**Aus dem Institut für Transfusionsmedizin und Immunologie der
Medizinischen Fakultät Mannheim
Direktor: Prof. Dr. med. Harald Klüter**

"The effect of titanium on the expression and activity of Matrix metalloproteinase 7 in differentially activated macrophages "

**Inauguraldissertation
zur Erlangung des medizinischen Doktorgrades
der
Medizinischen Fakultät Mannheim
der Ruprecht-Karls-Universität
zu
Heidelberg**

**vorgelegt von
David Hesselbarth**

**aus
Neuötting
Deutschland**

2019

Dekan: Herr Prof. Dr. med. Sergij Goerdt
Referentin: Frau Prof. Dr. rer. nat. Julia Kzhyshkowska

TABLE OF CONTENTS

Page

FIGURE INDEX 6

TABLE INDEX 7

ABBREVIATION LIST 8

1 INTRODUCTION 10

1.1 Matrix metalloproteinases 10

1.1.1 The classification of matrix metalloproteinases 10

1.1.2 The structure of matrix metalloproteinases 12

1.1.3 The regulation of matrix metalloproteinases 13

1.1.4 Functions of human matrix metalloproteinases 16

1.1.5 Cell and disease specific expression of matrix metalloproteinases . 18

1.1.6 Matrix metalloproteinases in implantation 21

1.1.7 Specific functions of matrix metalloproteinase 7 23

1.2 The monocyte/macrophage based model system established in our laboratory. 24

1.3 Aims and objectives of the study. 25

2 MATERIALS AND METHODS 26

2.1 Chemicals, reagents and kits 26

2.2 Consumables 26

2.3 Equipment 27

2.4 Kits 27

2.5 Buffers and solutions 28

2.6 Nucleic acid related methods 28

2.6.1 Polymerase chain reaction (PCR) 28

2.6.2 Agarose Gel electrophoresis 29

2.6.3 cDNA synthesis 29
2.6.4 Real time PCR with Taqman probe 30
2.7 Protein related techniques 32
2.7.1 Enzyme linked immunosorbent assay 32
2.8 Functional assay 33
2.8.1 Antibody based human MMP-7 activity assay 33
2.9 Statistical analysis 34

3 RESULTS 35

3.1 RT-PCR analysis of the effect of titanium on the MMP-7 mRNA expression in differentially activated macrophages 35
3.1.1 Selection and optimization of MMP-7 specific primers 35
3.1.2 Porous titanium induces MMP-7 mRNA in M0 and M2 macrophages 36
3.1.3 Polished titanium induces MMP-7 mRNA in M0, M1 and M2 macrophages. 39
3.2 ELISA analysis of the effect of titanium on the MMP-7 protein expression in differentially activated macrophages. 42
3.2.1 Dynamic accumulation of MMP7 secreted by macrophages during the differentiation on titanium disks 42
3.2.2 Porous titanium induces MMP-7 protein expression in M0 and M2 macrophages 43
3.3 Antibody based analysis of active MMP-7 protein secretion in differentially activated macrophages. 46
3.3.1 Analysis of active MMP-7 secretion in titanium stimulated macrophages. 46
3.3.2 Percentage of active MMP-7 secretion in macrophages of individual donors. 48
3.4 The effect of titanium on MMP7 mRNA, protein and activity 48
3.5 RT-PCR analysis of the effect of titanium on MMP-7 regulators in differentially activated macrophages 50
3.5.1 Involvement of TIMP-3 in regulation of MMP-7 activity 50
3.5.2 Involvement of CD151 in regulation of MMP-7 activity 52

4 DISCUSSION 54

4.1 The production of MMP-7 in response to titanium 54
4.2 The effect of elevated levels of MMP-7 on implant integration 59

ABSTRACT 61

REFERENCES 62

CURRICULUM VITAE 70

ACKNOWLEDGMENTS 71

FIGURE INDEX

Figure 1. The structure of human MMPs. 13

Figure 2. Schematic presentation of multilevel MMP regulation at the level of gene expression, zymogen activation and endogenous inhibition. 14

Figure 3. Various modes of MMP's zymogen activation..... 15

Figure 4. Electrophoretic analysis of amplified MMP-7 fragments in the agarose gel. 35

Figure 5. RT-PCR analysis of the effect of porous titanium on MMP-7 mRNA in differentially activated macrophages. 37

Figure 6. Statistical analysis of the effect of porous titanium on MMP7 gene expression. 38

Figure 7. RT-PCR analysis of the effect of polished titanium on MMP-7 mRNA in differentially activated macrophages. 40

Figure 8. Statistical analysis of the effect of polished titanium on MMP7 gene expression. 41

Figure 9. ELISA analysis of MMP-7 protein secretion in differentially activated macrophages. 43

Figure 10. ELISA analysis of the effect of porous titanium on MMP-7 protein in differentially activated macrophages of individual donors. 44

Figure 11. Statistical analysis of the effect of porous titanium on MMP-7 protein secretion. 46

Figure 12. Antibody-based analysis of active MMP-7 protein in differentially activated macrophages. 47

Figure 13. Statistical analysis of the effect of porous titanium on the active MMP-7 protein secretion. 47

Figure 14. The percentage of MMP-7 in the active form for 11 donors. 48

Figure 15. Statistical analysis of the effect of porous titanium on TIMP3 mRNA expression. 51

TABLE INDEX

Table 1. Domain structure and classification of human MMPs..... 11

Table 2. Mechanisms of MMP activation. 15

Table 3. Macromolecular substrates of MMPs 17

Table 4. The Expression of MMPs in different cell types under healthy and pathological conditions. 19

Table 5. Involvement of MMPs in different pathologies. 20

Table 6. Involvement of MMPs in implantation. 21

Table 7. Role of MMP-7 specfic activites in immune responses. 23

Table 8. Chemicals, reagents and kits 26

Table 9. Consumables..... 26

Table 10. Equipment used in the study. 27

Table 11. Kits..... 27

Table 12. Reagents for PCR 28

Table 13. PCR protocol 28

Table 14. Size ranges for Agarose gel 29

Table 15. Pipetting schema for primer optimization 30

Table 16. Primers and probe for real-time PCR designed in our lab. 31

Table 17. Ready-made mixes for real time PCR.. 31

Table 18. Reagents of RT-PCR..... 31

Table 19. RT-PCR protocol 31

Table 20. Fold-changes of MMP-7 gene expression, protein secretion, and activity affected by titanium. 49

Table 21. Factors regulating MMP-7. 57

ABBREVIATION LIST

°C	degrees centigrade
µg	microgram
µl	microliter
µm	micrometer
APMA	4-aminophenylmercuric acetate
Appr.	approximately
Arg	Arginin
Asp	Asprartat
BC	Buffy code
BSA	bovine serum albumin
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
CD14	CD14 positive macrophages
cDNA	complementary deoxyribonucleic acid
CR	complement receptor
Ct	threshold cycle
CXCL	C-X-C motif chemokine ligand
Cys	Cystein
ddH_2O	double distilled water
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides
EDTA	ethylene diamine tetra acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
Fc	fragment crystallisable
g	gram / relative centrifugal force
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gly	Glycin
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour(s)
HRP	horseradish peroxidase
IFNγ	interferon gamma
IgG	immunoglobulin G
IL	interleukin
L	liter
LPS	lipopolysaccharide
M	molar concentration
M0	macrophages non stimulated
M1	classically activated macrophages
M2	alternatively activated macrophages
M-CSF	macrophage colony-stimulating factor
met	metionin
min	minute(s)
ml	milliliter
mM	millimolar concentration
mm	millimeter
MMP	matrix metalloproteinase
mRNA	messenger RNA
N	equivalent concentration
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
nm	nanometer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pro	Prolin
pg	picogram

PMMA	polymethylmethacrylate
pmol	picomolar
RANK	receptor activator of nuclear factor kappa B
RANKL	receptor activator of nuclear factor kappa-B ligand
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
RT-qPCR	reverse transcription real-time polymerase chain reaction
s	second(s)
Std	standard
TAE	tris/Acetate/EDTA-buffer
TGF	transforming growth factor
TGFβR1	transforming growth factor, beta receptor I
TNFα	tumor necrosis factor alpha
U	conventional units
UV	ultraviolet
V	volt(s)
vs	versus

1 INTRODUCTION

1.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) form a class of proteolytic enzymes with a broad spectrum of substrates, dedicated to play an essential role in the stabilization of the human physiological tissue homeostasis (1, 2). However, in case of deregulated expression or activity, they are involved in several pathological processes, such as cancer metastasis, chronic inflammation, neurological disorders or the aseptic loosening of implants (3, 4). The first MMP was discovered in 1962 as a protease capable of degrading fibrillar collagen in tadpole tails during metamorphosis and was named interstitial collagenase (5). Step by step more proteases with similar structure and function were discovered. They were named according to the substrates they can degrade (e.g., gelatinase) or to their presumed function (e.g., matrilysins). Based on their similarity in structure and function, the International Union of Biochemistry and Molecular Biology organized this class of enzymes as a new family named Matrix metalloproteinases (6). This new term indicates the enzyme's capability of degrading extracellular matrix as well as its typical structure: containing a Zn^{2+} ion inside the active site (7).
In over 50 years of MMP research, scientists have been able to achieve several important discoveries in this field. A milestone was the identification of "tissue inhibitors of matrix metalloproteinases" (TIMPs). TIMPs inhibit MMPs and are essential for the balance of its activity and inactivity. Disruption of this tissue homeostasis may lead to numerous pathologies (8). Another important discovery was MMP's inactive form (pro-form), which was pioneering for the further understanding of MMP's activity regulation and could direct the attention to mechanisms of MMP activation (9). Structural determination of MMPs and the creation of the first MMP knockout mouse (MMP-7) complete the significant milestones in MMP research (6).
Members of the MMP family can degrade almost all components of extracellular matrix (ECM) (10), as well as plenty of chemokines, cytokines and growth factors (11). This broad spectrum of substrates indicates that MMPs are essential in the regulation of physiological and pathological processes (7, 11).

1.1.1 The classification of matrix metalloproteinases

Matrix metalloproteinases rank among the metzincin superfamily, that is a clan of zinc-dependent endopeptidases, which is complemented by astacins, serralysins, pappalysins, adamalysins, snapalysins and leishmanolysins (9, 12). Metzincin are characterized by their similar catalytic domains comprising a zinc-binding motif with the amino acid sequence HEXXHXXGXX(H/D) as well as a downstream methionine containing 1,4 β-turn named "met turn."
The classification of MMPs in the human genome encompasses 24 different genes encoding for 23 MMPs (Table 1). MMP-23 A&B encode two different genes but have the same amino acid sequence (13, 14). MMPs were named according to their order of discovery. However, some MMPs which were discovered in animals or plants had to be excluded as they could not be detected in the human species (MMP-4, -5, -6,-18,-22) (7).
The classification of MMP's found in the literature is not consistent and may lead to confusion. For instance, the mixture of structural and functional properties as well as the mixture of synonyms (e.g., gelatinase A for MMP-2) (9, 15).

Basically, MMPs can be categorized into secreted (MMP-1, -2, -3, -7, -8, -9, -10, -11, -12, 13,-19, -20, -21, -22, -27, -28) and membrane-anchored proteinases (MMP-14, -15, -16, -17, 23, 24,-25) (9). Furthermore, a classification according to structural properties or functional aspects is possible (Table 1) (13, 15). Regarding their structural properties, MMPs are divided into eight groups namely: minimal domain-MMPs, simple hemopexin-containing domain MMPs, gelatin-binding MMPs, furin-activated secreted MMPs, vitronectin-like insert MMP, type I transmembrane MMPs, glycosylphosphatidylinositol-anchor linked MMPs and type II transmembrane MMPs (16). The most conventional classification is based on the MMP substrate specificity and their cellular localization (Table 1). It encompasses five subgroups and additionally a sixth subgroup of heterogeneous MMPs which does not fit into the other groups (9, 11, 16-18)

Table 1. Domain structure and classification of human MMPs
Modified from Sbardella D, et al. and Visser R, et al. (11, 16-18)

Subgroups	MMP-number	Alternative name (functional properties)	Chromosome localization	Structural properties
Collagenases	MMP-1	Interstitial collagenase	11q22-q23	simple hemopexin-containing domain
	MMP-8	Neutrophil collagenase	11q21-q22	simple hemopexin-containing domain
	MMP-13	Collagenase-3	11q22.3	simple hemopexin-containing domain
Gelatinases	MMP-2	Gelatinase-A	16q13	gelatin-binding
	MMP-9	Gelatinase-B	20q11.2-q13.1	gelatin-binding
Stromelysins	MMP 3	Stromelysin-1 (transin-1)	11q23	simple hemopexin-containing domain
	MMP-10	Stromelysin-2	11q22.3-q23	simple hemopexin-containing domain
	MMP-11	Stromelysin-3	22q11.2	furin-activated secreted
Matrilysins	MMP-7	Matrilysin-1 (Pump-1)	11q21-q22	minimal domain-MMPs
	MMP-26	Matrilysin-2 (endometase)	11p15	minimal domain-MMPs
Membrane Type MMPs	MMP-14	MT1-MMP	14q11-q12	Transmembrane (Typ 1)
	MMP-15	MT2-MMP	15q13-q21	Transmembrane (Typ 1)
	MMP-16	MT3-MMP	8q21	Transmembrane (Typ 1)
	MMP-24	MT5-MMP	20q11.2	Transmembrane (Typ 1)
	MMP-17	MT4-MMP	12q24.3	GPI-anchored
	MMP-25	MT6-MMP	16p13.3	GPI-anchored
	MMP-23 A+B	Femalysin	1p36.3	Transmembrane (Typ 2)
Other MMPs	MMP-12	Macrophage	11q22.2-q22.3	simple

		metalloelastase		hemopexin-containing domain
	MMP-19	RASI-1	12q14	simple hemopexin-containing domain
	MMP-28	Epilysin	17q21.1	furin-activated secreted
	MMP-20	Enamelysin	11q22.3	simple hemopexin-containing domain
	MMP-21	None	1p 36	vitronectin-like insert
	MMP-27	None	11q24	simple hemopexin-containing domain

1.1.2 The structure of matrix metalloproteinases

All MMPs are synthesized as inactive pre-pro-enzymes and contain three conserved structural domains: a signal peptide (pre-domain), a pro-peptide (apart from MMP-23) and a catalytic domain with a Zinc²+ ion inside its active site (Figure 1) (13).
The pre-domain is an N-terminal signal peptide which is cleaved by the signal peptidase during entry into the endoplasmic reticulum. This enables the enzyme to be either secreted or anchored into the plasma membrane. Intracellular retention of the enzyme is possible in case of an inefficient action of the signal sequence (19).
The pro-peptide is approximately 80 amino acids long and possesses a highly conserved sequence (Pro-Arg-Cys-Gly-X-Pro-Asp) located upstream of the catalytic domain. The SH-thiol group, part of the cysteine in the pro-peptide, interacts with the Zn^{2+}-ion inside the active site, whereby the enzyme is kept in latency. This mechanism is called "cysteine switch." The enzyme is activated and acquires full proteolytic capacity when the pro-peptide is removed or disrupted (9, 20). Many physiological and pathophysiological pathways of this activation are known. The most common is the direct proteolysis by another MMP or protease (7).
The catalytic domains comprise approximately 165 residues which form together an upper and a lower subdomain. The active site is constituted by a zinc-binding conserved motif with the sequence HEXXHXXGXXH, where histidine (H), glutamic acid (E) and glycine (G) are invariant, and X is a variable residue. The three histidine residues coordinate the Zn^{2+} ion. A methionine contained met-turn follows the active site and forms a hydrophobic pillow for the catalytic Zn^{2+} ion (13).
Additionally, all MMPs, except MMP-7 and MMP-26, contain a C-terminal hemopexin-like domain (21). Hemopexin is a scavenger protein and eliminates free toxic haem. A number of proteins contain domains which resemble the hemopexin structurally and functionally, among them MMPs. They contain four hemopexin-like repeats, forming one globular hemopexin domain. This domain fulfills functions in activating and inhibiting MMPs, binding and cleavage of substrate, attachment and localization of MMPs at the cell surface, endocytosis and degradation of MMPs and homodimerization/-multimerization. It is mandatory for the degradation of triple helical collagen and plays a decisive role in MMP's substrate specificity (4, 21). The absence of the hemopexin domain in MMP-7 and MMP-26 may be one reason for their broad substrate specificity (22).

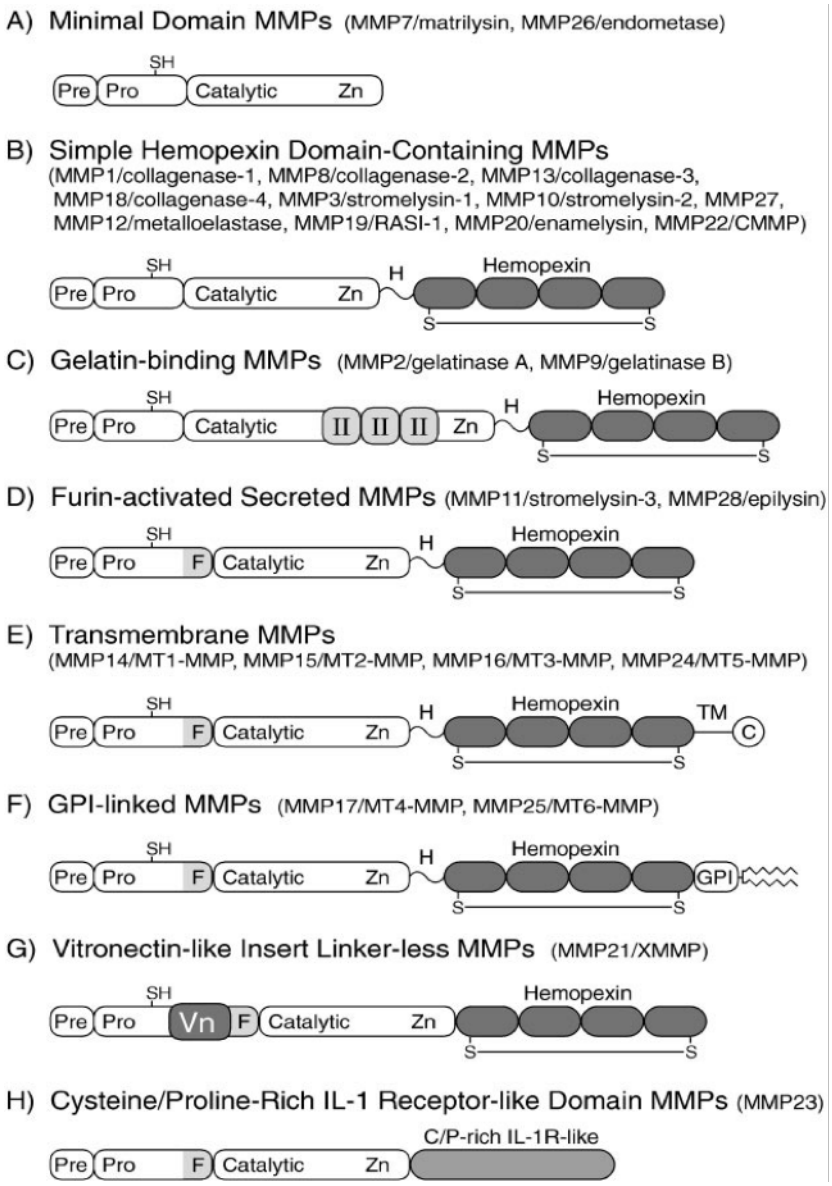


Figure 1. The structure of human MMPs. Domain structure of the MMPs. Pre: signal sequence; Pro: pro-peptide with a free zinc-ligating thiol (SH) group; F: furin-susceptible site; Zn: zinc-binding site; II: collagen-binding fibronectin type II inserts; H: hinge region; TM: transmembrane domain; C: cytoplasmic tail; GPI: glycophosphatidylinositol-anchoring domain; C/P: cysteine/proline; IL-1R: interleukin-1 receptor. The hemopexin/vitronectin-like domain contains four repeats with the first and last linked by a disulfide bond. With permission of Annual reviews nov 1, 2001 copyright 2001 (15).

1.1.3 The regulation of matrix metalloproteinases

MMPs are regulated on different levels of their synthesis including gene expression, epigenetic-, post-transcriptional- and activity regulation. The latter encompasses the initial zymogene activation as well as their endogenous inhibition (1, 23). Gaffney J et al. (2015) (Fig. 2) provide an excellent summary of the complex stepwise regulation which is influenced by numerous factors (1).

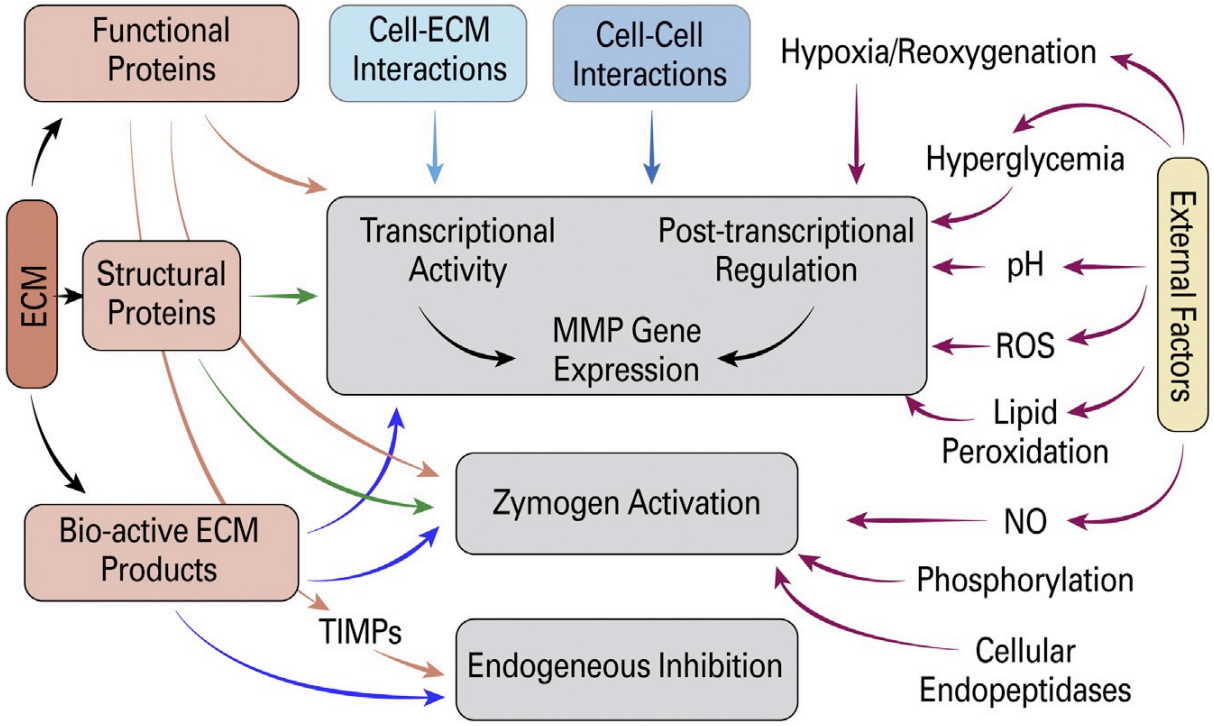


Figure 2. Schematic presentation of multilevel MMP regulation at the level of gene expression, zymogen activation and endogenous inhibition. With permission of Elsevier, Mai-July 2015, copyright 2015 (1).

MMPs are not only regulated by external factors as cytokines hormones and growth factors but also cell-cell, and cell-ECM interactions play an essential role in transcriptional and post-transcriptional regulation. Due to this fact, their expression is specified as a function of cell type and ECM conditions (1, 14). Cell specificity is achieved by the restricted expression of transcription factors only by selected cells (24).

Primarily MMPs are regulated at transcriptional level (1, 23, 24), which is a reasonable step as the whole process of protein biosynthesis is quite energy intensive. Thus an early intervention is more efficient than a later one (25). Fanjul-Fernández, M et al. (2009) took a closer look at the molecular mechanisms of transcription regulation (23). MMP promoters are downstream targets of early response signaling pathways. These early response proteins phosphorylate transcription factors which are subsequently able to bind promoters of MMP genes (23). The transcription factor binding sites inside the promoter are specific for each MMP. Due to their frequent repetition in MMP promoters, the PEA3-site, NF-κB-site, STAT-site and the AP-1 / -2 site appear to play essential roles in transcription regulation. Mainly the AP-1 site is vital as many signal-transduction pathways converge at it (14, 23).

Epigenetic regulation can be performed by DNA-methylation, histone modifying enzymes, and chromatin remodeling complexes. Depending on the cell type both DNA-methylation and altered chromatin structure can activate or repress MMP gene expression. (23, 24)

Although MMP regulation is mainly performed on the transcriptional level some posttranscriptional mechanisms including mRNA stability, post-transcriptional efficiency and micro-RNA based mechanisms contribute as well. MMP transcripts

possess 5' or 3' untranslated regions (UTRs) where UTR binding proteins can bind and either stabilize or destabilize the transcript (23, 26-28).
The next step in MMP control is the activation of the inactive pro-form via removal of the pro-peptide, realized either intracellularly, at the cell surface or within the extracellular space (Figure 3) (1). Mechanisms of the activation, listed in Table 2 can be discriminated either by their localization or by physiological/non-physiological activation.

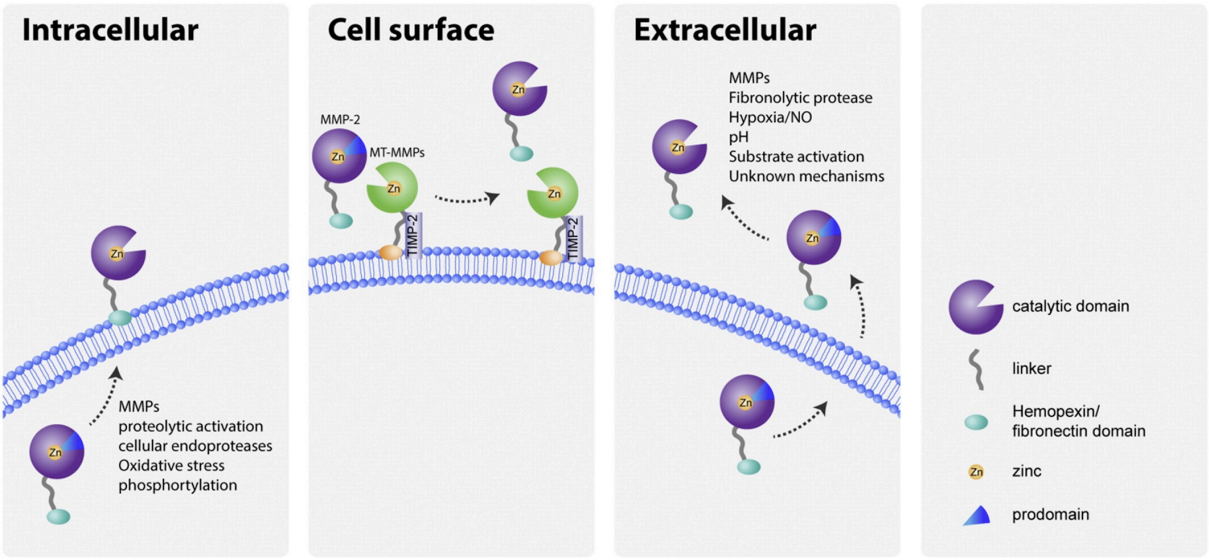


Figure 3. Various modes of MMP's zymogen activation. Intracellular and extracellular activation may take place via factors as indicated in the figure. Activation at the cell surface can occur by different mechanisms. In the case MMP-2, a complex comprising pro-MMP-2, MT1-MMP, and TIMP-2 is formed, enabling the cleavage of pro-MMP-2 by MT-1 MMP and to its activation. With permission of Elsevier, Mai-July 2015, copyright 2015 (1)

Table 2. Mechanisms of MMP activation.
Modified from Gaffney J et al. and Cauwe B et al. (1, 7)

Localization of activation	Mechanisms of activation
Intracellular	Proteolytic (serin/furin proteases, caspases & other MMPs) Oxidative stress Nitrosative stress Phosphorylation Alternative splicing
Cell surface	Activation via MT-MMPs
Extracellular	Proteolytic (Serin proteases & other MMPs) Thrombin, trypsin, NO, hypoxia, heavy metal ions

The most common physiological mechanism is the proteolysis of the pro-peptide by MMPs or other proteases (7). Additionally, oxidation of the thiol group by physiological oxidants (e.g., ROS) and disulfides (e.g., RNS), as well as allosteric activation via distortion of the catalytic site by binding a receptor or substrate can activate MMPs. Alternatively, spliced MMPs which have a lack of the pro-domain can be constitutively activated. Non-physiological activation can be attained likewise to the physiological ones via distortion of the catalytic site with the distinction that non-

physiological reagents such as organomercurials (APMA), chaotropic agents (urea), and detergents (e.g., SDS) activate MMPs. Additionally, alkylating agents and heavy metal ions can activate MMPs via modification of the cysteine in the pro-domain (7).
In order to ensure that MMP activity is neither too high nor too low, it is crucial to guarantee tight control. Otherwise, an impaired MMP activity can lead to irregular ECM turnover and numerous diseases (Table 5) (8). This control is mainly achieved by the tissue inhibitors of metalloproteinases (TIMPs) via blocking the enzyme's active site with a conserved anchor in a tight 1:1 complex (29). Thus, the balance between TIMPs and MMPs is decisive whether MMPs are active or not. However, also other factors such as α2-macroglobulin are capable of inhibiting MMPs (30, 31). Generally, all TIMPs have the capability of inhibiting all known MMPs, although their efficacy varies for each MMP (30). The opinion that a shift of the balance between MMPs and TIMPs in favor of TIMPs leads to fibrosis and vice versa to proteolysis is no longer generally accepted (30). Since it is known that MMPs function goes far beyond degrading just ECM and that it plays an essential role in activating or inactivating many biologically active proteins, it is suggested that MMPs can do both: directly inhibit ECM proteolysis and indirectly control ECM turnover (30). For instance, the growth factor TGFβ leads to increased deposition of ECM (32) and is activated directly by MMP-2, -9 and -13 (33, 34) as well as indirectly by MMP-7 (35). This implies that inhibition of MMPs via TIMPs indirectly decreases the TGFβ mediated ECM deposition and results in ECM degradation (30). As only some MMPs activate TGFβ, it has to be noted that the specific role of each TIMP is dependent on the MMP it is inhibiting. Given that the surrounding ECM influences expression and activity of MMPs their function is also dependent on the local tissue environment (30).

1.1.4 Functions of human matrix metalloproteinases

Initially, MMPs were identified as enzymes with crucial functions for the degradation of ECM, but more recently a growing number of non-ECM substrates including chemokines, cytokines, and growth factors have been identified (Table 3). MMPs do not only degrade these substrates; they can also activate them, which enables MMPs to contribute to many physiological and pathological processes. They play a vital role in inflammation as they recruit inflammatory cells or regulate their transmigration from vasculature to the site of inflammation in tissue. This recruitment and transmigration is achieved by cleavage of ECM and non-ECM substrates as well as the creation of chemokine gradients (chemotaxis) (11). They are also involved in tissue remodeling processes such as embryonic development, tissue morphogenesis, skeletal growth and wound repair (7, 11, 16).
However, MMPs and their function of cleaving ECM and non-ECM substrates have been attributed to numerous pathologies including tumor metastasis, arthritis, glomerulonephritis, periodontal disease, tissue ulcerations, cardiovascular disease, neurodegenerative diseases and peri-implant loosening (36, 37). MMP inhibition as a therapeutic approach was tried in the last years, but with little clinical success. One reason may be that the spectrum of the function of each MMP is still not understood completely. Therefore further studies are limited, as beneficial functions should not be modulated (36, 38). A complementation of the MMP's substrate spectrum is crucial in order to understand pathologies in which MMPs are involved.

Table 3. Macromolecular substrates of MMPs
Modified from Nissinen L et al. and Sbardella D et al. (11, 16).

Subgroup	MMP-number	ECM substrates	Non ECM substrates
Collagenases	MMP-1	Collagens I, II, III, VII,VIII, X; gelatin; aggrecan; link protein; entactin; tenascin; perlecan	α2-M; α-PI; α1-antichymotrypsin; IGFBP-2, 3, 5; proIL-1β; CTGF; α1-antitrypsin/α1-antichymotrypsin; IL-1β; Latent TNF-α; MCP-1, -2, -3, -4; SDF-1; VEGF
	MMP-8	Collagens I, II, III, V, VII, VIII, X; Fn; entactin; tenascin; gelatin; aggrecan; link protein	α1-antitrypsin; CXCL5; IL-8
	MMP-13	Collagens I, II, III, IV, VII, IX, X, XIV; aggrecan; gelatin; Fn; tenascin; osteonectin; Ln; perlecan	CTGF; ProTGF-β; MCP-3; α1-antichymotrypsin; plasminogen; Latent TNF-α; SDF-1
Gelatinases	MMP-2	Gelatin; collagens I, IV, V, VII, X, XI, XIV; Ln; Fn; elastin; aggrecan; osteonectin; link protein	ProTGF-β; FGF receptor I; MCP-3; IGFBP-5; proIL-1β; galectin-3; plasminogen IL-1β; SDF-1; MCP-3; IGFBP-3; Latent TNF-α; Pleiotrophin; CTGF; Decorin (35)
	MMP-9	Gelatin; collagens I, III, IV, V, VII, X, XII; elastin; entactin; aggrecan; Fn; link protein; vitronectin; N-telopeptide of collagen I	ProTGF-β; IL-2 receptor a; Kit-L; IGFBP-3; proIL-1β; ICAM-1; α1-PI; galectin-3; plasminogen; α1-antitrypsin; IL-1β; CXCL5; IL-8; SDF-1; Latent TGF-β; Latent TNF-α; IL-2Rα; IGFBP-1; VEGF
Stromelysins	MMP-3	Aggrecan; decorin; gelatin; Fn; Ln; collagens III, IV, V, IX, X, XI; tenascin; link protein; perlecan; osteonectin; entactin	IGFBP-3; proIL-1β; HB-EGF; CTGF; Ecadherin; α1-antichymotrypsin; α1-PI; α2-M; plasminogen; uPA; pro-MMP-1, 7, 8, 9, 13 α1-antitrypsin/α1-antichymotrypsin; IL-1β; Pro-IL-1β; MCP-1, -2, -3,-4; SDF-1; IGFBP-1, 3; Latent TGF-β; Pro-HB-EGF; Latent TNF-α; TNF-α; Osteopontin; VEGF; Decorin(35)
	MMP-10	Aggrecan; Ln; Fn; gelatin; collagens III, IV, V, IX, X, XI; tenascin; link protein;	Pro-1, 8, 10
	MMP-11	Fn; Ln; aggrecan; gelatins	α1-antitrypsin; α2-M; IGFBP-1
Matrilysins	MMP-7	Aggrecan; gelatin; Fn(39); Ln; elastin; entactin; collagens, III, IV(39), V, IX, X, XI;	Proα-defensin; b4 integrin; E-cadherin; CTGF; HB-EGF; RANKL; IGFBP-3; plasminogen; α1-

		tenascin; Decorin (35); link protein; Vitronectin;	antitrypsin; Pro-HB-EGF; Latent TNFα; Syndecan-1; Osteopontin; CellularmembraneboundFasL; VEGF; NGF (20);
	MMP-26	Gelatin; collagen IV; Fn; fibrinogen; vitronectin	pro-MMP-9; α1-antitrypsin
Membran Type MMPs	MMP-14	Collagen I, II, III; gelatin; aggrecan; Fn; Ln; fibrin; vitronectin; entactin; proteoglycans; Ln-5	Pro-MMP-2; Pro-MMP-13; CD44; MCP-3; tissue transglutaminase; SDF-1
	MMP-15	Fn; tenascin; nidogen; aggrecan; entactin; collagen; gelatin; perlecan; Ln; vitronectin	Pro-MMP-2; tissue transglutaminase
	MMP-16	Collagen III; aggrecan; gelatin; Fn; vitronectin.	Pro-MMP-2; tissue transglutaminase; VEGF
	MMP-24	Gelatin;fibronectin; vitronectin; collagen, aggrecan; PG	Pro-MMP-2
	MMP-17	Gelatin; fibrinogen	TNFα
	MMP-25	Gelatin; collagen IV; fibrin; Fn; Ln	ProMMP-2
	MMP-23	Gelatin	unkown
Other MMPs	MMP-12	Elastin; aggrecan; Fn; collagen IV; gelatin; vitronectin; entactin; osteonectin; Ln; nidogen	Plasminogen; apoliprotein(a); Latent TNF-α
	MMP-19	Collagen IV; gelatin; Fn; tenascin; aggrecan; enatctin; COMP; Ln; nidogen	IGFBP-3; proIL-1β; HB-EGF; CTGF; Ecadherin; α1-antichymotrypsin; α1-PI; α2-M; plasminogen; uPA; pro-MMP-1, 7, 8, 9, 13; VEGF
	MMP-28	unkown	Casein
	MMP-21	unkown	unkown
	MMP-20	Amelogenin; aggrecan; gelatin; COMP	unkown
	MMP-27	unkown	unkown

1.1.5 Cell and disease specific expression of matrix metalloproteinases

In order to understand pathologies in which MMPs are involved, it is essential to identify the cell types which express MMPs. MMPs are expressed most frequently in macrophages, endothelial cells, fibroblasts and adipocytes, although various other cells also produce MMPs (Table 4). Macrophages are key innate immune cells playing a decisive role in homeostasis as well as in inflammatory and healing processes. Effects of macrophages on their local tissue environment include trophic, homeostatic and defense functions, cytocidal interactions with neighboring cells, phagocytosis and remodeling of extracellular matrix (2). The latter is mainly attributed

to the matrix metalloproteinases secreted mostly by macrophages. Macrophages express twenty out of 23 human MMPs. Only for MMP-20, MMP-23, and MMP-24, there is no expression by macrophages reported in the literature (Table 4).

Table 4. The Expression of MMPs in different cell types under healthy and pathological conditions. Modified from Iyer RP et al. (6)

MMP type	Expressing cell type
MMP-1	endothelial cells, fibroblasts, macrophages, smooth muscle cells (40), cardiac fibroblasts (41), alveolar **macrophages** (42), adipocytes (43)
MMP-2	endothelial cells, fibroblasts, platelets, T-lymphocytes, smooth muscle cells (40), adipocytes (40), cardiac fibroblasts (41), **macrophages** (44)
MMP-3	endothelial, fibroblasts, **macrophages**, smooth muscle (40), adipocytes (43)
MMP-7	**macrophages**, gastric epithelial cells (45), mesangial cells(32), lung adenocarcinoma cells (46), pancreatic ductal adenocarcinoma cells (47), epithelial cells (22)
MMP-8	neutrophils, endothelial cells, fibroblasts, **macrophages**(5)
MMP-9	neutrophils, endothelial cells, eosinophils, **macrophages(48)**, T lymphocytes, adipocytes (40, 43) cardiac fibroblasts (41)
MMP-10	fibroblasts, T lymphocytes, **macrophages** (49), adipocytes (43)
MMP-11	fibroblasts, **macrophages** (50)
MMP-12	macrophages, stromal-cells, adipocytes (43)
MMP-13	fibroblasts, **macrophages** (51)
MMP-14	fibroblasts, **macrophages**
MMP-15	fibroblasts, **macrophages**
MMP-16	fibroblasts, **macrophages**, vascular smooth muscle cells
MMP-17	eosinophils, lymphocytes, **monocytes, macrophages** (52)
MMP-19	vascular smooth muscle cells, endothelial cells, **monocytes**, adipocytes (43)
MMP-20	endothelial cells
MMP-21	fibroblasts(53), **macrophages**(53)
MMP-23	Unknown
MMP-24	Unknown
MMP-25	neutrophils, **monocytes**
MMP-26	B-lymphocytes, **macrophages** (53)
MMP-27	fibroblasts, **macrophages** (54)
MMP-28	cardiomyocytes, **macrophages**, T-lymphocytes

Table 5 gives an overview of the involvement of MMPs in different pathologies. Collagenases (MMP-1, -8, -13) have been attributed to atherosclerosis, heart failure and aortic aneurysm. However, it seems that their role in tumorigenesis is of minor relevance. Gelatinases (MMP-2, -9) are mainly connected to inflammatory processes, autoimmune diseases, and neoplastic pathologies. Likewise, stromelysins (MMP-3, -10, -11) and the matrilysin MMP-7 were described to play a significant role in tumor diseases. Additionally, cardiovascular, neuronal and respiratory diseases are attributed to MMP-7. Diseases with the involvement of membrane-type-MMPs (MMP 14, -15, -16, -17, -24, -25) encompass neoplastic diseases, but they have also been described in cardiovascular, respiratory, nervous, and rheumatic diseases. The role of MMPs in specific tumor entities is not discussed as it would exceed the extent of this thesis (16).

The previously shown abundant spectrum of substrates of MMPs (Table 3), their nearly complete presentation in macrophages and the connection to pathologies (Table 5), gives reasons for further examinations of these enzymes in macrophages.

Table 5. Involvement of MMPs in different pathologies.
Modified from Sbardella D et al. **(16).**

System	Disease	MMP
Cardiovascular	Aortic aneurysm	MMP-1 MMP-2 (55) MMP-3 MMP-7 MMP-8 MMP-9 MMP-12 MMP-13 MMP-14
	Atherosclerosis	MMP-1 (56) MMP-2 MMP-3(56) MMP-8 MMP-9 MMP-10 MMP-11 MMP-12(56) MMP-13 MMP-14
	Myocardial infarction	MMP-2(57) MMP-3 MMP-7 MMP-9 (57) MMP-13 MMP-14
	Left ventricular hypertrophy	MMP-1 MMP-7 MMP-13 MMP-14
	Stroke	MMP-2 MMP-3 MMP-9 MMP-12 MMP-13 MMP-14
Nervous	Alzheimer	MMP-1 MMP-2 MMP-9 MMP-24
	Blood–brain barrier (BBB) disruption	MMP-2 MMP-7 MMP-9 MMP-14
	Brain injury	MMP-7 MMP-13
	Encephalomyelitis	MMP-8 MMP-9 MMP-12 MMP-25
	HIV-dementia	MMP-7
	Intracerebralhemorrhage (ICH)	MMP-12
	Meningitis	MMP-8 MMP-12
	Multiple sclerosis	MMP-2 MMP-7 MMP-9 MMP-12 MMP-19 MMP-25
	Parkinson	MMP-3
	Spinal cord injury	MMP-12
	Vascular dementia	MMP-1
Joint and muscular	Dupuytren	MMP-7
	Inflammatory myopathies	MMP-7
	Medial collateral ligament (MCL) injury	MMP-12
	Osteoarthritis (OA)	MMP-1 MMP-2 MMP-3 MMP-7 MMP-8 MMP-9 MMP-13 MMP-14 MMP-16 MMP-17
	Polymyositis	MMP-9
	Rheumatoid arthritis (RA)	MMP-1 MMP-2 MMP-3 MMP-8 MMP-9 MMP-12 MMP-13 MMP-14 MMP-19
Respiratory	Acute respiratory distress syndrome (ARDS)	MMP-3
	Asthma	MMP-1 MMP-8 MMP-9 MMP-12 MMP-25
	Emphysema/chronic obstructive pulmonary disease. (COPD)	MMP-1 MMP-8 MMP-9(58) MMP-12 MMP-14 MMP-25
	Idiopathic pulmonary fibrosis (IPF)	MMP-1 MMP-7 MMP-12
	Lung fibrosis	MMP-1 MMP-7 MMP-8 MMP-12
Digestive	Liver fibrosis	MMP-1 MMP-8 MMP-12 MMP-13 MMP-15

	Liver cirrhosis	MMP-8 MMP-9
Infectious diseases	Helicobacter pylori	MMP-1
	Mycobacterium tuberculosis	MMP-1 MMP-7
	Schistosomiasis	MMP-12
Autoimmune diseases	Autoimmune inner ear	MMP-9
	Bullosuspemphigoid	MMP-9
	C-protein induced myocarditis	MMP-9
	Lupus erythematosus	MMP-2 MMP-9, MMP-3(59)
	Sjögren Syndrome	MMP-9
Other diseases	Diabetic nephropathy	MMP-24 MMP-7(32)
	Endometriosis	MMP-1 MMP-2 MMP-3 MMP-7 MMP-13 MMP-24
	Periodontal inflammation	MMP-2 MMP-8 MMP-9 MMP-13 MMP-25
Wound healing		MMP-1 MMP-2 MMP-3 MMP-8 MMP-9 MMP-10 MMP-11 MMP-13 MMP-14 MMP-28

1.1.6 Matrix metalloproteinases in implantation

MMPs play a decisive role in ECM remodeling and destruction as they are capable of degrading almost all parts of the extracellular matrix (3, 10). The role of ECM remodeling is of particular interest in medical fields where implants are used frequently, for instance in the orthopedic field and odontology. A main complication in implantology is aseptic implant loosening, which has been associated with multiple factors as micromotion of the implant, hydrostatic fluid pressure or wear-generated particular debris. The latter is attributed to be the primary factor (3). Wear debris includes various components of the implant and is phagocytized by macrophages. This activates them and leads to secretion of multiple cytokines as well as MMPs contributing to periprosthetic osteolysis (3, 60). Evidence in support of the essential role of this pathology includes the fact that more than 70% of hip revisions and more than 44% of knee revisions are caused by aseptic implant loosening (61, 62).
Several studies showed that MMPs are induced by different implant material ranging from hip prosthesis and dental implants to experimentally generated wear debris. The cell spectrum expressing MMPs represents not only macrophages, although they represent a primary source of the enzyme (Table 6).

Table 6. Involvement of MMPs in implantation. Arrow up (↑) = induction of MMP by implant; arrow straight (→) = no effect on MMP expression by implant

MMP	Implant type/material	Effect on MMPs	Cells /tissue involved	References
MMP-1	• hip prosthesis	↑	• periprosthetic tissue of loose artificial hip joints	(63, 64)
	• wear debris (including also titanium)	↑	• osteoblasts	(65)
MMP-2	• Titanium alloy particels (implant	↑	• Peripheral monocytes	(66)

	tissue) • polymethylmethacrylate (PMMA) • hip prosthesis • dental implants (not indicated which material)	↑ → →	• Peripheral monocytes • periprosthetic tissue of loose artificial hip joints • salivary	(66) (64) (67)
MMP-3	• wear debris (including also titanium)	↑	• macrophages	(65)
MMP-7	• Titanium alloy particles (implant tissue) • polymethylmethacrylate (PMMA) • hip prosthesis	↑ ↑ →	• Peripheral monocytes • Peripheral monocytes • periprosthetic tissue of loose artificial hip joints	(66) (66) (64)
MMP-8	• hip prosthesis • dental implant • wear debris (including also titanium) • wear debris (including also titanium)	→ ↑ ↑ ↑	• periprosthetic tissue of loose artificial hip joints • Peri-implant-sulcus fluid • macrophages • osteoblasts	(64) (28) (65) (65)
MMP-9	• Titanium alloy particles (implant tissue) • Polymethylmethacrylate (PMMA) • hip prosthesis • Titanium	↑ ↑ ↑ ↑	• Peripheral monocytes • Peripheral monocytes • periprosthetic tissue of loose artificial hip joints • osteoclasts	(66) (66) (64) (68)
MMP-10	• hip prosthesis • wear debris (including also titanium)	↑ ↑	• periprosthetic tissue of loose artificial hip joints • macrophages	(64) (65)
MMP-11	• hip prosthesis	→	• periprosthetic tissue of loose artificial hip joints	(64)
MMP-12	• hip prosthesis	↑	• periprosthetic tissue of loose artificial hip joints	(64)
MMP-13	• hip prosthesis	↑	• periprosthetic tissue of loose artificial hip joints	(64)
MMP-14	• hip prosthesis	→	• periprosthetic tissue of loose	(64)

			artificial hip joints	
MMP-15	• hip prosthesis	→	• periprosthetic tissue of loose artificial hip joints	(64)
MMP-16	• hip prosthesis	→	• periprosthetic tissue of loose artificial hip joints	(64)
MMP-17	• hip prosthesis	→	• periprosthetic tissue of loose artificial hip joints	(64)
MMP-19	• hip prosthesis	→	• periprosthetic tissue of loose artificial hip joints	(64)

1.1.7 Specific functions of matrix metalloproteinase 7

As previously described, MMP-7 has a lack of hemopexin domain which is responsible for the enzyme's substrate specificity. Due to the absence of the hemopexin domain, MMP-7 has one of the broadest substrate spectrums among the family of MMPs and can degrade most proteins of the extracellular matrix (22). Such broad substrate specificity is essential for the migration of epithelial cells in embryogenesis and wound repair but on the other hand detrimental for pathological processes like tumor metastasis (69, 70), or the loosening of an implanted prosthesis (19). Functions of MMP-7 are not only attributed to the degradation of extracellular matrix; it regulates cell migration, tissue repair and plays a vital role in the maintenance of innate immunity (22) (Table 3). Zhang Zhi et al. could demonstrate that MMP-7 inactivates human α1-antitrypsin by cleavage, which was accompanied by a corresponding loss of its inhibitory capacity towards neutrophil elastase (71). The up-regulated activity of neutrophil elastase can enhance inflammation by the activation of IL1-β and IL-18 (72). MMP-7 activates Pro HB-EGF to its active form (34), which has been shown to contribute to cell proliferation and migration, wound healing as well as to cell survival (35, 73, 74). Hirotako Haro et al. developed a murine organ culture model with intervertebral disks. They could show that macrophage secreted MMP-7 activated soluble TNFα, which induced MMP-3 in chondrocytes. MMP-3 released a macrophage chemoattractant which resulted in macrophage infiltration into the disks as well as proteoglycan loss and disk resorption (75). Furthermore, in another mouse model, it has been demonstrated that MMP-7 cleaves Syndecan-1 which releases the chemokine CXCL-1, bound with Syndecan-1 in a complex. The shed complex can create a chemotactic gradient, guiding neutrophils into the alveolar space (76).

Table 7. Role of MMP-7 specfic activites in immune responses.

MMP	Model	Substrate	Response	Consequence	Reference
MMP-7	Human enzymes	α1-antitrypsin	Inactivation	Enhanced activity of neutrophil elastase	(77)
	mouse	Pro HB-EGF	Activation	Cell survival, proliferation, migration, wound healing	(34)

	mouse	Latent TNF-α	Activation	macrophage tissue infiltration (disks). enhanced disk resorption	(75)
	mouse	Syndecan 1	Activation (shedding)	Creating together with chemokine a chemotactic gradient for neutrophils	(78)

1.2 The monocyte/macrophage based model system for the analysis of macrophage response to titanium surfaces established in our laboratory.

Foreign body reaction to biomaterials is an immunologically and inflammatory-driven process in which macrophages play a central role. The paradigm of M1 and M2 macrophages is useful in order to distinguish pro-inflammatory M1 macrophages from anti-inflammatory and pro-fibrotic M2 macrophages. If the foreign body equilibrium is disturbed, macrophages are activated that may lead to a process of bone resorption resulting in the implant failure (79). Otherwise, the profibrotic functions of M2 macrophages, leading to a restricted range of motion, may result in implant failure as well (80).
Resident tissue macrophages are partially derived from circulating monocytes in the bloodstream. Their differentiation and activation in tissue is influenced by cytokines, growth factors, hormones and implant material (65). In order to understand the response of macrophages to implant material or other challenges, a model system based on the cultivation of M1 and M2 macrophages, challenged with different stimuli, was established by our laboratory.
A two-step gradient centrifugation was used to isolate human monocytes from buffy coats, which are the best source of functionally active monocytes. In the first step biocoll separating solution was used to isolate peripheral blood monocytes, and in the second step, the separation was performed with percoll, in order to enrich the cells. CD14+ cells were selected and cultured with serum medium, supplemented with 5 mmol/l glucose, 5ng/ml of M-CSF and 10^{-8} mol/l dexamethasone. For 6 days, the cells were incubated either on plastic or titanium disks as well as stimulated with different cytokines, in order to differentiate them in different types of macrophages. The cells were stimulated with interferon gamma (IFNγ), differentiating the monocytes into pro-inflammatory M1 macrophages or with Interleukin 4 (IL4), generating anti-inflammatory M2 macrophages. A third group obtained no stimulation (M0 macrophages). Cells were harvested on day 1, 3 and 6 of incubation to analyze molecular biomarkers (81).
In order to analyze the effect of titanium on the gene expression in macrophages, a microarray gene expression analysis was performed by Dr. Alexandru Gudima (realization of experiments) and Dr. Carsten Sticht (statistical analysis) in our laboratory. For microarray analysis, cells were harvested on day 6 of incubation and used for RNA isolation. 6 out of 8 donors based on integrity, number and RNA concentration were chosen for microarray analysis. For each donor, 6 different groups of macrophages were analyzed: M(Control), M(IFNγ), M(IL-4), M(Control) Ti, M(IFNγ) Ti, and M(IL-4) Ti (82).
Microarray analysis provided a list of genes whose expression changed in response to titanium disks. A cut off corresponding to a 2-fold change in gene expression was introduced in order to get more biologically meaningful results (in contrast to only

statistically significant). MMP-7 was one of the genes which were most up-regulated in macrophages cultured on porous titanium disks vs. control (82). Additionally, MMP-7's broad substrate spectrum, indicating for high biological relevance (21), and the fact that an up-regulation of MMP-7 in tissue next to titanium implants was also identified by others (83), made it to the first choice for validation in further experiments (RT-PCR, ELISA and analysis of active MMP7).

1.3 Aims and objectives of the study.

Titanium is a frequently used implant material applied in many different medical fields, such as orthopedics or oral implantology. Currently, the predominant complication in the sector of joint replacement is the aseptic loosening of endoprosthesis. The degradation of extracellular matrix by MMPs has been considered to be a main factor in the process of aseptic loosening.
The aim of this work was to examine the effect of human primary macrophage exposure to titanium on the MMP-7 production.

The specific objectives were:

- Identification of titanium induced changes in gene expression and secretion of MMP-7 in human primary M0, M1 and M2 macrophages.

- Analysis of titanium induced MMP-7 on three different levels of MMP-7 regulation (mRNA, Protein, activity).

- Analysis of TIMP-3 and CD151, regulators of MMP-7 activity, in response to titanium stimulation in human primary M0, M1 and M2 macrophages.

- Analysis of differential effects of porous and polished titanium on MMP-7 production by M0, M1, and M2 macrophages.

2 MATERIALS AND METHODS

This work was written using the operating system Windows 7 and the word processor Microsoft Office Word 2007. Graphical representations were made using Microsoft Office Excel 2007, GIMP 2.8 and GraphPad Prism 6.
All experiments, except the experiments with polished titanium, were performed with monocyte-derived macrophages of the same donors.

2.1 Chemicals, reagents and kits

Table 8. Chemicals, reagents and kits

Product	Company
Incomplete PCR buffer 10x	BIORON
Tris-Acetate EDTA (TAE) buffer 50x	Eppendorf
Agarose	Roth
Bovine Serum Albumin (BSA)	Sigma
Deoxyribonucleotides (dNTPs) 10M	Fermentas
DEPC Water	Thermo Scientific
Dimethylsulfoxide (DMSO)	Sigma
GeneRuler DNA ladder	Fermentas
Loadingdye 6x	Fermentas
$MgCl_2$	Sigma
GelRed Nucleic Acid Gel stain	Biotium
Oligo(dt) primer	Thermo Scientific
PBS Dulbecco, w/o Ca^{2+}, Mg^{2+}	Biochrom AG
PCR primers (designed in the lab)	Eurofinsn MWG Operon
PCR probes (designed in the lab)	Eurofins MWG Operon
Phosphate buffered saline (D-PBS), sterile 1x	Invitrogen
Sensimix II probe kit	Bioline
Taqpolymerase 5 u/µl	BIORON
Tween 20	Sigma

2.2 Consumables

Table 9. Consumables

Product	Company
Parafilm	American National Can
PCR tubes	Star Labs
Pipette tips	Eppendorf
Pipettes	Gilson, Eppendorf
Safe-Lock Eppendorf Tubes, 1.5ml	Eppendorf
Sterile pipettetips	Avantguard, Star Labs, Nerbeplus

Tubes	Falcon

2.3 Equipment

Table 10. Equipment used in the study.

Product	Company
Agarose electrophoresis unit i-Mupid	Erogentec
Centrifuge 5415 R	Eppendorf
Centrifuge Rotina 420	Hettich
Centrifuge Rotina 420R	Hettich
Cryofreezingcontainer	Nalgene
Deepfreezer (-80°C)	Sanyo
Electrophoresiscomb	Peqlab
Electrophoresis power supply	Peqlab
Elisa plate sealers	R&D systems
Elisa Plates	R&D systems
Freezer (-20°C)	Liebherr
Icemachine	Scotsman AF100
Incubator 37°C	Edmund Bühler GmbH
Magneticstirrer MR3000	Heidolph
Microwave oven	Sharp
Scalpel	Feather
Shaker KS 260 basic	IKA
Tecan Infinite M200	Tecan
Thermocycler DNA Engine PTC220 Dyad	MJ Research
Thermomixer 5436	Eppendorf
Benchtop UV Transilluminator	UVP
Vortex Genie 2 Water bath	Scientific Industries Memmert

2.4 Kits

Table 11. Kits

Product	Company
RevertAid H Minus First Strand Synthesis Kit	Fermentas
Human total MMP-7 quantikine ELISA kit	R&D systems
Human MMP-7 activity assay	Quickzyme

2.5 Buffers and solutions

50xTAE buffer

242g of Tris free base and 18.61g of Disodium EDTA were added to 700ml ddH_2O and stirred until they dissolved. 57.1ml Glacial Acetic Acid was added and the volume was adjusted to 1L.

1xTAE buffer

20ml of 50xTAE buffer was added to 980ml of ddH_2O.

Wash buffer for ELISA (0.05% Tween 20 in PBS)

500µl of Tween 20 was pipetted into 1L of PBS. The beaker was stirred on a magnetic stirrer for 30min and the solution was stored at RT.

2.6 Nucleic acid related methods

2.6.1 Polymerase chain reaction (PCR)

PCR was performed in order to test the quality of the cDNA and the quality of the primers before proceeding to real time PCR optimization.
DNA fragments from cDNA were amplified using primers specific to the gene of interest.

1. All of the following reagents, minus the polymerase and cDNA template, were added to an Eppendorf tube to make a master mix for the number of samples and mixed by vortexing:

Table 12. Reagents for PCR

Reagent	Amount (µL)
Buffer (w/o $+Mg^{2+}$) 10x	**2.5**
$MgCl_2$ (100 mM)	**0.4**
dNTPs (10 mM)	**0.5**
Forward primer (10 pmol/µL)	**1**
Reverse primer (10 pmol/µL)	**1**
Taq polymerase (5 U/µL)	**0.3**
cDNAtemplate	**1**
ddH_2O	**18.3**

2. The polymerase was added, and the solution was mixed by pipetting.
3. The master mix was divided amongst PCR tubes containing the template samples and mixed by pipetting.
4. The tubes were placed in a thermocycler and the following program was used for amplification of the target gene:

Table 13. PCR protocol

95°C	10 min	
95°C	15 sec	35x
60°C	1min	
4°C	∞	

*PCR products were visualized using agarose gel electrophoresis.

2.6.2 Agarose gel electrophoresis

1. Agarose solution was prepared with agarose powder and electrophoresis buffer (1x TAE). The percentage of agarose was selected according to the size of the DNA fragments:

Table 14. Size ranges for Agarose gel

% Agarose	DNA size (bp)
0.5	1,000-30,000
0.7	800-12,000
1.0	500-10,000
1.2	400-7,000
1.5	200-3,000
2.0	50-2,000

2. GelRed 0,01% nucleic acid gel stain was added to the agarose solution and mixed.
3. The solution was cooled down to 55-60°C with magnetic stirring and poured into the gel tray with comb.
4. The comb was removed carefully when the solution was solidified.
5. Gel tray was placed in the electrophoresis unit and filled with 1x TAE buffer.
6. 2µL of loading dye was added to 2µL of the PCR product and to 8µL of ddH_2O.The samples were loaded onto the gel.
7. 3µL of GeneRuler DNA Ladder was added to the first lane to verify the size of the target fragments.
8. Electrophoresis was carried out at constant voltage (130 V) for 50 min. The results were visualized by UV illumination (254 nm) and documented using a gel documentation system.

2.6.3 cDNA synthesis

cDNA synthesis was performed using RevertAid H Minus First Strand Synthesis Kit from Fermentas. Prior to cDNA synthesis all RNA samples were digested with DNAse I to remove possible contamination of genomic DNA.

<u>DNA digestion:</u>

1. DNase was digested by mixing the following in an RNase-free Eppendorf tube:

Reagent	Amount (µl)
Total RNA	5
DNase buffer with MgCl2 10X	1
RNAse free DNase I	1
Distilled water (RNase free)	3

2. Digestion was performed at 37°C for 40 min in a thermoblock followed by enzyme inactivation at 70°C for 10 min.
3. The samples were put on ice

cDNA synthesis:

1. 1 µl of Oligo dT primer and 1µl of distilled RNase free water were added to DNase I digested RNA samples.
2. Samples were incubated at 70°C for 5 min in order to perform primer annealing.
3. Subsequently the following components were added to the samples and mixed well:

Reagent	Amount (µl)
5X reaction buffer for polymerase	4
Ribolock RNase inhibitor	1
dNTP mix (10mM)	2
RevertAid H minus reverse transcriptase	1

4. Reaction was incubated at 42°C for 1h at a thermoblock.
5. The enzymatic activity was stopped by additional incubation at 70°C for 10 min.
6. cDNA samples were diluted 10 times with ddH2O.
7. The cDNA synthesis efficiency was checked by amplifiying the house-keeping gene GAPDH using PCR and gel electrophoreses as described above.

2.6.4 Real time PCR with Taqman probe

All primers and dual-labeled probes were ordered from Eurofins Genomics. Dual-labeled probes contained FAM on 5' end and BHQ1 quencher at 3' end of sequence. Primers were optimized by pipetting them with the following schema (Table 15).

Table 15. Pipetting schema for primer optimization
F: Forward Primer; R: Reverse Primer;

F50nM R50nM	F50nM R300nM	F50nM R600nM
F300nM R50nM	F300nM R300nM	F300nM R600nM
F600nM R50nM	F600nM R300nM	F600nM R600nM

The primer combination with the earliest and steepest curve was chosen for optimization of the probe which was pipetted in concentrations between 50nM-250nM with the optimized primers. The probe with the earliest and steepest curve was chosen.
The following primers and probe were used for further RT-PCR analysis (Table 16)

Table 16. Primers and probe for real-time PCR designed in our lab. All primers and probes were ordered from Eurofins MWG Operon.

Gene	Type	Sequence (5' to 3')	Concentration
MMP7	Forward	CTTCCTGTATGCTGCAACTCA	900nM
	Reverse	GGGATCTCCATTTCCATAGG	900nM
	Probe	TCCCATACCCAAAGAATGGCCAA	150nM
GAPDH	Forward	CATCCATGACAACTTTGGTATCGT	900nM
	Reverse	CAGTCTTCTGGGTGGCAGTGA	300nM
	Probe	AAGGACTCATGACCACAGTCCATGCC	200nM

Table 17. Ready-made mixes for real time PCR. All ready-made mixes were obtained from Life Technologies.

Gene	Assay code
CD151	HS00911635_g1
TIMP3	HS00165949_m1

The enzyme Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a housekeeping gene and was used as reference in all RT-PCR experiments. All of the following reagents, without cDNA template, were added to an Eppendorf tube to make a master mix for the number of samples and mixed by vortexing:

Table 18. Reagents of RT-PCR

Reagent	Amount (µl)
SensiMix II Probe kit	5
TaqMan MMP-7 primer mix	0.5
TaqMan GAPDH primer mix	0,5
cDNA	1
Distilled water	3

1. The master mix was divided amongst Eppendorf tubes containing the template samples and mixed by pipetting. The negative control contained H_20 instead of cDNA
2. Samples were transferred in triplicates to a 96 Well PCR plate
3. Amplification was performed using the LightCycler 480 instrument. The following program was used:

Table 19. RT-PCR protocol

1 cycle	95°C	10 min
50 cycles	95°C	15 sec
	60°C	60 sec

2.7 Protein related techniques

2.7.1 Enzyme linked immunosorbent assay

All reagents were brought to room temperature before using them.

1. The Capture Antibody was diluted to the working concentration (1:180) in PBS without carrier protein.
2. Immediately a 96-well micro plate was coated with 100µl per well of diluted capture antibody.
3. The plate was sealed and incubated overnight at room temperature.
4. The capture antibody was aspirated from the plate and each well was washed with 200µl wash buffer (PBS with tween 20 0.01%) 4 times.
5. Any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels.
6. The plate was blocked by filling to each well 100µl of Reagent Diluent (1% BSA in PBS, pH 7,2-7,4) and incubating it for 1 hour at room temperature.
7. The Reagent Diluent was aspirated from the wells.
8. 100µl of the Reagent diluent were added per well for standard and 50µl of Reagent diluent for samples.
9. 50µl of sample or 100µl of standard (pipetted in a serial dilution) were added in the Reagent diluent per well.
10. The plate was covered with an adhesive strip and incubated for 2 hours at room temperature.
11. Reagents were aspirated from the plate and each well was washed with 200µl wash buffer (PBS with tween 20 0.01%) 4 times.
12. 100µl of detection antibodies (1:180) were added in each well and the plate was incubated for 2 hours.
13. Reagents were aspirated from the plate and each well was washed with 200µl wash buffer (PBS with tween 20 0.01%) 4 times.
14. 100µl of a conjugated human specific cytokine (Streptavidin-HRP) was added to each well.
15. The plate was covered and incubated for 20 minutes at room temperature. Placing the plate in direct light was avoided.
16. Reagents were aspirated from the plate and each well was washed with 200µl wash buffer (PBS with tween 20 0.01%) 4 times.
17. 100µl of substrate solution (1:1 mixture of H_2O_2 and tetramethybenzidine) was added to each well.
18. The plate was covered and incubated for 10-20 minutes, depending on the blue color development, at room temperature. Direct light to the plate was avoided.
19. 50µl of stop solution (2N H2SO4) was added to each well to stop the reaction. The plate was gently tapped to ensure thorough mixing.
20. The optical density was determined of each well immediately, using a micro plate reader at 450nm.

2.8 Functional assay

2.8.1 Antibody based human MMP-7 activity assay

Day 1

Coating the RAG microtiter plate with anti-MMP-7:

1. The antibody stock-solution was diluted 100x with the assay buffer (100 µl stock + 10 ml assay buffer)
2. Immediately, a 96-well micro plate was coated with 100µl per well of diluted capture antibody.
3. The plate was incubated 2 hours at 37°C in a humidified chamber.
4. The coating solution was removed, and the plate was washed 4 times with wash buffer

Preparing the standard curve

1. 950 µl of assay buffer was added to the standard vial and gently mixed (32 ng/ml stock). The following standard curve was used:

32000 pg/ml = stock solution
16000 pg/ml = 250 µl 32 ng/ml + 250 µl buffer
8000 pg/ml = 250 µl 16 ng/ml + 250 µl buffer
4000 pg/ml = 250 µl 8 ng/ml + 250 µl buffer
2000 pg/ml = 250 µl 4 ng/ml + 250 µl buffer
1000 pg/ml = 250 µl 2 ng/ml + 250 µl buffer
500 pg/ml = 250 µl 1 ng/ml + 250 µl buffer
250 pg/ml = 250 µl 0.5 ng/ml + 250 µl buffer
125 pg/ml = 250 µl 250pg/ml + 250 µl buffer
62,5 pg/ml = 250 µl 125pg/ml + 250 µl buffer
31,25 pg/ml = 250 µl 62,5pg/ml + 250 µl buffer
15,62 pg/ml = 250 µl 31,25pg/ml + 250 µl buffer
0 pg/ml = 500 µl buffer

2. Samples were diluted in the assay buffer (1:2)
3. 100 µl of blank assay buffer, standard or sample were added to the wells.
4. The plate was incubated overnight at 4°C in a humidified chamber.

Day 2

1. Preparing p-Aminophenyl mercuric acetate (APMA)
   APMA was prepared in order to activate the MMP-7 proteins used as standard

   - 50 µl of Dimethyl Sulphoxide (DMSO) were added to the vial and vortexed until the solution was clear → concentrated APMA solution (1 M)
   - 5 µl from the 1 M APMA solution were added to a vial containing 5 ml of assay buffer at room temperature and vortexed → ready to use APMA solution (1 mM)

2. Wells were emptied
3. Wells were washed 4 times with the wash buffer

4. 50 µl of the ready to use APMA solution (1 mM) was pipetted into wells containing standards
5. 50 µl of assay buffer was pipetted into wells containing samples in which active MMP-7 was to be measured.
6. Detection reagent was prepared with the following reagents
   5.5 ml detection reagent:
   4070 µl assay buffer
   550 µl detection enzyme
   880 µl chromogenic substrate

7. 50 µl of the detection reagent were pipetted into all wells.
8. The plate was shaken for 20 seconds
9. The absorbance was measured at 405nm at t = 0, then covered and incubated at 37 ºC in a humidified chamber.
10. After 6 and 24 hours of incubation the absorbance was measured at 405nm again.
11. Depending on the sample's activity different standard lines for the calculation were used (for highly active samples the standard curve from 6h, for less active samples the standard curve from 24h).
12. A graph of the absorbance at 405nm for the standards was plotted and a best-fit standard curve was drawn. Using the most suitable standard curve, the A405 values of the samples could be calculated to pg/ml MMP-7, according to the polynomial equation that describes the curve.

2.9 Statistical analysis

The significance of a difference between two groups of experimental data in RT-PCR, ELISA, and activity assay analysis was determined using a paired two-tailed t-test calculated with Microsoft Office Excel 2007. A p-value of less than 0.05 was considered to be statistically significant.

3 RESULTS

3.1 RT-PCR analysis of the effect of titanium on the MMP-7 mRNA expression in differentially activated macrophages

3.1.1 Selection and optimization of MMP-7 specific primers

The analysis of MMP-7 mRNA expression requires primers with an optimal amplification rate in order to obtain an exponential amplification curve as well as high specificity to avoid amplification of MMP-7 similar sequences. In order to achieve this specificity, three primer pairs for the MMP-7 gene were pre-selected with the primer design software tool on the website www.genscript.com. As the first primer tests were not specific and showed smearing in agarose gel after PCR, other primers using stricter criteria for the pre-selection were selected. Criteria were primer length between 18-24bp, a GC content between 40-60%, non-complementarity as well as similar melting temperatures. To avoid primer-dimers, particularly at their 3' end primers should not be complementary (84). Figure 4 shows the results of agarose gel electrophoresis performed with the PCR products from the two best working primers showing the highest specificity. It displays clear bands with the expected amplicon sizes of 74bp and 103bp. These two bands are fragments from two different parts of the MMP-7 gene. Their size is predefined as the sequence of MMP-7 gene, and the sequence of the primers is known. The aim was to identify the fragment which has the highest efficiency and specificity. The Primer pair with the amplicon size of 103bp was chosen for RT-PCR as it exhibits the brightest refulgence indicating a high amplification efficiency. Specificity of the primers is demonstrated as there are no other bands or smearing visible. In a second step, the required concentrations of primers and probe were optimized. For this purpose, a concentration gradient in RT-PCR was performed, as indicated in material and methods. Concentrations with the earliest and steepest rise were selected and are shown in material and methods (Table 16. Primers and probe for real-time PCR)

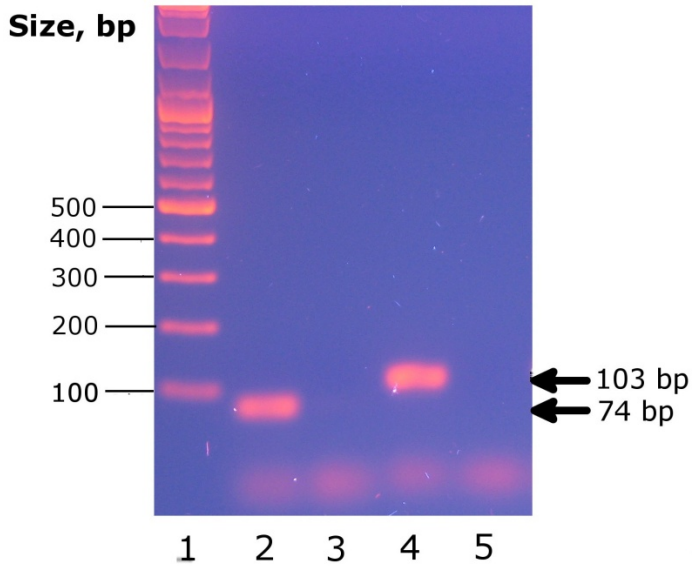
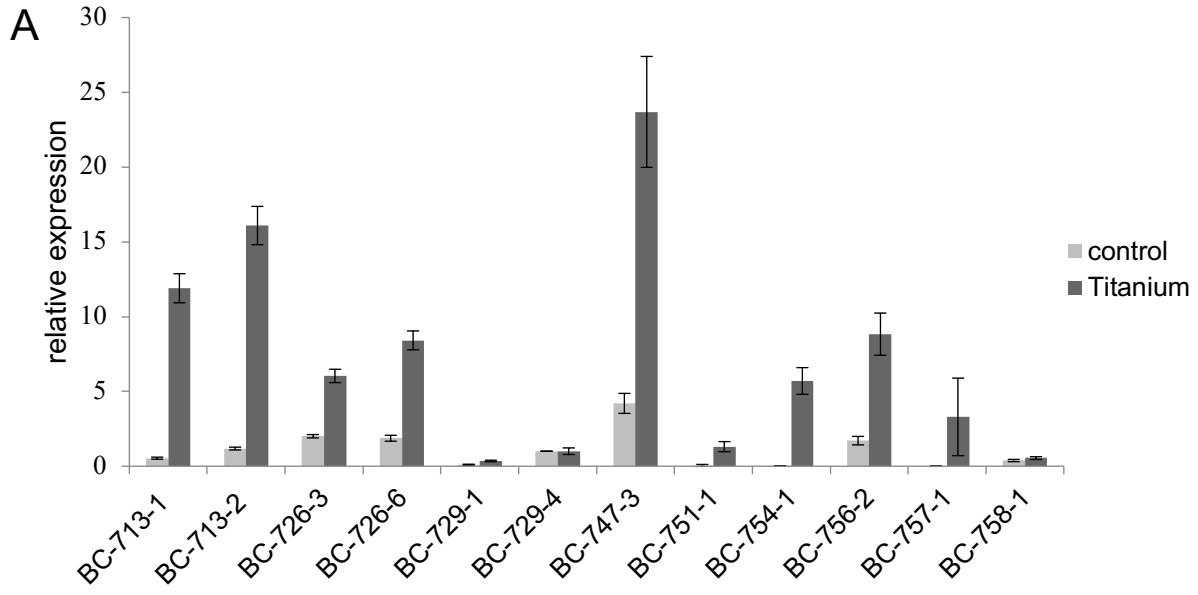


Figure 4. Electrophoretic analysis of amplified MMP-7 fragments in the agarose gel.
Efficiency and specificity of two different MMP-7 primer pairs were tested with PCR and agarose gel electrophoresis (lane 2 and lane 4). Negative controls were prepared with H_2O for each primer pair

(lane 3 and lane 5). A DNA-ladder (Lane 1) was used in order to identify the expected sizes of the MMP-7 gene fragments (74bp and 103bp). The primer pair in lane 4 was chosen for further analysis in RT-PCR, as it showed the brightest and clearest band as well as no smearing.

3.1.2 Porous titanium induces MMP-7 mRNA in M0 and M2 macrophages

In order to examine the effect of titanium surfaces on the expression of MMP-7 in differentially activated macrophages a test system established in our laboratory was used. For 6 days, human peripheral blood monocytes were cultured either on plastic or on titanium disks as well as stimulated with different cytokines in order to differentiate them in M1 (IFNγ), M2 (IL4) or M0 (no stimulation) macrophages.
Initially, the expression of MMP-7 was analyzed on the level of mRNA by RT-PCR from cells harvested on day 6 of cultivation. cDNA samples were partially provided by Alexandru Gudima (BC-726-3, BC-726-6, BC-729-1, BC-729-4, BC-747-3, BC-751-1, BC-754-1, BC-756-2, BC-757-1, BC-758-1). cDNA from the samples BC-713-1 and BC-713-2 were synthesized in own work. The expression of MMP-7 mRNA was examined for macrophage cultures from 12 individual donors.
Titanium induced MMP-7 mRNA in 11 out of 12 analyzed donors in M0 and in all 12 donors in M2 (Figure 5 A, C). The fold change induced by titanium ranged between 1,00 to 266,31 in M0 and between 5,6 to 507,99 in M2 macrophages (Table 20).
A fold-change of 2 means a change to 2 times. Interestingly, M1 macrophages exhibited a different pattern of MMP-7 expression. In these macrophages, titanium induced MMP-7 expression in 6 out of 12 donors, suppressed MMP-7 expression in 5 out of 12 donors and showed no effect in 1 out of 12 donors (Figure 5 B).



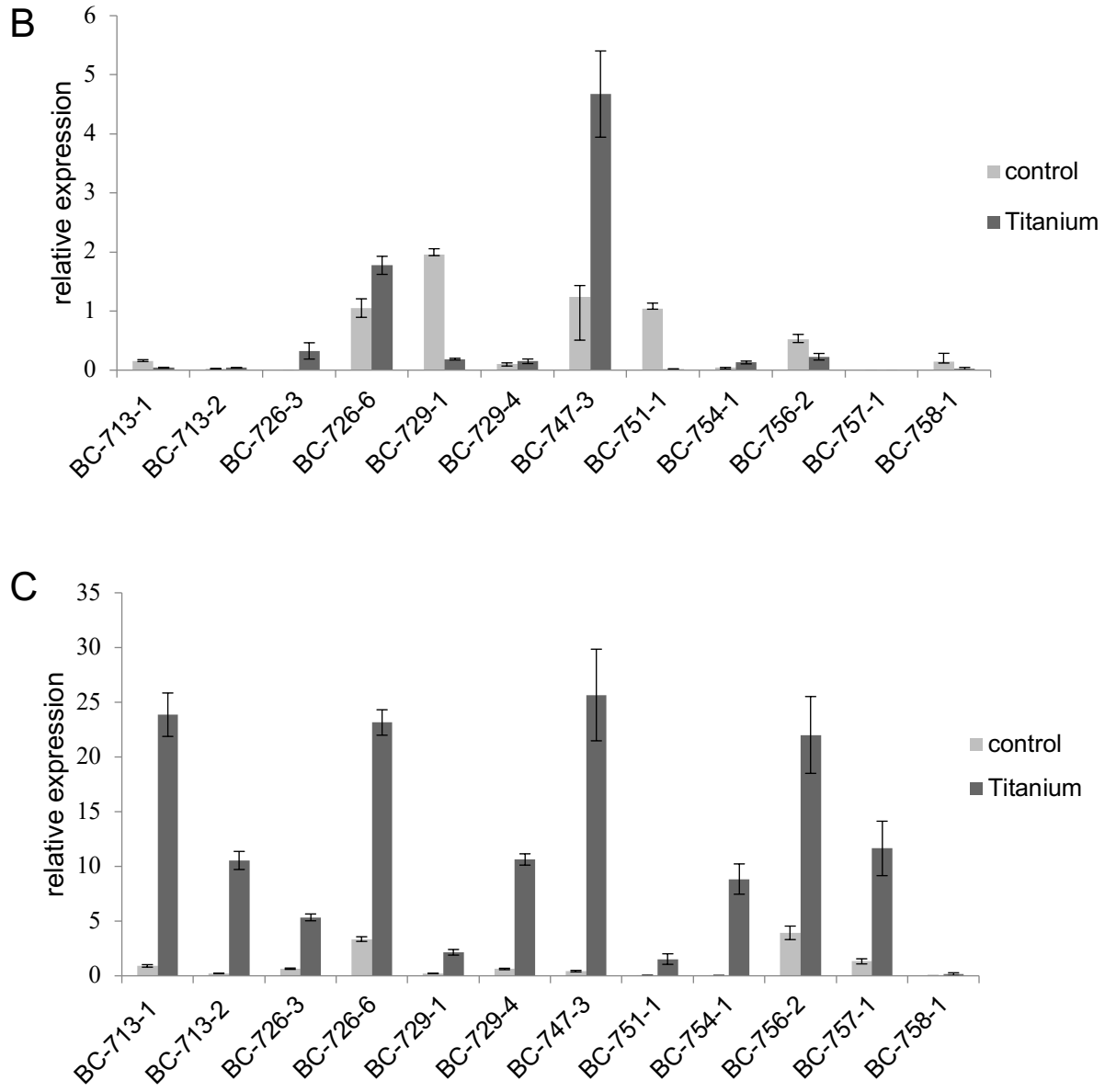


Figure 5. RT-PCR analysis of the effect of porous titanium on MMP-7 mRNA in differentially activated macrophages. Analysis of MMP-7 mRNA levels was performed for 3 subpopulations of macrophages of 12 individual donors: M0(A), M1(B) and M2(C), cultured on plastic (control, light gray) or on porous titanium disks (dark grey) for 6 days. Individual donors are indicated with buffy coat numbers. All experiments were performed in triplicates. Data were normalized to the control of M0 of BC 729-4 by dividing each sample's target/reference value through it. Hence M0 BC 729-4 (control) has a relative expression of 1. Error bars: Standard deviation

Given that the individual donors revealed clear differences between titanium and control groups, it was of interest to analyze whether the mean values can be discriminated with statistical significance. For this purpose, a two-tailed t-test was performed. M0 and M2 macrophages could be discriminated in titanium and control group with a significant difference of p=0,006 and p=0,0009. In turn, M1 macrophages displayed no difference between control and titanium samples (Figure 6). The general expression of MMP-7 in M1 macrophages cultured on plastic (control) was lower than in M0 and M2, however without showing statistical significance.

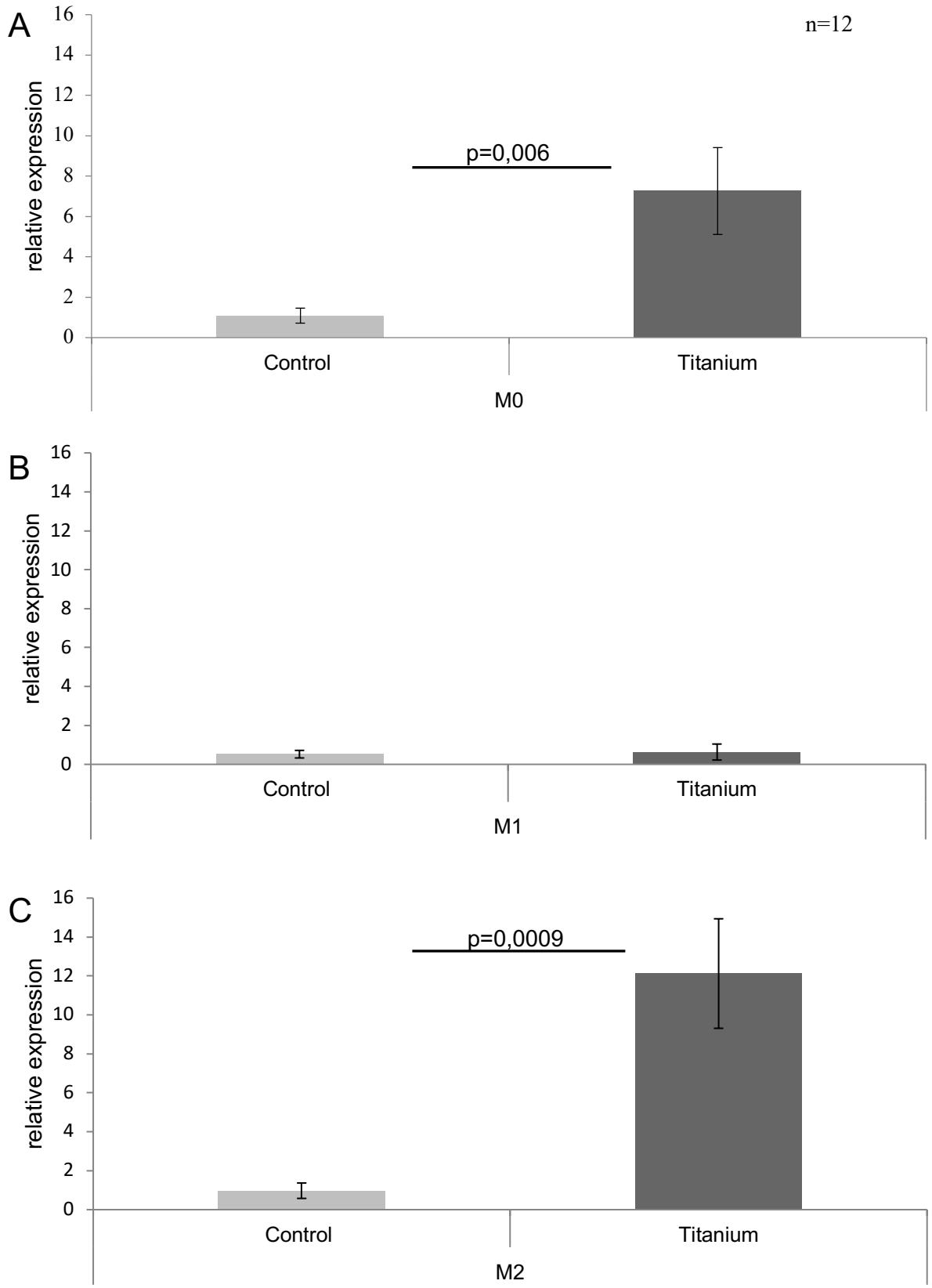
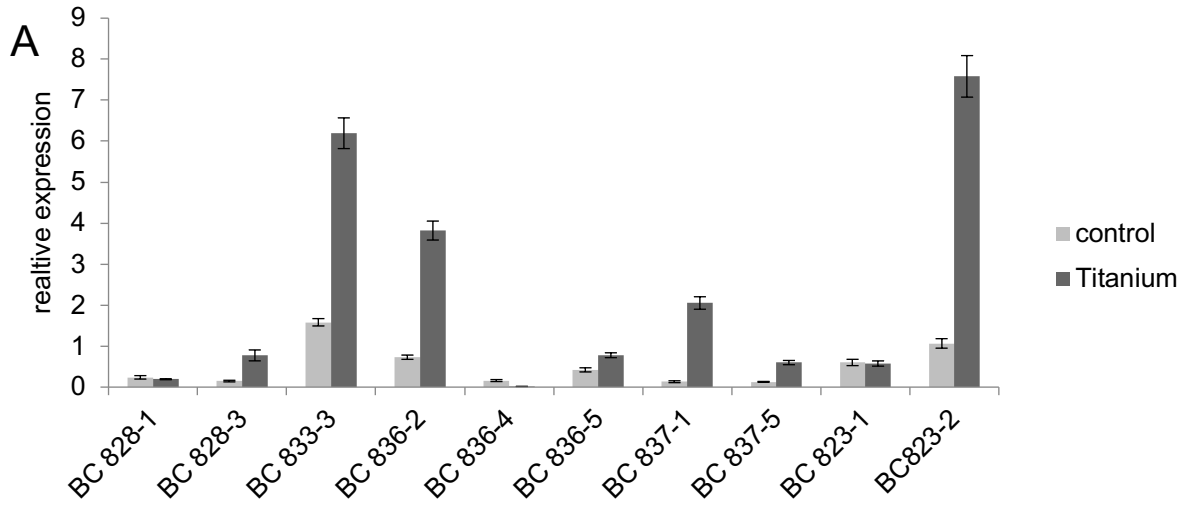


Figure 6. Statistical analysis of the effect of porous titanium on MMP7 gene expression.
Analysis of MMP-7 mRNA levels was performed for 3 subpopulations of macrophages of 12 individual donors: M0 (A), M1 (B) and M2 (C), cultured on plastic (control, light gray) or on porous titanium disks (dark grey) for 6 days. For statistical analysis Student's paired T-test was used. All experiments were performed in triplicates. Statistical analysis was performed out of individual donors in Figure 5. Error bars: Standard error of the mean.

3.1.3 Polished titanium induces MMP-7 mRNA in M0, M1 and M2 macrophages.

Due to the highly significant results of the experiment using titanium disks with pores, it was of interest if the results would be different using titanium with a polished surface, which is also widely used in implant industry. The experiment with polished titanium disks was designed in the same way as the experiment using porous titanium.
Similar to the experiment with porous titanium, a significant difference between titanium and control group in M0 and M2 macrophages was observed. MMP-7 was induced in 7 out of 10 cases in M0 and in 10 out of 10 in M2 macrophages (Figure 7 A, C). The three donors in M0 whose MMP-7 is reduced by titanium expressed both in control and titanium stimulated group a very low MMP-7 expression (relative expression ranging from 0,01 to 0,60), and also the reducing effect of titanium (decrease of fold ranging from 0,9 to 0,04) was meager in these three samples, indicating a minor relevance of the MMP-7 reduction by titanium in these donors (Figure 7 A). In contrast, in macrophages of donors where titanium induced MMP-7, an increase of fold ranging from 1,85 to 15,64 in M0 and from 1,85 to 137,54 in M2 could be determined. In contrast to the experiments with porous titanium, M1 macrophages expressed higher levels of MMP-7 in 8 out of 10 donors when cultured on polished titanium (Figure 7 B). The mean values in the M1 macrophage group demonstrated statistical significance, which is different from the results of porous titanium experiments where no statistical significance in the M1 group was shown (Figure 8).
Collectively, the performed experiments with porous and polished titanium suggest a different response of macrophage subtypes as a function of the implemented titanium type.



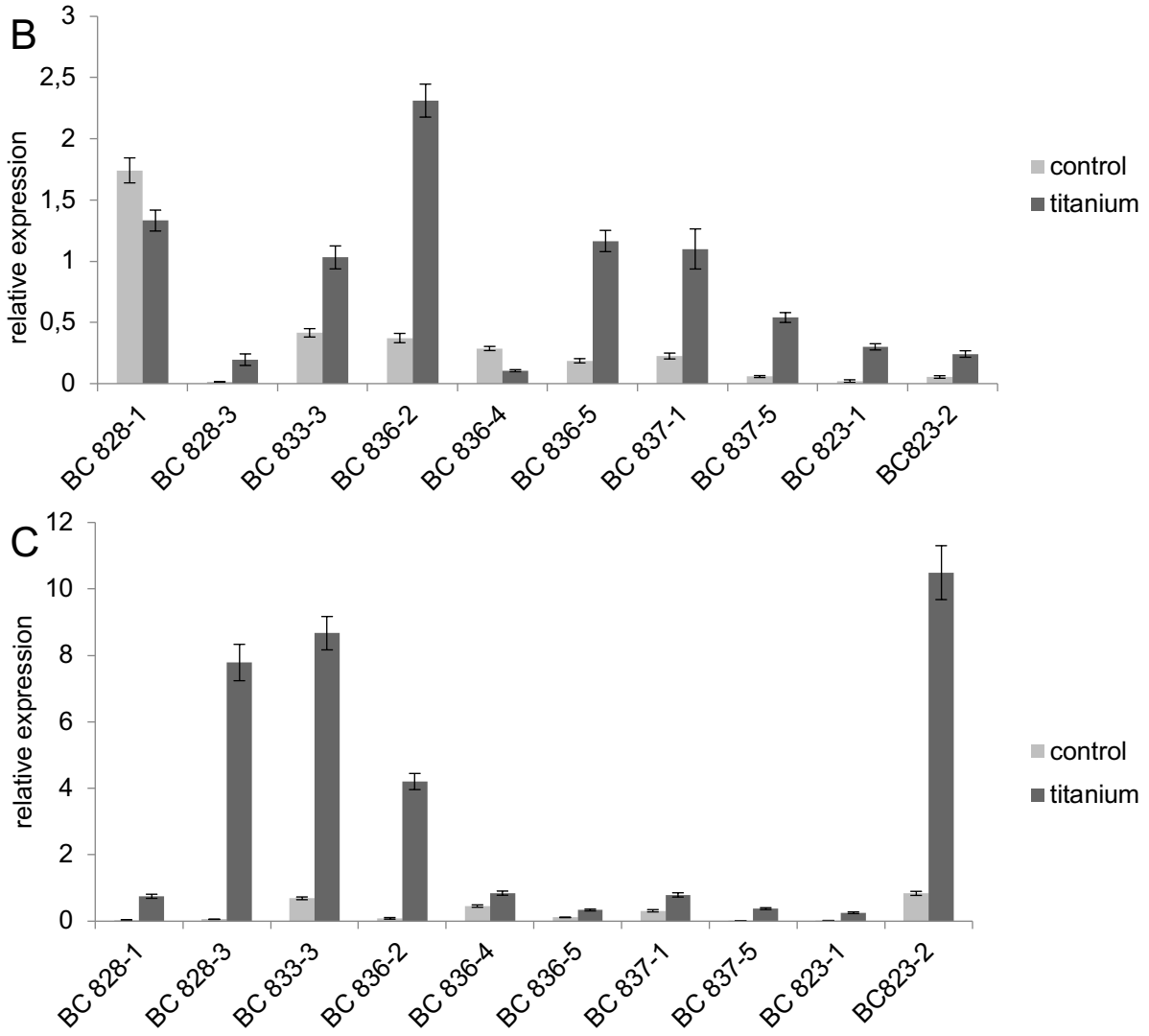


Figure 7. RT-PCR analysis of the effect of polished titanium on MMP-7 mRNA in differentially activated macrophages. Analysis of MMP-7 mRNA levels was performed for 3 subpopulations of macrophages of 10 individual donors: M0(A), M1(B) and M2(C), cultured on plastic (control, light gray) or on polished titanium disks (dark grey) for 6 days. Individual donors are indicated with buffy coat numbers. All experiments were performed in triplicates. Data were normalized to the control of M0 of BC 729-4 by dividing each sample's target/reference value through it. Hence M0 BC 729-4 (control) has a relative expression of 1. Error bars: Standard deviation

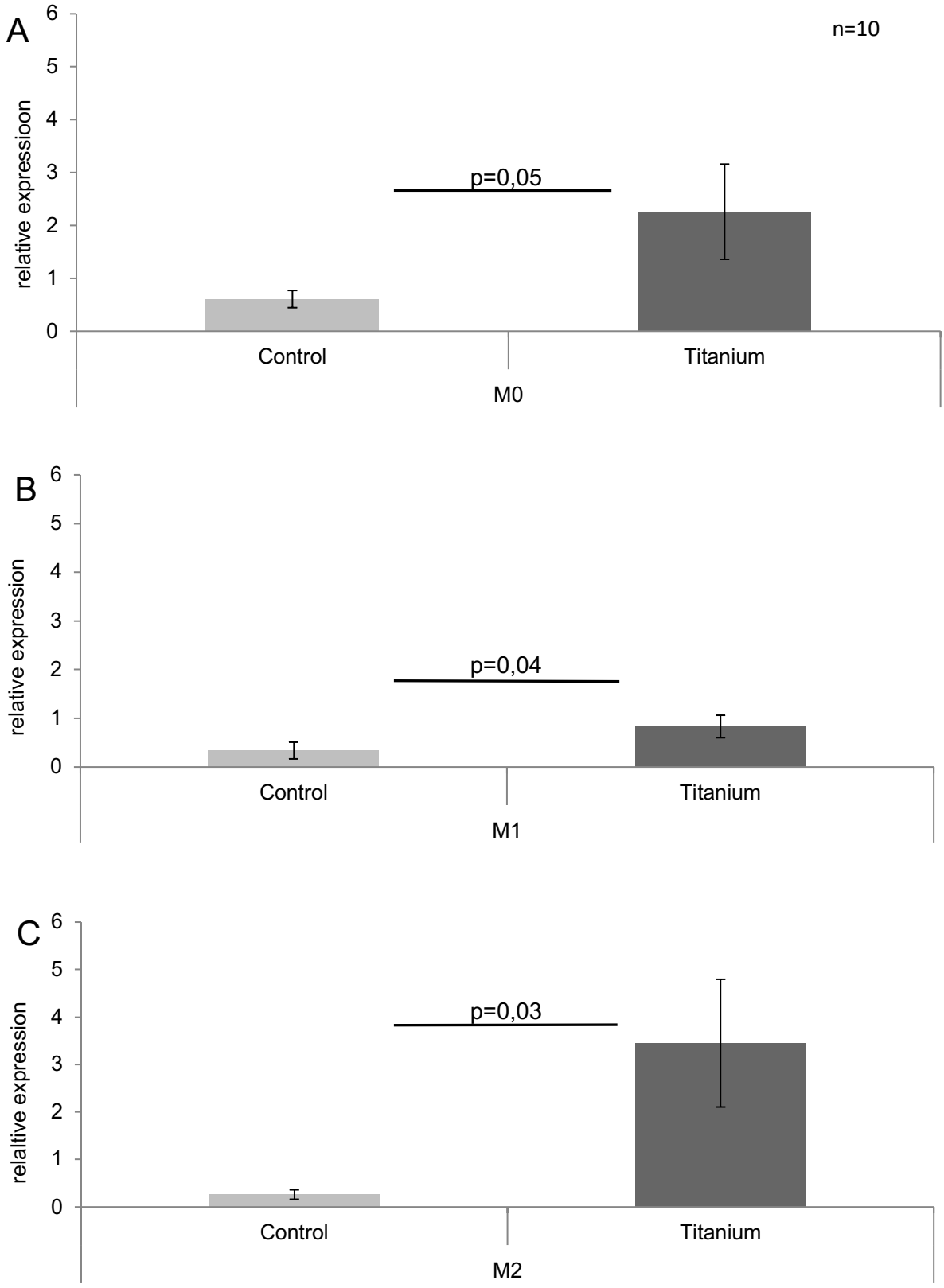


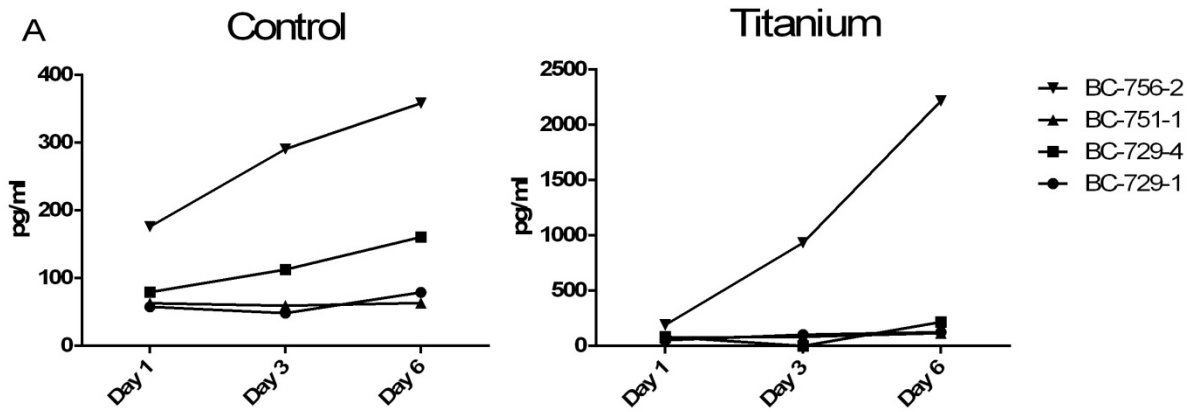
Figure 8. Statistical analysis of the effect of polished titanium on MMP7 gene expression. Analysis of MMP-7 mRNA levels was performed for 3 subpopulations of macrophages of 10 individual donors: M0(A), M1(B) and M2(C), cultured on plastic (control, light gray) or on polished titanium disks (dark grey) for 6 days. For statistical analysis Student's paired T-test was used. All experiments were performed in triplicates. Statistical analysis was performed out of individual donors in Figure 7. Error bars: Standard error of the mean.

3.2 ELISA analysis of the effect of titanium on the MMP-7 protein expression in differentially activated macrophages.

Since RT-PCR analysis revealed a significant effect of titanium on MMP-7 gene expression, the next step was to identify whether this effect is consistent on the protein level. Supernatants of macrophage cultures for the same 12 donors were harvested on day 1, 3 and 6 and MMP-7 concentrations were analyzed by ELISA.

3.2.1 Dynamic accumulation of MMP7 secreted by macrophages during the differentiation on titanium disks

In order to quantify at which time of incubation the highest amount of MMP-7 is expressed, supernatants of four donors stimulated with porous titanium were analyzed by ELISA which were harvested on day 1, 3 and 6 of macrophage cultivation. As displayed in Figure 9, MMP-7 protein increased in all macrophage subpopulations during the period of incubation. The highest amount of MMP-7 protein levels could have been stated on day 6. In order to quantify this increase during the duration of incubation, fold changes of MMP-7 secretion from day 1 to day 6 were calculated. In the control group, an increase of fold ranging from 1,37 up to 2,03 in M0 (Figure 9A) was observed, from 2,71 up to 7,18 in M1 (Figure 9B) and from 1,00 up to 14,98 in M2 macrophages (Figure 9C). In samples stimulated with porous titanium, the increase of fold ranged from 1,56 up to 11,60 in M0, from 0,75 up to 1,99 in M1, and from 6,47 up to 28,12 in M2 macrophages. M1 macrophages of one donor stimulated with titanium, secreted less MMP-7 on day 6 than on day 1. MMP-7 in M0 and M2 macrophages, stimulated by porous titanium, showed a stronger increase of fold by the duration of incubation than their controls. In contrast, MMP-7 of M1 macrophages was stronger induced in the control group. On day 1, titanium did not affect the MMP-7 secretion, and the effect on MMP-7's fold-change was mostly pronounced between day 3 and day 6.



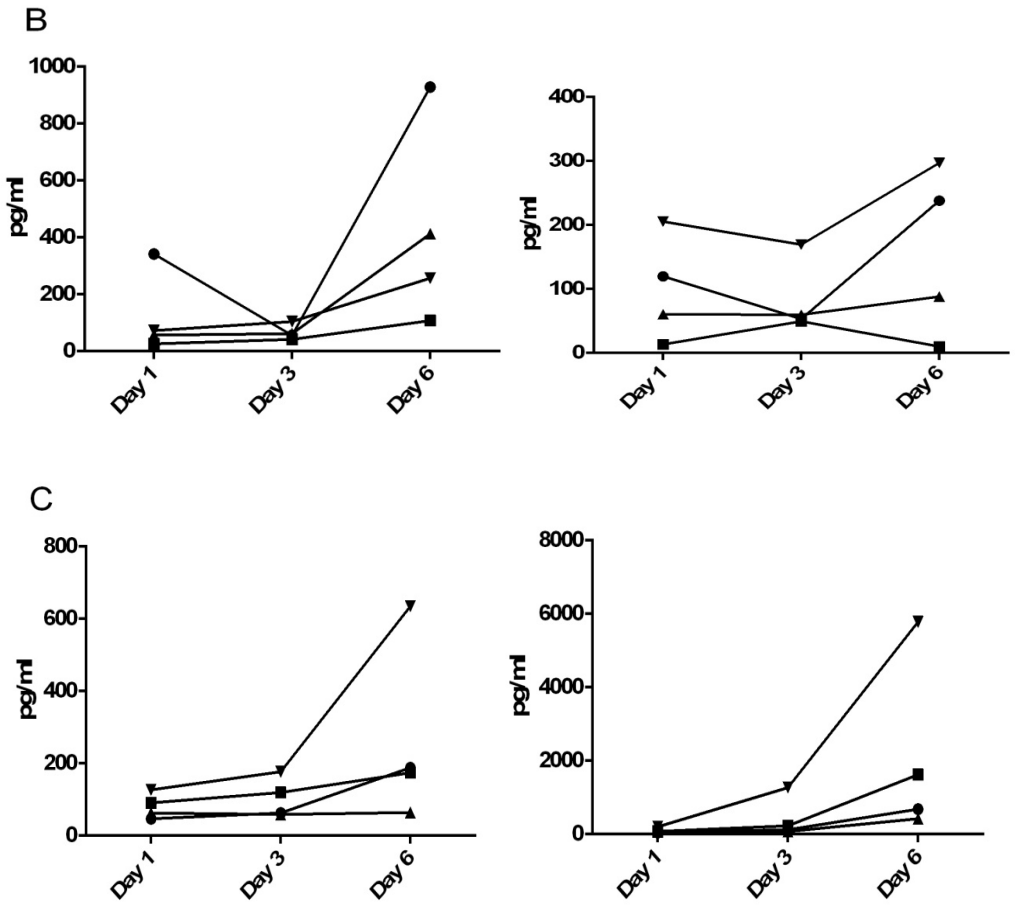


Figure 9. ELISA analysis of MMP-7 protein secretion in differentially activated macrophages. Analysis of MMP-7 protein levels was performed for 3 subpopulations of macrophages of 4 individual donors: M0(A), M1(B) and M2(C), cultured on plastic (control, left column) or on porous titanium disks (right column) for 6 days. Individual donors are indicated with buffy coat numbers. Supernatants were collected on day 1, 3 and 6 of macrophage cultivation. All experiments were performed in duplicates.

3.2.2 Porous titanium induces MMP-7 protein expression in M0 and M2 macrophages

Since MMP-7 secretion was mostly pronounced on day 6, it was of interest how protein levels of the other donors were affected by titanium after 6 days of incubation. Regarding M0 and M2 macrophages, donors showed a similar response to titanium on protein level when compared to the mRNA level. Titanium induced MMP-7 protein in 11 out of 12 donors analyzed in M0 and in 10 out of 12 in M2 (Figure 10 A, C). In M1 macrophages 5 out of 12 donors showed higher secretion of MMP-7, while 6 out of 12 suppressed the secretion of MMP-7 in response to titanium. One donor showed no effect on titanium (Figure 10 B).

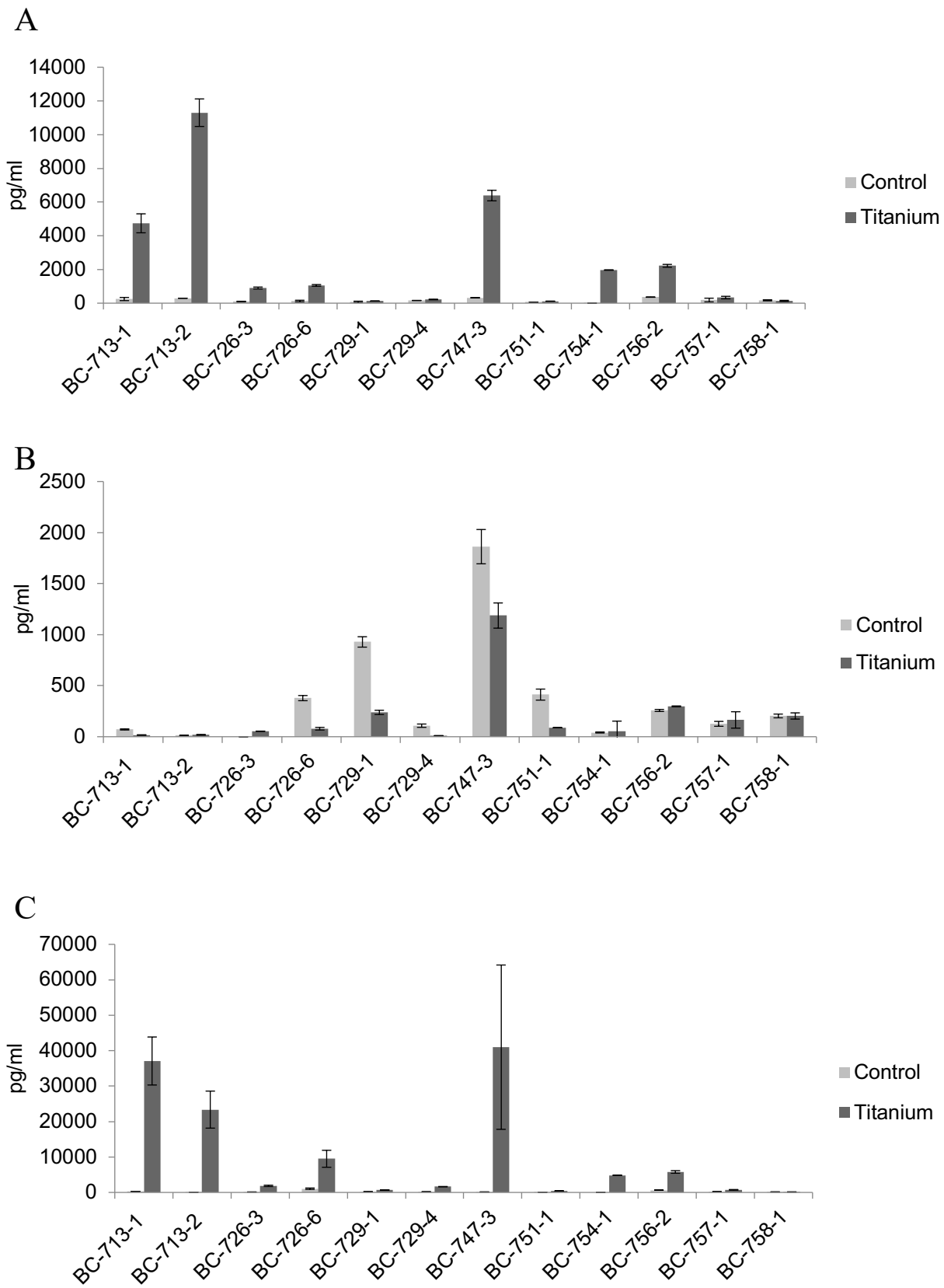
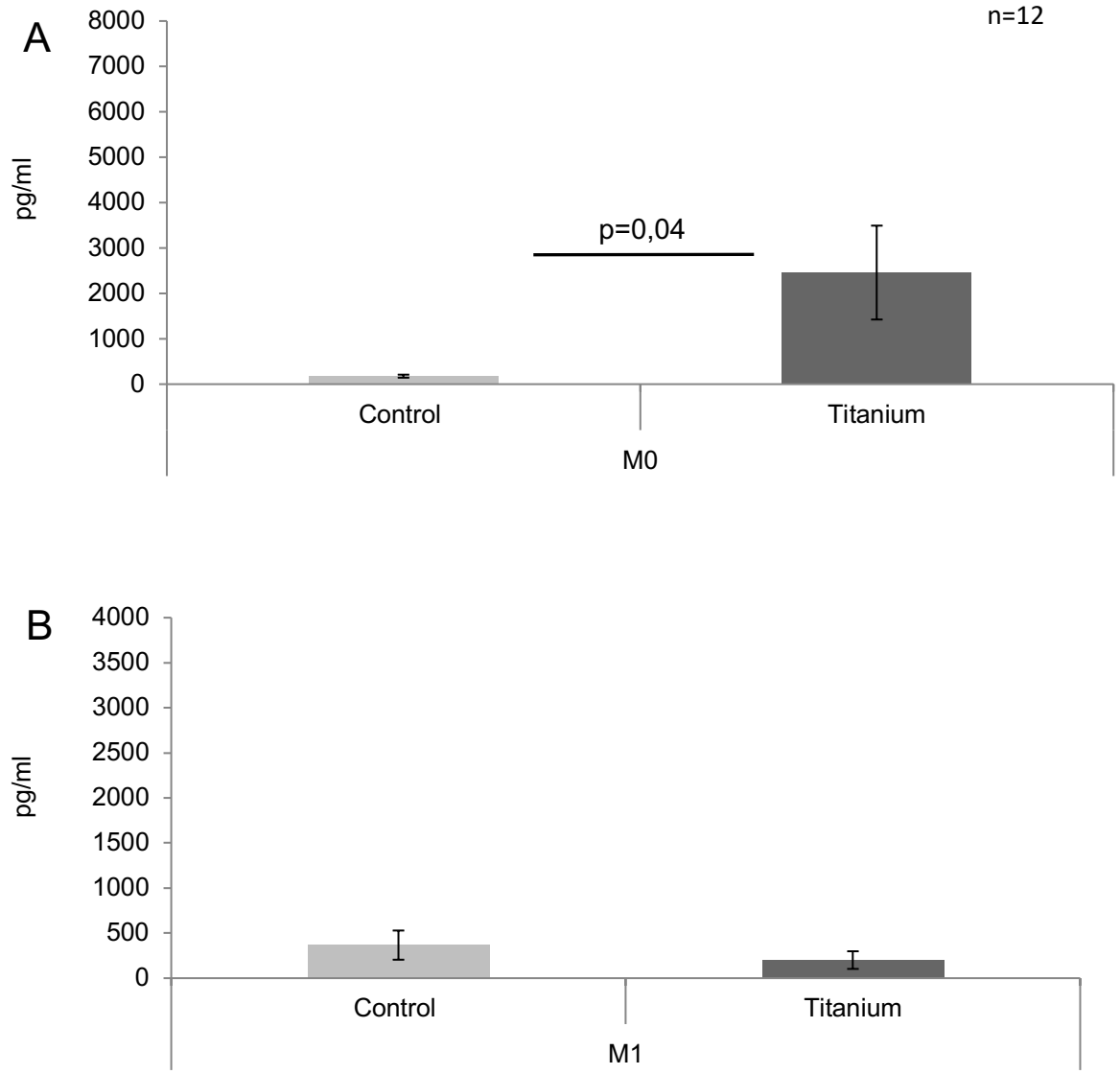


Figure 10. ELISA analysis of the effect of porous titanium on MMP-7 protein in differentially activated macrophages of individual donors. Analysis of MMP-7 protein levels was performed for 3 subpopulations of macrophages of 12 individual donors M0(A), M1(B) and M2(C), cultured on plastic (control, light gray) or on porous titanium disks (dark grey) for 6 days. Individual donors are indicated with buffy coat numbers. The analysis was performed from supernatants collected on day 6 of macrophage cultivation. All experiments were performed in duplicates. Error bars: Standard deviation.

Statistical analysis for the secreted levels of MMP-7 as measured by ELISA demonstrated a significant induction by titanium in M0 (p=0,04) and M2 macrophages (p=0,03) (Figure 11). The Data of M0 and M2 macrophages corresponds to the results of the RT-PCR analysis, where a significant induction by titanium was noted as well. In the M1 group, a non-significant reduction of MMP-7 was observed (p=0,06). However, the levels of secreted MMP-7 in M1 are so low that they have probably no biological relevance.



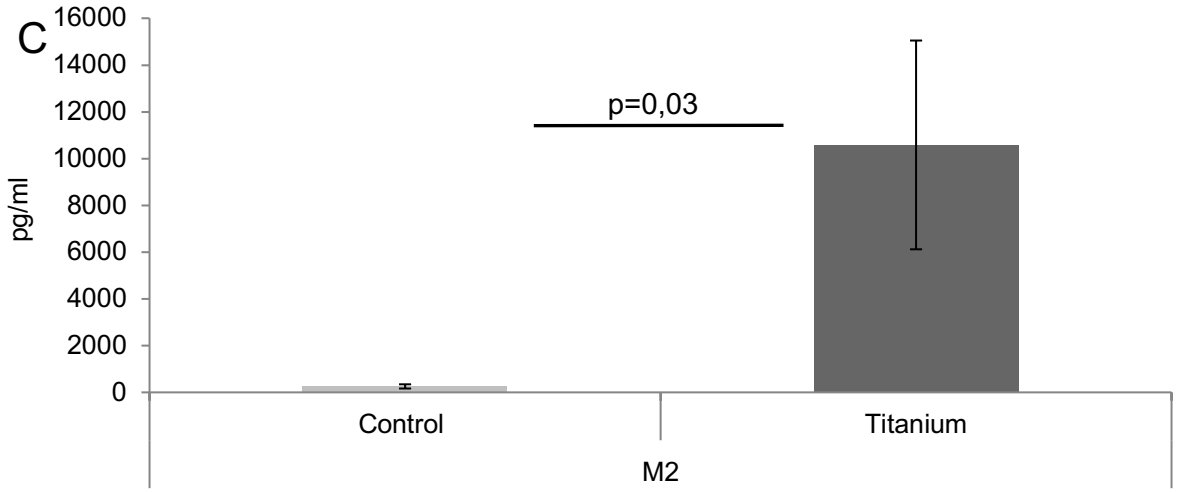


Figure 11. Statistical analysis of the effect of porous titanium on MMP-7 protein secretion. Analysis of MMP-7 protein levels was performed for 3 subpopulations of macrophages of 12 individual donors: M0(A), M1(B) and M2(C), cultured on plastic (control, light gray) or on porous titanium disks (dark grey) for 6 days. Statistical analysis was performed from supernatants collected on day 6 of macrophage cultivation (Figure 10). For statistical analysis Student's paired T-test was used. All experiments were performed in duplicates. Error bars: Standard error of the mean.

3.3 Antibody-based analysis of active MMP-7 protein secretion in differentially activated macrophages.

3.3.1 Analysis of active MMP-7 secretion in titanium stimulated macrophages.

As MMP-7 can be secreted in an active and an inactive form (46), it was of interest to examine if titanium stimulated macrophages also express the active form of MMP-7. Therefore, an activity assay, based on ELISA method was performed. Supernatants from macrophages cultured with and without titanium were analyzed after incubation with a pro-enzyme which, upon activation by active MMP-7, can release color from a chromogenic peptide substrate. Active MMP-7 concentration was measured photometrically. It was identified that cultivation of M0 and M2 macrophages on titanium disks resulted in the production of elevated levels of active MMP-7 in 10 out of 11 donors and in 5 out of 11 donors in M1 (Figure 12).
Absolute levels of active MMP-7 showed a strong variation among macrophage subtypes. M2 macrophages stimulated with titanium expressed the highest levels of MMP-7 among the macrophage subtypes (mean: 687,3 pg/ml; range of between 0-2916,2 pg/ml). M1 macrophages expressed the lowest levels of MMP-7 out of all macrophage subtypes with a mean concentration of 37,7 pg/ml and a range of between 0-174,1 pg/ml under titanium stimulation. M0 macrophages ranged in the middle with a mean activity of 285,5 pg/ml and a range of between 0-2134,8 pg/ml under titanium stimulation. Active MMP-7 concentrations secreted from macrophages without titanium stimulation were low in general (mean activity: M0 (18,3), M1(40,6) and M2 (20,8)) Range was between 0-197,1 pg/ml.
Active MMP-7 was induced in M0 and M2 macrophages derived from monocytes isolated from 10 out 11 individual donors, however without statistical significance (p=0,18 for M0 and p=0,06 for M2, (Figure 13)).

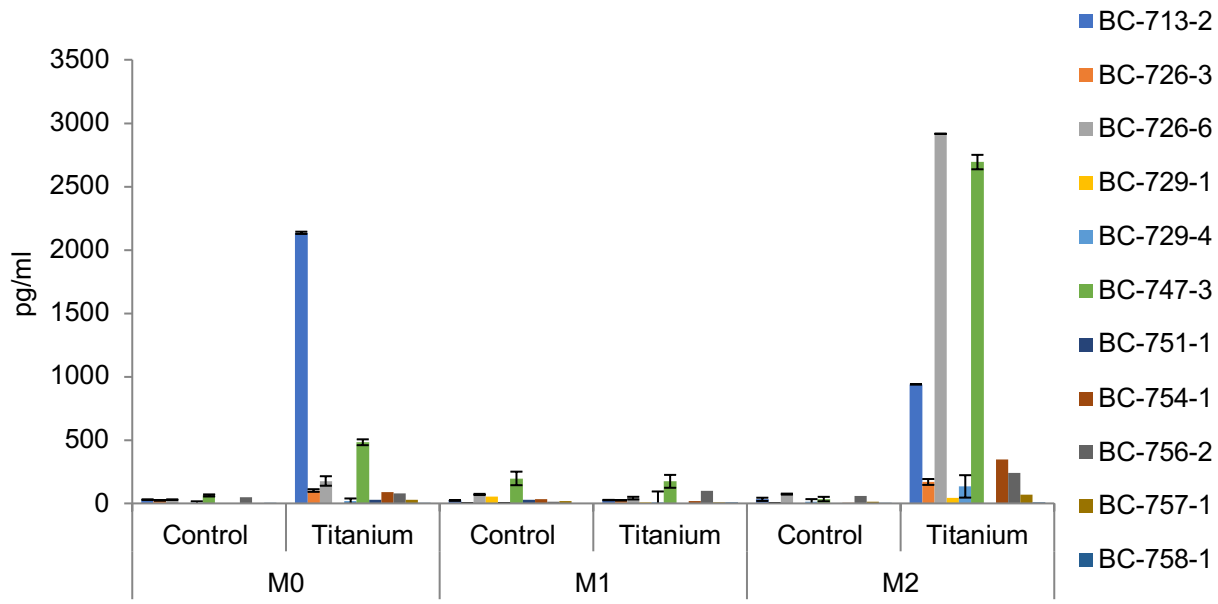


Figure 12. Antibody-based analysis of active MMP-7 protein in differentially activated macrophages. Analysis of MMP-7 protein levels was performed for 3 subpopulations of macrophages of 11 individual donors: M0, M1, and M2 cultured on plastic (control) or porous titanium disks for 6 days. Individual donors are indicated with buffy coat numbers. The analysis was performed from supernatants collected on day 6 of macrophage cultivation. A405 was measured at 6h and 24h after capturing MMP-7 with specific antibodies and incubating it with a chromogenic substrate which was degraded only by active MMP-7. Concentrations of active MMP-7 were calculated with a standard curve. For statistical analysis Student's paired T-test was used. All experiments were performed in duplicates. Error bars: Standard deviation.

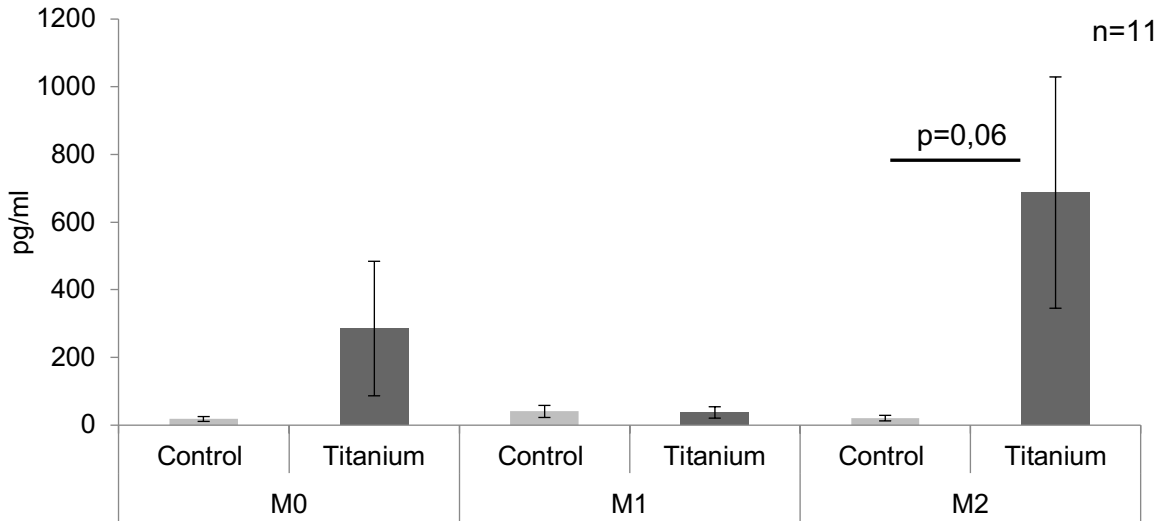


Figure 13. Statistical analysis of the effect of porous titanium on the active MMP-7 protein secretion.
Analysis of MMP-7 protein levels was performed for 3 subpopulations of macrophages of 11 individual donors: M0, M1, and M2 cultured on plastic (control) or porous titanium disks for 6 days. Statistical analysis was performed from the supernatants collected on day 6 of macrophage cultivation (Figure 12). A405 was measured at 6h and 24h after capturing MMP-7 with specific antibodies and incubating it with a chromogenic substrate which was degraded only by active MMP-7. Concentrations of active MMP-7 were calculated with a standard curve. For statistical analysis Student's paired T-test was used. All experiments were performed in duplicates. Error bars: Standard error of the mean.

3.3.2 Percentage of active MMP-7 secretion in macrophages of individual donors.

In order to identify whether titanium in addition to the induction of MMP-7 secretion affects MMP-7 activation, the percentage of active MMP-7 both in control and titanium stimulated group was calculated (Figure 14). This is necessary as higher levels of active MMP-7 in titanium stimulated macrophages (Figure 12) may also be a result of higher levels of total MMP-7 in this group. Therefore, the total MMP-7 protein of an individual donor, measured in ELISA, was divided by its active form of MMP-7, measured in the activity assay, and was multiplied with one hundred.
Active form of MMP-7 was between 0,7% and 84,2% in control macrophages and between 2,7% and 53,1% in macrophages cultivated on titanium. MMP-7 protein of macrophages was considered as activated by titanium when the percentage of activation in macrophages cultured on titanium was higher than in macrophages cultured on plastic. In macrophages cultured on titanium 6 out of 11 analyzed donors in M0, 5 out of 11 in M1 and 7 out of 11 in M2 exhibited induction of their activity under titanium stimulation (Figure 14).
Mean values between control and titanium stimulated groups of all macrophage subtypes showed no significant difference (data not shown).

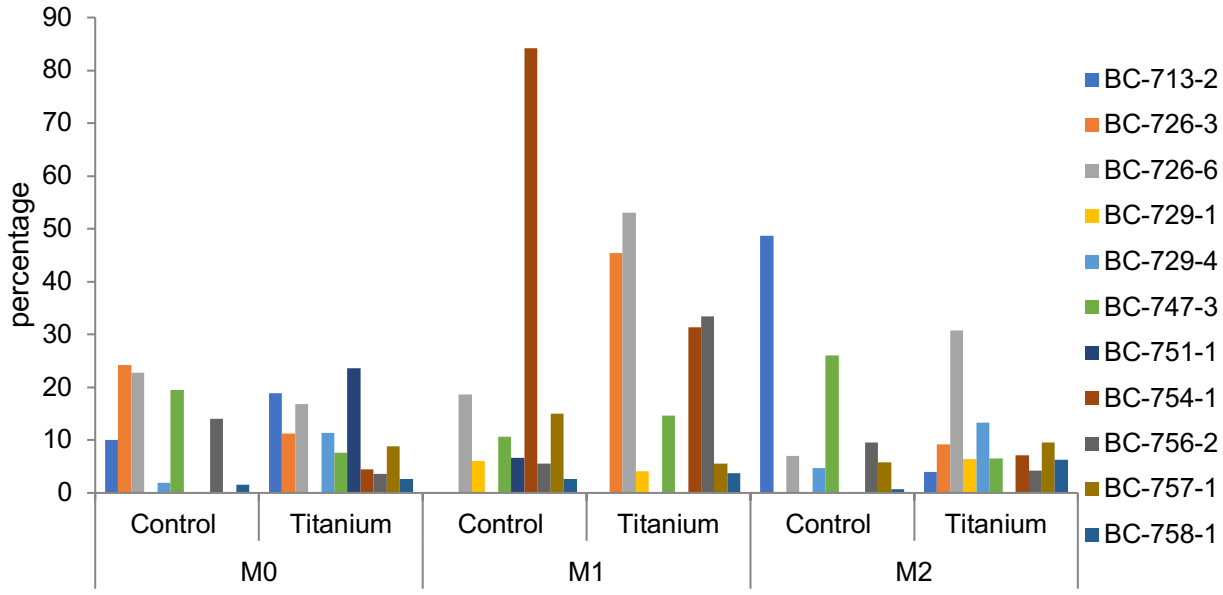


Figure 14. The percentage of MMP-7 in the active form for 11 donors.
Percentage of active MMP-7 was calculated by dividing the concentrations of active MMP-7 protein, measured in the activity assay, by the total protein concentrations, measured in ELISA.

3.4 The effect of titanium on MMP-7 mRNA, protein and activity

In order to identify the extent to which titanium affects mRNA, protein and active protein expression, fold-changes between titanium and control groups were calculated (Table 20). Empty fields indicate donors who could not be calculated, as either the control or the titanium stimulated sample showed no expression. Therefore,

macrophages of some donors reacted on titanium, but fold-changes could not be calculated.
As MMP-7 expression is regulated on different levels, it is of interest on which level of regulation the titanium induction is more pronounced. For this purpose, fold changes in mRNA, total protein and active protein were calculated separately and subsequently compared with each other.
Induction of MMP-7 mRNA in macrophages derived from individual donors in general correlated with the induction on protein level, and partially on the activity level (Table 20).
M1 macrophages exhibit only very low fold-changes so that they have probably no biological relevance.
MMP-7 was only considered as “induced” on a certain level of regulation if the fold-change on this level was higher than on the previous level. This is because MMP-7 protein is a result of MMP-7 mRNA and active MMP-7 a result of pro-MMP-7, measured in ELISA.
In M0 macrophages of 11 out of 12 donors, titanium induced MMP-7 on mRNA level, although the fold change of one donor could not be calculated as the control sample showed no expression (BC-757-1). 6 out of 11 of these donors were also induced on protein level and 1 out of these 6 donors also on activity level (BC-713-2). In M2 macrophages, of 12 out of 12 donors, titanium induced MMP-7 on mRNA level, although the fold change of one donor could not be calculated, as the control sample showed no expression (BC-758-1). 6 out of 12 of these donors were also induced on protein level and 1 out of these 6 donors also on activity level (BC-726-6). (Table 20).

Induction was considered as “more pronounced” on a certain level of regulation when the fold change was more than the double of the previous level of regulation. In M0 macrophages of 7 out of 12 donors analyzed, and in M2 macrophages of 8 out of 12 donors analyzed, the induction on MMP-7 on the mRNA level was more pronounced than on the protein level, whereas the other donors exhibited a higher induction on protein level.

Table 20. Fold-changes of MMP-7 gene expression, protein secretion, and activity affected by titanium. Values were calculated by dividing the MMP-7 expression of macrophages cultured on titanium disks by the MMP7 expression of macrophages cultured on plastic (control).

	M0			M1			M2		
	mRNA	**Protein**	**activity**	**mRNA**	**Protein**	**activity**	**mRNA**	**Protein**	**activity**
BC-713-1	22,09	19,30	-	1,70	0,23	-	26,32	130,52	-
BC-713-2	13,61	38,30	72,23	1,70	1,45	1,12	46,21	353,36	29,20
BC-726-3	3,01	9,10	4,25	-	-	-	8,26	15,08	-
BC-726-6	4,49	8,14	6,01	1,69	0,21	0,59	6,89	9,03	39,77
BC-729-1	3,41	1,59	-	0,10	0,26	0,16	9,73	3,64	-
BC-729-4	1,00	1,36	6,55	1,38	0,09	-	17,10	9,34	9,38
BC-747-3	5,64	19,77	7,69	3,77	0,64	0,88	60,49	312,01	78,92
BC-751-1	27,19	1,85	-	0,01	0,21	0,00	75,74	6,53	-
BC-754-1	266,31	1123,18	-	3,25	1,32	0,49	507,99	-	136,45
BC-756-2	5,16	6,19	1,57	0,43	1,15	0,16	5,60	9,11	3,98
BC-757-1	-	1,78	-	-	1,30	0,48	8,80	3,77	6,23
BC-758-1	1,43	0,80	1,41	0,19	1,00	1,39	-	0,95	8,61

3.5 RT-PCR analysis of the effect of titanium on MMP-7 regulators in differentially activated macrophages

Since the effect of titanium on the level of MMP-7 protein production and the activation of MMP-7 were different for individual donors, it was of interest to analyze whether titanium influences the regulators of MMP-7 and orchestrates the activity of MMP-7. One inhibitor (TIMP-3) and one activator (CD151) of MMP-7 were chosen for RT-PCR analysis. It was hypothesized, that these MMP regulators are induced or suppressed by titanium. TIMP-3 and CD151 were chosen, as human macrophages express them and the difference in the expression levels of TIMP-3 and CD151 were previously identified using Affymetrix gene expression analysis. In the Affymetrix analysis an up-regulation of CD151 and a down-regulation of TIMP-3 was identified in macrophages cultured on titanium. However, only with statistical significance for CD151 (Alexandru Gudima, Affymetrix data) (82).

3.5.1 Involvement of TIMP-3 in the regulation of MMP-7 activity

TIMP-3 has the broadest spectrum of MMP inhibition out of all TIMP's (18), and it inhibits MMP-7 by binding it in a 1:1 stoichiometry. It was hypothesized that TIMP-3 is down-regulated in macrophages cultured on titanium leading to the up-regulation of MMP-7 activity.
Using RT-PCR it was found that TIMP-3 expression is not significant decreased in M1 and M2 macrophages cultured on titanium (Figure 15), (2,02 fold change in M2 and 9,36 fold change in M1). In M0 macrophages, cultivation on titanium had a stimulating effect on TIMP-3 expression ($p=0,08$). As there was a tendency but no significance for down-regulation in M1 and M2, individual donors which were activated by titanium were examined (Figure 14). Active MMP-7 was induced in M2 macrophages of 7 out 11 donors (Figure 14). In 4 out of these 7 donors (BC-726-3, BC-726-6, BC-729-1, BC-754-1) TIMP-3 was down-regulated, suggesting a mechanism of activity regulation (Figure 16). In M1 macrophages 5 out of 11 donors were induced by titanium (Figure 14). 2 out of 5 of these donors (BC 747-3, BC 758-1) showed a down-regulation of TIMP-3.

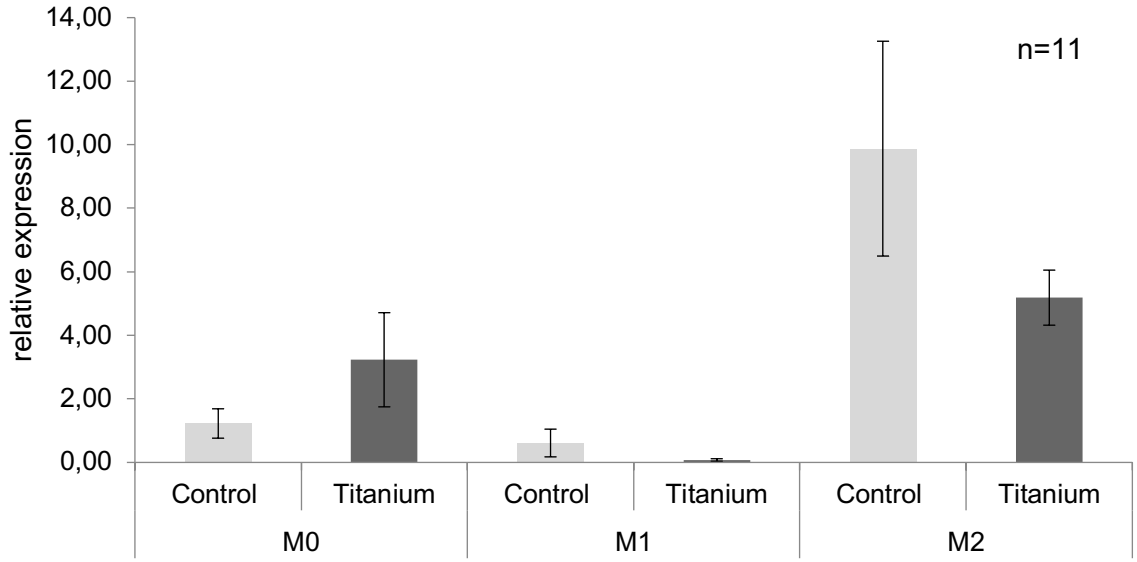


Figure 15. Statistical analysis of the effect of porous titanium on TIMP3 mRNA expression. Analysis of MMP-7 mRNA levels was performed for 3 subpopulations of macrophages of 11 individual donors: M0, M1 and M2 cultured on plastic (control, light gray) or porous titanium disks (dark grey) for 6 days. For statistical analysis Student's paired T-test was used. All experiments were performed in triplicates. Error bars: Standard error of the mean.

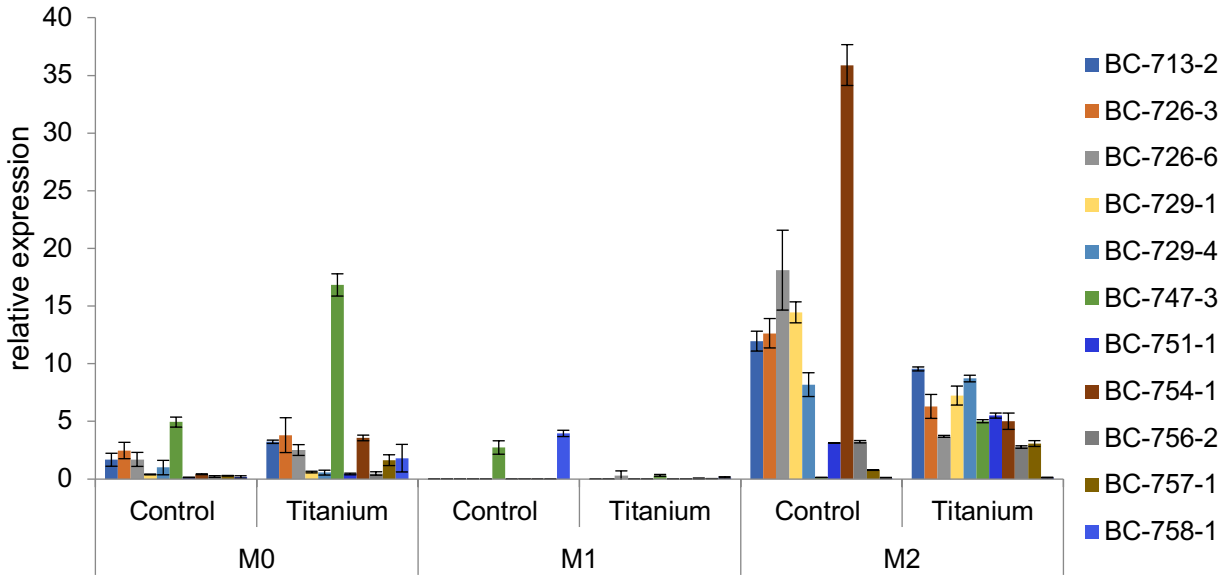


Figure 16. RT-PCR analysis of the effect of porous titanium on TIMP-3 mRNA expression in differentially activated macrophages. Analysis of TIMP-3 mRNA levels was performed for 3 subpopulations of macrophages of 11 individual donors: M0, M1 and M2 cultured on plastic (control) or porous titanium disks for 6 days. Individual donors are indicated with buffy coat numbers. All experiments were performed in triplicates. Data were normalized to the control of M0 of BC 729-4 by dividing each sample's target/reference value through it. Hence M0 BC 729-4 (control) has a relative expression of 1. Error bars: Standard deviation.

3.5.2 Involvement of CD151 in the regulation of MMP-7 activity

Shiomi et al. were able to provide evidence for high proteolytic activity of MMP-7 in case of co-localisation with CD151. However, as CD151 has no proteolytic activity itself, it was considered that it activates MMP-7 indirectly by binding it to the cell surface and facilitating the proteolysis of its pro-peptide by other MMP activators (85). It was of interest whether differentially expressed levels of CD151 could explain the up-regulation of activity of MMP-7 in macrophages cultured on titanium.
In M0 macrophages cultured on titanium, a slightly enhanced (2,55 vs.1,93) but not statistically significant (p=0,38) expression of CD151 was detected suggesting a potential contribution of CD151 to the MMP-7 regulation (Figure 17). Likewise, for CD151 individual donors which are activated by titanium (Figure 14) were examined. Active MMP-7 was induced in M0 macrophages of 6 out 11 donors (Figure 14). In 3 out of these 6 donors (BC-729-4, BC 751-1, 758-1) an upregulation of CD151 was observed (Figure 18). In M1 macrophages, no difference in CD151 mRNA levels was found. In M2, a not significant down-regulation of CD151 gene expression was detected in macrophages cultured on the titanium disks (p=0,37).

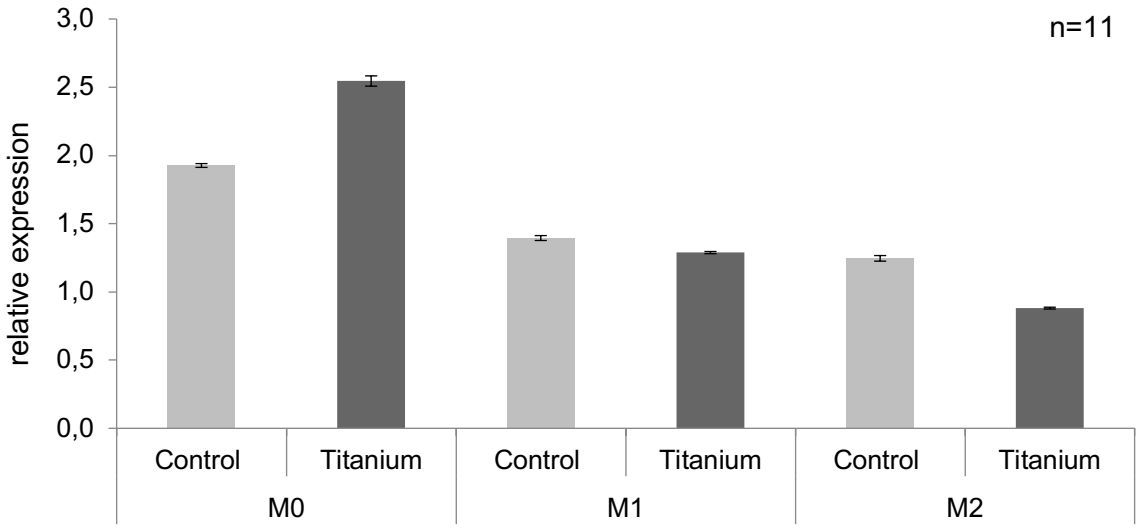


Figure 17. Statistical analysis of the effect of porous titanium on CD151 gene expression for macrophages. Analysis of MMP-7 mRNA levels was performed for 3 subpopulations of macrophages of 11 individual donors: M0, M1 and M2 cultured on plastic (control, light gray) or porous titanium disks (dark grey) for 6 days. For statistical analysis Student's paired T-test was used. All experiments were performed in triplicates. Error bars: Standard error of the mean.

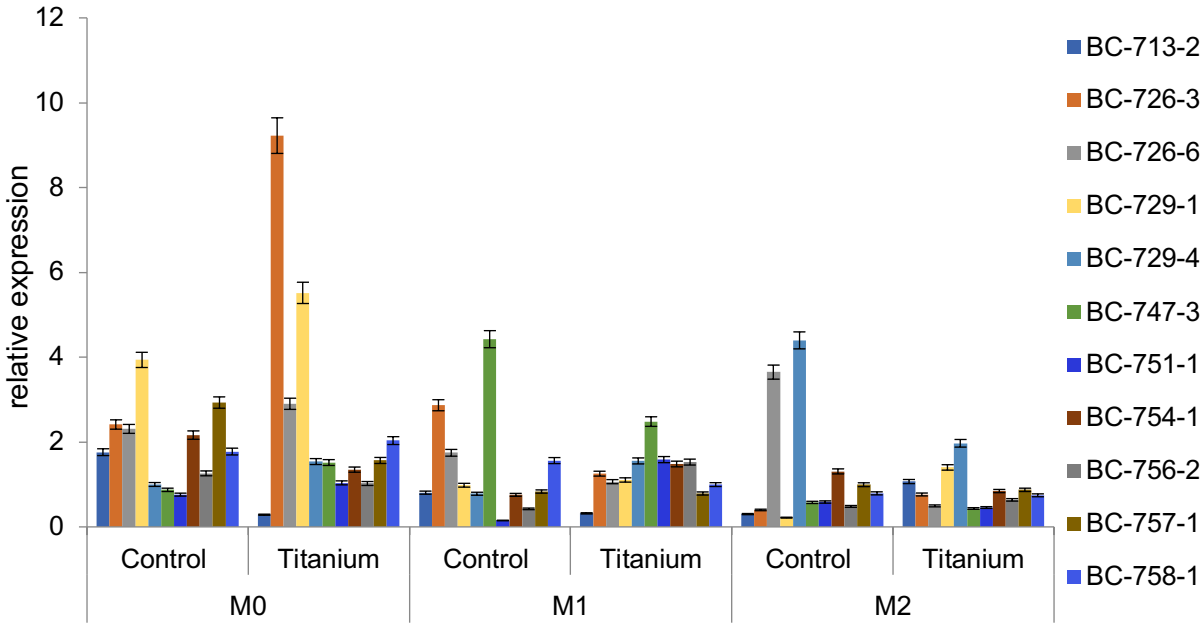


Figure 18. RT-PCR analysis of the effect of porous titanium on CD151 mRNA expression in differentially activated macrophages. Analysis of CD151 mRNA levels was performed for 3 subpopulations of macrophages of 11 individual donors: M0, M1 and M2 cultured on plastic (control) or porous titanium disks for 6 days. Individual donors are indicated with buffy coat numbers. All experiments were performed in triplicates. Data were normalized to the control of M0 of BC 729-4 by dividing each sample's target/reference value through it. Hence M0 BC 729-4 (control) has a relative expression of 1. Error bars: Standard deviation.

4 DISCUSSION

4.1 The production of MMP-7 in response to titanium

Titanium is the metal of choice regarding the implementation of prosthetics, dental implants, medical devices and in general for intraosseous use in medical fields. The material has been used for decades and offers advantages with respect to biocompatibility, corrosion resistance and mechanical properties (86). Nevertheless, aseptic implant failure of titanium implants can occur and may cause severe problems for health and life quality of the patient (87). It has been reported that 3,4-9% of orthopedic implants fail (88, 89). Regarding the reasons for failure, aseptic implant loosening is responsible for more than 80% of the carried out revision operations in the field of endoprosthesis (90). MMP's, in general, are considered to play a crucial role in periprosthetic loosening (3). Macrophages play a vital role in inflammatory processes and present a primary source of MMPs (Table 4). Thus, it is of interest to analyze how this cell type expresses MMPs after being exposed to titanium. The response of differentially activated pro-inflammatory M1 macrophages, anti-inflammatory M2 macrophages and M0 macrophages has been analyzed. The focus was on MMP-7 as preliminary Affymetrix chip microarray analysis (performed by Alexandru Gudima (82)), showed the strongest induction by titanium out of all MMPs. Additionally, MMP-7's broad substrate spectrum indicates for a high biological relevance (21).
The quantitative RT-PCR performed in the current study has confirmed the microarray analysis and demonstrated a significant induction of MMP-7 in M0 and M2 macrophages on mRNA and protein level. The active form of MMP-7 was induced in M0 and M2 macrophages derived from monocytes isolated from 10 out of 11 individual donors. Although there was no statistical significance ($p=0,18$ for M0 and $p=0,06$ for M2) the results indicate for relevant higher concentrations of active MMP-7 secreted by macrophages stimulated with titanium. The non-significant results can be explained by the high variability of MMP-7 concentrations among macrophages derived from individual donors, resulting in large standard errors. Nevertheless, a tendency for higher levels of active MMP-7 both in M0 and M2 macrophages was observed.

In order to identify whether titanium affects MMP-7 activation, the percentage of activation was calculated as described in results.
Titanium induced MMP-7 in 50-60% of the donors (6 out of 11 analyzed donors in M0, 5 out of 11 in M1 and 7 out of 11 in M2), meaning these donors showed a higher percentage of activation in titanium stimulated macrophages compared to macrophages without stimulation. Statistical significance was not proven. The activation of MMP-7 is crucial as mainly the enzyme in its active form can cleave its numerous substrates (15). However, also MMPs in its pro-form (MMP-1, -9, -13, -14) were described as functionally active (6). If these findings from other MMPs are transferable to MMP-7 is not clear and has to be analyzed in further experiments.

Other studies have also reported the up-regulation of MMP-7 expression and other MMPs by titanium. Yasuharu Nakashima et al. were able to demonstrate an enhanced production of MMP-1, MMP-2, and MMP-9 in primary human macrophages exposed to titanium alloy particles. In the same experiment, they also presumed the detection of MMP-7 in an analysis by zymography, due to its molecular weight and

the caseinolytic activity (66). Shubayev et al. reported an increase of MMP-7, -2 and -9, detected by immunohistochemical analysis of rat tissue after the implementation of titanium implants (91). However, the levels of MMP-7 secreted by differentially activated macrophages cultured on titanium, as well as the effect of titanium on the three primary levels of regulation of MMP-7 production (gene expression, protein secretion, activation) have not been examined until now.
Fold-changes between titanium and control groups were calculated, in order to identify the extent to which titanium affects mRNA, protein and active protein expression. It was also of interest if protein induction corresponds to mRNA induction and on which level of regulation the induction is most pronounced (Table 20).
Both in M0 and M2 macrophages of 6 out of 12 donors, an induction of MMP-7 on mRNA followed by an induction on protein level was shown. One donor showed an induction on all three levels of regulation. Fold-changes of M1 macrophages were so low that they have probably no biological relevance.
MMP-7 is to a greater extent induced on mRNA level (7 out of 12 in M0 and 8 out of 12 in M2) which corresponds to the literature where it is described that the main part of MMP regulation is mostly attributed to the transcriptional level (1, 23, 24). However, it is not clear for which cell types these findings are relevant. Induction by titanium is shown on both levels of regulation. Whether MMP-7 is mainly induced on mRNA or protein level may also be donor specific. However, the main part of MMP-7 induction is located on mRNA and protein level and not on activity level.

As it was demonstrated in the experiments that MMP-7 is up-regulated differentially by titanium on all levels of regulation, it is of interest by which mechanisms and on which level of regulation titanium is able to induce MMP-7. Table 22 shows regulators of MMP-7 on different levels of regulation expressed by macrophages and other cell types.
General mechanisms of MMP regulation, for instance, early response signal pathways on the transcriptional level, mRNA stabilization on post-transcriptional level or proteolytic activation have been reported previously.
However, there are no studies so far reporting mechanisms where titanium interferes with the mRNA or protein syntheses of MMPs. Nevertheless, it is possible that titanium enhances the phosphorylation of transcription factors like AP-1, PEA3 NFK-B and STAT, which are key transcription factors and essential in the regulation of MMP genes (23, 24). For instance, for MMP-1 it has been reported that inflammatory cytokines increase the levels of different AP-1 proteins via the MAPK signaling pathway (23). Similar mechanisms for MMP-7 may be possible. As titanium induces MMP-7 also on the protein level (Table 20).
an influence on AU-rich binding proteins which play a central role in mRNA stabilization can be suggested. These proteins, of which approximately 20 have been identified to date, can either promote or impair rapid mRNA turnover or translation (92). MMP-9 contains several copies of AU-rich elements (23). Other MMPs containing these proteins have to be identified in further experiments. Also, Micro RNA and UTR binding proteins play important roles in the post-transcriptional regulation of MMP-7 (23, 26-28). A modification of these structures by titanium can be suggested as well.
Statistical significance of MMP-7 activation by titanium could not be proven. However, some donors were activated by titanium (Figure 14), leading to an examination of MMP-7 activity regulators. In the experiments TIMP-3 and CD151, regulators of MMP-7 activity were examined. They were selected for RT-PCR analysis, as preliminary Affymetrix gene chip analysis showed an up-regulation for

CD151 and a downregulation for TIMP-3 in titanium stimulated samples (A. Gudima, Affymetrix data (82)). It was hypothesized that a downregulation of TIMP-3 and an up-regulation of CD151 in titanium stimulated macrophages is a mechanism of activity regulation in macrophages stimulated with titanium.

Analysis of the MMP-7 inhibitor TIMP-3 did not show a statistical significance of down-regulation in macrophages (Figure 15). However, a tendency of down-regulation was observed in M1 and M2 macrophages, leading to an examination of those donors which were activated by titanium (7 out of 11 in M2 and 5 out 11 in M1, Figure 14). 4 out of 7 of these donors in M2 and 2 out of 5 of these donors in M1 showed a downregulation of TIMP-3, suggesting a mechanism of activity regulation in these macrophage subtypes by titanium (Figure 14, Figure 16).
Analysis of the MMP-7 activator CD151 did not show a significant up-regulation in macrophages, however a tendency of induction by titanium in M0 macrophages (Figure 17). Looking at individual donors in M0 macrophages, 6 out of 11 donors were activated by titanium and 3 out of 6 of these donors exhibited an upregulation of CD151 (Figure 14, Figure 18).
Though, the analysis of CD151 mRNA does not show how much of the molecule will be localized to the cell surface where it is biologically relevant. For this analysis, immunostaining is required. Further studies analyzing regulating factors of MMP-7 that depend on the presence of titanium should be performed. Collectively, TIMP-3 and CD151 might be regulators of MMP-7 activity specific for macrophage subtypes as well as for individual donors.
Further possible mechanisms of MMP-7 activation by titanium is the modification of the cysteine in the pro-domain, as it has already been described for heavy metal ions (7).
In macrophages of samples where active MMP-7 was detected but no induction by titanium has been shown on the activation level, an auto-activation of MMP-7 can be expected which is independent of the presence of titanium (39).
Additional factors which can affect MMP-7 expression up-regulation are shown in Table 22. All these factors may orchestrate MMP-7 expression and activity in vivo. The influence of titanium on them should also be examined in vivo experiments.

ELISA analysis showed that MMP-7 increased by the time of incubation. After 6 days, a maximum of MMP-7 protein both in control and titanium groups was detected. The increase of fold was stronger in the titanium stimulated samples (Figure 9), which is consistent with, identified in this study, the ability of titanium to induce MMP-7. The increase of MMP-7 during the time of macrophage cultivation on titanium disks corresponds to the results obtained by Filippov et al. demonstrating that the concentration of MMP-7 in cultured macrophages was augmented by time and reached a plateau after 5 days (77). Therefore, titanium supports the accumulation of MMP-7 protein during the macrophage maturation. However, the variations of induction by titanium on the different levels of regulation (Table 20) suggest that titanium can affect expression or functional activation of the regulating factors (Table 21). For instance, Lipopolysaccharides (LPS) found in the outer membrane of gram-negative bacteria can induce MMP-7 mRNA and Protein in macrophages. Whether there are similar mechanisms for gram-positive bacteria which are more prevalent in implant infections has to be examined in further studies.

Table 21. Factors regulating MMP-7.

Level & kind of regulation	Receptor/Stimuli	Mechanism	Localization/ Method	Cells/tissue	Ref.
Activity ↑	CD 151	Suggestion: Promotion of autoactivation by compartmentalization	Pericellular/ cell surface	Human rectal carcinoma, yeast	(85)
Activity ↑	trypsin	Proteolytic cleavage at E^{77}-Y^{78} within the propeptide	Purified ProMMP in SDS- Page	Purified MMP-7 from Human rectal carcinoma cells	(93, 94)
50% of Activity ↑	Plasmin & leukocyte elastase	Proteolytic cleavage of propeptide	Purified ProMMP in SDS-Page	Purified MMP-7 from Human rectal carcinoma cells	(94)
Activity ↑	MMP-3	Proteolytic cleavage of propeptide	Purified ProMMP in SDS-Page	Purified MMP-7 from Human rectal carcinoma cells	(94)
Activity ↑	4-aminophenylmercuric acetat (APMA)	Proteolytic cleavage at E^{77}-Y^{78} within the propeptide	Purified ProMMP in SDS-Page	Purified MMP-7 from Human rectal carcinoma cells as well as myeloma cells	(93, 94)
Activity ↑	High temperature (53°C incubation: max activity after 6h; 37°C incubation: max activity after 24h)	Proteolytic cleavage at E^{77}-Y^{78} within the propeptide	Purified ProMMP in SDS-Page	Purified MMP-7 from mouse myeloma cells	(93)
Activity ↑	Slow autoactivation at 4°C storage	unknown	Purified ProMMP in SDS-Page	Purified MMP-7 from mouse myeloma cells	(93)
Activity ↑	Sulfated GAG	Promote autolytic cleavage of propeptide	Recombinant human proMMP-7	Recombinant human proMMP-7	(95)

Effectivity ↑	Sulfated GAG	Provide anchor and compartmentalize MMP-7 to specific substrates	Recombinant human proMMP-7	Recombinant human proMMP-7	(95)
Protein ↑	H.pylori cag^+	ERK 1/2 mediates up-regulation	IHC, Western Blot	AGS human gastric epithelial cells	(45)
mRNA ↑	LPS	unknown	intracellular	Peripheral human blood monocytes	(75)
Protein ↑	LPS	unknown	Extracellular/immunoprecipitation	Peripheral human blood monocytes	(75)
Protein ↑	zymosan	unknown	Extracellular/immunoprecipitation	Peripheral human blood monocytes	(75)
mRNA ↑	hypoxia	unknown	Intracellular/ RT-PCR	RAW 264.7 macrophage cell line	(78)
Protein ↓	Dexamethason	unknown	Extracellular/immunoprecipitation	Peripheral human blood monocytes	(75)
Protein ↓	All trans retinoic acid	unknown	Extracellular/immunoprecipitation	Peripheral human blood monocytes	(75)
Protein ↓	IL-4	unknown	Extracellular/immunoprecipitation	Peripheral human blood monocytes	(77)
Protein ↓	IL-10	unknown	Extracellular/immunoprecipitation	Peripheral human blood monocytes	(75)
Protein ↓	IFNγ	unknown	Extracellular/immunoprecipitation	Peripheral human blood monocytes	(75)
mRNA ↑	Nitroglycerin	unknown	Intracellular/ RT-PCR	Monocyte derived macrophages	(79)

In this study, polished and porous titanium disks were analyzed as both surfaces are widely used in medical fields. Polished titanium disks are commonly used in dental applications (96), whereas porous titanium is applied for femoral and tracheal prosthesis (97, 98). Kujala et al. reported promising results for porous titanium with respect to osseointegration (99).
Both polished and porous titanium induced MMP-7 in monocyte-derived macrophages. However, a significant expression of MMP-7 in pro-inflammatory M1 macrophages was observed only on polished titanium, which may be important for pathological processes of implant loosening. Regarding MMP-7 and the process of implant failure, further studies should be performed in order to evaluate other factors participating in the process of implant failure.

4.2 The effect of elevated levels of MMP-7 on implant integration

Elevated levels of MMP-7 may have differential and partially contrary effects on extracellular matrix, cells, and cytokines. MMP-7 regulates cell migration or contributes to inflammatory processes by activating TNFα, for instance (Table 3, Table 7). One of MMP-7 main functions is the degradation of extracellular matrix, especially as it has, due to its lack of the hemopexin region, the broadest spectrum among all MMPs (Table 3) (21). On the other hand, the activation of TGFβ by MMP-7 may lead to fibrosis (35). The central question is whether the turnover of ECM supports osseointegration of titanium implants or if it contributes to the implant failure. Shubayev et al. proposed that elevated levels of MMP-7 are essential for the implantation of titanium as MMP-7 regulates the turnover of proteoglycans and contributes to the solubilization of osteoid. Proteoglycans are crucial for the adhesion of titanium implants to bone (91). In contrast, the majority of studies favors the hypothesis of MMP-7 contribution to the implant failure (3, 19, 37, 66). Implant failure is caused either by excessive ECM degradation and thereby implant loosening or by matrix accumulation resulting in fibrosis with a limited function of the implant. Both matrix degradation and accumulation has been attributed to MMP-7 (16, 63, 66).
Matrix degradation by MMP-7 may result in numerous pathologies like aortic aneurysm, plaque rupture or aseptic implant loosening (Table 5). Matrix accumulation gives rise to fibrosis in different organs. A potential contribution of MMP-7 was found in kidney (66), lung (63, 100) or liver (101), for instance. Isao Takei et al. were able to demonstrate the expression of MMP-7 mRNA in synovium-like interface tissues between bone and prosthesis which was collected from patients with aseptic loose artificial hip joints (19). Fibrosis around implants can be detrimental in case it leads to capsule contracture, restricting the function of the implant (80). In total knee arthroplasty, the pathology of arthrofibrosis has been attributed to the synovial membrane and the infrapatellar fat pad. Fibrosis in these structures may prevent a normal range of motion and can lead to a dramatic reduction of the quality of life for patients (64). However, there is no study which connects elevated MMPs and fibrosis around implant material. The experiments in this study have shown an induction of MMP-7 by titanium and posed the question whether MMP-7 will contribute either to matrix accumulation or degradation.
In order to answer this question, the paradigm of M1 and M2 macrophages was used to distinguish acute inflammatory macrophages as M1 and anti-inflammatory/healing macrophages as M2 (61). The paradigm is an effective operationalization to make macrophage biology more comprehensible (62).
Porous and polished titanium induced MMP-7 in M0 and M2 whereas in M1 only an induction by polished titanium has been observed, suggesting the induction depends on the type of titanium used as well as on the macrophage activation state. Macrophage subtypes that have not been exposed to titanium showed no difference in MMP-7 expression, suggesting that only titanium induced MMP-7 and not the macrophage subtype itself. IL-4 suppresses MMP-7 (96), but titanium supports its high expression.
Differential induction of MMP-7 in M0, M1, and M2 by titanium needs to be considered in the context of different roles, macrophage subtypes playing in homeostasis and pathologies. In addition, it is of interest if these different expression patterns can be observed in connection with pro-fibrotic and matrix degrading functions of MMP-7.
The M1 macrophage subtype is characterized by the production of high levels of pro-inflammatory cytokines, reactive nitrogen, oxygen intermediates (62), and the ability

to kill intracellular pathogens (61). In case of prolonged or extensive activity, they can be detrimental to health, sustaining inflammation by the secretion of IL-1-beta, TNFα, IL-12, IL-18 and IL-23 (62). Additionally, they are capable of differentiating into osteoclasts and thereby contributing to osteolysis (102). By contrast, M2 macrophages are characterized by their involvement in tissue remodeling, immune regulation, tumor promotion, phagocytic activity and parasite control (62). M2 macrophages have been implicated in fibrosis (103), as they can secrete fibrotic factors like TGFβ and Galactin-3 (37). TGFβ promotes collagen synthesis in myofibroblasts and can lead to encapsulation of foreign bodies (84, 103), while the cellular actions of Galactin-3 lead to cell proliferation, cell adhesion and eventually to fibrosis (104). TGFβ can also be activated by MMP-7 (35).
The differential spectrum of function of macrophages, in which M1 macrophages contribute to matrix degradation and osteolytic processes, whereas M2 macrophages are responsible for fibrosis, suggests different functions of MMP-7 as a function of the macrophage subtype expressing it.
It can be hypothesized that MMP-7, synthesized by M1 macrophages, leads to matrix degradation, potentially resulting in aseptic implant loosening and MMP-7 expressed by M2 macrophages gives rise to matrix accumulation resulting in fibrosis. M0 macrophages which are not yet differentiated to M1 or M2 may have the potential to cause both implant loosening and fibrosis. A significant up-regulation of MMP-7 by M1 macrophages can be observed in samples stimulated with polished titanium disks, whereas an up-regulation in M2 macrophages was shown in both polished and porous titanium groups. The matrix-degrading or pro-fibrotic function of the enzyme can be modulated by other factors which are synthesized by the specific macrophage subtype in concert with MMP-7.
However, considering the absolute levels of the active enzyme (mean concentrations under titanium stimulation: M2: 687,3 pg/ml vs. M1: 37,7 pg/ml), MMP-7 secreted by M2 macrophages seems to be biological more relevant. Comparison of absolute MMP-7 levels with other studies is difficult, as they usually measured serum levels or analyzed different cell types. In a study of Abbas et al. mean plasma levels of MMP-7 were 1960 pg/ml in patients with atherosclerosis, reflecting conditions of chronic inflammation. Immunostaining in this study showed that MMP-7 was located next to macrophage infiltration (105).
In the experiments in our laboratory, the concentration of active MMP-7 secreted by M2 macrophages stimulated with titanium reached levels above 2000 pg/ml in some donors (max. 2916,2 pg/ml) underlining the biological relevance.
It should be noted that the expression and function of MMP-7 also depends on the tissue microenvironment in which multiple factors influence the enzyme. These conditions are difficult to simulate in the in vitro experiments. However, the study provides relevant data for further analysis in this field. The differences on the reactions of macrophages derived from the individual donors suggest the possibility to use macrophage-based test system to predict the patient's individual risk of foreign body reaction towards titanium. Patients with a strong induction of MMP-7 can be expected to have a higher risk for implant failure. Additionally, the paradigm of M1 and M2 macrophages allows to discriminate the risk for either aseptic loosening or fibrosis. However, more extensive studies in clinical settings from patients both with successful and failed implants should be performed in order to identify the correlation between MMP-7 production by macrophages and clinical complications in patients with titanium implants.

ABSTRACT

Implant material is widely used in medical fields such as orthopedics, odontology or cardiology. Due to its excellent properties regarding corrosion resistance and biocompatibility, titanium has been used successfully for decades. However, in the orthopedic field, studies report failure rates between 3,4-9%. One of the main reasons for implant failure is aseptic implant loosening which has been reported to be responsible for 80% of revision surgeries. Matrix metalloproteinase and especially MMP-7 play a significant role in the pathology of implant failure. MMP-7 has, due to the lack of its hemopexin domain, one of the broadest substrate spectrums among all MMPs. It can degrade the extracellular matrix as well as activate a large number of cytokines. These cytokines, in turn, can cause numerous pathologies (e.g., fibrosis or chronic inflammation). Macrophages are the principal cell type involved in the orchestration of the foreign body response. In order to analyze the foreign body response to titanium, which is one of the most used materials for implants in humans, the aim of this work was to examine the effect of human primary macrophage exposure to titanium on the MMP-7 production. Using Affymetrix microarray assays it was previously identified in our laboratory, that titanium induces MMP-7 gene expression in macrophages (82). The specific aims of the current project included 1) quantification of titanium induced changes in gene expression and secretion of MMP-7 in human primary M0, M1 and M2 macrophages; 2) comparative analysis of titanium induced MMP-7 production on 3 different levels (mRNA, protein secretion, activity); and 3) analysis of TIMP-3 and CD151, regulators of MMP-7 activity, in response to titanium stimulation. Analysis of differential effects of porous and polished titanium on MMP-7 production by M0, M1, and M2 was performed in the model system established in our laboratory where human primary M0, M1, and M2 macrophages are differentiated out of peripheral blood monocytes cultured on titanium disks was used. RT-PCR analysis revealed that both polished and porous titanium up-regulate MMP-7 mRNA in M0 and M2 macrophages. Additionally, polished titanium was able to induce MMP-7 in M1 macrophages. Protein analysis by ELISA confirmed that secretion of MMP-7 correlates with the up-regulated levels of mRNA in M0 and M2. Analysis of active MMP-7 demonstrated that titanium induced release in M0 and M2 in some donors, however without statistical significance. The inducing effect of titanium on MMP7 production was differential on each level of regulation with a principal induction on mRNA and protein level. It was shown that MMP-7 increased by the time of incubation both under titanium stimulation as well as without titanium. The maximum MMP-7 levels were detected on day 6 of incubation. Exposure of macrophages to titanium resulted in changes of expression of the MMP-7 regulators TIMP3 and CD151 in some donors, however without statistical significance, suggesting that there are also other mechanisms responsible for the post-translational regulation of MMP-7. Collectively, the results demonstrate that MMP-7 is statistically significant induced in human primary macrophages by titanium on mRNA and protein levels, and in part of donors on the levels of released active MMP-7. MMP-7 production was induced in macrophages by both porous and polished titanium, while the strongest effect was found in M2 macrophages. As the up-regulation of MMP-7 may be essential for the success of the integration of a titanium implant, a macrophage-based model system is suggested as a predictive tool for implant failure enabling a personalized therapeutic approach.

REFERENCES

1. Gaffney J, Solomonov I, Zehorai E, Sagi I. Multilevel regulation of matrix metalloproteinases in tissue homeostasis indicates their molecular specificity in vivo. Matrix biology : journal of the International Society for Matrix Biology. 2015.

2. Gordon S, Pluddemann A, Martinez Estrada F. Macrophage heterogeneity in tissues: phenotypic diversity and functions. Immunol Rev. 2014;262(1):36-55.

3. Syggelos SA, Aletras AJ, Smirlaki I, Skandalis SS. Extracellular matrix degradation and tissue remodeling in periprosthetic loosening and osteolysis: focus on matrix metalloproteinases, their endogenous tissue inhibitors, and the proteasome. BioMed research international. 2013;2013:230805.

4. Klein T, Bischoff R. Physiology and pathophysiology of matrix metalloproteases. Amino acids. 2011;41(2):271-90.

5. Craig VJ, Polverino F, Laucho-Contreras ME, Shi Y, Liu Y, Osorio JC, et al. Mononuclear phagocytes and airway epithelial cells: novel sources of matrix metalloproteinase-8 (MMP-8) in patients with idiopathic pulmonary fibrosis. PloS one. 2014;9(5):e97485.

6. Iyer RP, Patterson NL, Fields GB, Lindsey ML. The history of matrix metalloproteinases: milestones, myths, and misperceptions. American journal of physiology Heart and circulatory physiology. 2012;303(8):H919-30.

7. Cauwe B, Opdenakker G. Intracellular substrate cleavage: a novel dimension in the biochemistry, biology and pathology of matrix metalloproteinases. Critical reviews in biochemistry and molecular biology. 2010;45(5):351-423.

8. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochimica et biophysica acta. 2010;1803(1):55-71.

9. Mannello F, Medda V. Nuclear localization of matrix metalloproteinases. Progress in histochemistry and cytochemistry. 2012;47(1):27-58.

10. Verma S, Kesh K, Ganguly N, Jana S, Swarnakar S. Matrix metalloproteinases and gastrointestinal cancers: Impacts of dietary antioxidants. World journal of biological chemistry. 2014;5(3):355-76.

11. Nissinen L, Kahari VM. Matrix metalloproteinases in inflammation. Biochimica et biophysica acta. 2014;1840(8):2571-80.

12. Gomis-Ruth FX. Catalytic domain architecture of metzincin metalloproteases. J Biol Chem. 2009;284(23):15353-7.

13. Tallant C, Marrero A, Gomis-Ruth FX. Matrix metalloproteinases: fold and function of their catalytic domains. Biochimica et biophysica acta. 2010;1803(1):20-8.

14. Overall CM, Lopez-Otin C. Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nature reviews Cancer. 2002;2(9):657-72.

15. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Bi. 2001;17:463-516.

16. Sbardella D, Fasciglione GF, Gioia M, Ciaccio C, Tundo GR, Marini S, et al. Human matrix metalloproteinases: an ubiquitarian class of enzymes involved in several pathological processes. Molecular aspects of medicine. 2012;33(2):119-208.

17. Peng WJ, Yan JW, Wan YN, Wang BX, Tao JH, Yang GJ, et al. Matrix metalloproteinases: a review of their structure and role in systemic sclerosis. Journal of clinical immunology. 2012;32(6):1409-14.

18. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circulation research. 2003;92(8):827-39.

19. Hegde RS, Bernstein HD. The surprising complexity of signal sequences. Trends in biochemical sciences. 2006;31(10):563-71.

20. Ali TK, Al-Gayyar MM, Matragoon S, Pillai BA, Abdelsaid MA, Nussbaum JJ, et al. Diabetes-induced peroxynitrite impairs the balance of pro-nerve growth factor and nerve growth factor, and causes neurovascular injury. Diabetologia. 2011;54(3):657-68.

21. Piccard H, Van den Steen PE, Opdenakker G. Hemopexin domains as multifunctional liganding modules in matrix metalloproteinases and other proteins. Journal of leukocyte biology. 2007;81(4):870-92.

22. Burke B. The role of matrix metalloproteinase 7 in innate immunity. Immunobiology. 2004;209(1-2):51-6.

23. Fanjul-Fernandez M, Folgueras AR, Cabrera S, Lopez-Otin C. Matrix metalloproteinases: evolution, gene regulation and functional analysis in mouse models. Biochimica et biophysica acta. 2010;1803(1):3-19.

24. Yan C, Boyd DD. Regulation of matrix metalloproteinase gene expression. Journal of cellular physiology. 2007;211(1):19-26.

25. Rassow J HK, Netzker R, Deutzmann R. Biochemie. 2. Auflage ed2008.

26. Chou J, Shahi P, Werb Z. microRNA-mediated regulation of the tumor microenvironment. Cell cycle. 2013;12(20):3262-71.

27. Chou J, Lin JH, Brenot A, Kim JW, Provot S, Werb Z. GATA3 suppresses metastasis and modulates the tumour microenvironment by regulating microRNA-29b expression. Nature cell biology. 2013;15(2):201-13.

28. Thierbach R, Maier K, Sorsa T, Mantyla P. Peri-Implant Sulcus Fluid (PISF) Matrix Metalloproteinase (MMP) -8 Levels in Peri-Implantitis. Journal of clinical and diagnostic research : JCDR. 2016;10(5):ZC34-8.

29. Parks WC, Shapiro SD. Matrix metalloproteinases in lung biology. Respir Res. 2001;2(1):10-9.

30. Arpino V, Brock M, Gill SE. The role of TIMPs in regulation of extracellular matrix proteolysis. Matrix biology : journal of the International Society for Matrix Biology. 2015.

31. Yamamoto K, Murphy G, Troeberg L. Extracellular regulation of metalloproteinases. Matrix biology : journal of the International Society for Matrix Biology. 2015.

32. McLennan SV, Kelly DJ, Schache M, Waltham M, Dy V, Langham RG, et al. Advanced glycation end products decrease mesangial cell MMP-7: a role in matrix accumulation in diabetic nephropathy? Kidney international. 2007;72(4):481-8.

33. D'Angelo M, Billings PC, Pacifici M, Leboy PS, Kirsch T. Authentic matrix vesicles contain active metalloproteases (MMP). a role for matrix vesicle-associated MMP-13 in activation of transforming growth factor-beta. J Biol Chem. 2001;276(14):11347-53.

34. Wei-Hsuan Yu JFW, Jr., John D. McNeish, and Ivan Stamenkovic. CD44 anchors the assembly of matrilysin/MMP-7 with heparin-binding epidermal growth factor precursor and ErbB4 and regulates female reproductive organ remodeling. 2002.

35. Opanashuk LA, Mark RJ, Porter J, Damm D, Mattson MP, Seroogy KB. Heparin-binding epidermal growth factor-like growth factor in hippocampus: modulation of expression by seizures and anti-excitotoxic action. J Neurosci. 1999;19(1):133-46.

36. Amar S, Smith L, Fields GB. Matrix metalloproteinase collagenolysis in health and disease. Biochimica et biophysica acta. 2017.

37. Vernon MA, Mylonas KJ, Hughes J. Macrophages and renal fibrosis. Seminars in nephrology. 2010;30(3):302-17.

38. Overall CM, Kleifeld O. Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. Nature reviews Cancer. 2006;6(3):227-39.

39. Crabbe T, Willenbrock F, Eaton D, Hynds P, Carne AF, Murphy G, et al. Biochemical characterization of matrilysin. Activation conforms to the stepwise mechanisms proposed for other matrix metalloproteinases. Biochemistry. 1992;31(36):8500-7.

40. Rohrborn D, Eckel J, Sell H. Shedding of dipeptidyl peptidase 4 is mediated by metalloproteases and up-regulated by hypoxia in human adipocytes and smooth muscle cells. Febs Lett. 2014;588(21):3870-7.

41. Mao S, Wang Y, Zhang M, Hinek A. Phytoestrogen, tanshinone IIA diminishes collagen deposition and stimulates new elastogenesis in cultures of human cardiac fibroblasts. Experimental cell research. 2014;323(1):189-97.

42. Wallace AM, Loy LB, Abboud RT, D'Armiento JM, Coxson HO, Muller NL, et al. Expression of matrix metalloproteinase-1 in alveolar macrophages, type II pneumocytes, and airways in smokers: relationship to lung function and emphysema. Lung. 2014;192(4):467-72.

43. O'Hara A, Lim FL, Mazzatti DJ, Trayhurn P. Microarray analysis identifies matrix metalloproteinases (MMPs) as key genes whose expression is up-regulated in human adipocytes by macrophage-conditioned medium. Pflugers Archiv : European journal of physiology. 2009;458(6):1103-14.

44. Ishida Y, Kuninaka Y, Nosaka M, Kimura A, Kawaguchi T, Hama M, et al. Immunohistochemical analysis on MMP-2 and MMP-9 for wound age determination. International journal of legal medicine. 2015.

45. Crawford HC, Krishna US, Israel DA, Matrisian LM, Washington MK, Peek RM. Helicobacter pylori strain-selective induction of matrix metalloproteinase-7 in vitro and within gastric mucosa. Gastroenterology. 2003;125(4):1125-36.

46. Zhang J, Luo J, Ni J, Tang L, Zhang HP, Zhang L, et al. MMP-7 is upregulated by COX-2 and promotes proliferation and invasion of lung adenocarcinoma cells. European journal of histochemistry : EJH. 2014;58(1):2262.

47. Fukuda A, Wang SC, Morris JPt, Folias AE, Liou A, Kim GE, et al. Stat3 and MMP7 contribute to pancreatic ductal adenocarcinoma initiation and progression. Cancer cell. 2011;19(4):441-55.

48. Watanabe R, Maeda T, Zhang H, Berry GJ, Zeisbrich M, Brockett R, et al. MMP (Matrix Metalloprotease)-9-Producing Monocytes Enable T Cells to Invade the Vessel Wall and Cause Vasculitis. Circulation research. 2018;123(6):700-15.

49. Nishioka J, Iwahara C, Kawasaki M, Yoshizaki F, Nakayama H, Takamori K, et al. Di-(2-ethylhexyl) phthalate induces production of inflammatory molecules in human macrophages. Inflammation research : official journal of the European Histamine Research Society [et al]. 2012;61(1):69-78.

50. Pettersen JS, Fuentes-Duculan J, Suarez-Farinas M, Pierson KC, Pitts-Kiefer A, Fan L, et al. Tumor-associated macrophages in the cutaneous SCC microenvironment are heterogeneously activated. The Journal of investigative dermatology. 2011;131(6):1322-30.

51. Lavin B, Gomez M, Pello OM, Castejon B, Piedras MJ, Saura M, et al. Nitric oxide prevents aortic neointimal hyperplasia by controlling macrophage polarization. Arteriosclerosis, thrombosis, and vascular biology. 2014;34(8):1739-46.

52. English WR, Puente XS, Freije JM, Knauper V, Amour A, Merryweather A, et al. Membrane type 4 matrix metalloproteinase (MMP17) has tumor necrosis factor-alpha convertase activity but does not activate pro-MMP2. J Biol Chem. 2000;275(19):14046-55.

53. Skoog T, Ahokas K, Orsmark C, Jeskanen L, Isaka K, Saarialho-Kere U. MMP-21 is expressed by macrophages and fibroblasts in vivo and in culture. Experimental dermatology. 2006;15(10):775-83.

54. Cominelli A, Gaide Chevronnay HP, Lemoine P, Courtoy PJ, Marbaix E, Henriet P. Matrix metalloproteinase-27 is expressed in CD163+/CD206+ M2 macrophages in the cycling human endometrium and in superficial endometriotic lesions. Molecular human reproduction. 2014;20(8):767-75.

55. Schmitt R, Tscheuschler A, Laschinski P, Uffelmann X, Discher P, Fuchs J, et al. A potential key mechanism in ascending aortic aneurysm development: Detection of a linear relationship between MMP-14/TIMP-2 ratio and active MMP-2. PloS one. 2019;14(2):e0212859.

56. Hu W, Wei R, Wang L, Lu J, Liu H, Zhang W. Correlations of MMP-1, MMP-3, and MMP-12 with the degree of atherosclerosis, plaque stability and cardiovascular and cerebrovascular events. Experimental and Therapeutic Medicine. 2017.

57. L. Zhou D-QK. Correlation between acute myocardial infarction complicated with cerebral infarction and expression levels of MMP-2 and MMP-9. European Review for Medical and Pharmacological Sciences. 2019;23:297-302.

58. Wells JM, Parker MM, Oster RA, Bowler RP, Dransfield MT, Bhatt SP, et al. Elevated circulating MMP-9 is linked to increased COPD exacerbation risk in SPIROMICS and COPDGene. JCI Insight. 2018;3(22).

59. Lee JM, Kronbichler A, Park SJ, Kim SH, Han KH, Kang HG, et al. Association between Serum Matrix Metalloproteinase- (MMP-) 3 Levels and Systemic Lupus Erythematosus: A Meta-analysis. Disease markers. 2019;2019:9796735.

60. Sundfeldt M, Carlsson LV, Johansson CB, Thomsen P, Gretzer C. Aseptic loosening, not only a question of wear: a review of different theories. Acta orthopaedica. 2006;77(2):177-97.

61. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000prime reports. 2014;6:13.

62. Laboratoiries B-R. macrophage-polarization-mini-review 2019 [01.11.2019]. Available from: https://www.bio-rad-antibodies.com/macrophage-polarization-minireview.html#top.

63. Hamai K, Iwamoto H, Ishikawa N, Horimasu Y, Masuda T, Miyamoto S, et al. Comparative Study of Circulating MMP-7, CCL18, KL-6, SP-A, and SP-D as Disease Markers of Idiopathic Pulmonary Fibrosis. Disease markers. 2016;2016:4759040.

64. Abdul N, Dixon D, Walker A, Horabin J, Smith N, Weir DJ, et al. Fibrosis is a common outcome following total knee arthroplasty. Scientific reports. 2015;5:16469.

65. Jonitz-Heincke A, Lochner K, Schulze C, Pohle D, Pustlauk W, Hansmann D, et al. Contribution of human osteoblasts and macrophages to bone matrix degradation and proinflammatory cytokine release after exposure to abrasive endoprosthetic wear particles. Molecular medicine reports. 2016;14(2):1491-500.

66. Zhou D, Tian Y, Sun L, Zhou L, Xiao L, Tan RJ, et al. Matrix Metalloproteinase-7 Is a Urinary Biomarker and Pathogenic Mediator of Kidney Fibrosis. Journal of the American Society of Nephrology : JASN. 2016.

67. Gomes AM, Douglas-de-Oliveira DW, Ferreira SD, Silva TAD, Cota LOM, Costa FO. Periodontal disease, peri-implant disease and levels of salivary biomarkers IL-1beta, IL-10, RANK, OPG, MMP-2, TGF-beta and TNF-alpha: follow-up over 5 years. J Appl Oral Sci. 2019;27:e20180316.

68. Zhang Y, Lin Y, Xiao L, Feng E, Wang W, Lin L. The effects of icariine concentration on osteoclasts bone resorption induced by titanium particles in vitro. Regenerative biomaterials. 2015;2(3):197-202.

69. Parks WC SS. Matrix metalloproteinases in lung biology. Respir Res. 2001;2:10-9.

70. Powell WC, Knox JD, Navre M, Grogan TM, Kittelson J, Nagle RB, et al. Expression of the metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. Cancer Res. 1993;53(2):417-22.

71. Zhang Z, Winyard PG, Chidwick K, Murphy G, Wardell M, Carrell RW, et al. Proteolysis of human native and oxidised alpha 1-proteinase inhibitor by matrilysin and stromelysin. Biochimica et biophysica acta. 1994;1199(2):224-8.

72. Meyer-Hoffert U, Wiedow O. Neutrophil serine proteases: mediators of innate immune responses. Curr Opin Hematol. 2011;18(1):19-24.

73. Higashiyama S, Abraham JA, Klagsbrun M. Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. J Cell Biol. 1993;122(4):933-40.

74. Marikovsky M, Breuing K, Liu PY, Eriksson E, Higashiyama S, Farber P, et al. Appearance of heparin-binding EGF-like growth factor in wound fluid as a response to injury. Proc Natl Acad Sci U S A. 1993;90(9):3889-93.

75. Haro H, Crawford HC, Fingleton B, Shinomiya K, Spengler DM, Matrisian LM. Matrix metalloproteinase-7-dependent release of tumor necrosis factor-alpha in a model of herniated disc resorption. The Journal of clinical investigation. 2000;105(2):143-50.

76. Li Q, Park PW, Wilson CL, Parks WC. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. Cell. 2002;111(5):635-46.

77. Filippov S, Caras I, Murray R, Matrisian LM, Chapman HA, Jr., Shapiro S, et al. Matrilysin-dependent elastolysis by human macrophages. The Journal of experimental medicine. 2003;198(6):925-35.

78. Burke B, Giannoudis A, Corke KP, Gill D, Wells M, Ziegler-Heitbrock L, et al. Hypoxia-Induced Gene Expression in Human Macrophages. The American Journal of Pathology. 2003;163(4):1233-43.

79. Trindade R, Albrektsson T, Tengvall P, Wennerberg A. Foreign Body Reaction to Biomaterials: On Mechanisms for Buildup and Breakdown of Osseointegration. Clinical implant dentistry and related research. 2014.

80. Tang L, Eaton JW. Inflammatory responses to biomaterials. Am J Clin Pathol. 1995;103(4):466-71.

81. Kzhyshkowska J, Gudima A, Moganti K, Gratchev A, Orekhov A. Perspectives for Monocyte/Macrophage-Based Diagnostics of Chronic Inflammation. Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie. 2016;43(2):66-77.

82. Gudima A. Analysis of reactions of macrophages to titanium and biodegradable coating materials [Dissertation zur Erlangung des Doctor scientiarum humanarum]. Mannheim: Ruprecht-Karls-Universität Heidelberg; 2017.

83. Shubayev VI, Branemark R, Steinauer J, Myers RR. Titanium implants induce expression of matrix metalloproteinases in bone during osseointegration. The Journal of Rehabilitation Research and Development. 2004;41(6).

84. Klopfleisch R. Macrophage reaction against biomaterials in the mouse model - Phenotypes, functions and markers. Acta biomaterialia. 2016;43:3-13.

85. Shiomi T, Inoki I, Kataoka F, Ohtsuka T, Hashimoto G, Nemori R, et al. Pericellular activation of proMMP-7 (promatrilysin-1) through interaction with CD151. Laboratory investigation; a journal of technical methods and pathology. 2005;85(12):1489-506.

86. Goutam M, Giriyapura C, Mishra SK, Gupta S. Titanium allergy: a literature review. Indian J Dermatol. 2014;59(6):630.

87. Landgraeber S, Jager M, Jacobs JJ, Hallab NJ. The pathology of orthopedic implant failure is mediated by innate immune system cytokines. Mediators of inflammation. 2014;2014:185150.

88. Makela KT, Matilainen M, Pulkkinen P, Fenstad AM, Havelin L, Engesaeter L, et al. Failure rate of cemented and uncemented total hip replacements: register study of combined Nordic database of four nations. BMJ. 2014;348:f7592.

89. Beckmann NA, Mueller S, Gondan M, Jaeger S, Reiner T, Bitsch RG. Treatment of severe bone defects during revision total knee arthroplasty with structural allografts and porous metal cones-a systematic review. J Arthroplasty. 2015;30(2):249-53.

90. Otto M, Kriegsmann J, Gehrke T, Bertz S. [Wear particles: key to aseptic prosthetic loosening?]. Pathologe. 2006;27(6):447-60.

91. Shubayev VI, Branemark R, Steinauer J, Myers RR. Titanium implants induce expression of matrix metalloproteinases in bone during osseointegration. The Journal of Rehabilitation Research and Development. 2004;41(6):757.

92. Moore AE, Chenette DM, Larkin LC, Schneider RJ. Physiological networks and disease functions of RNA-binding protein AUF1. Wiley Interdiscip Rev RNA. 2014;5(4):549-64.

93. Herberts P, Malchau H. Long-term registration has improved the quality of hip replacement: a review of the Swedish THR Register comparing 160,000 cases. Acta Orthop Scand. 2000;71(2):111-21.

94. Robertsson O, Knutson K, Lewold S, Lidgren L. The Swedish Knee Arthroplasty Register 1975-1997: an update with special emphasis on 41,223 knees operated on in 1988-1997. Acta Orthop Scand. 2001;72(5):503-13.

95. Ra HJ, Harju-Baker S, Zhang F, Linhardt RJ, Wilson CL, Parks WC. Control of promatrilysin (MMP7) activation and substrate-specific activity by sulfated glycosaminoglycans. J Biol Chem. 2009;284(41):27924-32.

96. Cochran DL. A comparison of endosseous dental implant surfaces. J Periodontol. 1999;70(12):1523-39.

97. Mantelli P, Fioruzzi A, Bisogno L, Fioruzzi C, Fusco U, Olivieri M, et al. Short femoral stem and porous titanium: winning combination? Acta Biomed. 2014;85 Suppl 2:71-4.

98. Debry C, Dupret-Bories A, Vrana NE, Hemar P, Lavalle P, Schultz P. Laryngeal replacement with an artificial larynx after total laryngectomy: the possibility of restoring larynx functionality in the future. Head Neck. 2014;36(11):1669-73.

99. Kujala S, Ryhänen J, Danilov A, Tuukkanen J. Effect of porosity on the osteointegration and bone ingrowth of a weight-bearing nickel–titanium bone graft substitute. Biomaterials. 2003;24(25):4691-7.

100. Nakatsuka Y, Handa T, Nakashima R, Tanizawa K, Kubo T, Murase Y, et al. Serum matrix metalloproteinase levels in polymyositis/dermatomyositis patients with interstitial lung disease. Rheumatology (Oxford). 2019.

101. Jiang J, Wang J, Shen Z, Lu X, Chen G, Huang Y, et al. Serum MMP-7 in the Diagnosis of Biliary Atresia. Pediatrics. 2019.

102. Goodman SB, Gibon E, Pajarinen J, Lin TH, Keeney M, Ren PG, et al. Novel biological strategies for treatment of wear particle-induced periprosthetic osteolysis of orthopaedic implants for joint replacement. Journal of the Royal Society, Interface / the Royal Society. 2014;11(93):20130962.

103. Wynn TA, Barron L. Macrophages: master regulators of inflammation and fibrosis. Seminars in liver disease. 2010;30(3):245-57.

104. Harvey A, Montezano AC, Lopes RA, Rios F, Touyz RM. Vascular Fibrosis in Aging and Hypertension: Molecular Mechanisms and Clinical Implications. The Canadian journal of cardiology. 2016;32(5):659-68.

105. Zirlik A, Abbas A, Aukrust P, Russell D, Krohg-Sørensen K, Almås T, et al. Matrix Metalloproteinase 7 Is Associated with Symptomatic Lesions and Adverse Events in Patients with Carotid Atherosclerosis. PloS one. 2014;9(1).

CURRICULUM VITAE

PERSONAL DETAILS

Name and Surname:	Hesselbarth, David
Date of birth:	04.06.1988
Place of birth:	Altötting, Germany
Family status:	single
Father:	Hesselbarth, Frank
Mother:	Hesselbarth, Barbara

EDUCATIONAL BACKGROUND

1998-2007	Maria Ward Gymnasium Altötting
29.6.2007	Abitur

UNIVERSITY BACKROUND

2011	Begin of the studies “Human Medicine” at the University of Heidelberg, Medical Faculty Mannheim
17.09.2013	First part of medical exams (M1)
2013 – Jun 2018	Main studies „Human Medicine“
2014 – present	Member of the “Junior Scientific Master Class”, Medical Faculty Mannheim, Prof. Hammes
2015 - 2016	Studies of „Human Medicine“ at the University of Montpellier
05.05.2017	Second part of medical exams (M2)
21.06.2018	Third part of medical exams (M3)
2018-present	Resident physician “Department of Cardiology and Angiologie I” Heart Center University of Freiburg

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor Prof. Dr. Julia Kzhyshkowska for guiding me through every step of this MD thesis. Without her continuous support, enthusiasm, encouragement and guidance, this work would not have been possible.
I would like to acknowledge Professor Dr. med. Harald Klüter, director of the Institute for Transfusion medicine and Immunology, Medical Faculty Mannheim of the University of Heidelberg, for permitting me to work in his institute and providing me with all the necessary facilities to carry out this work.

I am heartily thankful to Dr. Alexandru Gudima for his advice, guidance and help throughout my years in this group, for his excellent technical assistance and helping me in getting acquainted with the lab.

I am sincerely thankful to Ms. Christina Schmuttermaier for her initial training, advice and assistance with experiments.

I am also grateful to Prof. Dr. Karen Bieback for giving me precious advice and assisting me in RT-qPCR data analysis.

I am also thankful to Mrs. Tanya Armbruster for her advices in scientific writing.

I would like to sincerely thank my friends and colleagues Shuiping Yin, Vladimir Riabov, Julia Michel, Tengfei Liu, Dandan Xu, Kondaiah Moganti, Michael Balduff, Laura Matuschik and Amanda Mickley for their advices and for providing many enjoyable moments in and outside the lab.

Finally, my warmest thanks go to my family for their constant support and encouragement.