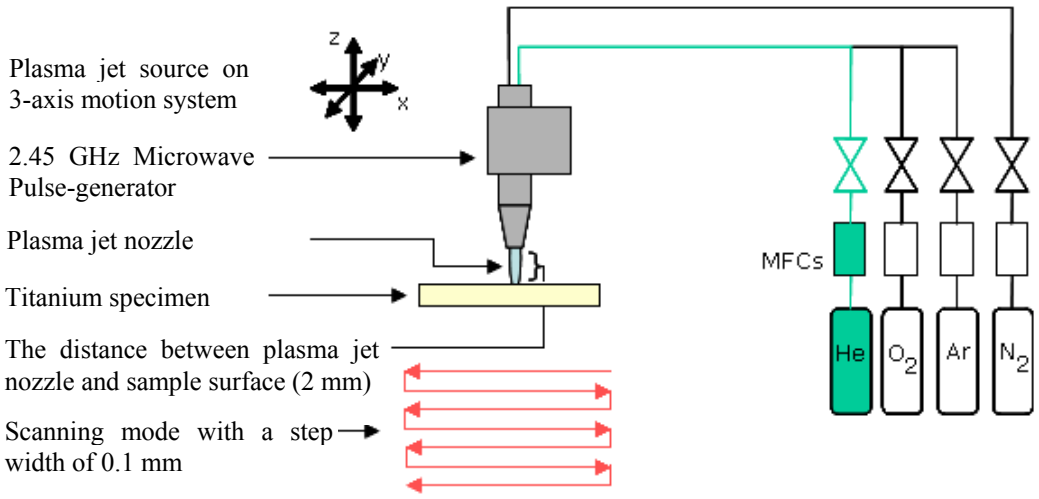

Fig. 2: The drawing describes the plasma source and specimens' treatment

[*1] Leibniz Institute of Surface Modification, Leipzig, Germany.

[*2] Steinmeyer MC-G047, Feinmess Dresden GmbH, Germany.

[*3] Mass flow controllers, Bronkhorst, Ruurlo, The Netherlands.

The operational temperatures for all CAP parameters were measured dynamically by the means of infrared thermography[*1] on the surfaces of the Ti discs and were performed at room temperature with a thermal resolution of ±0.1 °C, an optical frame of 160 × 120 pixels, and a frame rate of 100 Hz (Rupf *et al*., 2011).

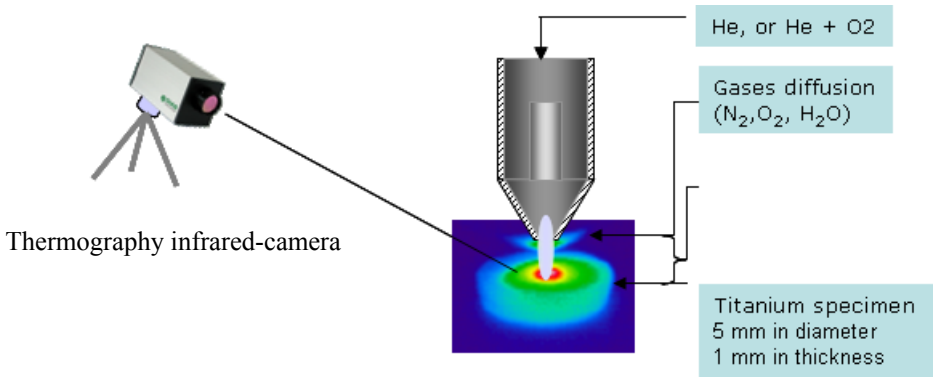

Fig. 3: The drawing describes the plasma source, specimens and thermography infrared-camera

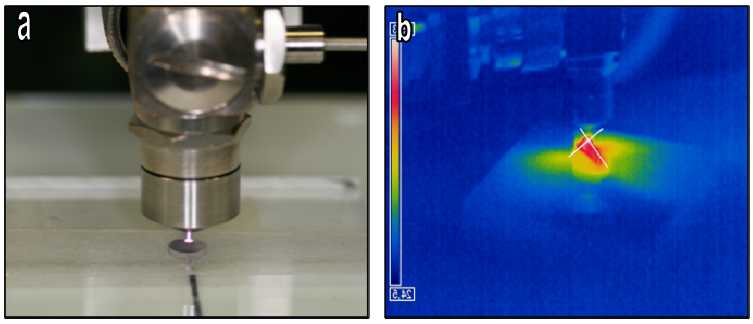

Fig. 4: a: Plasma source and b: Thermography infrared-camera

[*1] Optris PI, Optris GmbH, Berlin, Germany.

Diode Laser

The laser treatment was applied in a scanning mode by using a diode laser[*1], set at a mean power of 2.5 W, and pulse repetition of 25 ms pulse and a pause of 50 ms (Fig. 5, Table 2-b).

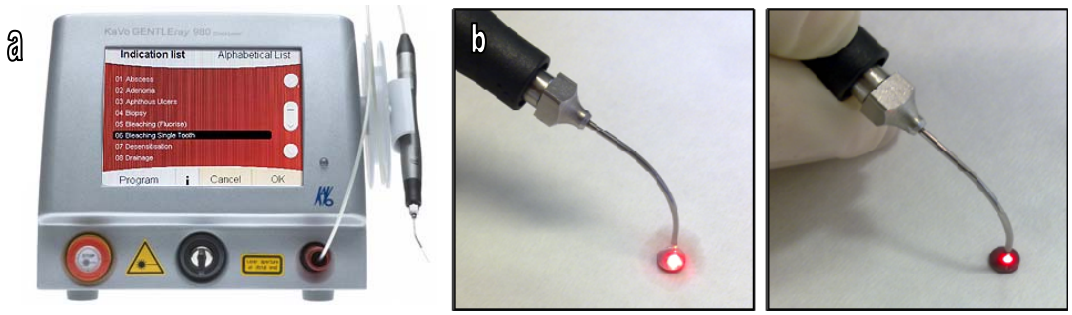

Fig. 5: a: Diode laser device, b: Laser treatment of Ma Ti discs (left) and MS Ti discs (right)

Air-abrasive powder

The air-abrasion treatment was performed in a circular motion by using a dental powder blaster (PROPHYflex 2, KaVo) and amino acid glycine powder (Fig. 6, Table 2-b).

Chlorhexidine

The specimens were submerged in a 200 µl chlorhexidine digluconate solution 0.2%[*2] (Fig. 7) for 60 s (CHX1) or for 200 s (CHX2) (Table 2-b).

Fig. 6: Left: Air-flow Perio Subgingival.
Right: Prophyfex 2, KaVo

Fig. 7: Chlorhexamed® forte 0.2% GlaxoSmithKline

[*1] 980 nm, GENTLEray 980 classic, KaVo, Biberach, Germany.

[*2] Chlorhexamed® forte 0.2% GlaxoSmithKline, Munich, Germany.

3.5. Analysis of biofilm

ParoCheck analysis

ParoCheck[*1] is a commercial microarray system that allows simultaneous detection of up to 20 different oral bacterial species, based on species-specific highly conserved regions, from the 16S rRNA gene (Eberhard et al., 2008). The Ti discs were assayed in a 20 µl reaction mixture containing 1 µl template, 0.2 µl Taq DNA polymerase, and 18.8 µl master mix supplied with the Parocheck kit[*2]. The DNA preparation was performed directly from the plaque biofilms accumulated on the Ti discs and carried out in accordance with the manufacturer's instructions.[*3]

Live/dead staining

Live/dead staining is a technology that permits quantitative distinguishing between live and dead bacteria. The live/dead kit[*4] consists of two stains, green (SYTO®9) and red (propidium iodide), which differ in their abilities to penetrate bacterial cells and stain nucleic acids. The acid's green fluorescing is able to enter every cell, while the red fluorescing only enters the cells with damaged cytoplasmic membrane and can reduce SYTO® 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes have only SYTO® 9 and fluoresce green, while dead bacteria with damaged membranes have both the dyes but fluoresce red.

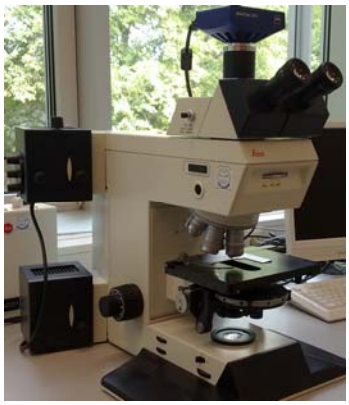

Fig. 8: Fluorescence microscope

The stain was prepared by diluting X µl of SYTO 9 and X µl of propidium iodide in X ml of distilled water. The specimens were placed in 48 well plates and 100 µl of the reagent mixture were added to each of them, followed by incubation, at room temperature, in the dark, for 15 min. Each of the specimens was carefully positioned on a glass slide, and covered with mounting oil and stored in a dark space at 4 °C until further processing. The samples were

[*1] ParoCheck®; Greiner Bio-One GmbH, Frickenhausen, Germany.

[*2] ParoCheck kit containing buffers, MgCl2, dNTPs, DNase-free water and fluorophore-labeled primers.

[*3] The analysis was carried out by Dr. med. Rita Grigorean, IGD Saar GmbH, Zentrum für Innovative Genetische Diagnostik, Homburg, Deutschland.

[*4] Back Light Bacterial Viability Kit L7012, Molecular Probes, Carlsbad, USA.

evaluated under a reverse light fluorescence microscope[*1] (Fig. 8), equipped with a digital camera[*2] and filter sets, by using the image processing software AxioVision 4.8[*3]. Five images were taken at 100 × magnification from the center and the four quarters of the specimen. Live/dead images of the Ti surfaces were separated to red and green color channels, and the median areas of the red and green fluorescence were calculated. Lastly, the green fluorescence values were expressed as a percentage of the red fluorescence (Rupf *et al.,* 2011; Idlibi *et al*., 2013).

To determine the thickness of the biofilms, five Ma and five MS Ti discs were explored by CLSM[*4]. For this purpose, the biofilms were analyzed by using a 10 × 63 oil immersion objective. Each biofilm was scanned at four representative areas. The Z-series of optical sections were generated by vertical sectioning at 6 µm distances through the biofilms. Image analysis and biofilm thickness measurement were performed by using the LSM software ZEN 2008 (Zeiss).

Microbiology

The viability of biofilms was tested by contact inoculation of the treated and untreated Ti plates with universal brain heart infusion blood agar (Rodac plates)[*5]. Then, the Rodac plates were incubated at 37 °C (5% CO_2) for 24 h.

Fig. 9: Agar Rodac plates

After 24 h, the plates were pictured and the impression area of each specimen on the blood agar was classified as positive (presence of colonies) or negative (no colonies detected). Also, each impression area was analyzed by using the image-J software to calculate: 1) The colony area; and 2) The colony-free area.

[*1] Leitz DMR, Leica, Wetzlar, Germany.

[*2] AxioCam MRm Rev. 3, Carl Zeiss Microlmaging, Goettingen, Germany.

[*3] Carl Zeiss Microlmaging, Goettingen, Germany.

[*4] Confocal laser scanning microscopy; Zeiss LSM 710, Carl Zeiss MicroImaging GmbH, Jena, Germany.

[*5] BHI, Sigma-Aldrich, Taufkirchen, Germany.

Detection of total protein

The specimens were transferred to a fresh 2 ml Eppendorf tube, and overlaid with 50 µl of RIPA buffer[*1] and sonicated for 10 min at 4 °C; then 100 µl d H_2O as well as 150 µl of Micro BCA working reagent were added to it; finally, the samples were incubated at 55 °C for 60 min.

Standard protein solutions (albumin) from 0-100 µg/ml were prepared and treated identically to the samples. The absorbance of protein standard and treated specimens was measured at 562 nm in a multifunctional microplate reader[*2] by tenfold determination of each well. Total protein content of the biofilms was calorimetrically estimated (Micro BCA assay, Pierce Biotechnology, Rockford, IL, USA).

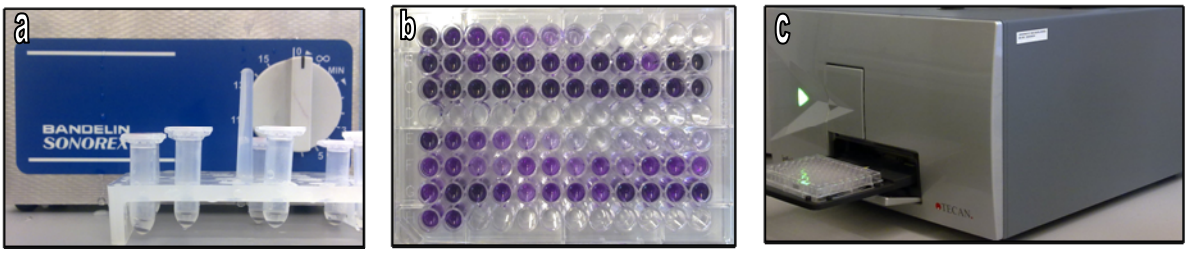

Fig. 10: From left to right: a: Bandelin Sonorex device, b: 96 Well Microplate, c: Tecan Infinite m200

Scanning electron microscopy (SEM)

The controlled and treated Ti discs were washed gently with phosphate buffer saline (PBS) to remove the cells not attached to the surface and fixed in glutaraldehyde[*3] for 2 h; afterward, they were rinsed five times for 10 min in PBS.

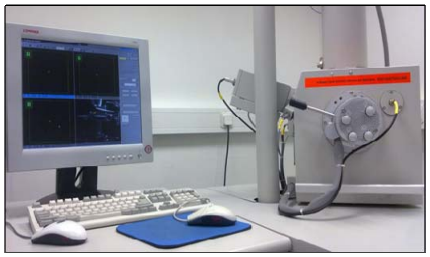

Fig. 11: Scanning electron microscope

[*1] 150 mM NaCl, 1.0% Octylphenyl-polyethylene glycol, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, Sigma-Aldrich, Steinheim, Germany.

[*2] Tecan Infinite 200, Magellan V6.6, Tecan, Grödig, Austria.

[*3] 2.5% in phosphate buffered saline (PBS); PAA Laboratories GmbH, Pasching, Austria.

Subsequently, the samples were dehydrated in an increasing series of ethanol (50–90% 10 min each; then 100% 3 x 15 min). Finally, the samples were dried in 1,1,1,3,3,3-hexamethyl-disilazane[*1]. HMDS was vaporized at room temperature in a clean bench. All the samples were mounted on the SEM-sample stubs and sputtered with platinum. The SEM analysis was carried out in a FEI XL30 ESEM FEG[*2] at magnifications of 50, 1000, 5000, and 10000. The Ti surfaces were scanned to investigate residual biofilm, bacteria, and structural changes of biofilm and surface alterations caused during the treatment.

3.6. Statistical analysis

400 Ti discs (200 Ma and 200 MS) were equally divided between 20 groups (10 Ma and 10 MS), which 20 biofilm specimens were assigned to each group (Table 2-b). All analyzes (live/dead, microbiology, detection of total protein, and scanning electron microscopy) were conducted five times.

The data were analyzed by using the SPSS software (SPSS statistics base 17.0, Chicago, USA) as follows: 1) All the values were expressed as mean and standard deviation. 2) Each of the treated groups (GC, CAP1, CAP2, CAP3, DL, AA, CHX1, and CHX2) was compared with the untreated control by using Student t-test (two-tailed, independent). 3) One way Analysis of Variance (ANOVA) followed by Sidak post hoc test was used to compare the means of the four plasma-treated groups (CAP1, CAP2, CAP3, and CAP4). 4) Base on the treatment duration, repeated measures were employed for comparison of plasma groups with the positive control groups (CAP1 (196.25 s) was compared to CHX2 (200 s), and CAP3 (49.04 s) was compared to DL (60 s), AA (60 s), and CHX1 (60 s)) by using Student t-test (two-tailed, independent). For all comparison $p < 0.05$ was considered statistically significant.

[*1] HMDS, Acros Organics, Geel, Belgium.

[*2] FEI Company, Eindhoven, The Netherlands.

4. Results

4.1. Surface analysis of Ti discs

The Ma Ti discs showed small formations (Fig. 12-a). The SEM micrographs of the MS Ti discs were processed by using a 3D image reconstruction software (Alicona MeX 5.1) to analyze the roughness, height, and profile of the MS Ti discs. The microstructures had a mean roughness of 2.04 µm, average height of profile 1.2 µm, and the maximum measured distance between the highest and the lowest level reached 21.3 µm (Figs. 12-b and 13, Table 3).

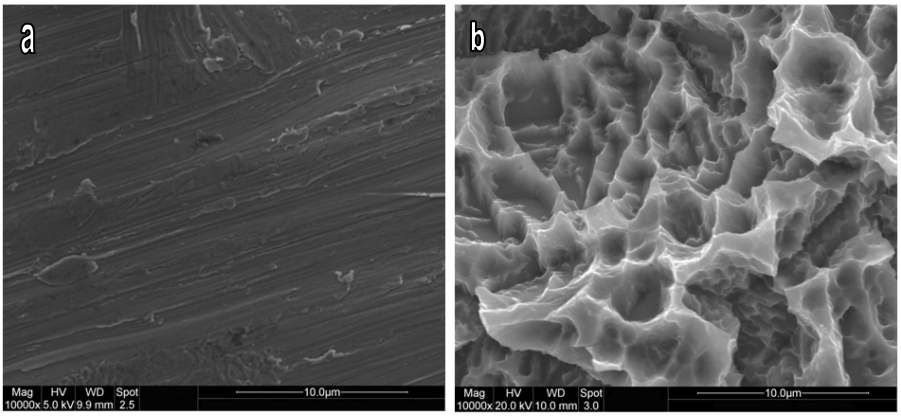

Fig. 12: The SEM micrographs of the Ma Ti discs (a) and the MS Ti discs (b) at 10000 x magnification

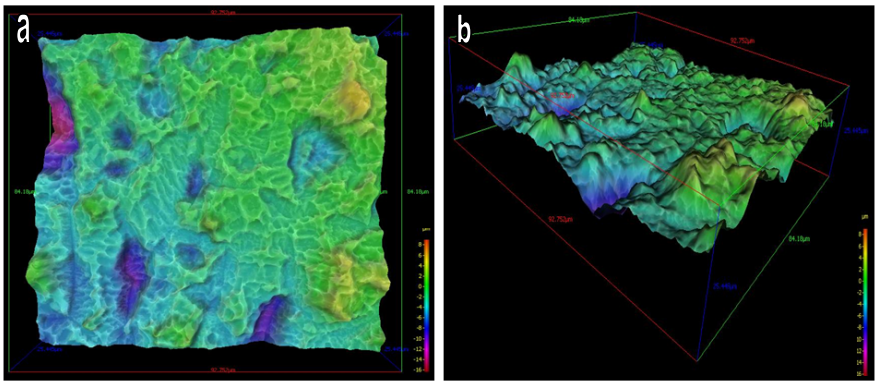

Fig. 13: 3D horizontal (a) and lateral (b) colure reconstruction surface profiles of the MS Ti discs (SEM data, visualized by using 3D image reconstruction software (Alicona MeX 5.1))

Table 3: Profile analysis using a 3D image reconstruction software (Alicona MeX 5.1)

MS Ti discs	1	2	3	4	5	6	M	SD
Pa; average height of profile	1.7	1.4	1.4	1.5	1.7	2.2	1.6	0.3
Pt; maximum peak to valley height of profile	31.1	16.7	24.4	15.5	16.1	24.3	21.4	6.3
Ra; average roughness of profile	2.1	2.1	2	1.9	2.1	2	2	0.1
Rt; maximum peak to valley roughness of profile	11.1	7.9	9.9	7.6	9.2	8.3	2.1	1.3

4.2. Analysis of three days *in situ* oral biofilm

The SEM results showed that all the MS Ti discs (one of each volunteer) were entirely filled up with biofilm, while only two Ma Ti discs (two volunteers) showed surfaces entirely covered with biofilms; one specimen was covered approximately 80% and the last two specimens were covered approximately 60%. The center and the edge of the MS and Ma Ti discs had always thicker biofilm accumulation as in the middle (Figs. 17-a1 and 17-b1).

100 x 10 mag. FM and 10000 SEM micrographs showed domination of cocci-shaped bacteria (Figs. 15 a1-a3 and b1-b3, Figs. 17-a3 and 17-b3). The lower layers had only cocci-shaped bacteria, while bacilli-shaped bacteria were visible in the upper layers of the biofilms, especially on the MS Ti discs (Fig. 16-b1, CLSM results).

There was significant differences between the amount of the accumulated biofilms on the MS and Ma Ti discs $p = 0.0003$, the amount of the accumulated biofilms on the MS Ti discs (54.6 ± 10.4 µg) exceeded twice the amount of the biofilms accumulated on the Ma Ti discs (22.2 ± 5.1 µg).

The proportion of red fluorescent was slightly more on the MS discs as the Ma discs' $p = 0.225$. All contact inoculation of the MS and Ma discs showed bacterial growth to cover the entire impression area on blood agar, but the bacterial colonies of the MS discs were larger and showed more proliferation than the Ma Ti discs.

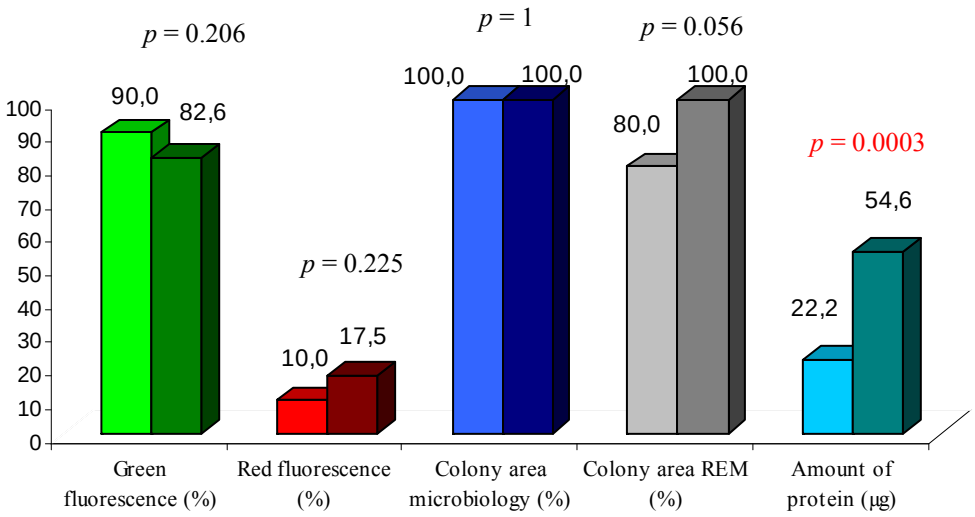

Fig. 14: Comparison between the accumulated biofilms on the Ma (light colour) and the MS Ti discs (dark colour)

The ParoCheck analysis showed the domination of cocci-positive gram bacteria, but anaerobia, negative gram, and bacilli are also present. The following species were detected in the 72 h *in situ oral* biofilms: *Streptococuss mitis, Streptococuss gordonii, Streptococuss constellatus, Viellonella parvula, Eikenella corrodens, Capnocytophaga sp,* Actinomyces *odontolyticus, Fusobacterium nucleatum, Actinomyces viscosus*, and only by one volunteer the *Peptostreptococcus micros* (Table 4).

Table 4: The detected pathogens on Ma and MS Ti discs of 72 h old oral biofilms (ParoCheck analysis)

Bacteria	**MS**	**Ma**	**Description of Bacteria**
Streptococuss mitis group	(+, +, +, +, +)	(+, +, +, +, +)	Gram positive, facultative anaerobe, are part of the oral flora, usually arranged in short chains in the shape of cocci.
Streptococuss gordonii group	(+, +, +, +, +)	(+, +, +, +, +)	Gram positive, grow in pairs or bead like chains, are integral members of the human oral flora and plays an integral role in initiating colonization for other colonizers to adhere to biofilm.
Viellonella parvula	(+, +, +, +, +)	(+, +, +, +, +)	Gram negative cocci, anaerobe are part of the oral flora.
Eikenella corrodens	(+, +, +, +, +)	(+, +, +, +, +)	Gram negative facultative anaerobic bacillus. It grows in aerobic and anaerobic conditions.
Streptococuss constellatus group	(+, +, +, +, +)	(+, +, +, +, +)	Gram positive, part of the human oral normal flora, The cells are small, normally 0.5-1μm in diameter and form short chains.
Capnocytophaga sp	(+, +, +, +, +)	(+, +, +, +, +)	Gram-negative bacteria, normally found in the oropharyngeal tract of mammals, they are involved in the pathogenesis of some animal bite wounds as well as periodontal diseases.
Actinomyces odontolyticus	(+, +, +, +, +)	(+, +, +, +, +)	Gram positive, rod shaped bacilli, anaerobe, part of the human oral normal flora.
Fusobacterium nucleatum	(+, +, +, +, +)	(+, +, +, +, +)	Gram-negative, rod shaped bacilli, anaerobic. It is a key component of periodontal plaque due to its abundance and its ability to coaggregation with other species in the oral cavity.
Actinomyces viscosus	(-, -, +, -, +)	(-, -, +, -, +)	Gram-positive, is part of the human oral flora, rod-shaped filamentous bacteria, occurs in high numbers in the dental plaque, cemental caries, and tonsillar crypts.
Peptostreptococcus micros	(-, -, -, -, -)	(-, -, -, +, -)	Gram-positive, anaerobic. The cells are small, spherical, and can occur in short chains, in pairs or individually.

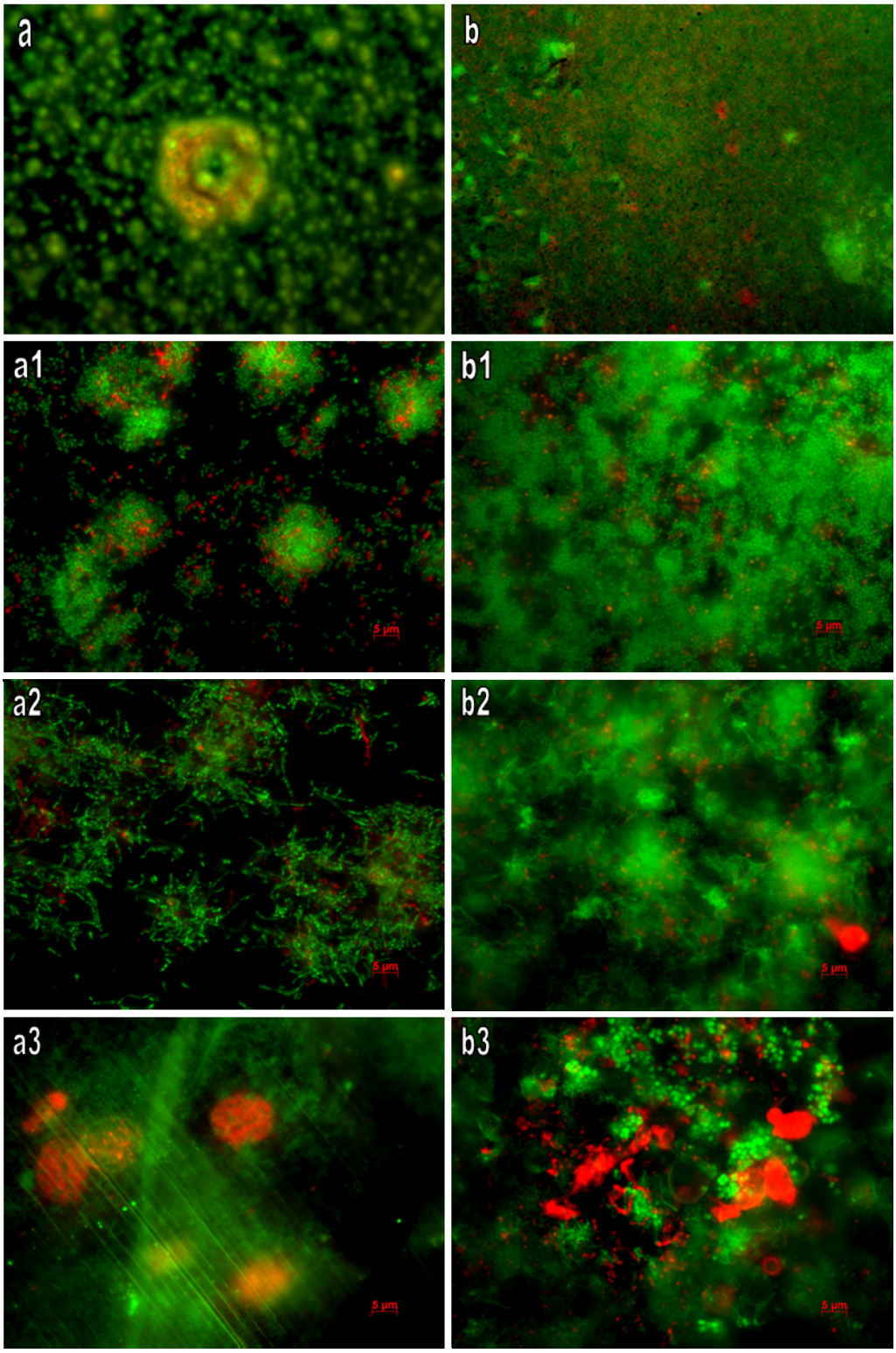

Fig. 15: Fluorescence micrographs of the untreated *in situ* oral biofilms on the Ma (left) and MS (right) Ti discs, a and b at 10 x magnification describe the ratio of the covered area on Ti discs, a1, a2, a3 and b1, b2, b3 at 100 x 10 magnifications exhibit different forms of bacteria, nuclei of keratinized cells and the ratio of green and red fluorescence

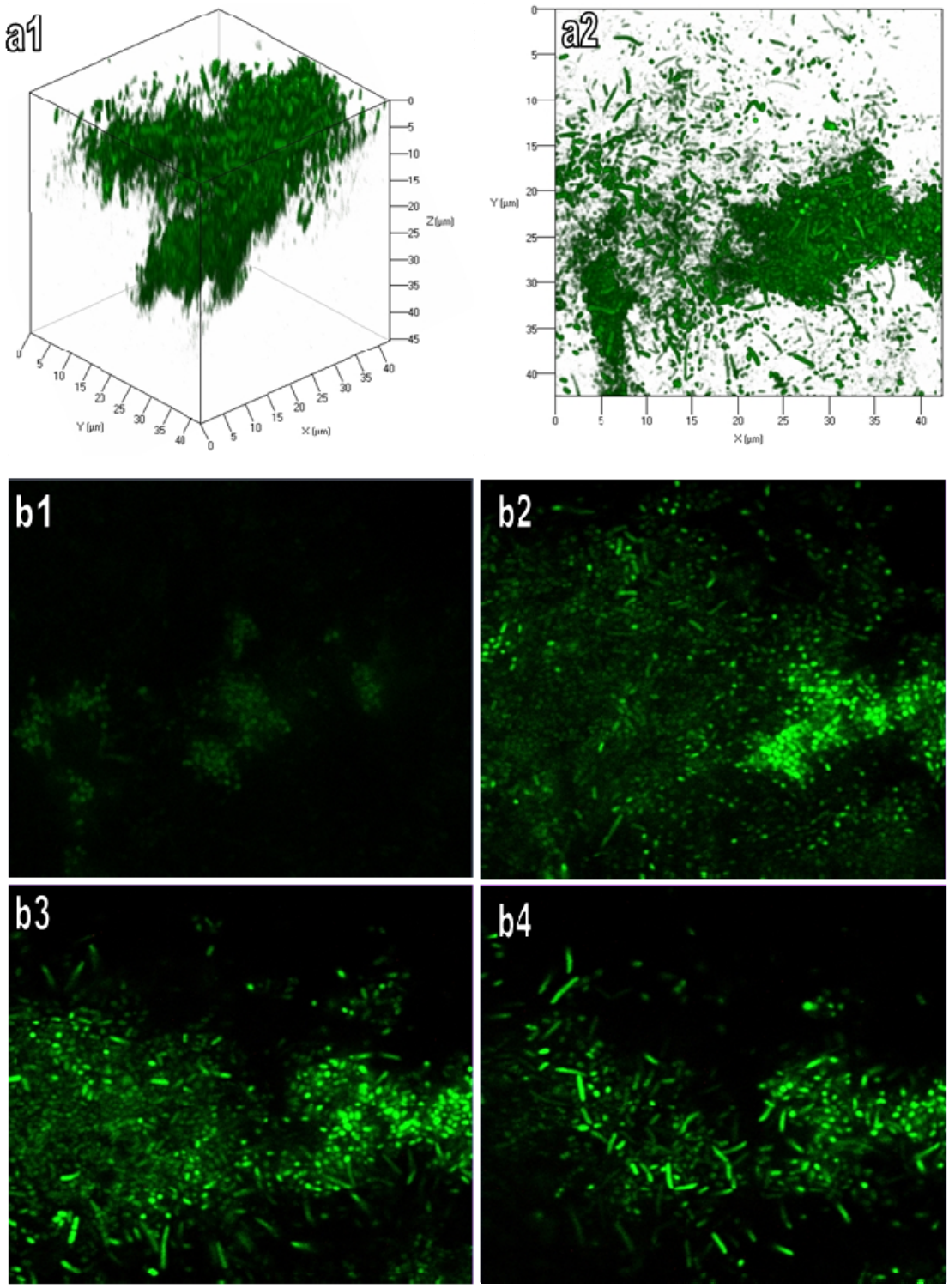

Fig. 16: Fluorescence micrographs captured with confocal laser microscope of the untreated control oral biofilms formed on the MS Ti discs, a1 and a2 are horizontal and lateral sections, b1-b4 are four sagittal sections from the bottom to the top. The lower sections showed only cocci form bacteria, while bacilliform bacteria become clear in the upper sections

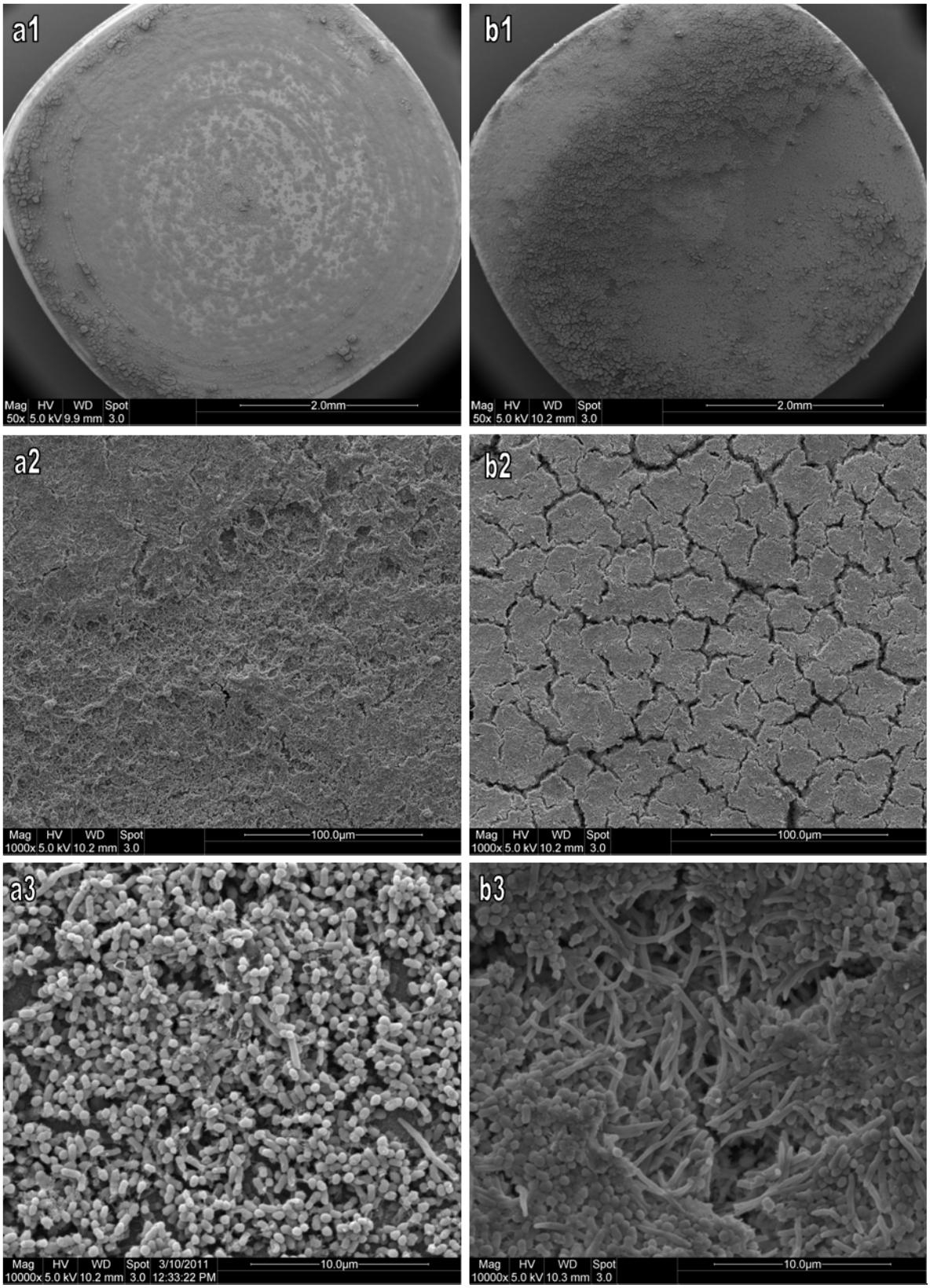

Fig. 17: SEM micrographs of the untreated 72 h oral biofilms formed on the Ma (left) and MS (right) Ti discs, a1 and b1 at 50 × magnification describe the ratio of covered areas, a2 and b2 at 1000 × magnification, a3 and b3 at 10000 × magnification show the biofilm structure (Idlibi *et al*., 2013)

4.3. Analysis of biofilm after treatment

4.3.1. **Live/dead staining**

- Machined titanium discs:

The average percentage cover of green fluorescence on the UC, GC and AA was 90.04%, 78.02% and 72.7%, respectively. Two out of the five Ti discs after the treatment with AA did not show residual biofilms. All CAP groups, DL, CHX1 and CHX2 resulted in a significant decrease in the percentage cover of green fluorescence, in comparison with UC (CAP1 15.11%, CAP2 38.6%, CAP3 35.96%, CAP4 35.13%, DL 8.15%, CHX1 13.76%, and CHX2 12.18%, $p = 0.0$ for all comparison). The average percentage cover of green fluorescence after the treatment with CAP1 differed significantly from CAP2, CAP3, and CAP4 ($p < 0.05$, Table 5-2b), whereas no significant difference was observed between CAP2, CAP3, and CAP4 ($p > 0.05$, Table 5-2b). No significant difference was observed between CAP1 and CHX2 ($p = 0.626$), while DL and CHX1 differed significantly from CAP3 ($p < 0.05$, Table 5-3).

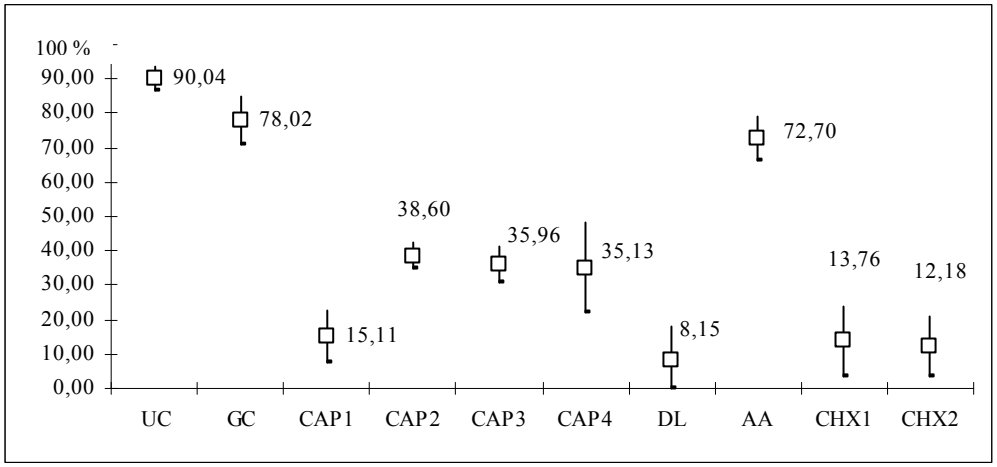

Fig. 18: Average percentage (mean and 95 % confidence intervals) of green fluorescence on the Ma Ti discs determined after the various treatments of the *in situ* biofilms. Fivefold determination (Idlibi *et al.,* 2013)

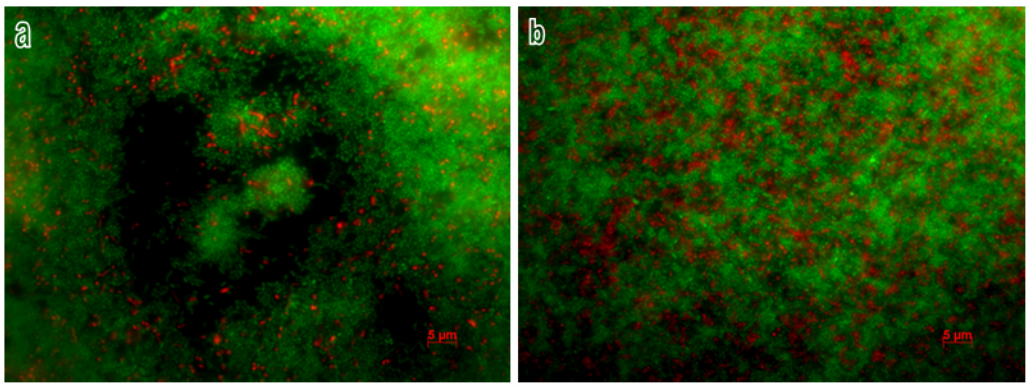

Fig. 19: Fluorescence micrographs of the Ma Ti discs at 1000 x magnification a: UC, b: GC (Idlibi *et al.*, 2013)

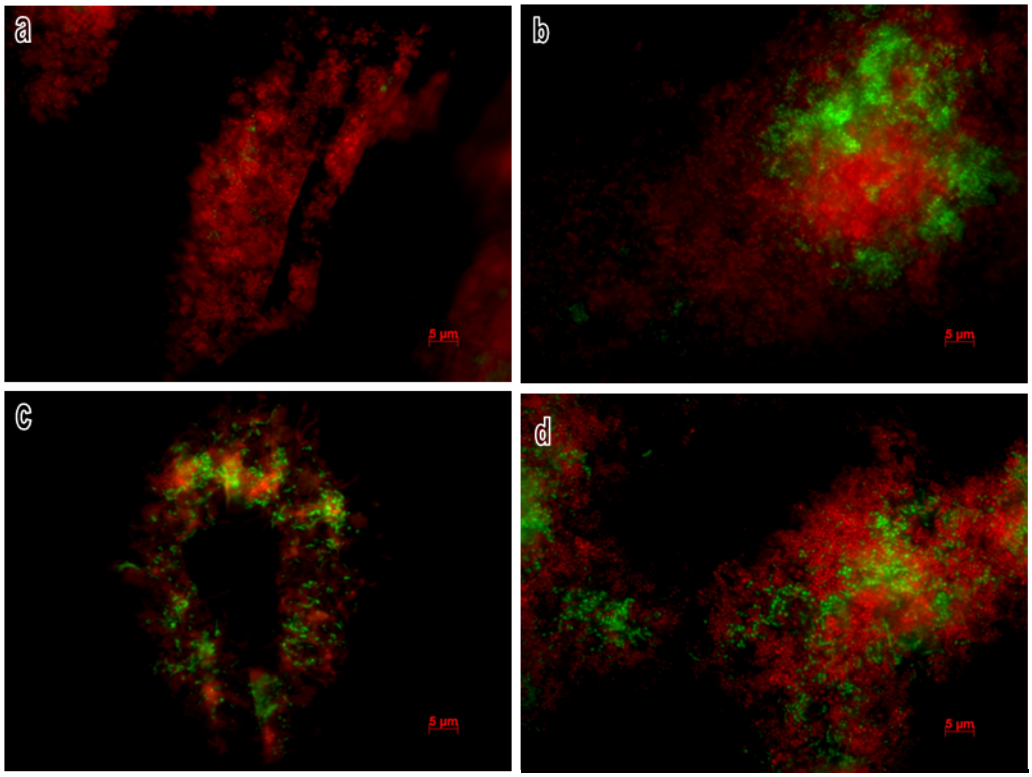

Fig. 20: Fluorescence micrographs of the Ma Ti discs at 1000 X magnification, a: CAP1, b: CAP2, c: CAP3, d: CAP4. Notice an increase in the proportion of red fluorescence (Idlibi *et al*., 2013)

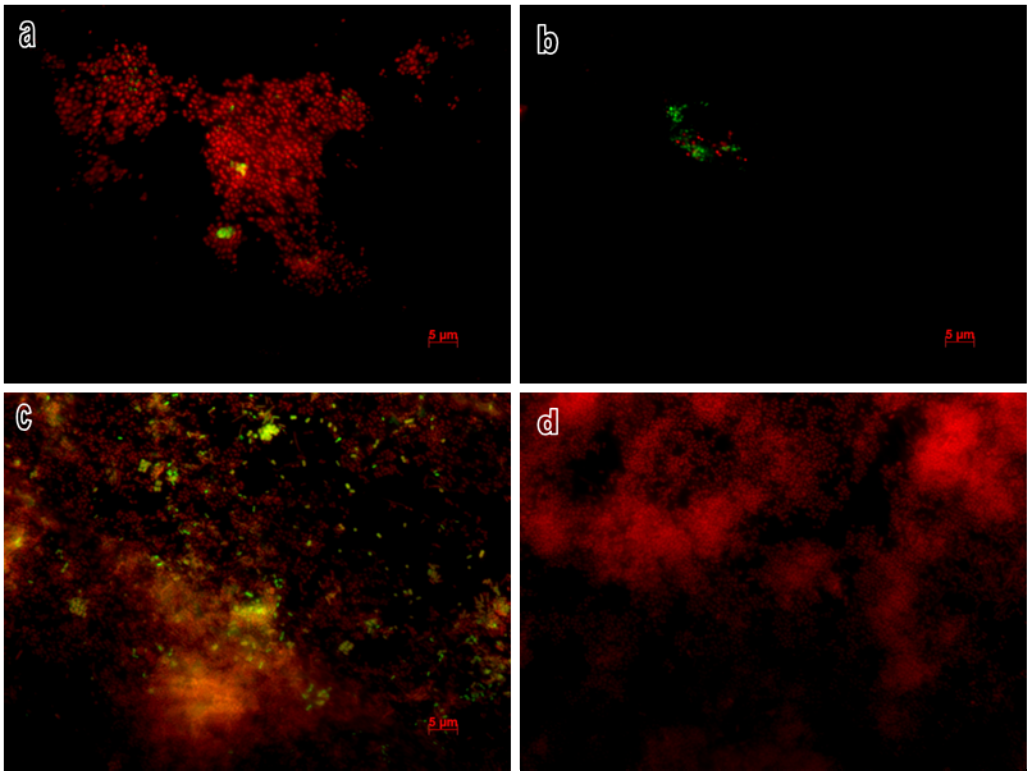

Fig. 21: Fluorescence micrographs of the Ma Ti discs at 1000 X magnification, a: DL, b: AA, c: CHX1, d: CHX2. Notice an increase in the proportion of red fluorescence after the treatment with DL and CHX, and a decrease in the amount of biofilms after the treatment with AA (Idlibi *et al*., 2013)

- Microstructured titanium discs:

The average percentage cover of green fluorescence on GC (75.5%) and AA (68.79%) did not differ significantly ($p < 0.05$, Table 6-1) from UC (82.55%). All CAP experimental groups, DL, CHX1 and CHX2 resulted in a significant decrease of green fluorescence compared to UC (CAP1 13.95%, CAP2 24.74%, CAP3 33.41%, CAP4 36.76%, DL 8.48%, CHX1 7.36%, and CHX2 3.96%, $p = 0.00$ for all comparisons, Table 6-1).The lowest recorded percentage cover of green fluorescence was achieved by treatment with CHX2 (3.96%), only one of the five replicates did not show any green fluorescence from residual biofilms.

The average percentage cover of green fluorescence after treatment with CAP1 did not differ significantly from CAP2 and CAP3 ($p < 0.05$), while differed significantly from CAP4 ($p = 0.037$, Table 6-2b). No significant difference was observed between CAP1 and CHX2 ($p = 0.078$), while DL and CHX1 differed significantly from CAP3 ($p < 0.05$, Table 6-3).

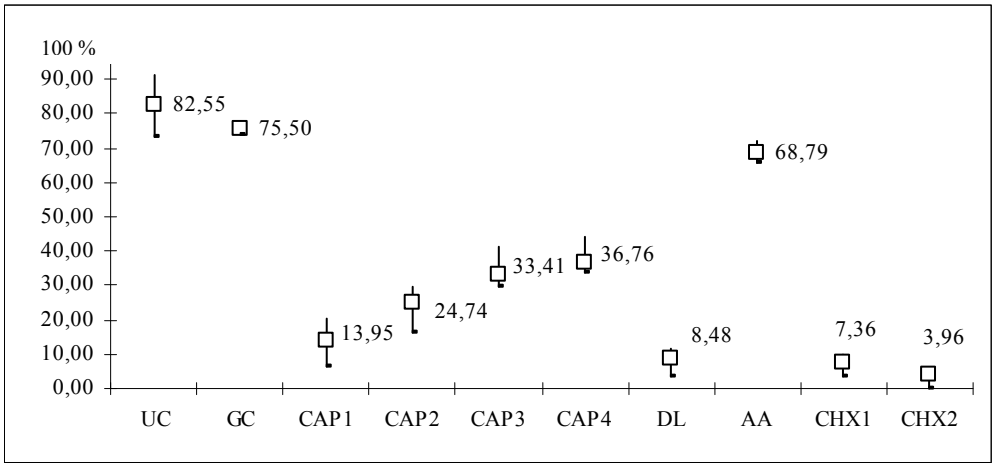

Fig. 22: Average percentage (mean and 95 % confidence intervals) of green fluorescence of biofilms on the MS Ti discs determined after the various treatments of the *in situ* oral biofilms. Fivefold determination

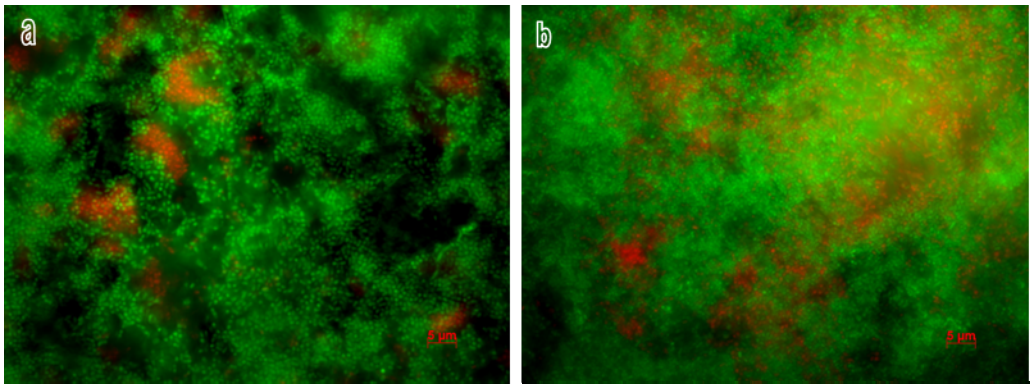

Fig. 23: Fluorescence micrographs of the Ma Ti discs at 1000 x magnification a: UC, b: GC

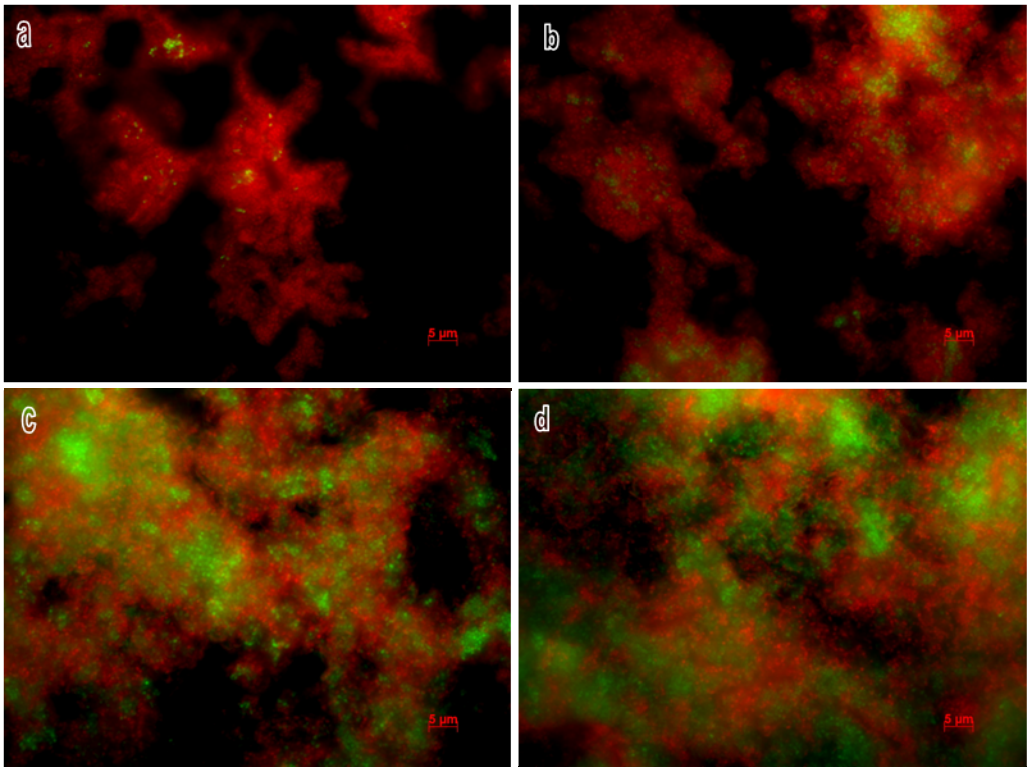

Fig. 24: Fluorescence micrographs of the MS Ti discs at 1000 × magnification, a: CAP1, b: CAP2, c: CAP3, d: CAP4. Notice an increase in the proportion of red fluorescence after the treatment with CAP

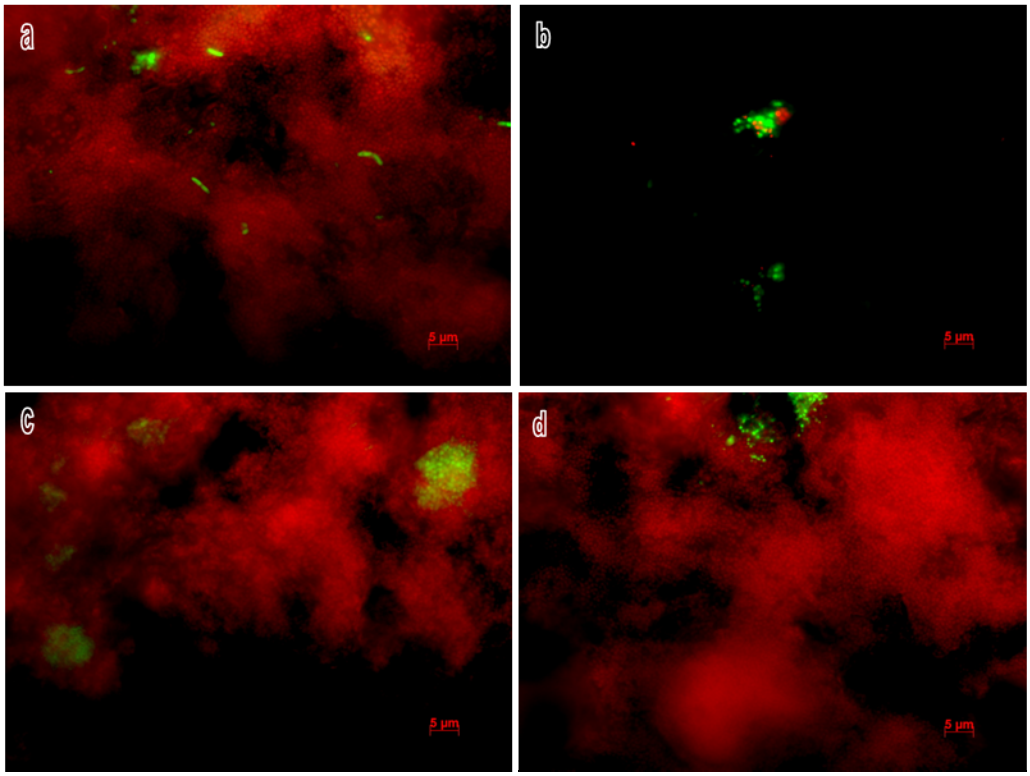

Fig. 25: Fluorescence micrographs of the MS Ti discs at 1000 × magnification, a: DL, b: AA, c: CHX1, d: CHX2. Notice an increase in the proportion of red fluorescence after the treatment with DL and CHX, and a decrease in the amount of biofilms after the treatment with AA

4.3.2. Microbiology

- Machined titanium discs:

All contact inoculation of UC and GC showed bacterial growth covering the entire impression area for all replicates (Figs. 26 and 27, Table 7). No bacterial growth was detected on any of the inoculated areas after the treatment with CAP1 and CAP3 (Figs. 26-3 and 26-5). One of the five replicates of CAP4 and CHX2 showed bacterial growth with an area of 0.33% and 2.51%, respectively (Figs. 26-6 and 26-10). Four of the five inoculated areas on blood agar showed bacterial growth after the treatment with CAP2, DL, AA and CHX1, with an average coverage by microbial growths of 2.59%; 2%; 3.71%, and 15.49%, respectively (Figs. 26-4; 26-7, 26-8, and 26-9). The results after the treatment with CAP1 and CAP3 differed significantly from CAP2 ($p = 0.021$, Table 7-2b). No significant difference was observed between CAP1 and CHX2 ($p = 0.347$) and between CAP3 and CHX1 ($p = 0.322$), while CAP3 differed significantly from DL ($p = 0.023$, Table 7-3).

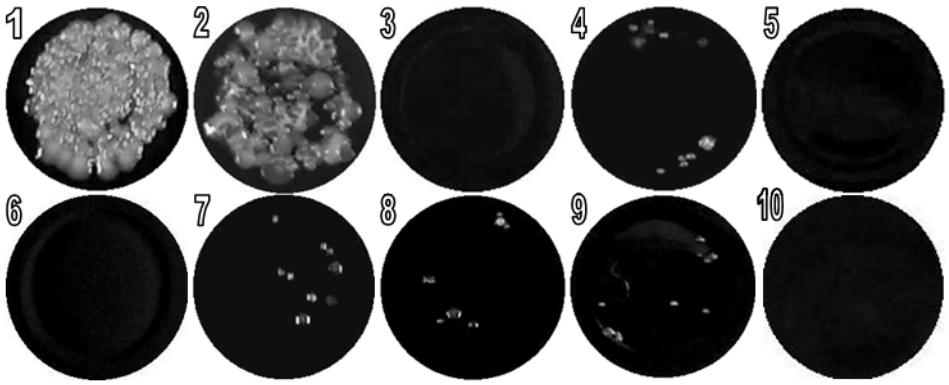

Fig. 26: Photographs of contact areas on the blood agar of the Ma Ti discs. 1: UC; 2: GC; 3: CAP1; 4: CAP2; 5: CAP3; 6: CAP4; 7: DL; 8: AA; 9: CHX1; 10: CHX2. Notice the microbial growth on the blood agar of 1, 2, 4, 7, 8 and 9. (Idlibi *et al*., 2013)

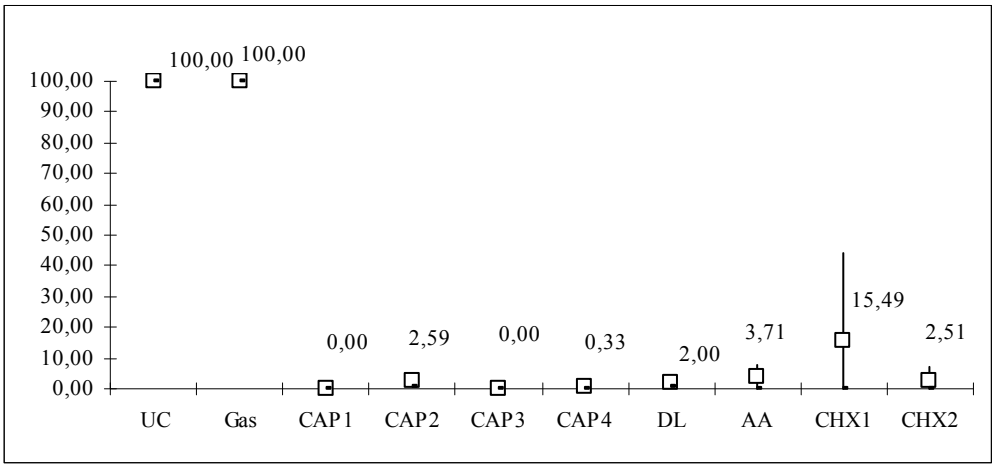

Fig. 27: Average percentage of colony areas grew on blood agar after inoculation with the Ma Ti Discs determined after the various treatments of the *in situ* formed biofilms. Fivefold determination

- Microstructured titanium discs:

All contact inoculation of UC and GC showed bacterial growth covering the entire impression area for all replicates (Figs. 28-1 and 28-2). No bacterial growth was detected on any of the five inoculated areas after the treatment with CAP1, CAP3, and CAP4 (Figs. 28-3, 28-5, and 28-6). Three replicates of CAP2 showed bacterial growth with colony area of 4.4% (Figs. 28-4). All inoculated areas after the treatment with DL, AA, CHX1, and CHX2 showed microbial growth with an average coverage of 8.43%, 21.74%, 52.5%, and 13.7%, respectively (Figs. 28-7, 28-8, 28-9, and 28-10). The results after the treatment with CAP1, CAP3 and CAP4 differed significantly from CAP2 ($p < 0.05$ for all comparisons, Table 8-2b). Also CAP1 differed significantly from CHX2 ($p = 0.006$), and CAP3 from CHX1 ($p = 0.003$), while no significant difference was observed between CAP1 and DL ($p = 0.129$, Table 8-3).

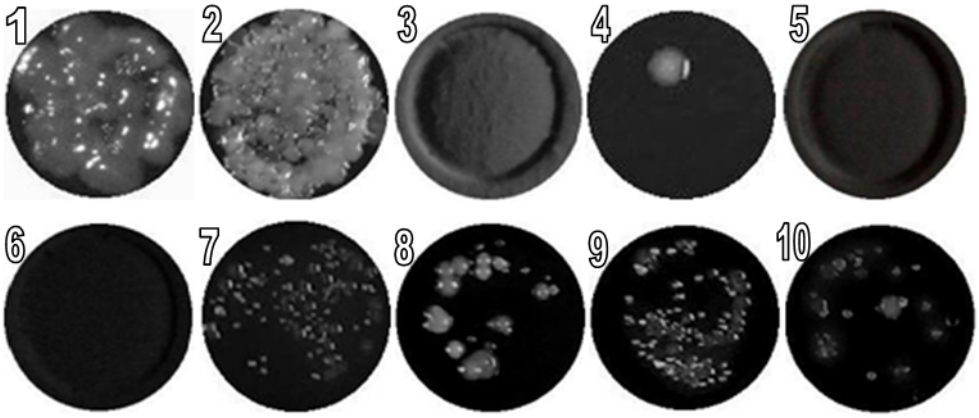

Fig. 28: Photographs of contact areas on the blood agar of the MS Ti discs. 1: UC; 2: GC; 3: CAP1; 4: CAP2; 5: CAP3; 6: CAP4; 7: DL; 8: AA; 9: CHX1; 10: CHX2. Notice growing of the bacteria on the blood agar of 1, 2, 4, 7, 8, 9 and 10

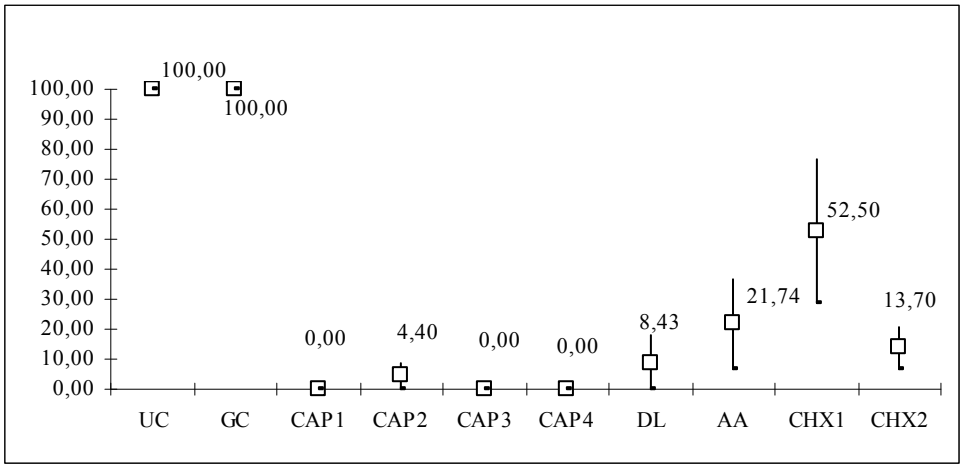

Fig. 29: Average percentage of colony areas grew on blood agar after inoculation with the MS Ti Discs determined after the various treatments of the *in situ* formed biofilms. Fivefold determination

4.3.3. Detection of total protein

- Machined titanium discs:

All experimental parameters of CAP, DL and AA led to a significant reduction in the amount of protein, compared with UC ($p < 0.05$, Table 9-1). The mean values of residual protein after the treatment with CAP groups 1-4 were 6.29 µg, 11.14 µg, 11.14 µg, and 13.44 µg, respectively. Three of the five Ti discs, treated with AA, had levels of protein below detection, while the remaining two replicates showed a mean residual protein of 0.83 µg. The gas treatment and submersion of the Ti discs in chlorhexidine for 60 s (CHX1) and for 200 s did not significantly decrease the total amount of protein, compared with UC ($p > 0.05$, Table 9-1); the mean values of residual protein were 20.2 µg for GC, 16.62 µg for CHX1 and 15.06 µg for CHX2.

No significant differences were observed in the amount of the residual biofilms between CAP parameters ($p > 0.05$ for all comparisons, Table 9-2b). Also no significant differences were observed between CAP3 and DL ($p = 0.384$), while AA differed significantly from CAP3 ($p = 0.002$, Table 9-3).

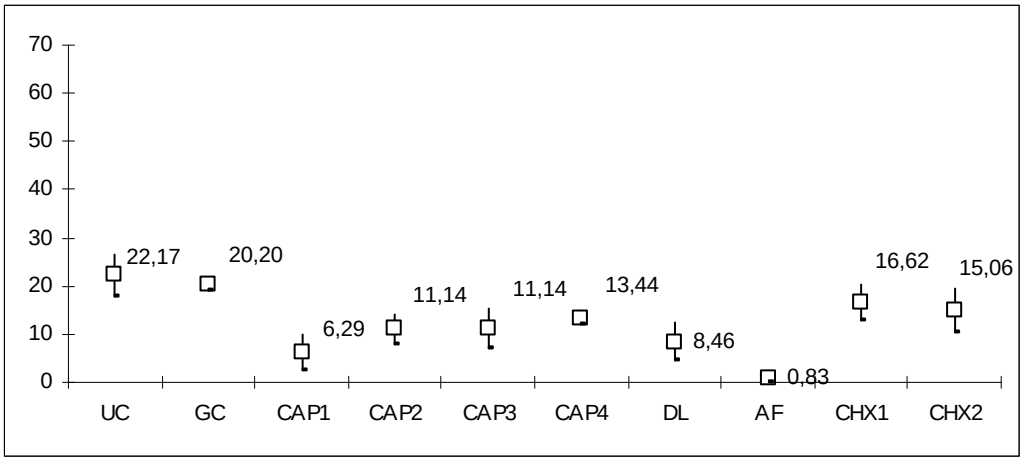

Fig. 30: Average protein amounts (mean and 95 % confidence intervals) determined after the various treatments of the *in situ* oral biofilms formed on the Ma Ti discs. Fivefold determination (Idlibi *et al.,* 2013)

- Microstructured titanium discs:

All experimental parameters of CAP, DL, AA, and CHX2 led to a significant reduction in the amount of protein, compared with UC ($p < 0.05$, Table 10-1). The mean values of residual protein after the treatment with CAP parameters were 15.71 µg, 21.31 µg, 32.5 µg, and 34.26 µg. Two of the five replicates, treated with AA, had levels of protein below detection, while the remaining three replicates showed a mean residual protein of 2 µg ($p = 0.00$). Calculations of the amount of protein after the treatment with DL resulted in the mean value of 24.49 µg. The submersion of the Ti discs in CHX for 60 s (CHX1) did not lead to significant decreases in the total amount of protein; mean value: 39.04 µg and $p = 0.051$, while the submersion for 200 s (CHX2) led to the mean value of protein 34.04 µg and differed significantly from UC ($p = 0.006$). No significant difference was observed after the treatment with GC ($p = 0.684$), in comparison with UC; the mean value reached 51.66 µg (Table 10-1).

No significant difference was observed in the amount of the residual protein between CAP1 and CAP2 ($p = 0.905$), or between CAP3 and CAP4 ($p = 1$), while CAP1 differed significantly from CAP3 ($p = 0.044$) and CAP4 ($p = 0.023$, Table 10-2b). No significant difference was observed between CAP3 and DL ($p = 0.327$), while AA differed significantly from CAP3 ($p = 0.00$, Table 10-3).

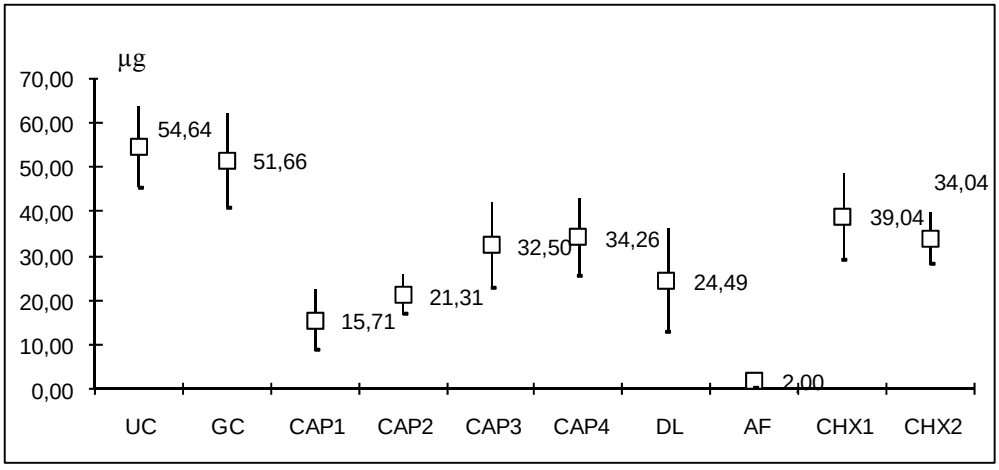

Fig. 31: Average protein amounts (mean and 95 % confidence intervals) determined after the various treatments of the *in situ* formed biofilms on the MS Ti discs. Fivefold determination

4.3.4. Scanning electron microscope

- Ma and MS titanium discs:

The SEM images of the UC showed a predominance of coccoid-shaped bacteria and the presence of rod-shaped bacteria (Figs. 32-a, 33-a). All the Ti discs, treated with CAP, showed degradation of the structure of the biofilms, reduced amounts of biofilm, and a substantial decrease in the number of bacteria in the superficial layer (Figs. 34-35). The greatest effect was observed after the treatment with CAP1 (Figs. 34-a, and 35-a), while no substantial difference was apparent between the other CAP treatments (Figs. 34 b-d, and 35 b-d). DL led to a decrease in the amount of biofilms (Figs. 36-a, and 37-a). The greatest efficacy was observed after the treatment with AA; low magnification (x 50) gave the impression that the Ti discs were completely cleaned, but at higher magnification (x 10000) small bacterial colonies spread on the surface of the specimen could be observed (Figs. 36-b, and 37-b). The Ti discs, treated with CHX1 and CHX2, showed rarefication in the biofilm matrix and a decrease in the bacteria in the superficial layer (Figs. 36 c-d, and 37 c-d). The GC did not show any alterations in the amount of bacteria or the biofilm structure (Figs. 32-b, and 33-b).

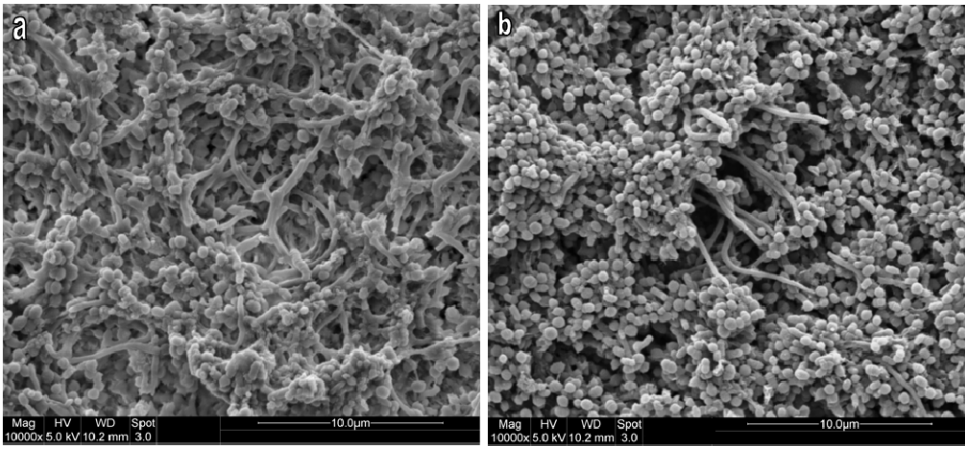

Fig. 32: SEM micrographs of the Ma Ti discs at 10000 x magnification, a: UC, b: GC (Idlibi *et al*., 2013)

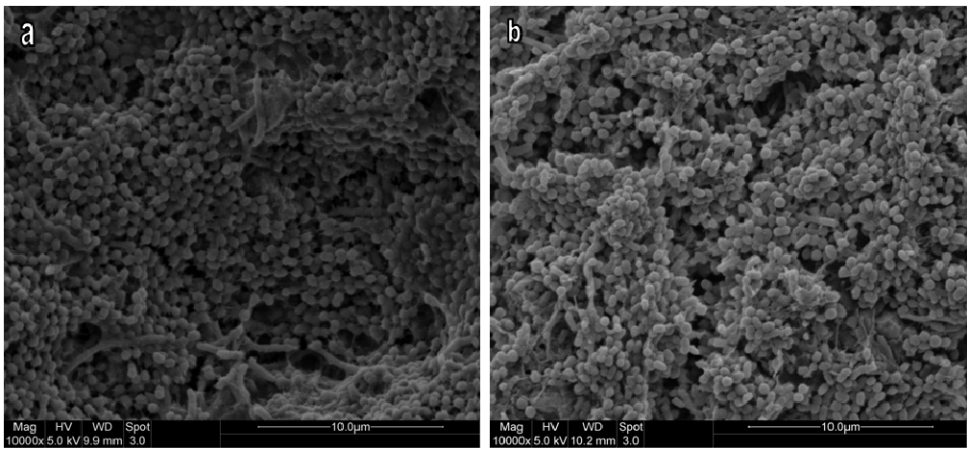

Fig. 33: SEM micrographs of the MS Ti discs at 10000 x magnification, a: UC, b: GC

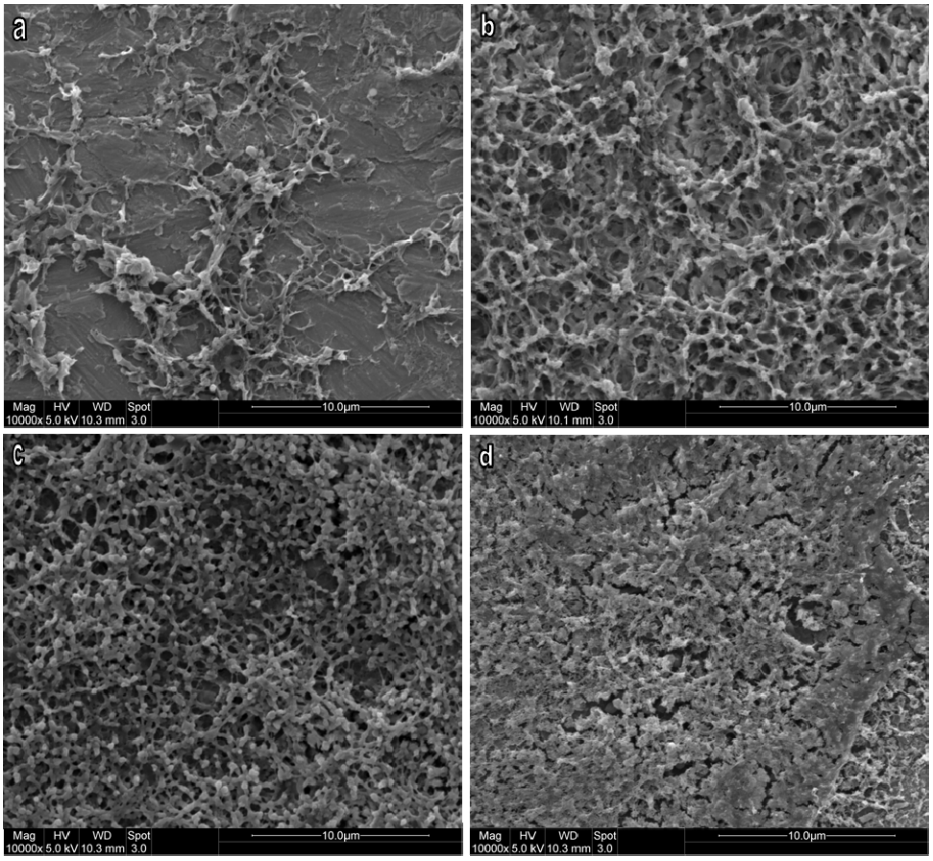

Fig. 34: SEM micrographs of the Ma Ti discs at 10000 x magnification, after the treatment with a: CAP1, b: CAP2, c: CAP3, d: CAP4 (Idlibi *et al*., 2013)

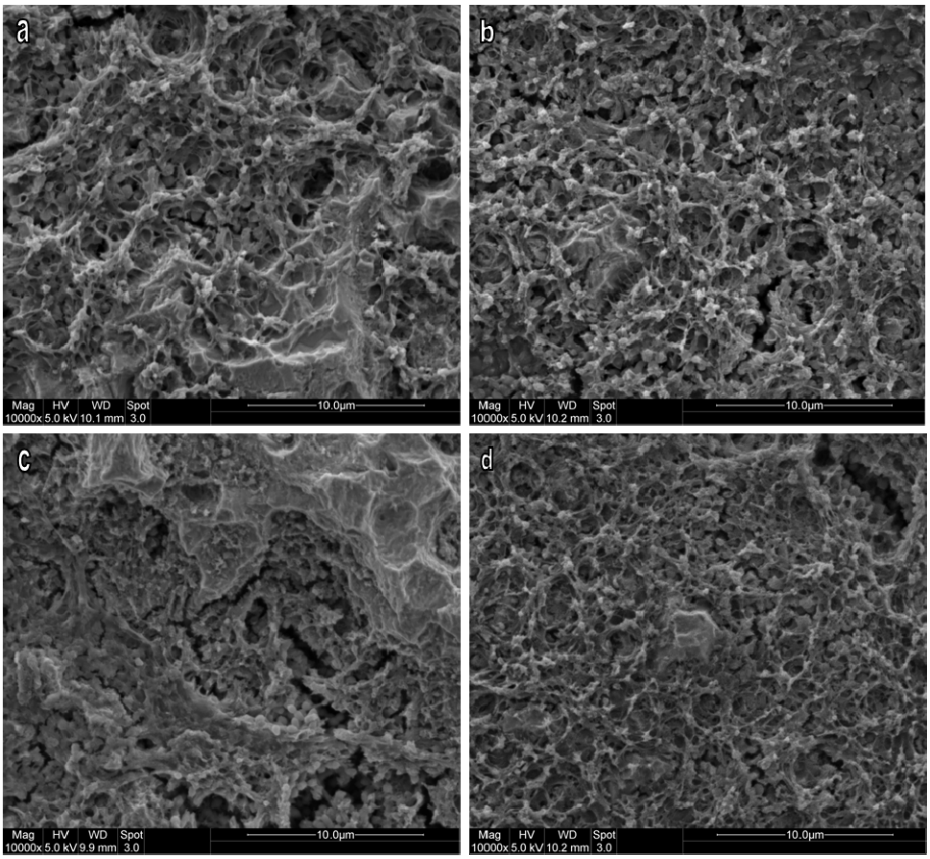

Fig. 35: SEM micrographs of the MS Ti discs at 10000 x magnification, after the treatment with a: CAP1, b: CAP2, c: CAP3, d: CAP4

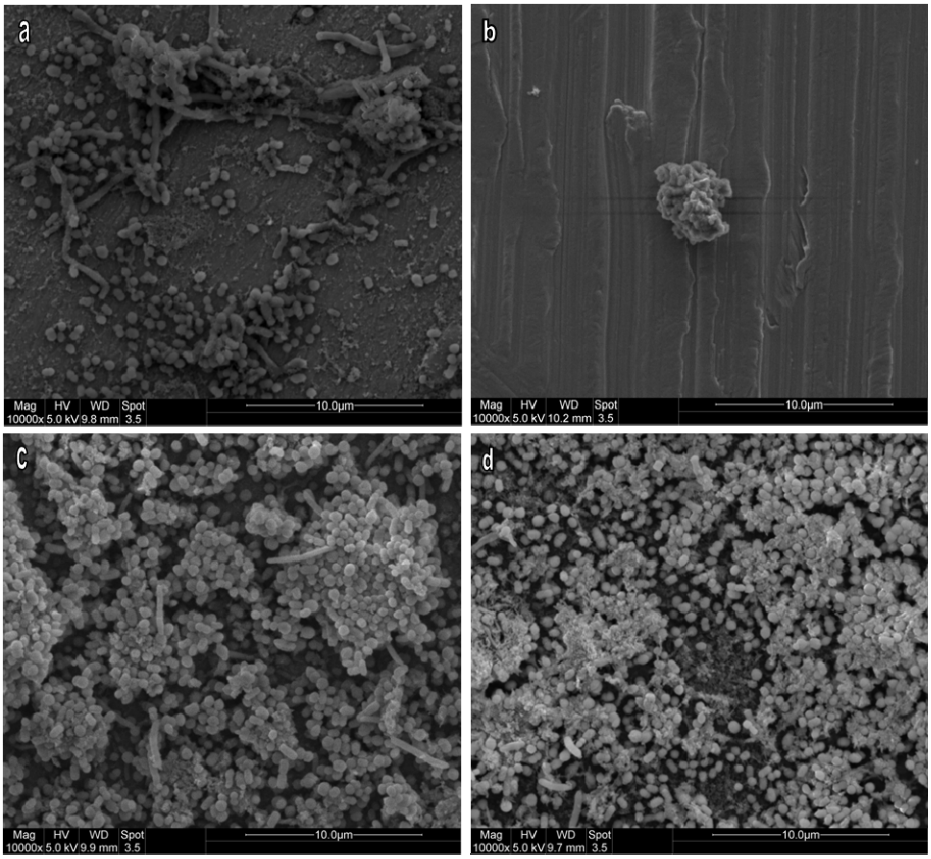

Fig. 36: SEM micrographs of the Ma Ti discs at 10000 x magnification after the treatment with a: DL, b: AA, c: CHX1, d: CHX2 (Idlibi *et al*., 2013)

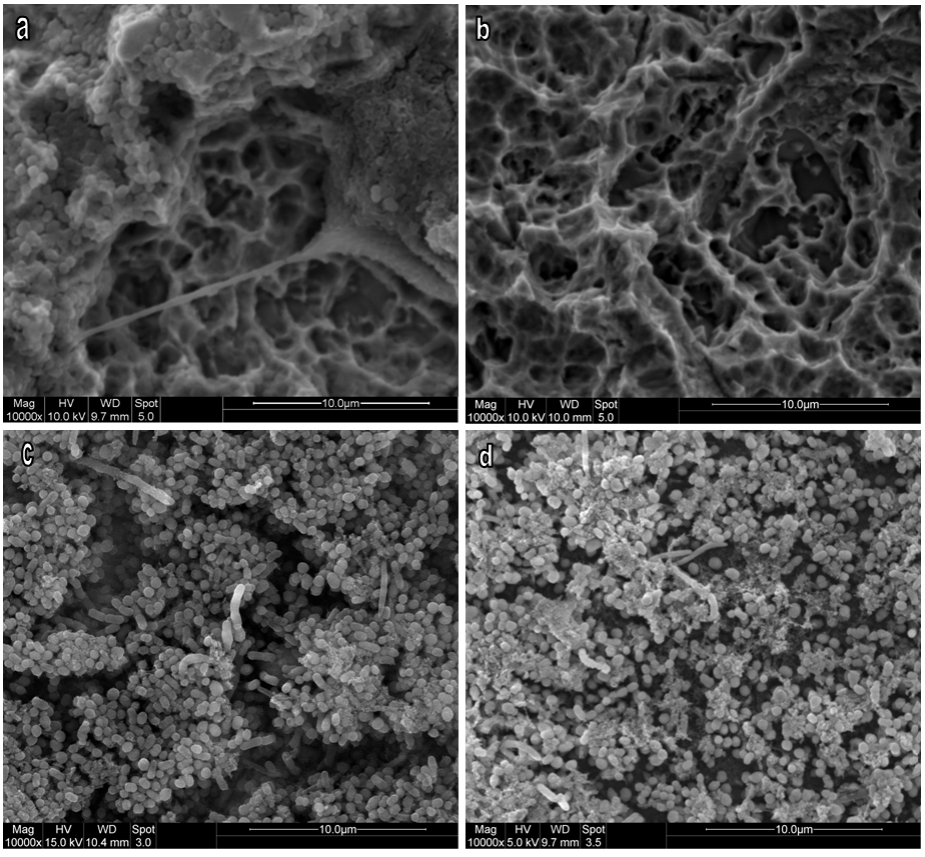

Fig. 37: SEM micrographs of the MS Ti discs at 10000 x magnification after the treatment with a: DL, b: AA, c: CHX1, d: CHX2 (Idlibi *et al*., 2013)

4.4. Temperature monitoring

The surface temperature increased instantaneously and reached the maximum in the plasma jet's center within five seconds. Increase of the surface temperature during the CAP treatment correlated with the presence of biofilms on the Ti discs, increase of the surface roughness, and mean power (Figs. 38 and 39, Tables 11, 12, and 13).

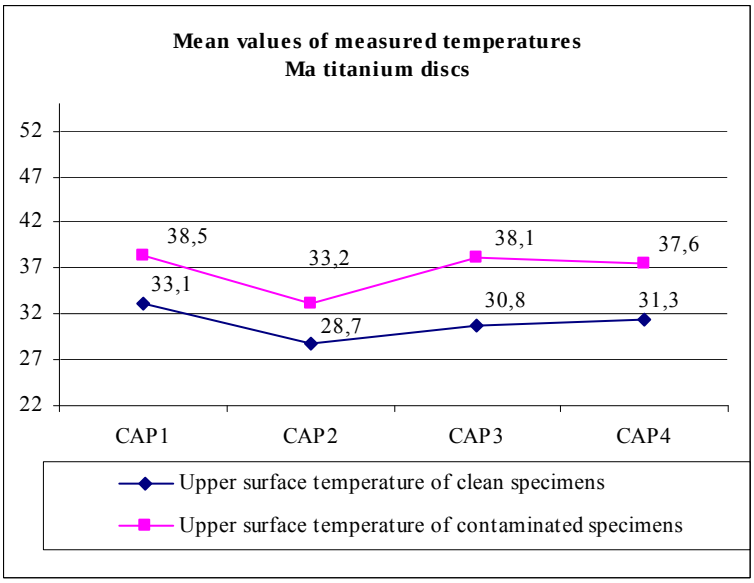

Fig. 38: Mean temperature values on the upper surfaces of the clean and contaminated Ma Ti discs during the treatment with cold atmospheric plasma

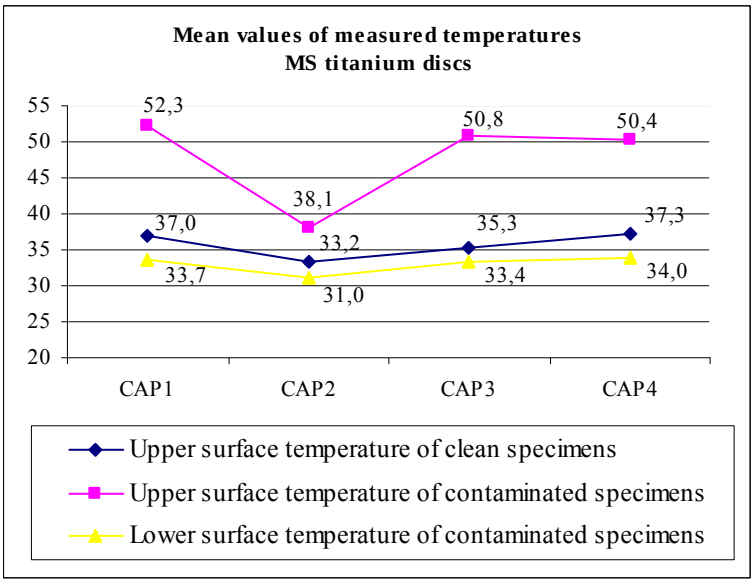

Fig. 39: Mean temperature values on the upper and the lower surfaces of the clean and contaminated MS Ti discs during the treatment with cold atmospheric plasma

4.5. Effects of cold plasma on the surface structure of titanium surfaces

The SEM examination failed to demonstrate any visible morphological differences between the CAP-treated and control-untreated Ma and MS Ti surfaces. In particular, no thermal side effects, such as melting or loss of the porosity, were observed (Figs. 40 and 41).

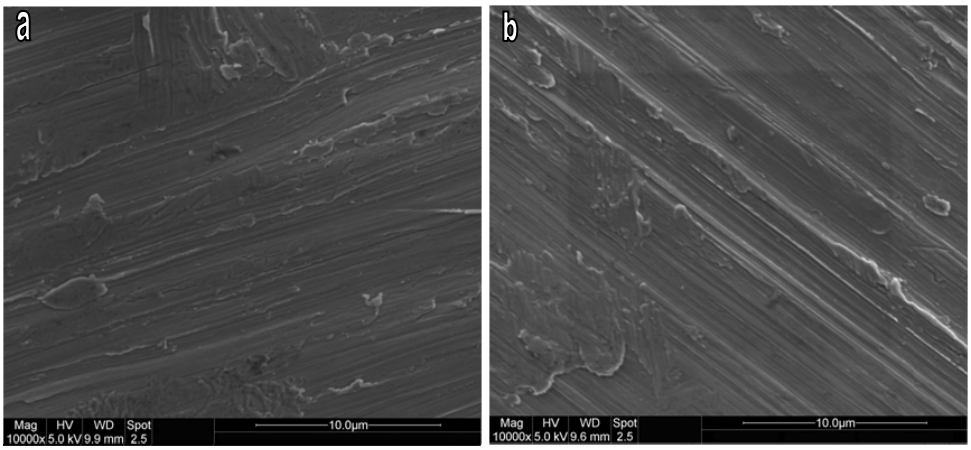

Fig. 40: SEM micrographs of the Ma Ti discs at 10000 x magnification; a: control untreated and b: after treatment with CAP1

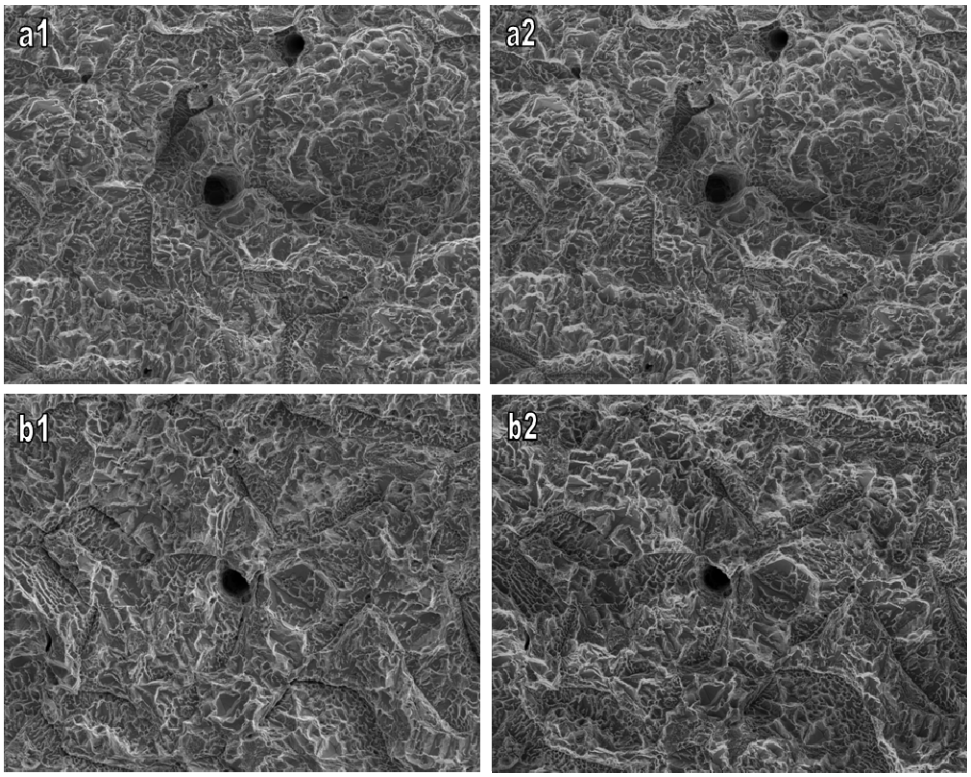

Fig. 41: SEM micrographs of the MS Ti discs at 10000 x magnification; a1: control untreated; a2: is the same area after treatment with CAP1. b1: control untreated; and b2: is the same area after treatment with CAP2

5. Discussion

The present experimental investigation has demonstrated that cold atmospheric plasma is suitable for inactivating and destroying the three-day *in situ* oral biofilms formed on the machined and microstructured titanium discs at acceptable temperatures, without causing any damage to titanium surfaces. The efficacy of CAP was evaluated at two different mean powers, two different gas mixtures, and two different treatment durations. The untreated biofilms as negative control and the treated biofilms groups (diode laser, air-abrasion, and chlorhexidine) as positive controls were used to estimate the efficacy of CAP.

All plasma parameters exhibited great abilities to inhibit bacterial growth on blood agar and resulted in a significant decrease in the total amount of biofilms and the average percentage of viable bacteria, compared with the untreated control. There was a correlation between increasing the effectiveness of plasma and increasing the mean power and/or treatment duration. The antiseptic efficacy of CAP was comparable with diode laser and chlorhexidine.

5.1 Titanium specimens and the biofilm formation

The used Ti discs in the current study represent the most prevalent types of implant surfaces. The first is machined, typifying the supragingival part of dental implants which is usually used to prevent the formation of oral biofilms. The second is microstructured, typifying the subgingival part of dental implants which is used to enhance the osseointegration between bone and implant surfaces. The microstructure of the Ti discs had a mean roughness of 2 µm, with a maximum measured distance between the highest and the lowest level of 21 µm.

The volunteers adapted their oral hygiene to create reproducible amounts of viable biofilms like those that accumulate around dental implants in the oral cavity. They stopped using mouth rinses two weeks before the study started. The oral hygiene measures were carried out without using toothpastes and no brushing of the Ti discs. The Ti discs were positioned ca. 1 mm deeply in the silicon impression material to avoid any self-cleaning movement that may happen by contacts with cheek mucosa (Fig. 1). The splints were removed from the oral cavity during brushing, eating, and drinking. Moreover, biofilm inhibiting drinks, such as wine or tea, were avoided (Rupf *et al*., 2011; Idlibi *et al.,* 2013).

5.2 Analysis methods

Two methods were used to check the viability of biofilms: The first being the live/dead staining technique that allowed living and dead bacteria to be distinguished quantitatively. The kit consists of two nucleic acid stains: green (SYTO9) and red (propidium iodide). These stains differ in their abilities to penetrate bacterial cell membranes and stain nucleic acids. Acids green fluorescing is able to enter all cells, while the red fluorescing only enters cells with damaged cytoplasmic membranes and can reducing SYTO®9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes have only SYTO®9 and will fluoresced green, while dead bacteria with damaged membranes have the both dyes but fluoresce red. Despite the fact that this technique can be applied rapidly and gives an idea of viability changes, there are some limitations which should be taken into consideration: (1) Bacteria in intermediate states, represented by dormant or prelytic cells, may appear green, but are not viable or cultivable. Additionally, staining of bacterial cells does not always produce distinct “green” and “red” populations. It may make difficult the interpretation of results (Berney *et al*., 2007); (2) It is quite simple to picture separate bacteria on plane surfaces, but quantitative interpretation of images becomes increasingly difficult with irregular surfaces containing numerous numbers of compact bacterial aggregates, as is the case in this study of natural biofilms formed *in situ*; and (3) The kit stains both DNA and RNA. Non-bacterial components, such as eukaryotic cells, are therefore also stained green or red (Berney *et al*., 2007). In conclusion, it can be assumed that red fluorescence represents non-viable bacteria; however, it is not necessarily true that all green fluorescence indicates viable bacteria. Therefore, it seems appropriate to use another technique to investigate the viability of bacteria in the residual biofilms. For this purpose, contact inoculation of control and the treated specimens were performed on blood agar, a non-selective growth medium, thereby providing a complete nutrient environment for microbes and supporting the growth of a wide range of organisms.

The detection of total protein was used to define the amount of the residual biofilm and to estimate the ability of the technique to eliminate the oral biofilms. SEM was used to investigate the presence of biofilms or the cleanliness of the specimens, as well as to investigate morphological changes in the residual biofilm.

5.3 Three-day *in situ* oral biofilms grown on titanium surfaces

All the volunteers showed similar colonization patterns of *Streptococcus mitis, Streptococcus gordonii, Streptococcus constellatus*, *Actinomyces odontolyticus, Actinomyces viscosus*, *Fusobacterium nucleatum*, *Viellonella parvula, Eikenella corrodens* and *Capnocytophaga* on the Ma and MS Ti surfaces. The *Streptococci* were always the predominant colonizing microbes, *Actinomyces species* and *Streptococci* are considered to be early colonizers on intraoral hard tissues which prepare the environment for the adhesion and growth of late colonizers. *Veillonella spp*. are anaerobic gram-negative *cocci* and considered to be early colonizers of the normal oral human flora. *Fusobacterium nucleatum* has been reported to be a late colonizer that is only prominent in dental plaques after early colonizers are settled on the teeth (Socransky & Haffajee, 1998; Foster & Kolenbrander, 2004; Al-Ahmad *et al*., 2010).

No differences in composition were observed between the biofilms accumulated on the Ma or MS Ti surfaces in relation to the colonization of the investigated bacteria. Our results are in agreement with previous observations which showed no significant differences in biofilm composition formed on different implant surfaces, or between the biofilms formed on implant surfaces and intra-oral hard tissues (Al-Ahmad *et al*., 2010; Foster & Kolenbrander, 2004).

The personal factor and the roughness of Ti discs played a large role in determining the amount of the accumulated biofilms. Results of protein detection showed significant differences in the amount of the accumulated biofilms between volunteers; $p = 0.001$ of the Ma Ti discs and $p = 00$ of the MS Ti discs. Results of protein analysis showed a significantly higher biofilm accumulation on the MS Ti discs than on the Ma Ti discs $p = 0.0003$ (Fig. 14). According to the SEM examination, all the MS Ti discs were entirely filled up with biofilms and the biofilms were even visually detectable after air drying without optical enlargement, while the covered areas on the Ma Ti discs ranged between 60-100%. These results are consistent with other *in vivo* studies, which have reported a close relationship between the surface roughness and accumulation of dental plaques (Nakazato *et al*., 1989; Quirynen *et al*., 1989 and 1990; Quirynen *et al*., 1993; Bollen *et al*., 1997; Rimondini *et al*., 1997; Al-Ahmad *et al*., 2010; Bürgers *et al*., 2010). The rough and irregular surfaces create a safe haven for the microorganisms which are to be more protected against shear forces. Furthermore, they increase the potential area for bacterial adhesion (Nyvad and Fejerskov, 1987).

5.4 Decontamination of titanium discs

Incomplete removal of biological contamination, including plaque biofilm and bacteria, is considered one of the most important factors that may affect the healing of peri-implant diseases after conservative or regenerative therapy. To the best of our knowledge, no available study has evaluated the efficacy of helium cold atmospheric plasma to remove and kill *in situ* oral biofilms formed on Ti surfaces. The efficacy of all treatment techniques, which were compared in this study, differed according to their respective abilities to remove oral biofilms, their bactericidal properties, or both.

All CAP parameters accomplished a significant reduction of green fluorescence percentage on the Ma and MS Ti discs, compared with the UC ($p < 0.05$) (Tables 5-1 and 6-1, Figs. 18, 20, 22, and 24). The average percentage of residual green fluorescence on the Ma Ti discs is inversely related to the increase of mean power and treatment duration. The greatest effect on the Ma Ti discs was achieved after the treatment with CAP1, which differed significantly from CAP2, CAP3, and CAP4 ($p < 0.05$, Tables 5-2a and 5-2b). No significant differences were observed between CAP2 and CAP3, or between CAP2 and CAP4, in spite of the differences in treatment duration (Table 5-2b). On the MS Ti discs, CAP1 showed more capacity to dead biofilm as CAP2 or CAP3, but the differences between the groups were not significant ($p > 0.05$, Table 6-2b).

The lowest percentage of green fluorescence was recorded after treatment with DL (60 s) on the Ma Ti discs, and CHX2 (200 s) on the MS Ti discs. The results showed that CAP1 (196 s) is comparable with CHX2 (200 s) in its effect against oral biofilms on the Ma Ti discs, no significant differences between the treatments were observed ($p > 0.05$, Table 5-3). Conversely, DL (60 s) and CHX1 (60 s) have more efficacy than CAP3 (49 s) in reducing green fluorescence on the Ma and MS Ti discs ($p < 0.05$, Tables 5-3 and 6-3).

The results of blood agar inoculation revealed no bacterial growth after treatment with CAP1 and CAP3 of the Ma and MS Ti discs. Only one of the five trials of CAP4, with the Ma Ti discs, displayed bacterial growth in a small area, while all trials of the MS Ti discs were negative. On the other hand, CAP2 showed bacterial growth on four impression areas of the Ma Ti discs and all agar inoculation of the MS Ti discs, but all the colonies were small and their areas did not exceed 2.6% on the Ma Ti discs and 3.8% on the MS Ti discs (Tables 7-1 and 8-1).

On the basis of microbiology results, we can conclude that the residual green fluorescence, after the treatment with CAP parameters, did not exhibit proliferation capability, excluding a little amount of bacteria after the treatment with CAP2. If we exclude the single positive specimen of CAP4 on the Ma Ti discs, we can report that all biofilms treated with 5 W did not display any bacterial growth on blood agar. Also, CAP3 with 49 s of treatment duration was enough to attain the same efficacy level as CAP1 with the treatment duration of 196 s (Tables 7-1 and 8-1). The results of inoculation on blood agar after the treatment with CAP showed no differences between the Ma and MS Ti discs, in spite of the MS Ti discs having more amounts of accumulated biofilms (the amount of biofilm accumulated on the MS discs reached approximately twice the amount on Ma discs).

The results showed that CAP1 (196 s) was comparable to CHX2 (200 s), and CAP3 was comparable to CHX1 (60 s) and DL (60 s) in their effects on the Ma and MS Ti discs (Tables 7-3 and 8-3). According to microbiology results, CAP produced the best results with regard to the inhibition growth of oral microorganisms on blood agar.

The results of colorimetric determination showed that all the tested CAP parameters on the Ma and MS Ti discs, compared to UC, led to a significant reduction in the amount of biofilms, but in different proportions (Tables 9-1 and 10-1, Figs. 30 and 31). The comparison of the amount of residual protein on the MS Ti discs indicated an association between the effectiveness of CAP with increases in the treatment duration, which led to more biofilm elimination as is evident by comparing CAP1 with CAP3, or CAP1 with CAP4. The removed amount of biofilm by CAP1 was approximately two times more than the amount removed by CAP3 on the Ma and MS Ti discs. CAP1 differed significantly from CAP3 on the MS Ti discs ($p = 0.04$). Increase of the mean power showed more capacity to decrease biofilm; the removed amount of biofilm by CAP1 was approximately twice as much as the amount of biofilm removed by CAP2 in the case of the Ma Ti discs, and approximately 30% in the case of the MS Ti discs. However, statistical comparison failed to find significant differences between CAP1 and CAP2 on the Ma or MS Ti discs ($p > 0.05$, Tables 9-2b and 10-2b). The admixture of oxygen to helium did not improve the effectiveness of CAP3, while no significant differences were observed between CAP3 and CAP4 on the Ma or MS Ti discs ($p > 0.05$, Tables 9-2b and 10-2b).

There were no significant differences in the amount of residual protein between CAP3 (49 s) and DL (60 s) on the Ma or MS Ti discs ($p > 0.05$, Tables 9-3 and 10-3). AA was the best

effective cleaning method in the current study, three of the five replicates of the Ma Ti discs and two of the five replicates of the MS Ti discs had levels of protein below detection. AA differed significantly from CAP3 on the Ma and MS Ti discs ($p < 0.05$, Tables 9-3 and 10-3).

Scanning electron microscopy analysis revealed distinct micro-morphological alterations of the adherent microorganisms and the biofilm matrix after the CAP treatment. All Ti discs showed destruction of biofilm structure and the amount of biofilm was reduced. The greatest effect was observed after the treatment with CAP1, then CAP2 (Figs. 34: a-b and 35: a-b), while no substantial difference was noticed between CAP3 and CAP4 (Figs. 34: c-d and 35: c-d). All plasma-treated groups were characterized by substantial decreases in the number of bacteria in the superficial layer of the residual biofilm. However, complete removal of biofilms from the Ti surfaces after plasma treatment could not be observed. CHX1 and CHX2 groups showed rarefication in the biofilm matrix and decreases in the bacteria in the superficial layer (Figs. 36 c-d and 37 c-d). Gas control did not display alterations in the biofilm structure (Figs. 32b and 33b). The application of AA showed the best efficacy at removing oral biofilms; though the 50-fold magnification give an impression that the Ti discs were 100% clean, the 10000 fold magnification showed small bacterial colonies on the specimen surface. However, it was demonstrated that half of the specimens treated with AA showed viable residual biofilms (green fluorescence or bacterial growth on blood agar); treatments for 60 s were not always enough to achieve complete biofilm elimination (Figs. 36-b and 37-b).

The current results of cold atmospheric plasma are comparable with other *in vitro* studies. The application of atmospheric-pressure glow-discharge plasma led to the killing and inhibition of *Streptococcus mutans* cultured on the agar nutrient in petri dishes (Goree *et al*., 2006; Sladek *et al*., 2007). Tissue-tolerable plasma was found to be effective against *Pseudomonas aeruginosa* biofilms growth on polystyrene and silicone materials (Hübner *et al*., 2010). Rupf *et al*., (2010) evaluated the antimicrobial efficacy of non-thermal atmospheric plasma against adherent oral microorganisms (*Escherichia coli, Lactobacillus casei, Streptococcus mutans, and Candida albicans*) on agar plates and dentin slices. The plasma treatment of agar plates caused complete growth inhibition of *Escherichia coli* and *Lactobacillus casei*. A time-dependent reduction of growth was observed on the irradiated surfaces for *Streptococcus mutans*.

Rupf *et al*., (2011) combined cold atmospheric plasma with air/water spray to investigate the disinfection and removal of *in situ* formed biofilms on the microstructured Ti discs. The Ti discs were exposed to human oral cavities from two volunteers for 24 and 72 h, respectively. The plasma treatment of biofilms was carried out *ex vivo* by using the same plasma source and working under the same conditions of the current study. The mean power was adjusted by setting the pulse frequency at 3 or 5 W with the treatment time as 196 s. Group (A) was treated only with plasma; while in the case of Group (B), after the plasma treatment, specimens were washed with air/water spray for 5 s. Group (C) was treated with plasma and washed with air/water spray, and then second plasma treatment was repeated. No microbial growth was detected on Rodac plates of any of the 24 h biofilm specimens on the Ti discs treated by cold atmospheric plasma, or in combination with air/water spray. In case of the 72 h biofilm samples, bacterial colonies were detected on two out of five Rodac culture plates after the first round of cold plasma at 3 W, and subsequent air/water spray treatment, and all other Rodac plates displayed no bacterial growth. Fluorescence microscopy and scanning electron microscopy analyses revealed distinct micromorphological alterations of the adherent microorganisms and the biofilm matrix. A strong decrease of biofilm viability (green fluorescence) and significant reductions in biofilm protein amounts were recorded for both 24 h and 72 h biofilms. Higher mean plasma power (5 W) resulted in the extended thinning of the biofilms. However, a treatment of biofilms with the plasma jet alone was not sufficient, under the chosen conditions, to achieve a complete removal of the biofilms. The plaque biofilm was disinfected and reduced in thickness with disintegration of the remnant biofilm structure, and superficial bacteria as well as most bacteria of the deeper biofilm layers were destroyed after the plasma treatment alone. Additional application of mechanical cleaning by the air/water spray, however, almost completely reduced the biofilm remnants.

It can be concluded that the efficacy of cold atmospheric plasma was manifested through two relevant therapeutic effects: the first one is physical, caused by burning and devastation of the biofilm and its components (Manner 2008; Dobrynin *et al*., 2009), while the second one is biological and it results from reactive species such as oxygen and nitric radicals (Laroussi 2002; Kieft *et al*., 2005; Vleugels *et al*., 2005). The physical effect was associated with an increase of mean power and treatment duration, and correlated with an increase of the eliminated biofilm and destructed bacteria. Consequently, the efficacy of CAP ranges between disinfection of microorganisms and removal of biofilm (Idlibi *et al*., 2013).

5.5 The mechanical and thermal effects of cold atmospheric plasma

The SEM analysis of plasma-treated Ti discs failed to reveal any micromorphologically detectable changes of the machined or microstructured Ti surfaces. This does not exclude, however, the chemico-physical changes of the Ti surfaces due to the plasma application, including UV radiation, jet stream of chemical radicals and electrons leading to an increase in the hydrophilicity on the Ti surfaces (Yoshinari *et al*., 2010).

The maximum surface temperature reached approximately 52 °C during the treatment of contaminated MS Ti discs with 5 W mean power (Table 12). The surface temperatures of the MS Ti discs were, in all cases, higher than the surface temperatures of the Ma Ti discs (clean or contaminated Ti discs). Also, the surface temperatures of the Ma or MS Ti discs during the treatment with 5 W were always higher than surface temperature during the treatment with 3 W in all cases (Tables 11 and 12). It was observed that the surface temperatures of the contaminated Ti discs were always higher than the surface temperatures of the clean Ti discs at the same surface type and the same mean power (Tables 11 and 12). The surface temperatures of the clean Ti discs had approximate average values, while the measured temperatures on the contaminated Ti discs were disparate and had larger standard deviation values (Tables 11 and 12). These differences in the measured temperatures may be due to the presence of biofilms on the Ti discs and to the differences in the amount of the accumulated biofilms between the volunteers.

Because of the high surface temperature during the treatment of the contaminated MS Ti discs with 5 W mean power, we have measured the surface temperature on the lower side of the contaminated MS Ti discs. The results approached the values recorded on the upper surfaces of the clean Ti discs that were treated with the same parameters. This indicates that the temperature recorded during the treatment with 5 W mean power of the contaminated MS Ti discs is not the temperature of the Ti discs, but the temperature of burning biofilms (Table 13).

In any case, the available data regarding the thermal tolerance of peri-implant bone suggested a limit of 44 °C (Eriksson & Alberktsonn, 1984). The temperature at the jet contact point can be established, as shown by limiting the output below the critical temperature range. However, the potential damage to peri-implant tissue must be conducted before the plasma jet treatment is introduced to dental practice.

5.6 Conclusion

Concerning these aspects, the plasma jet source used in the present study appears to be suitable to disintegrate the matured oral *in situ* biofilms formed on the machined and microstructured Ti surfaces without causing any thermal or mechanical damages. The comparison with other studies suggested that cold atmospheric plasma was comparable to diode laser and Chlorhexamed 0.2% in its antibacterial effects. Cold atmospheric plasma might be used alone, when there is no urgent need for mechanical decontamination, or after mechanical decontamination, to ensure disinfection.

Further investigations are necessary to evaluate the potential use of cold atmospheric plasma under *in vivo* conditions and the combination of cold atmospheric plasma with other mechanical debridement methods such as plastic curettes, ultrasonic, or air-abrasion, as well as their possible role in the clinical setting to improve the re-osseointegration of implants. From a clinical point of view, it should also be taken into account that a large number of different implant types and surface characteristics complicate a generalization of the present results.

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7. Publications and Acknowledgement

7.1 Publications

Removing biofilms from microstructural Ti *ex vivo*: a novel approach using atmospheric plasma technology

Rupf S; Idlibi AN; Al-Marrawi F; Hannig M; Schubert A; von Müller L; Spitzer W; Holtmann H; Lehmann A; Rueppel A; Schindler A (2011) *PloS ONE* 6; e25893

Abstract:

The removal of biofilms from microstructured titanium used for dental implants is a still unresolved challenge. This experimental study investigated disinfection and removal of *in situ* formed biofilms from microstructured titanium using cold atmospheric plasma in combination with air/water spray. Titanium discs (roughness (Ra): 1.96 μm) were exposed to human oral cavities for 24 and 72 h (n = 149 each) to produce biofilms. Biofilm thickness was determined using confocal laser scanning microscopy (n = 5 each). Plasma treatment of biofilms was carried out *ex vivo* using a microwave-driven pulsed plasma source working at temperatures from 39 to 43 °C. Following plasma treatment, one group was air/water spray treated before re-treatment by second plasma pulses. Vital microorganisms on the titanium surfaces were identified by contact culture (Rodac agar plates). Biofilm presence and bacterial viability were quantified by fluorescence microscopy. Morphology of titanium surfaces and attached biofilms was visualized by scanning electron microscopy. Total protein amounts of biofilms were colorimetrically quantified. Untreated and air/water treated biofilms served as controls. Cold plasma treatment of native biofilms with a mean thickness of 19 μm (24 h) to 91 μm (72 h) covering the microstructure of the titanium surface caused inactivation of biofilm bacteria and significant reduction of protein amounts. Total removal of biofilms, however, required additional application of air/water spray, and a second series of plasma treatment. Importantly, the microstructure of the titanium discs was not altered by plasma treatment. The combination of atmospheric plasma and non-abrasive air/water spray is applicable for complete elimination of oral biofilms from microstructured titanium used for dental implants and may enable new routes for the therapy of periimplant disease.

Disinfection and removal of biofilms on microstructured Ti by cold atmospheric plasma

Rupf S; Idlibi AN; Umanskaya N; Hannig M; Nothdurft F; Lehmann A; Schindler A; von Müller L; Spitzer W (2012) *Journal of Dental Implantology* 2:126-137

Introduction: Biofilms on dental implants play an important role in the genesis of inflammatory peri-implant disease. Decontamination of microstructured titanium is still a challenge for the dental practitioner. Cold atmospheric plasma jets offer disinfecting capabilities at biologically acceptable temperatures. This experimental study investigated disincentive and destructive effects of cold atmospheric plasma on oral biofilms formed *in situ* on microstructured titanium surfaces.

Material and Methods: microstructured titanium discs (sandblasted/etched; n = 120) were exposed to the oral environment of two healthy volunteers for 24 h to produce biofilms. Plasma treatment was carried out by means of a meander like CC line by line scanning with a pulsed microwave driven (2.45 GHz) plasma jet (2.5 s/mm²; 2.0 l/min He; 3 W or 5 W microwave mean power). Following plasma treatment some Ti discs were air/water sprayed and subsequently subjected to a second plasma treatment. Non-irradiated biofilms; biofilms treated with chlorhexidine and microstructured titanium discs without biofilms served as controls. Disinfection of biofilms was assessed by contact agar samples (RODAC technique) and fluorescence microscopy (vital/dead staining). Biofilm morphology was visualized by scanning electron microscopy; biofilm coverage was measured by fluorescence microscopy. Total protein was quantified colorimetrically.

Results: Depending on the plasma jet power the microstructured titanium surface temperature at the plasma contact point varied between 39 and 43 °C. After plasma treatment only; disintegration as well as reduction of biofilm viability and of total protein was observed. The additional application of air/water spray resulted in a further reduction of biofilm viability and of total protein. Fluorescence and protein amounts were reduced comparable to control Ti discs without biofilms after a second plasma treatment. The microstructured surface of the samples was not altered by plasma treatment.

Destruction of oral *in situ* biofilms on machined titanium surfaces by cold atmospheric plasma

Idlibi AN; Al-Marrawi F; Hannig H; Lehmann A; Rueppell A; Schindler A; Jentsch H and Rupf S (2013) *Biofouling* 29:369-379

Abstract:

The decontamination of implant surfaces represents the basic procedure in the management of peri-implant diseases, but it is still a challenge. The study aimed to evaluate the degradation of oral biofilms grown *in situ* on machined titanium discs by cold atmospheric plasma (CAP). 200 titanium discs were exposed to the oral cavities of five healthy human volunteers for 72 h. The resulting biofilms were divided randomly between the following treatments: CAP (which varied in mean power, treatment duration, and/or the gas mixture), and untreated and treated controls (diode laser, air-abrasion, chlorhexidine). The viability, quantity, and morphology of the biofilms were determined by live/dead staining, inoculation onto blood agar, quantification of the total protein content, and scanning electron microscopy. Exposure to CAP significantly reduced the viability and quantity of biofilms compared with untreated control. The efficacy of treatment with CAP correlated with the treatment duration and plasma power. No single method achieved complete biofilm removal; however, CAP may provide an effective support to established decontamination techniques for treatment of peri-implant diseases.

7.2 Short lectures

Biofilm Removal from Ti Surfaces by Non-Thermal Atmospheric Plasma-Jet (24 Stunden *in situ* oralen Biofilmen)

Rupf S; Idlibi AN; Lehmann A; Rueppell A; Hannig M; and Schindler A

International Association for Dental Research (IADR) (Barcelona 2010)

Biofilm destruction of early biofilms on microstructured Ti by cold atmospheric plasma (24 Stunden *in situ* oralen Biofilmen)

Rupf S; Idlibi AN; Umanskaya N; Hannig M**;** Nothdurft F; Lehmann A; Schindler A; Müller L; Spitzer W

25. Jahrestagung der deutsche Gesellschaft für Implantologie (Dresden 2011)

Destruktion und Entfernung von Biofilmen auf mikrostrukturierten Titanoberflächen mit kaltem atmosphärischem Plasma (72 Stunden *in situ* oralen Biofilmen)

Idlibi AN.; Al-Marrawi F.; Hannig M.; Schindler A.; Rupf S

Jahrestagugng der deutsche Gesellschaft für Parodontologie (Baden-Baden 2011)

Destruktion und Entfernung von Biofilmen auf maschinierten Titanoberflächen mit kaltem atmosphärischem Plasma (72 Stunden *in situ* oralen Biofilmen)

Idlibi AN; Al-Marrawi F; Hannig M; von Müller L; Rueppell A; Schindler A; Rupf S

Deutscher Zahnärztetag (Frankfurt 2011)

7.3 Acknowledgement

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8. Lebenslauf

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Seit 11.2012	Zahnarzt in der Gemeinschaftspraxis Dr. Hans-Friedrich Hicks-Monreal & Dr. Michael Menzel / Pirmasens
04.2013	Deutsche Approbation

Publikationen

Removing biofilms from microstructural Ti *ex vivo*: a novel approach using atmospheric plasma technology

Rupf S; Idlibi AN; Al-Marrawi F; Hannig M; Schubert A; von Müller L; Spitzer W; Holtmann H; Lehmann A; Rueppel A; Schindler A (2011) PloS ONE 6; e25893

Disinfection and removal of biofilms on microstructured Ti by cold atmospheric plasma

*Rupf S; Idlibi AN; Umanskaya N; Hannig M**;** Nothdurft F; Lehmann A; Schindler A; von Müller L; Spitzer W (2012) ZZI*

Destruction of oral *in situ* biofilms on machined titanium surfaces by cold atmospheric plasma

Idlibi AN; Al-Marrawi F; Hannig H; Lehmann A; Rueppell A; Schindler A; Jentsch H and Rupf S (2013) Biofouling 29:369-79

9. Appendix

Table 5: Live/dead staining (Ma Ti discs)

Table 5-1: Calculations of Means, standard deviations and standard error for all values. CAP-treated groups and positive controls (DL, AA, CHX) were compared with untreated controls by applying t-tests

	N	M	S D	S E	t-test for Equality of Means					95% Confide Interval	
					t	df	Sig.[*1]	M Diff.	SE Diff.	Lower	Upper
UC	5	90.03	4.15	1.86							
GC	5	78.02	8.01	3.59	UC-GC	2.98	8	0.018	12.01	4.04	2.71	21.32
CAP1	5	15.11	8.32	3.72	UC-CAP1	18.03	8	0.000	74.93	4.16	65.34	84.51
CAP2	5	38.6	4.53	2.03	UC-CAP2	18.73	8	0.000	51.44	2.75	45.11	57.77
CAP3	5	35.96	6.07	2.72	UC-CAP3	16.44	8	0.000	54.08	3.29	46.5	61.66
CAP4	5	35.13	14.66	6.56	UC-CAP4	8.06	8	0.000	54.91	6.82	39.2	70.63
DL	5	8.15	11.38	5.09	UC-DL	15.12	8	0.000	81.89	5.42	69.4	94.37
AA	3	72.7	7.025	4.06	UC-AA	4.49	8	0.004	17.34	3.86	7.9	26.78
CHX1	5	13.76	11.46	5.12	UC-CHX1	13.99	8	0.000	76.28	5.45	63.72	88.85
CHX2	5	12.18	9.94	4.45	UC-CHX2	16.16	8	0.000	77.86	4.82	66.75	88.97

Table 5-2a: Multiple comparisons of CAP groups (One Way ANOVA)

	Sum of Squares	df	Mean Square	F	Sig.[*1]
Between Groups	1758.22	3	586.07	6.86	0.003
Within Groups	1366.23	16	85.39		
Total	3124.45	19			

Table 5-b: Multiple comparisons of CAP groups (Sidak test)

Group (I)	Group (J)	M Differ. (I-J)	SE Differ.	Sig. (2-tailed)[*1]	95% Confidence Interval	
					Lower Bound	Upper Bound
CAP1	CAP2	-23.49	5.84	0.006	-41.01	-5.96
	CAP3	-20.85	5.84	0.015	-38.37	-3.33
	CAP4	-20.01	5.84	0.021	-37.54	-2.5
CAP2	CAP3	2.64	5.84	0.998	-14.89	20.16
	CAP4	3.47	5.84	0.993	-14.05	21
CAP3	CAP4	-0.84	5.84	1.000	-16.69	18.36

Table 5-3: Comparisons of CAP (CAP1/CAP3) with the positive controls (DL and CHX) by applying t-tests

	t-test for Equality of Means					95% Confidence Interval	
	t	df	Sig. (2-tailed)[*1]	Mean Differ.	SE Differ.	Lower	Upper
CAP3- CHX1	3.830	8	0.005	22.21	5.8	8.83	35.58
CAP3- DL	4.822	8	0.001	27.81	5.77	14.51	41.11
CAP1- CHX2	5.407	8	0.626	2.93	5.98	-10.43	16.3

[*1] The mean difference is significant at the 0.05 level.
N: Number of specimens; M: Mean; SD: Standard Deviation; SE: Standard Error; M Diff: Mean Difference; SE Diff: Standard Error Difference.

Table 6: Live/dead staining (MS Ti discs)

Table 6-1: Calculations of Means, standard deviations and standard error for all values. CAP-treated groups and positive controls (DL, AA, CHX) were compared with untreated controls by applying t-tests

	N	M	S D	S E	t-test for Equality of Means					95% Confide Interval	
						t	df	Sig.[*1]	M Diff.	SE Diff.	Lower	Upper
UC	5	82.55	11.39	5.09								
GC	5	75.5	2.42	1.08	UC-GC	1.35	8	0.213	7.05	5.21	-4.96	19.06
CAP1	5	13.95	10.36	4.63	UC-CAP1	9.96	8	0.00	68.60	6.88	52.72	84.48
CAP2	5	24.74	12.80	5.73	UC-CAP2	7.54	8	0.00	57.81	7.67	40.14	75.49
CAP3	5	33.41	9.3	4.16	UC-CAP3	7.47	8	0.00	49.14	6.58	33.97	64.31
CAP4	5	36.76	13.01	5.82	UC-CAP4	5.92	8	0.00	45.79	7.73	27.96	63.62
DL	5	8.48	6.16	2.75	UC-DL	12.79	8	0.00	74.07	5.79	60.72	87.42
AA	3	68.79	6.31	3.64	UC-AA	1.87	8	0.108	13.76	7.3	-4.09	31.61
CHX1	5	7.36	4.77	2.13	UC-CHX1	13.62	8	0.00	75.19	5.52	62.45	87.92
CHX2	5	3.96	3.9	1.74	UC-CHX2	14.6	8	0.00	78.59	5.38	66.18	91.01

Table 6-2a: Multiple comparisons of CAP groups (One Way ANOVA)

	Sum of Squares	df	Mean Square	F	Sig.[*1]
Between Groups	1557.76	3	519.25	3.94	0.028
Within Groups	2108.13	16	131.76		
Total	3665.9	19			

Table 6-2b: Multiple comparisons of CAP groups (Sidak test)

Group (I)	Group (J)	M Differ. (I-J)	SE Differ.	Sig. (2-tailed)[*1]	95% Confidence Interval	
					Lower Bound	Upper Bound
CAP1	CAP2	-10.79	7.26	0.641	-32.55	10.98
	CAP3	-19.46	7.26	0.095	-41.23	2.31
	CAP4	-22.81	7.26	0.037	-44.57	-1.04
CAP2	CAP3	-8.67	7.26	0.821	-30.44	13.09
	CAP4	-12.02	7.26	0.527	-33.79	9.74
CAP3	CAP4	-3.35	7.26	0.998	-25.11	18.42

Table 6-3: Comparisons of CAP (CAP1/CAP3) with the positive controls (DL and CHX) by applying t-tests

	t-test for Equality of Means					95% Confidence Interval	
	t	df	Sig. (2-tailed)[*1]	Mean Differ.	SE Differ.	Lower	Upper
CAP3-CHX1	5.57	8	0.001	26.05	4.67	15.27	36.83
CAP3-DL	5	8	0.001	24.93	4.99	13.43	36.44
CAP1-CHX2	6.8	8	0.031	9.99	4.95	-1.42	21.4

[*1] The mean difference is significant at the 0.05 level.
N: Number of specimens; M: Mean; SD: Standard Deviation; SE: standard Error; M Differ: Mean Difference; SE Differ: Standard Error Difference.

Table 7: Microbiology (Ma Ti discs)

Table 7-1: Calculations of Means, standard deviations and standard error for all values. CAP-treated groups and positive controls (DL, AA, CHX) were compared with untreated controls by applying t-tests

	N	M	S D	S E	t-test for Equality of Means					95% Confide Interval	
						t	df	Sig.[*1]	M Diff.	SE Diff.	Lower	Upper
UC	5	100	0.0	0.0								
GC	5	100	0.0	0.0	UC-GC				unable to compare[*2]			
CAP1	5	0.0	0.0	0.0	UC-CAP1				unable to compare*[2]			
CAP2	5	2.59	2.28	1.02	UC-CAP2	95.49	8	0.0	97.41	1.02	95.05	99.76
CAP3	5	0.0	0.0	0.0	UC-CAP3				unable to compare*[2]			
CAP4	5	0.33	0.74	0.33	UC-CAP4	302.03	8	0.0	99.67	0.33	98.91	100.43
DL	5	2	1.60	0.72	UC-DL	136.80	8	0.0	98	0.72	96.35	99.65
AA	3	3.71	4.39	1.96	UC-AA	49.05	8	0.0	96.29	1.96	91.76	100.82
CHX1	5	15.49	32.82	14.68	UC-CHX1	5.76	8	0.0	84.51	14.68	50.67	118.36
CHX2	5	2.50	5.60	2.51	UC-CHX2	38.90	8	0.0	97.49	2.51	91.72	103.27

Table 7-2a: Multiple comparisons of CAP groups (One Way ANOVA)

	Sum of Squares	df	Mean Square	F	Sig.[*1]
Between Groups	23.50	3	7.83	5.45	0.009
Within Groups	22.99	16	1.44		
Total	46.49	19			

Table 7-2b: Multiple comparisons of CAP groups (Sidak test)

Group (I)	Group (J)	M Differ. (I-J)	SE Differ.	Sig. (2-tailed)[*1]	95% Confidence Interval	
					Lower Bound	Upper Bound
CAP1	CAP2	-2.56	0.76	0.021	-4.87	-0.32
	CAP3	0.0	0.76	1.000	-2.27	2.27
	CAP4	-0.33	0.76	0.999	-2.60	1.94
CAP2	CAP3	2.59	0.76	0.021	0.32	4.87
	CAP4	2.26	0.76	0.051	-0.01	4.54
CAP3	CAP4	-0.33	0.76	0.999	-2.60	1.94

Table 7-3: Comparisons of CAP (CAP1/CAP3) with the positive controls (DL and CHX) by applying t-tests

	t-test for Equality of Means					95% Confidence Interval	
	t	df	Sig. (2-tailed)[*1]	Mean Differ.	SE Differ.	Lower	Upper
CAP3-CHX1	-1.01	8	0.322	-15.49	14.68	-49.33	18.36
CAP3-DL	-2.8	8	0.049	-2	0.716	-3.65	-0.35
CAP1-CHX2	-1	8	0.347	-2.51	2.51	-8.29	3.27

[*1] The mean difference is significant at the 0.05 level.
N: Number of specimens; M: Mean; SD: Standard Deviation; SE: Standard Error; M Differ: Mean Difference; SE Differ: Standard Error Difference.
[*2] The standard deviations of both groups are zero.

Table 8: Microbiology (MS Ti discs)

Table 8-1: Calculations of Means, standard deviations and standard error for all values. CAP-treated groups and positive controls (DL, AA, CHX) were compared with untreated controls by applying t-tests

	N	M	S D	S E	t-test for Equality of Means					95% Confide Interval	
						t	df	Sig.[*1]	M Diff.	SE Diff.	Lower	Upper
UC	5	100	0.0	0.0								
GC	5	100	0.0	0.0	UC-GC				unable to compare[*2]			
CAP1	5	00	0.0	0.0	UC-CAP1				unable to compare[*2]			
CAP2	5	4.37	4.86	2.17	UC-CAP2	44.01	8	0.0	95.63	2.17	90.62	100.64
CAP3	5	0.0	0.0	0.0	UC-CAP3				unable to compare[*2]			
CAP4	5	0.0	0.0	0.0	UC-CAP4				unable to compare[*2]			
DL	5	8.32	10.98	4.91	UC-DL	18.67	8	0.0	91.68	4.91	80.36	102.99
AA	3	21.74	16.89	7.55	UC-AA	10.36	8	0.005	78.26	7.55	60.85	95.68
CHX1	5	52.49	27.46	12.28	UC-CHX1	3.87	8	0.0	47.51	12.28	19.19	75.83
CHX2	5	13.70	8.15	3.65	UC-CHX2	23.68	8	0.0	86.3	3.65	77.89	94.70

Table 8-2a: Multiple comparisons of CAP groups (One Way ANOVA)

	Sum of Squares	df	Mean Square	F	Sig.[*1]
Between Groups	71.68	3	23.89	4.05	0.026
Within Groups	94.44	16	5.90		
Total	166.12	19			

Table 8-2b: Multiple comparisons of CAP groups (Sidak test)

Group (I)	Group (J)	M Differ. (I-J)	SE Differ.	Sig. (2-tailed)[*1]	95% Confidence Interval	
					Lower Bound	Upper Bound
CAP1	CAP2	-4.37	1.54	0.048	-8.98	0.23
	CAP3	0.0	1.54	1.00	-4.61	4.61
	CAP4	0.0	1.54	1.00	-4.61	4.61
CAP2	CAP3	4.37	1.54	0.048	-0.24	8.98
	CAP4	4.37	1.54	0.048	-0.24	8.98
CAP3	CAP4	0.0	1.54	1.00	-4.61	4.61

Table 8-3: Comparisons of CAP (CAP1/CAP3) with the positive controls (DL and CHX) by applying t-tests

	t-test for Equality of Means					95% Confidence Interval	
	t	df	Sig. (2-tailed)[*1]	Mean Differ.	SE Differ.	Lower	Upper
CAP3-CHX1	-4.27	8	0.003	-52.49	12.28	-80.81	-24.17
CAP3-DL	-1.7	8	0.129	-8.32	4.91	-19.64	2.99
CAP1-CHX2	-3.759	8	0.006	-13.70	3.64	-22.11	-5.3

[*1] The mean difference is significant at the 0.05 level.

N: Number of specimens; M: Mean; SD: Standard Deviation; SE: Standard Error; M Differ: Mean Difference; SE Differ: Standard Error Difference.

[*2] The standard deviations of both groups are zero.

Table 9: Detection of total protein (Ma Ti discs)

Table 9-1: Calculations of Means, standard deviations and standard error for all values. CAP-treated groups and positive controls (DL, AA, CHX) were compared with untreated controls by applying t-tests

	N	M	S D	S E	T-test for Equality of Means					95% Confide Interval	
						t	df	Sig.[*1]	M Diff.	SE Diff.	Lower	Upper
UC	5	22.17	5.12	2.29								
GC	5	20.2	1.08	0.48	UC-GC	0.84	8	0.424	1.97	2.34	-3.43	7.37
CAP1	5	6.29	4.35	1.94	UC-CAP1	5.29	8	0.001	15.88	3	8.96	22.81
CAP2	5	11.14	3.53	1.58	UC-CAP2	3.97	8	0.004	11.03	2.78	4.62	17.44
CAP3	5	11.14	4.86	2.17	UC-CAP3	3.5	8	0.008	11.02	3.16	3.75	18.3
CAP4	5	13.44	1.61	.719	UC-CAP4	3.64	8	0.007	8.73	2.4	3.2	14.27
DL	5	8.46	4.35	1.94	UC-DL	4.54	8	0.002	13.71	3	6.78	20.63
AA	3	0.83	1.13	0.51	UC-AA	9.1	8	0.0	21.34	2.35	15.93	26.75
CHX1	5	16.62	4.09	1.83	UC-CHX1	1.83	8	0.095	5.55	2.93	-1.21	12.31
CHX2	5	15.06	5.14	2.3	UC-CHX2	2.13	8	0.060	7.11	3.24	-0.37	14.59

Table 9-2a: Multiple comparisons of CAP groups (One Way ANOVA)

	Sum of Squares	df	Mean Square	F	Sig.[*1]
Between Groups	135.92	3	45.31	3.15	0.054
Within Groups	229.91	16	14.37		
Total	365.84	19			

Table 9-2b: Multiple comparisons of CAP groups (Sidak test)

Group (I)	Group (J)	M Differ. (I-J)	SE Differ.	Sig. (2-tailed)[*1]	95% Confidence Interval	
					Lower Bound	Upper Bound
CAP1	CAP2	-4.85	2.4	0.310	-12.04	2.34
	CAP3	-4.86	2.4	0.309	-12.04	2.33
	CAP4	-7.15	2.4	0.052	-14.33	0.04
CAP2	CAP3	-0.004	2.4	1.000	-7.19	7.18
	CAP4	-2.3	2.4	0.926	-9.48	4.89
CAP3	CAP4	-2.3	2.4	0.927	-9.48	4.9

Table 9-3: Comparisons of CAP (CAP1/CAP3) with the positive controls (DL and CHX) by applying t-tests

	t-test for Equality of Means					95% Confidence Interval	
	t	df	Sig. (2-tailed)[*1]	Mean Differ.	SE Differ.	Lower	Upper
CAP3-DL	0.92	8	0.384	2.68	2.91	-4.03	9.41
CAP3-AA	4.63	8	0.002	10.32	2.23	5.18	15.46

[*1] The mean difference is significant at the 0.05 level.
N: Number of specimens; M: Mean; SD: Standard Deviation; SE: standard Error; M Differ: Mean Difference; SE Differ: Standard Error Difference.

Table 10: Detection of total protein (MS Ti discs)

Table 10-1: Calculations of Means, standard deviations and standard error for all values. CAP-treated groups and positive controls (DL, AA, CHX) were compared with untreated controls by applying t-tests

	N	M	S D	S E	t-test for Equality of Means					95% Confide Interval	
						t	df	Sig.*1	M Diff.	SE Diff.	Lower	Upper
UC	5	54.66	10.37	4.64								
GC	5	51.66	12.03	5.38	UC-GC	0.42	8	0.684	3	7.1	-13.38	19.38
CAP1	5	15.72	7.85	3.51	UC-CAP1	6.7	8	0.0	38.94	5.82	25.53	52.35
CAP2	5	21.30	5.11	2.29	UC-CAP2	6.45	8	0.0	33.36	5.17	21.44	45.28
CAP3	5	32.54	10.92	4.88	UC-CAP3	3.29	8	0.011	22.12	6.73	6.59	37.65
CAP4	5	34.26	9.72	4.34	UC-CAP4	3.21	8	0.012	20.4	6.36	5.74	35.06
DL	5	24.48	13.38	5.98	UC-DL	3.99	8	0.004	30.18	7.59	12.73	47.63
AA	3	2.02	1.96	.878	UC-AA	11.16	8	0.0	52.64	4.72	41.76	63.52
CHX1	5	39.04	11.12	4.97	UC-CHX1	2.3	8	0.051	15.62	6.8	-0.05	31.29
CHX2	5	34.04	6.7	3	UC-CHX2	3.74	8	0.006	20.62	5.52	7.89	33.35

Table 10-2a: Multiple comparisons of CAP groups (One Way ANOVA)

	Sum of Squares	df	Mean Square	F	Sig.*1
Between Groups	1193.8	3	397.93	5.28	0.01
Within Groups	1206.11	16	75.38		
Total	2399.91	19			

Table 10-2b: Multiple comparisons of CAP groups (Sidak test)

Group (I)	Group (J)	M Differ. (I-J)	SE Differ.	Sig. (2-tailed)*1	95% Confidence Interval	
					Lower Bound	Upper Bound
CAP1	CAP2	-5.58	5.49	0.905	-22.04	10.88
	CAP3	-16.82	5.49	0.044	-33.28	-0.36
	CAP4	-18.54	5.49	0.023	-35	-2.08
CAP2	CAP3	-11.24	5.49	0.299	-27.70	5.22
	CAP4	-12.96	5.49	0.174	-29.42	3.5
CAP3	CAP4	-1.72	5.49	1.00	-18.18	14.74

Table 10-3: Comparisons of CAP (CAP1/CAP3) with the positive controls (DL and CHX) by applying t-tests

	t-test for Equality of Means					95% Confidence Interval	
	t	df	Sig. (2-tailed)*1	Mean Differ.	SE Differ.	Lower	Upper
CAP3-DL	1.04	8	0.327	8.06	7.72	-9.74	25.87
CAP3-AA	6.15	8	0.0	30.52	4.96	19.08	41.96

*1 The mean difference is significant at the 0.05 level.
N: Number of specimens; M: Mean; SD: Standard Deviation; SE: standard Error; M Differ: Mean Difference; SE Differ: Standard Error Difference.

Table 11: Measured temperatures on the upper surfaces of the clean and contaminated Ma Ti discs

Cleaned	**1**	**2**	**3**	**4**	**5**	**M**	**SD**
CAP1	30.7	33	35.5	33.6	32.5	33.1	1.7
CAP2	27.9	29.2	29	29	28.5	28.7	0.5
CAP3	28.7	29.7	34.1	30.5	31.1	30.8	2.1
CAP4	29.8	29.6	34	30	33	31.3	2
Contaminated	**1**	**2**	**3**	**4**	**5**	**M**	**SD**
CAP1	42.8	42.8	35.4	35.4	36	38.5	4
CAP2	34.1	34.1	32.6	32.6	32.7	33.2	0.8
CAP3	36.3	36.3	40	40	38	38.1	1.8
CAP4	34.7	34.7	43.9	43.3	31.6	37.6	5.6

Table 12: Measured temperatures on the upper surfaces of the clean and contaminated MS Ti discs

Cleaned	**1**	**2**	**3**	**4**	**5**	**M**	**SD**
CAP1	34.7	38.8	37.6	36.2	37.7	37	1.6
CAP2	33.8	33.6	32.9	33.6	32.1	33.2	0.7
CAP3	34.6	33.2	38.8	34.7	35.1	35.3	2.1
CAP4	35.4	38.8	37.9	37.2	37	37.3	1.3
Contaminated	**1**	**2**	**3**	**4**	**5**	**M**	**SD**
CAP1	56	45.8	52.7	56	50.9	52.3	4.2
CAP2	34	38.4	41.8	41	35.3	38.1	3.4
CAP3	50.4	41	52.2	52.8	57.8	50.8	6.2
CAP4	50.5	40.1	55.1	54.4	51.9	50.4	6.1

Table 13: Measured temperatures on the lower surfaces of the contaminated MS Ti discs

MS	**1**	**2**	**3**	**4**	**5**	**M**	**SD**
CAP1	31.6	34	35.4	33.1	34.1	33.6	1.9
CAP2	32.6	30	30	31.6	31	30.8	1.5
CAP3	32.6	33.1	34.3	34.1	32.9	33.3	0.9
CAP4	33.3	34.4	33.5	34.6	34.1	33.7	0.6