D I P L O M A T H E S I S

INFLUENCE OF MECHANICAL LOADING ON THE EXPRESSION OF MATRIX METALLOPROTEINASES IN CHONDROCYTES OF THE HUMAN GROWTH PLATE IN VITRO

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Declaration of Autorship

I certify that the work presented here is, to the best of my knowledge and belief, original and the result of my own investigations, except as acknowledged, and has not been submitted, either in part or whole, for a degree at this or any other university.

Graz, Signature

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„There are no such things as limits to growth
because there are no limits to the human capacity for
intelligence, imagination and wonder.“
(Ronald Reagan)

Dedicated to my grandfather.

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ABBREVATIONS

ALP	alkaline phosphatase
ATP	adenosine-5-triphosphate
BMP	bone morphogenetic protein
bp	base pair
BRIC	Bioresorbable Implants for Children
CDMP	cartilage-derived morphogenetic protein
cDNA	complementary deoxyribonucleic acid
COMP	cartilage-oligomeric-matrix-protein
CT	comparative threshold cycle
CTGF	connective tissue growth factor
Ctrl	control
CXCL	chemokine (C-X-C motif) ligand
DMEM/F-12	Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham
DNA	deoxyribonucleic acid
EB	ethidium bromide
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FGF	fibroblast growth factor
FCS	fetal calf serum
FGFR	fibroblast growth factor receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoid
GDF	growth differentiation factor
GH	growth hormone
Glu	glutamic acid
Gly	glycine
GP	growth plate
GR	GelRed Nucleic Acid Stain
h	hour

HB-EGF	heparin-binding epidermal growth factor-like growth factor
HGF	hepatocyte growth factor
His	histidine
HZ	hypertrophic zone
Hz	hertz
IFG	insulin-like growth factor
Ig	immunoglobuline
Ihh	Indian hedgehog
IL	interleukin
l	liter
MC	mesenchymal cells
mg	milligram
ML	mechanical loading
µl	microliter
min	minute
ml	milliliter
ML	Mechanical Loading
MMP	matrix metalloproteinase
MPa	Megapascal
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
MT-MMP	membrane-type matrix metalloproteinase
MV	mean value
MZ	mineralization zone
NTC	No Template negative Control
PBS	phosphate-buffered saline
PDGF	platelet derived growth factor
Ph1	Phase 1
PHL	Power High Load
PTHrP	parathyroid hormone-related peptide
PZ	proliferative zone
qRT-PCR	quantitative real-time polymerase chain reaction

RNA	ribonucleic acid
rpm	rounds per minute
RQ	relative quantity
rRNA	ribosomal ribonucleic acid
RT-	Reverse Transcriptase minus
RZ	reserve zone
S	Svedberg Units
SD	standard deviation
SDF	stroma cell-derived factor
SE	standard error
sec	second
Ser	serine
T_3	triiodothyronin
T_4	thyroxid
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TGF	transforming growth factor
TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumor necrosis factor
V	volt
VEGF	vascular endothelial growth factor

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ABSTRACT

Aim

Children have the unique capability of bone remodeling after fractures. The underlying molecular mechanisms have not been investigated yet. We hypothesize that the growth plate and Matrix Metalloproteinases (MMP) within it play an exceedingly important role during remodeling and that they are influenced by mechanical stimuli. Knowledge of the molecular mechanisms of bone remodeling in children could help to improve current treatment approaches and prevent malalignments after fractures.

Materials and Methods

Chondrocytes, isolated from the growth plate of the supernumerary digit of children with polydaktylism, were cultured and subsequently subjected to mechanical loading at 1 Hertz and 10% elongation for 1 h, 4 h and 24 h with a Flexcell Tension Plus System (Flexcell International, Hillsborough, USA). The expression of MMP-2, -3, -9 and -13 was analyzed in a quantitative real-time polymerase chain reaction. Wilcoxon signed rank test was used to determine statistical significant results ($p < 0.05$).

Results

The expression of MMP-2 was significantly ($p < 0.05$) up-regulated after 1 h and 24 h compared to control. Although not statistically significant, expression rate of MMP-13 was also increased after 1 h and 24 h. The expression of MMP-3 was significantly ($p < 0.05$) up-regulated after 24 h. No changes were observed in the expression of MMP-9.

Conclusion

For the first time, the current study examined the influence of mechanical loading on the expression of MMP-2, -3, -9 and -13 in chondrocytes of the human growth plate in vitro. The data presented herein contribute to a better understanding of the remodeling process, since we showed that mechanical loading significantly up-regulates MMP-2 and-3 expression and increases MMP-13 expression in chondrocytes of the human growth plate in vitro. Therefore, we conclude that MMPs might be involved in bone remodeling.

Key words

Growth Plate; Matrix Metalloproteinases; Mechanical Loading.

ZUSAMMENFASSUNG

Ziel

Das kindliche Skelett besitzt die einzigartige Fähigkeit des Remodelings nach einer Fraktur. Die molekularen Mechanismen sind jedoch noch nicht ausreichend erforscht. Wir stellen die Hypothese auf, dass der Wachstumsfuge und den epiphysealen Matrix Metalloproteinasen (MMP) eine bedeutende Rolle während des Remodelings zukommen und diese durch mechanische Belastung beeinflusst werden. Wissen um die molekularen Mechanismen des Knochenremodelings könnten zur Verbesserung der gegenwärtigen Behandlungsansätze beitragen und Fehlstellungen nach Frakturen verhindern.

Materialien und Methoden

Chondrozyten aus der Wachstumsfuge überschüssiger Zehen und Finger von Kindern mit Polydaktylie wurden isoliert und kultiviert. Diese Zellen wurden mit dem Flexcell Tension Plus Systems® mit 1 Hertz und 10% Elongation für 1, 4 und 24 Stunden belastet. Mittels qRT-PCR wurde die Expression von MMP-2, -3, -9 und -13 analysiert. Zur Ermittlung statistisch signifikanter Ergebnisse ($p < 0.05$) wurde der Wilcoxon-Rangsummentest angewandt.

Ergebnisse

Die Expression von MMP-2 war im Vergleich zur Kontrollgruppe nach 1 Stunde und 24 Stunden signifikant ($p < 0.05$) erhöht. Obwohl statistisch nicht signifikant, stieg die Expression von MMP-13 nach 1 Stunde und 24 Stunden ebenfalls an. Expression von MMP-3 war nach 24 Stunden signifikant ($p < 0.05$) erhöht. Keine Änderung konnte in der Expression von MMP-9 beobachtet werden.

Fazit

Unsere Studie untersuchte zum ersten Mal den Einfluss mechanischer Belastung auf die Expression der MMPs-2, -3, -9 and -13 in Chondrozyten der menschlichen Wachstumsfuge in vitro. Unsere Daten tragen zum besseren Verständnis des Remodelingprozesses bei und zeigen, dass mechanische Belastung die Expression von MMP-2 sowie MMP-3 signifikant und von MMP-13 in Chondrozyten der menschlichen Waschstumsfuge in vitro erhöht. Wir schlussforlgern, dass MMPs eine wichtige Rolle im Prozess des Remodelings innehaben.

Schlüsselworte

Wachstumsfuge; Matrix Metalloproteinasen; Mechanische Belastung.

1. INTRODUCTION

In the Republic of Austria more than 830.000 accidents happen each year, 170.000 of which are affecting children. One-third requires medical attendance due to its severeness. The Austrian Road Safety Board provides region-related statistics of injured children accounting for an estimated number of 7.000 children being treated as inpatients in Styria (Tabel 1, below). [1]

	Number				Percentage (%)		
	Traffic	Work, School	Home, Leisure, Sport	Total	Traffic	Work, School	Home, Leisure, Sport
Killed	633	182	1772	2587	24	7	68
Injured with permanent disability	1591	1105	5861	8557	19	13	68
Injured with inpatient treatment	12324	14928	136066	163318	8	9	83
Total amount if injured persons	49791	176055	610500	836000	6	21	73

Tab. 1. Casualties – according to setting [1]

Twenty-five percent of all recorded injuries happen to children (Table 2, see following page). [1, 2] Hereby, bone injuries are ranging from 20% to 25% [3, 4], of which approximately 55% are affecting long bones [3-5]. The risk of sustaining a fracture during adolescence is estimated at 15% to 45%. [3, 5] In case of an affected growth plate (GP), the prevalence of growth disturbances is higher over one`s lifetime. The GP, which is responsible for the longitudinal growth of a bone, is subjected to a constant morphogenesis and belongs to the weakest spots of the juvenile skeleton. Especially during times of rapid growth, it is the most fragile and thus, trauma frequently occurs. [3, 5, 11] Whenever mechanical strain applied to long bones exceeds the stability of the GP, a fracture is the most common result. [3, 5, 6] According to the Salter-Harris classification, type I and II fractures through the GP usually heal without severe consequences, whereas type III and IV often lead to axial malalignments. [7-11]

Age	Number					Percentage (%)			
	Traffic	Work, School	Sport	Home, Leisure	Total	Traffic	Work, School	Sport	Home, Leisure
0-14	3197	40573	41500	80700	166000	2	24	25	49
15-24	15570	39836	50300	38900	144600	11	28	35	27
25-59	24637	77128	92700	140400	334900	7	23	28	42
60+	6365	2024	18600	147200	174200	4	1	11	85

Tab. 2. Accidents and injuries – according to indicators [1]

Representing a frequent complication during postfracture healing in pediatric traumatology, bone growth disturbances and malalignments are a quite common phenomenon in children. [13, 18] Clinical observations show that growth disturbances and remodeling are both related to the location of the fracture, type of dislocation, fracture distance to the GP as well as to the age of the affected children. [13, 15-17] Molecular mechanisms of remodeling have not been sufficiently investigated. With knowledge of the molecular mechanisms of remodeling, therapeutic support - for instance the use of adequate stimuli like mechanical loading - could come into focus of treatment options. Accordingly, the outcome in cases with malalignments near to a GP of low potential and in case of older children with almost closed GP could significantly be improved as well. The prevention of traumatic-induced long-term damage based on better knowledge of factors that influence remodeling means a relevant financial economization for the health care system like personal and socio-economic benefits and an improvement in quality of life for those patients. Moreover, this would also mean a shorter in-patient treatment for our young patients and therefore, prevent related stress factors.

2. THEORETICAL PRINCIPLES

2.1. Essentials of Bone Development

Bone development is explained by two models: the direct, intramembranous ossification and the indirect, chondral ossification. In both processes, bone arises via replacement of precursor tissue. Primary woven bone formation is always followed by tissue replacement with secondary lamellar bone. [19-23]

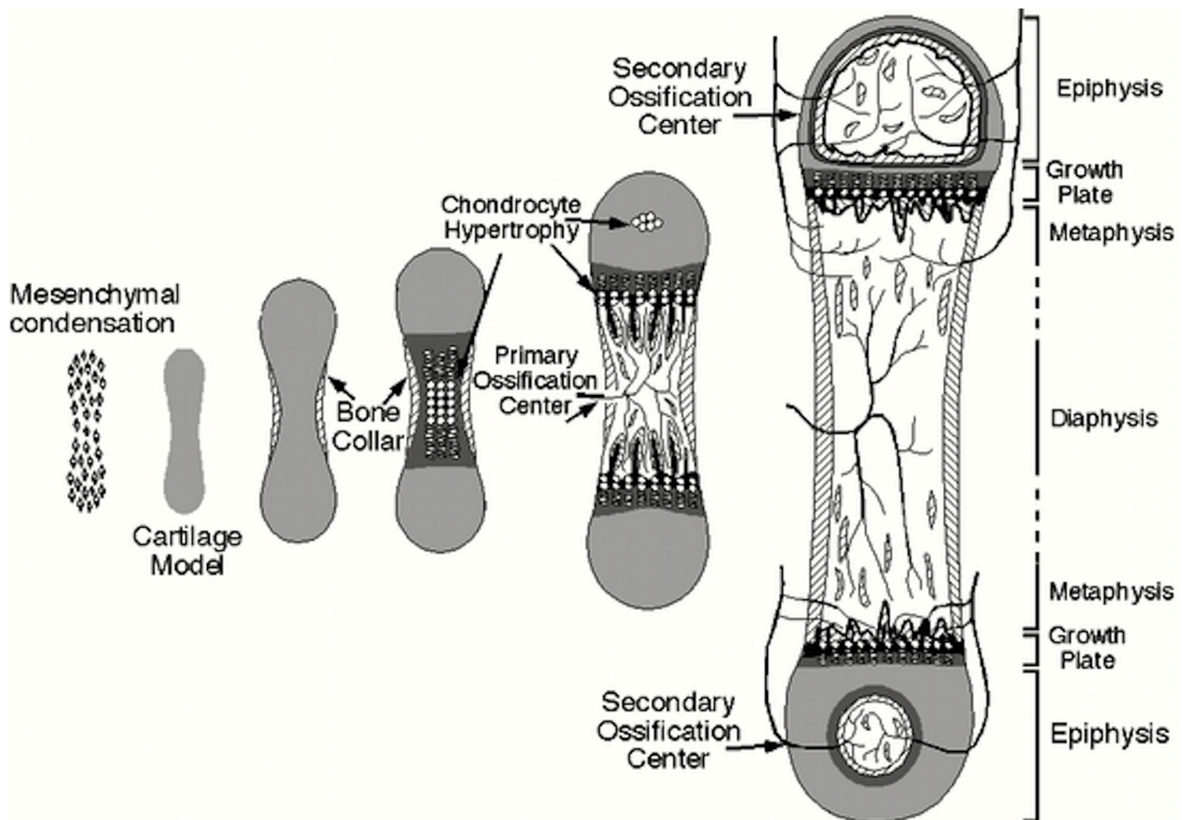


Fig. 1. Bone development. Schematic diagram showing the initial stages of endochondral ossification.
(www.endotext.org, accessed on 02/12/2011,
http://1.2.3.10/bmi/www.endotext.org/parathyroid/parathyroid1/figures/figure9.jpg)

2.1.1. Intramembranous Ossification

Intramembranous ossification is characterized by direct transformation of embryonic connective tissue into bone. Compared to chondral ossification, hyaline cartilage is not present here. [24]

At the initial stages of intramembranous ossification, mesenchymal cells (MC), located in a highly vascularized area of primitive connective tissue, proliferate, aggregate and condense. Once condensated, these MCs differentiate into osteogenic cells, then synthesize collagen fibrils and secret a thin matrix substance. Occupying intercellular space, this matrix substance forms bone spicules by means of calcification. Mesenchym between these spicules develops to bone marrow. Superficial layers of tissue, that do not ossify, form periosteum and endosteum. Gaining in growth, adjacent spicules merge and form fusing trabeculae. Woven bone matrix is synthesized that eventually matures to lamellar bone. [8, 22, 24, 25]

Intramembranous ossification is a process by which ossification occurs at numerous sites simultaneously, which distinguishes this process from chondral bone formation. Eventually, the separated areas will grow together as they expand. Intramembranous ossification occurs during the embryonic development of flat bones, for instance in craniofacial bones, sternum and clavicle. This process plays an important role both in fracture healing and in development of the periochondral cuff of long bones. [8, 22, 26-28]

2.1.2. Chondral Ossification

Skeletal development starts with a hyaline cartilage model that serves as pre-existing tissue. This way of bone formation is called chondral ossification. [23] In contrast to intramembranous ossification, chondral formation of bone is not a direct process. In chondral ossification MCs condensate and differentiate into chondrocytes and periochondral cells. Since long bones gain growth both in length and width, two types of indirect ossification, periochondral and endochondral ossification, are distinguished. [8, 19-21, 29, 30]

2.1.2.1. Perichondral Ossification

Perichondral ossification takes place at the diaphysis of long bones. Perichondral cells align at the surface of the cartilage template and osteoblasts of the perichondrium produce cartilage matrix that is added on the outer surface. Gradually, the diaphyseal bone collar forms. Growth in thickness, called appositional growth, is induced. [21, 23, 26, 29-33]

2.1.2.2. Endochondral Ossification

In contrast to periochondral ossification, endochondral ossification starts from the inner of the cartilage matrix. This pathway of ossification takes place at the epiphyses and apophyses of long bones. Within this process, cartilage, that is an avascular tissue, is gradually converted into a highly vascular bony tissue. In the first step, blood vessels grow in, accompanied by MCs. Chondrocytes - not to be confused with chondroblasts as in intramembranous ossification - form cartilaginous templates using condensed MCs. [19, 20, 23, 26, 30-32, 34-37] These cells proliferate and secernate a matrix that is rich of type I collagene and aggrecan. By proliferation and production of matrix, the cartilage gains in growth. In the following, chondrocytes mature from proliferating chondrocytes to non-proliferating hypertrophic cells. These cells synthesize type X collagene, regulate the mineralization of the surrounding matrix and induce the ingrowth of blood vessels by production of vascular endothelial growth factor (VEGF). The proliferative and hypertrophic cells undergo apoptosis and haematopoetic stem cells, chondroclasts and osteoblasts migrate into the vascularized area. MCs differentiate into osteoblasts, gain in growth and secondary centers of ossification are formed. Gradually, resorption of calcified cartilage and resorption of bone by

osteoclasts lead to replacement of the cartilage model by bone matrix. In the end, the cartilage model is replaced by new bone. Layers of cartilage between the centers of ossification, known as GP, are formed. [22, 25, 30, 38-41, 48-55]

During growth, the GP is the site of endochondral ossification. Afterwards, bone remodeling continuously takes place in order to adapt to the local and systemic environment. [26] Examination of the GP reveals chondrocytes that are arranged in columns. These chondrocytes take part in the process of endochondral ossification. Several zones, that are discussed in detail in chapter 2.2.1., can be identified. [20-22, 27, 28] Endochondral ossification is structurally organized in five distinct zones that can be seen at the light-microscope level similar to those seen in the GP: zone of proliferation, maturation, hypertrophy, cartilage degeneration and osteogenic zone. [29, 31, 32 36] Growth of cartilage in length is based on continuous cell proliferation and further secretion of extracellular matrix (ECM) in-between chondrocytes . This process is called interstitial growth. [19, 20, 23, 26, 29-36] When skeletal maturity is reached, endochondral ossification only recapitulates if a fracture occurs. Then, bone tissue develops from an initially formed cartilaginous callus. [26]

Intramembranous and chondral ossification can occur side by side, especially in the case of fractures. While the growth of a long bone in length takes place via endochondral ossification, intramembranous ossification occurs at the same time on the surface in order to maintain proper shape and thickness of the bone anatomy. Thus, the final bone shape is determined by a combination of these two processes. [42-55]

The above mentioned well orchestrated process of enchondral ossification can be easily disrupted by fractures involving the GP. In GPs, local peripheral injuries lead to formation of bone bridges through destruction of the epiphyseal circulation and neovascularization. Xian et al. [42] showed that in GPs of rats, injuries were followed by formation of a bone bridge via the pathway of intramembranous ossification. Recent data suggested that endochondral ossification occurred during bone repair as a major mechanism, as well. Arasapam et al. [45] and Chung et al. [46, 47] demonstrated that both cartilage and bone were present in the GP while reparation of a bone injury took place. Accordingly, both intramembranous and endochondral ossification mechanisms occurred during bone reparation.

2.2. The Growth Plate

2.2.1. Macroscopic Anatomy of the Growth Plate

During growth, long bones consist of the epiphysis (terminal part), the GP and the metaphysis (intermediate part) at both the proximal and distal end, separated by the diaphysis (shaft). Key player during growth of children is the GP, which is the cartilaginous zone between the epiphyseal and the metaphyseal region of the bone. It extends across the full horizontal section of long bones and is comprised by layers of chondrocytes at various stages of differentiation, that are embedded in a highly hydrated ECM. The GP can be subdivided into four horizontal zones that synchronize chondrocyte differentiation in a spatio-temporal process. [30, 33, 34, 56-60] Growing in length by the GP is based on a simple principle: Chondrocytes proliferate and differentiate to bone tissue. Further length gaining comes to an end just after ossification has started. The zones are not clearly separated from each other but act as a well elaborated system merged by smooth transitions. Growing in length is a complex interplay and a well orchestrated process between the different zones and synthesis of ECM. [8, 21, 22, 41,56, 59, 61]The four main horizontal layers from the epiphyseal to the metaphyseal end are [8, 21, 22, 41, 59, 61]:

1.) The Resting Zone

2.) The Proliferative Zone

3.) The Hypertrophic Zone

4.) The Mineralization Zone

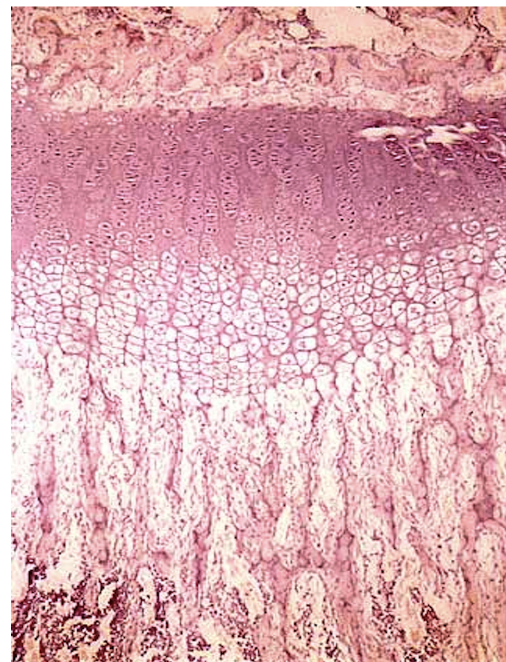


Fig. 2. Zones of the growth plate
(www.bioscience.org, accessed on 02/12/2011,
http://www.bioscience.org/1998/v3/d/naski/fig5.jpg)

2.2.2. Microscopic Anatomy of the Growth Plate

2.2.4.1. Resting Zone

The resting or reserve zone (RZ) is located at the epiphyseal end of the GP. The RZ consists of inert chondrocytes that are widespread through the matrix resulting in a high ECM to chondrocytes ratio. This zone is rather inactive regarding matrix and cell turnover but it is of importance as nutritive and supply storage. Within the RZ the synthesis of proteoglycans and collagen type II is exceedingly high. [21, 22, 59, 61-63]

2.2.4.2. Proliferative Zone

The proliferative zone (PZ) is the main area of cell division, production of ECM proteins and chondrocyte differentiation. Stem cells of the PZ primarily synthesize collagen type II as well as the leading proteoglycan aggrecan. The PZ is the only zone with mitotic activity and has the highest rate of ECM synthesis and turnover. Within the PZ the chondrocytes have a flat shape and are arranged in columns parallel to the longitudinal axis of the bone but are kept apart from each other by longitudinal septa of cartilage matrix. Each column consists of a varying number of chondrocytes, subject to the actual mitotic activity of the column. These cells act as glycogen storages and accumulate a considerable quantity of intracellular organelles. In the lower part of the PZ, where it continuously blends with the hypertrophic zone (HZ), the cells are deprived in their ability to proliferate. The GP is considered to be monopolar since all the growth is directed away from the PZ. [21, 22, 33, 41, 59-64]

2.2.4.3. Hypertrophic Zone

The HZ shows a diminished proliferation rate with an established apoptosis program for hypertrophic chondrocytes. In the HZ the chondrocytes grow in size, enlarging their cell volume five to seven folds, become pre-hypertrophic and then finally differentiate. The mitochondria of the hypertrophic cells primarily store and release calcium rather than produce adenosine-5-triphosphate (ATP). These cells also have the highest concentration of glycolytic enzymes and produce alkaline phosphatase (ALP), collagen type X and crystalline

hydroxylapatite for proper mineralization. Hypertrophic chondrocytes prepare the in columns arranged bone matrix for calcification and for angiogenesis, which is of great importance for the invasion of osteoprogenitor cells, osteoblasts, chondroclasts and osteoclasts. Volume changes of the HZ correlate to longitudinal bone growth. [21, 22, 33, 41, 59-64]

2.2.4.4. Mineralization Zone

The mineralization zone (MZ) is characterized by calcification. In the MZ, chondroblasts go into apoptosis and entice macrophages. Vascular channels from the metaphysis invade the dead columnar cells and thus, the ECM becomes mineralized. The mineralization is initiated by matrix vesicles that comprise Annexin V. This molecule is activated by collagen X, and opens calcium channels to deposit calcium for mineralization. Around ⅔ of the mineralized longitudinal septa are degraded by chondroclasts, ⅓ remains as template for osteoblasts, that deposit bone matrix. These template septa consist of mineralized cartilage as well as bone matrix and are called primary bone trabeculae. In the metaphysis these trabeculae change to bone lamellae. Various MMPs are responsible for this transformation of cartilage into bone matrix. Differentiated chondrocytes undergo apoptosis and therefore, space is formed for invading blood vessels that are attracted by VEGF-activated MMP-9. This process is an important step in establishing bone tissue. [21, 22, 33, 41, 59-65]

Adjacent to the MZ and the GP is the primary spongiosa zone. It resembles the lower mineralization area and consists of mineralized cartilage as well as bone formation. The hypertrophic chondrocytes within the central cartilage undergo apoptosis, whereby invasion of blood vessels is stimulated. The primary spongiosa zone is transformed by removing the cartilage septa and by replacing its woven architecture with lamellar bone. Osteoprogenitor cells remove the cartilaginous material and produce osteoid. Accordingly, secondary trabeculae are formed. A peripheral ring of fibrocartilage, that supports structural stability, encircles the GP. The ossification groove of Ranvier supplies MSCs that provide cells of the RZ. These cells allow the GP to extend in width during bone growth. The perichondrial fibrous ring of LaCroix blends with the periosteum, providing mechanical support to the GP. [21, 22, 33, 41, 59, 61, 63, 67, 68]

2.2.4.6. The Extracellular Matrix

Bone tissue is a connective tissue and hence, contains cells, fibers and ECM. The ECM, which is subjected to constant flux, plays an important role in tissue architecture, cell migration and tissue homoeostasis. Matrix synthesis and degradation are located in the PZ as well as the HZ, in addition to high mitotic and proliferative activity in those zones. The primary contents of a well organized ECM are proteoglycans, collagens, non-collagenous proteins and water. The latter is essential for the load-bearing capacity of the cartilage. Collagens of the ECM are the types II, X and XI. These collagens are generated and emitted by corresponding chondrocytes from respective GP zones, aggrecan, other proteogylcans as well as non-collagenous proteins. Type II collagen is a structural unit that builds a fibrillar system needed to resist tensile load. Type X collagen is only localized in the HZ and participates in bone mineralization. Type XI collagen surrounds the surface of type II collagen and mediates the interaction of type II collagen with other elements of the ECM. Aggrecan is the leading proteoglycan molecule and provides the osmotic properties by which compressive loads are resisted. Smaller interstitial proteoglycans are Decorin and Biglycan that influence cellular proliferation and migration. The cartilage-oligomeric- matrix-protein (COMP) is a critical non-collagenous protein that belongs to the Thromospondin family. It is a calcium-binder and substantial component of the ECM. [21, 22, 33, 41, 59, 61, 63, 69-71]

While a fracture is rebuilt, chondrocytes mainly accumulate type II collagen and Aggrecan in the ECM. During hypertrophy, chondrocytes deposit ECM that mainly consists of type X collagen and later on - after partial mineralization - of type I collagen. Osteoblasts and osteoclasts, that are accountable for this process, continue degrading regenerated tissue into mature bone. Various proteases are involved in the proteolytic degradation of the ECM. Here, MMPs are the key players. Remodeling of the ECM is highly important for both development and fracture repair. [59, 61, 65, 69, 71]

2.2.5. Regulation of the Growth Plate

Bone growth can be itemized according to two aspects: gaining width and growing in length. Concerning gaining width, the regulatory effect of the GP is still quite unaccounted for, while the regulation of longitudinal growth consists of three factors [33, 41]:

1) systemic factors
2) local factors
3) mechanical factors.

Homeostasis of the skeleton as well as the GP is regulated by hormones and local mediators. Since their variety is huge, only the most important are listed below (Table 3) and will be discussed further in chapters 2.2.5.1. and 2.2.5.2. [33, 36, 41, 59, 72] Due to an exceedingly high relevance of mechanical loading and its influence on the expression of MMPs, mechanical factors will be discussed separately in chapter 2.4.

Systemic Factors	Local Factors
Glucocorticoids (GC)	Bone Morphogenetic Proteins (BMPs)
Growth Hormone (GH)	Fibroblast Growth Factors (FGFs)
Insulin-like Growth Factor (IGF) I and II	Indian hedgehog (Ihh)
Sex Hormones (Estrogen and Androgen)	Matrix Metalloproteinases (MMPs)
Thyroid Hormones (T_3 and T_4)	Parathyroid Hormone-related Peptide (PTHrP)
	Vascular Endothelial Growth Factors (VEGFs)

Tab. 3. Factors influencing the growth plate [33, 36, 41, 59, 72]

2.2.5.1. Systemic Factors

a) Glucocorticoids

Glucocorticoids (GC) are commonly used as anti-inflammatory and immunosuppressive drugs. High-dose treatment over a longer period of time often has side effects like growth retardation and reduction of bone density. [73] GC have an inhibitory effect resulting in a delayed senescence and a lower extension of the GP. This slow longitudinal growth of long bones is caused by a higher apoptosis rate of terminal hypertrophic chondrocytes, inhibited chondrocyte proliferation by regulation of protein synthesis and thus, inhibited ECM synthesis. Moreover GC interfere with the Growth Hormone-Insulin-like Growth Factor-axis, stimulate osteoclasts and inhibit osteoblasts. A high GC level has a negative effect on VEGF and restricts therefore ingrowth of vessels into the metaphysis. [41, 72, 74-78]

b) Growth Hormone and Insulin-like Growth Factor I and II

Prenatally, Insulin-like Growth Factors (IGFs) I and II regulate the longitudinal growth of long bones. Prior to birth, IGF I and II act independently of the Growth Hormone (GH). Postnatally, GH and IGF I are the key hormones in the hypothalamus-hypophysis-system for growth. They are critical for puberty induced longitudinal bone growth. The hypophysis secrets GH in a pulsatile rhythm. In pituitary adenomas there is an overproduction of GH, resulting in acromegaly or gigantism. GH leads to an increased mitotic activity and finally, to an extension of the GP. Moreover GH directly acts on chondroprogenitor cells in the RZ by stimulating their multiplication and differentiation. This process triggers local IGF I secretion. IGF I incites and controls hypertrophying and differentiation processes of chondrocytes. IGF I is mainly produced in the liver, then, bound to carrier proteins and transported to the GP. [41, 72, 79]

c) Sex Hormones

Sex hormones are of the utmost importance during puberty, regarding control of longitudinal growth. Their dose-dependent effects on longitudinal growth depends on individual age and sex. Based on the conversion of androgens to estrogen, effects of both hormones on bone growth can hardly be distinguished. The main effects of estrogen are its inhibitory as well as stimulating effect on longitudinal growth and its key role in closing the GP. The inhibitory effect of estrogen antagonizes GH and decreases the proliferation rate of chondrocytes in the GP. During the physeal closure process, ingrowth of vessels into the GP takes place and proliferation of chondrocytes stops. Neovascularization and down-regulation of proliferation happen via two chondrocyte-linked receptors, estrogen receptor α and β. Androgens, particularly testosterone, indirectly affect cartilage tissue through their partial conversion to estrogen. Androgens promote maturation of cartilage and accelerate longitudinal growth and bone maturation. Estrogen as well as androgens have direct influence on chondrocytes of the GP via corresponding receptors. [41, 72, 80, 81]

e) Thyroid Hormones

The thyroid hormones, triiodothyronin (T_3) and thyroxid (T_4) are produced in thyroid epithelial cells. T_3 is the precursor of T_4 and both are crucial mediators for regular bone maturation. Children with congenital hypothyroidism or those, who have a T_3 deficit, suffer from bone deformation during growth resulting in growth retardation. By contrast, hyperthyroidism results in excessive growth and in earlier closure of the GP. Both T_3 and T_4 interact with the GH-IGF system by stimulating GH. The thyroid hormones act locally on the GP by recruiting cells from the RZ. T_4 can elicit the expression of the collagens type II and X, which is induced by the bone morphogenetic protein (BMP) 2. Furthermore, T_4 can induce the expression of ALP and the hypertrophy of chondrocytes, that is necessary for mineralization of the cartilaginous matrix. [41, 72, 82, 83]

2.2.5.2. Local Factors

a) Bone Morphogenetic Proteins

BMPs are a group of multi-functional growth factors, belonging to the transforming growth factor-β superfamily. Some BMPs are called cartilage-derived morphogenetic proteins (CDMPs); several others are growth differentiation factors (GDFs). BMPs in vertebrates are of great importance due to their roles in cartilage and bone development. They have an osteoinductive ability that also includes the differentiation of MSCs to osteoblasts. BMP-2 and -6 are produced in hypertrophic chondrocytes and BMP-7 in proliferating chondrocytes. BMP-2 is the only molecule in the large family of BMPs that autonomously incites cartilage and bone development. BMP signaling stimulates proliferation as well as differentiation of chondrocytes by increasing the expression of Indian hedgehog (Ihh). With this increase in expression and the opposite effect on terminal hypertrophic chondrocytes as induced by Fibroblast Growth Factor (FGF), BMP becomes an antagonist to FGF. [41, 72, 84-88]

b) Fibroblast Growth Factors

FGFs are of great importance in the process of proliferation and differentiation of various cells and tissues. FGFs also play a significant role in mesenchyme condensation and embryonic bone development. Most of the FGFs are glycoproteins that dock on heparan sulfates. This binding is the reason why FGFs accumulate in the ECM. FGFs serve as ligand for a family of five FGF receptors (FGFR). FGFR-1 to -3 have a key role in bone development. FGFR-1 is expressed on pre-hypertrophic and hypertrophic chondrocytes, FGFR-2 has its place on perichondral cells and FGFR-3 is found on proliferating chondrocytes of the GP. FGFR-3 acts as an inhibitor of chondrocyte proliferation and interacts with Parathyroid Hormone-related Peptide (PTHrP)- and Ihh-signaling pathways. Altogether, FGFs cause a decreased proliferation of chondrocytes and a suppressed expression of Ihh. These two factors lead to a decrease in length of the columnar cartilage. [41, 52, 72, 85, 88, 90, 91]

c) Indian hedgehog and Parathyroid Hormone-related Peptide

Ihh belongs to the hedgehog family of secreted proteins and is synthesized by pre-hypertrophic as well as hypertrophic chondrocytes. It acts particularly during enchondral bone ossification. Ihh takes part in differentiation, proliferation as well as maturation of chondrocytes and controls these processes by feedback control on PTHrP. Ihh positively regulates PTHrP and thus, modulates the rate of chondrocytes undergoing hypertrophy. Additionally, it is responsible for maintaining a normal amount of proliferating chondrocytes. Nevertheless, Ihh also stimulates - independently of PTHrP - the differentiation of periarticular chondrocytes. By transmitting developmental signals from pre-hypertrophic and hypertrophic chondrocytes to pre-osteoblasts and osteoblasts, Ihh links chondrogenesis to osteogenesis. [41, 72, 92, 95]

PTHrP and its receptor are of great importance for the regulation of enchondral bone development and longitudinal bone growth. PTHrP is released from perichondral cells and from chondrocytes of the PZ. PTHrP delays differentiation of proliferating chondrocytes and slows down hypertrophy. PTHrP signals its receptor to impede the differentiation of proliferating terminal chondrocytes. Accordingly, PTHrP receptor regulates the differentiation of chondrocytes and takes part in the development of the GP. With PTHrP, Ihh coordinates the length of the PZ and of the columnar chondrocytes. These processes maintain the GP stable in both width and length. The synthesis of PTHrP is stimulated by Ihh, whereas PTHrP inhibits Ihh production - they have feedback control on each other. [41, 72, 96, 97]

d) Matrix Metalloproteinases

Due to an exceedingly important relevance of MMPs in this work, they will be highlighted separately in chapter 2.5.

e) Vascular Endothelial Growth Factor

VEGF is a signaling protein and belongs to a sub-family of growth factors. VEGF is involved in vasculogenesis and angiogenesis. Neovascularization depending on VEGF is a crucial link between cartilage production (chondrogenesis) and bone formation (osteogenesis). Cartilage, basically avascular, relies upon the supply system of vascular cell arcades that are connected to the blood vessels of the metaphyseal bone. VEGF is expressed by hypertrophic chondrocytes. This implicates that hypertrophic chondrocytes trigger neovascularization. In addition to its important role in angiogenesis, VEGF also plays a key role in differentiation of chondrocytes, proper function of chondroclasts and mineralization as well as degradation of the ECM. In summary, VEGF is not only important for the ingrowth of blood vessels into the GP but also for physeal morphogenesis and enchondral bone formation. [41, 72, 98-102]

2.3. Essentials of Growth Plate Injuries

Fractures during childhood usually present particular advantages in healing and are rather easy to treat comparing to those in adults. [17, 18] These advantages include short time to union as well as the unique capacity of spontaneous remodeling. Primarily malpositioned healed injuries get compensated secondarily. [12, 13, 18]

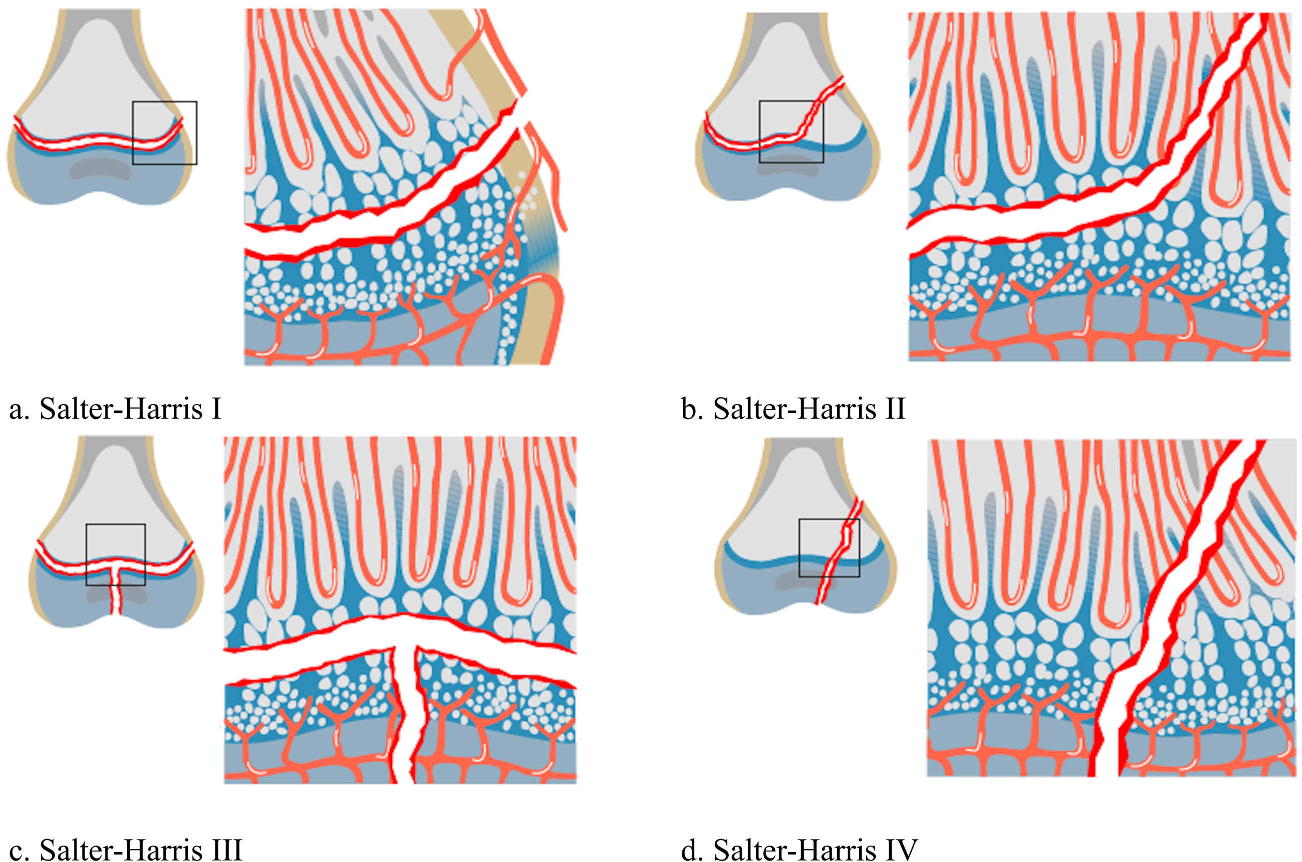
GP injuries are quite common in children, accounting for 15% of all childhood long bone fractures. [103] As the GP is responsible for the longitudinal growth of the juvenile skeleton, the GP belongs to the most fragile spots of a maturing bone. [11] If mechanical strain exceeds the stability of the GP, a fracture will be the consequence. [3, 5, 6] Fractures through the GP can elicit growth disturbances, malalignments and axial deviations, since each destruction of the germinative chondrocyte layer leads to remaining growth disturbances. These growth disturbances are especially related to type of fracture and growth potential of the affected GP. [16-18] Fractures with involvement of the GP are generally categorized according to either Aitken [54] or Salter-Harris [7, 8, 15, 54, 56, 58, 103, 104]. Since the Salter-Harris classification is the most commonly used classification, it is portrayed in the following chapter.

2.3.1. Salter-Harris Classification

The Salter-Harris classification from the year 1963 takes into account injury mechanism, run of fracture line and prognostic relevance of fractures through the GP. [7, 8, 15, 54, 56, 58, 103, 104] As a radiological classification it distinguishes five fracture types as seen in the following table 4 [7, 8, 10, 12, 15, 54, 56, 58, 103, 104] and figures 3a-d [18]. Type II is the most common one, [10, 12] percentages indicate the occurrence frequency.

Type I	Epiphysiolysis; transverse fracture through the growth plate	13.2%
Type II	Epiphysiolysis with metaphyseal fragment	53.6%
Type III	Epiphysiolysis with epiphyseal fragment; joint involvement	10.9%
Type IV	Epiphyseal fracture with metaphyseal and epiphyseal fragment	6.5%
Type V	Crushed growth plate; caused by axial compression	< 1%

Tab. 4. Salter-Harris classification [7, 8, 15, 54, 56, 58, 103, 104]



a. Salter-Harris I

b. Salter-Harris II

c. Salter-Harris III

d. Salter-Harris IV

Fig. 3. Salter-Harris I-IV [18]

2.3.2. Developmental Stages of the Growth Plate

Physeal fractures frequently result in severe problems regarding the further growing process of long bones, since the GP has limited regeneration ability. [105] Growth disturbances only occur if the affected GP is not yet closed. [14, 15, 18] Three stages of growth are established [8]:

1.) Active growth phase

Until the 10th to 12th year of age, proliferation and mineralization processes are said to be balanced. The GP is open and the bone gains in length. [8, 41, 59-61, 106]

2.) Resting phase

After the growth spurt and towards the end of the growth phase, the GP becomes thinner and hormonal influences result in a resting phase. Proliferation processes are finished but mineralization still goes on. The bone stops growing for a short period but the GP still keeps its growth potential. [8, 41, 59-61, 106]

3.) Closure phase

The short period of resting proceeds to the closure phase. Proliferation fails and finally stops. Mineralization dominates the GP. Bone bridges are formed, spread a priori on the metaphyseal, then on the epiphyseal part of the bone and finally bring the GP to closure. Usually, the process of mineralization starts peripherally. The moment of final physeal closure is influenced by sex of the child and growth potential of the particular GP and thus, individually varies. The growth in height is completed with the definite physeal closure. [8, 41, 59-61, 106]

2.3.3. Growth Disturbances

Fractures through the GP may result in severe problems for the further growth of long bones, since the GP has limited regeneration abilities only. [105] Growth disturbances can occur if the GP is still open and either the whole or partial GP can be affected. [14, 15, 18]. Injuries to epiphyseal plates, that are either nearly mature or are already closed, do not lead to growth disturbances. [105] Nevertheless, they are quite common clinical posttraumatic phenomenons in children, representing a frequent complication during fracture healing in pediatric traumatology. Especially growth disturbances of the lower extremity are crucial since they have an essential influence on the whole musculoskeletal system, causing pelvic obliquity, back problems or even scoliosis. [13, 16-18] Eventually, these malalignments represent an extraordinary risk factor for developing an early arthrosis. [107, 108] Clinical observations show that growth disturbances correlate with the age of the injured child and the severity of the injury, irrespectively of the Salter-Harris grade. [109] There are two forms of posttraumatic growth disturbances: a stimulating and an inhibiting one, discussed in detail in the following chapters.

2.3.3.1. Stimulating Growth Disturbances

Stimulating growth disturbances can be expected after any fracture of a growing bone. Especially, injuries during the actual skeletal growth phase (< 10th year of life) result in posttraumatic persistence of longitudinal growth. Fractures through the GP prior to maturity, in the inactive phase of the GP, can result in initial lengthening. This can later be compensated by premature closure of the affected GP. According to current literature, stimulating growth disturbances are attributable to hyperaemia at the fracture side. However, they frequently occur after surgical intervention. [8, 13, 105, 109]

2.3.3.2. Inhibiting Growth Disturbances

Fractures through the GP in its closure phase (> 10th year of life) result in premature closure and inhibiting growth disturbances. A premature closure is precipitated by direct or indirect vessel damage and normally leads to a shortening of the affected bone side. In most cases a partial closure of the GP occurs and a bridge callus arises as part of the reparation process of the injured area. Fractures that cross the PZ of the GP and therefore disrupt its structural organization, lead to the formation of a bone bridge. The bone bridge can persist during further growth and, without therapeutic intervention, lead to malalignments and axial deviations. The most feared complication of a fracture through the GP is a premature partial or total closure that leads to sudden growth stagnation of the affected GP. [8, 13, 56, 105, 109]

2.3.4. Remodeling

The juvenile skeleton has the unique capability of remodeling after a fracture occurred. Remodeling means that injuries, that have primarily healed in a malposition, are autonomously compensated by epiphyseal and periosteal mechanisms. Clinical observations showed that remodeling has been related to the potential of the affected GP, the location of the fracture, the type of dislocation, the fracture distance to the GP and the age of the patient. This remodeling capacity significantly influences any fracture management in the case of children, since it is one of the greater challenges in pediatric traumatology to estimate which fracture needs reduction and which can be treated in terms of a conservative approach. The mechanisms of remodeling are a consequence of appositional periosteal formation on the concavity of the deformed bone, resorption on the convex side and asymmetrical growth of the GP. The most crucial cells for remodeling are osteoblasts and osteoclasts. The ECM is of great importance in terms of remodeling after fractures, since its degradation occurs during mineralization of chondrocytes and their restructuring to bone substance (enchondral ossification). Remodeling can last from months to years but it definitively is the major part to achieve stability. Bone stability is essential to gain full physical activity. [8, 13-18, 50]

2.4. Mechanical Loading

Besides systemic and local factors, mechanical factors complete the complex system of GP regulation. The GP is subjected to large gravitational and muscular loads. There is clinical and experimental evidence that mechanobiological factors strongly influence bone ossification as well as the morphogenesis of the GP. [110-114] Therefore, mechanical factors are essential in normal bone growth. Under normal physiological conditions, the GP is primarily subjected to compressive loads. According to the Hueter-Volkmann principle, the growth rate of the GP is in indirect ratio to the axial forces that affect the GP. [110-115] The Hueter-Volkmann principle deals with the modulation of growth. As a conclusion, compression forces retard growth and conversely, reduced pressure as well as tensile forces exacerbate or respectively, accelerate growth. Excessive loads can lead to progressive deformities. [110-114]

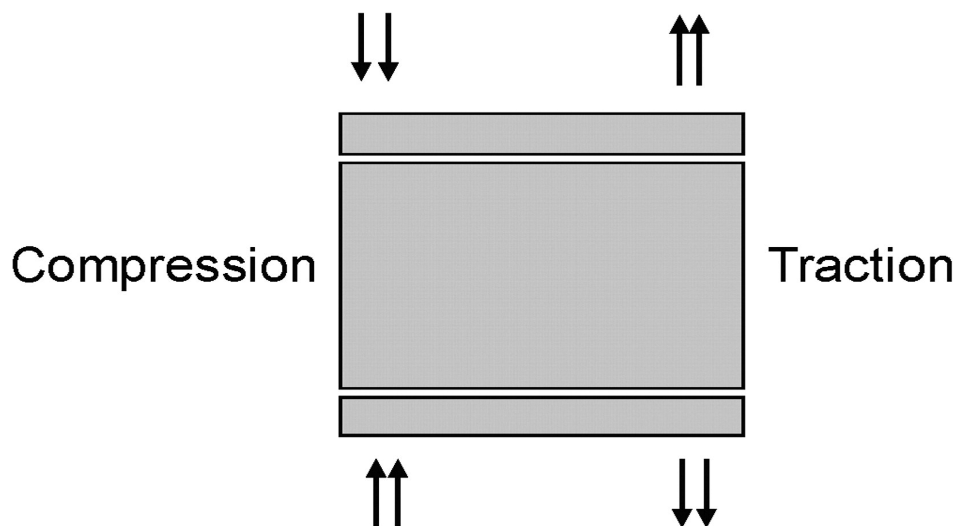


Fig. 4. Illustration based on the Hueter-Volkmann principle. Compression exerted on the GP slows growth, whereas traction accelerates it.

(Kim H, Kim HS, Moon ES, Yoon CS, Chung TS, Song HT, Suh JS, Lee YH, Kim S. Scoliosis imaging: what radiologists should know. Radiographics. 2010 Nov;30(7):1823-42.)

While longitudinal growth reacts to sustained forces [110-114], remodeling replies to transient loading [115]. Fluid exchange in the GP takes place only slowly, due to the low permeability of the adjacent bone. Hydroxy-apatite crystals and water are accountable for buffering the pressure that inflicts on the bone. Accordingly, mechanical strain also has direct influence on cells. Pressure compresses cells, tension stretches them. Therefore, mechanical forces induce a shift in the osmotic system. Longitudinal growth is inhibited in the case of excessive strain on the GP. On the opposite, redundant growth is the consequence of too much axial tension. [33, 41, 59, 70, 116-120] Stokes et al. [110-113] and Cancel et al. [116] pointed out that the GP is influenced by static sustained loads. Moreover, these authors showed that an increase in compressive forces reduces longitudinal growth, thus confirming the Hueter-Volkmann principle. An even ratio between bone resorption and formation is the case in balanced physiological loads only. As a result, mechanical forces have a huge impact on longitudinal growth, the formation of bone as well as the degradation of the GP. [33, 41, 59, 70, 116-120] On the basis of clinical observations and according to the Hueter-Volkmann principle, it can be concluded that particularly mechanical stimuli lead to a secondary correction of malalignments. [110]

2.5. Matrix Metalloproteinases

2.5.1. Introduction

The MMP family consists of over 20 zinc- and calcium-dependent enzymes [121] that act as proteases and are either secreted or anchored on the cell surface by a transmembrane segment, called membrane-type MMPs (MT-MMPs). MMPs are involved in various physiological as well as pathological processes. Hormones, growth factors and transformational cell process control the expression of these enzymes during transcription. MMPs are able to degrade all kinds of ECM proteins and also have influence on proliferation, differentiation and apoptosis of cells. Moreover MMPs are involved in remodeling of cartilaginous tissue, regulation of angiogenesis and activity modulation of relevant growth factors and their receptors. The degradation of the ECM is crucial for remodeling after fractures, whereby the remodeling process is largely imputable to MMPs. It is also largely susceptible to mechanical forces, as clinical observations already have shown. [69, 121-126] According to these facts, it is of interest, how mechanical loading is capable to influence the expression of particular MMPs in chondrocytes of the juvenile GP.

2.5.2. The Structure and Activity of MMPs

MMPs have a highly conserved sequence of their deoxyribonucleic acid (DNA) and consist of several homologous regions. They are synthesized as prepro-MMPs and secreted as inactive pro-MMPs. Their conversion to the active enzyme is conferred by plasminogen activator or pro-hormone convertase. The activity of MMPs is controlled by the tissue inhibitors of matrix metalloproteinases (TIMPs). [124-126] MMPs share three common domains: the pro-peptide domain, the highly conserved catalytic domain and the haemopexin-like C-terminal domain. [121, 124, 125, 127, 128]

a) The catalytic domain comprises of the zinc binding motif (His-Glu-X-Gly-His-X-X-Gly-X-X-His-Ser, wherby X is any amino acid) that promotes the binding of substrates. In contrast to other MMPs, MMP-2 and -9 have three tandem fibronectin type II repeats within the catalytic domain to mediate a better gelatin binding. [121, 124, 125, 127, 128]

b) The pro-peptide domain occupies the active site of the zinc molecule, restricting the catalytic enzyme from binding with substrates. Accordingly, latency of pro-MMPs is maintained. [121, 124, 125, 127, 128]

c) The C-terminal haemopexin-like domain confers substrate-binding specifity of the enzymes and is a vital part for collagenases to cleave collagens. Moreover, all MMPs, with exception of MMP-7 and -29, have a hinge region that connects the catalytic with the haemopexin domain by a flexible proline-rich hinge peptide. [121, 124, 125, 127, 128]

2.5.3 Classification of MMPs

MMPs are active at a neutral pH-level, catalyze the normal formation and resorption of ECM proteins and are therefore essential for maintaining tissue allostasis. [124] Since MMPs were initially described with focus on their ability to degrade ECM proteins, they have a descriptive name according to their preferred substrate, e.g. collagenases, gelatinases and stromelysins. A numbering system corresponds to the chronological order of their discovery. [121, 125, 129, 130]

a) Collagenases

They are able to degrade triple-helical fibrillar collagens into ¼ and ¾ parts. Collagens are important elements of cartilage as well as bone and MMPs only are known to degrade them. MMP-1, -8, -13 and -18 are collagenases. [124, 127, 128]

b) Gelatinases

Main substrates of the gelatinases are type IV collagen and gelatin. MMP-2 and -9 are gelatinases. [124, 127, 128]

c) Stromelysins

They are able to degrade ECM proteins but are unable to degrade the triple-helical fibrillar collagens. MMP-3, -10 and-11 are stromelysins. [124, 127, 128]

MMPs have specific but also coinciding substrate specifities. A division into substrate classes is therefore quite artificial since some MMPs fall out of the classification.

In the following Table 5, selected human MMPs and their substrates are described according to Somerville et al. [121]:

Protein name	Alternative name	Collagenous substrates	Non-collagenous ECM substrates	Non-structural ECM component substrates
MMP-2	Gelatinase-A	Collagen types I, IV, V, VII, X, XI, XIV and gelatin	Aggrecan, elastin, fibronectin, laminin, nidogen, proteoglycan link protein and versican	Active MMP-9, active MMP-13, FGF-R1, IGF-BP3, IGF-BP5, IL-1β, recombinant TNF-α peptide and TGF-β
MMP-3	Stromelysin-I	Collagen types II, IV, IX, X and gelatin	Aggrecan, casein, decorin, elastin, fibronectin, laminin, nidogen, perlecan, proteoglycan, proteoglycan link protein and versican	α_1-antichymotrypsin, α_1-proteinase inhibitor, antithrombin III, E-cadherin, fibrinogen, IGF-BP3, L-selectin, ovostatin, pro-HB-EGF, pro-IL-β, pro-MMP-1, pro-MMP-8, pro-MMP-9, pro-TNF-α and SDF-1
MMP-9	Gelatinase-B	Collagen types IV, V, VII, X and XIV	Fibronectin, laminin, nidofen, proteoglycan link protein and versican	CXCL5, IL-1β, IL2-R, plasminogen, pro-TNF-α, SDF-1 and TGF-β
MMP-13	Collagenase-3	Collagen types I, II, III, IV, V, IX, X, XI and gelatin	Aggrecan, fibronectin, laminin, perlecan and tenascin	Plasminogen activator 2, pro-MMP-9, pro-MMP-13 and SDF-1

Tab. 5. MMP-2, -3, -9 and -13 and their substrates [121]

2.5.4. Regulation of MMP Activity

The in vivo activity of MMPs is strictly regulated at many levels. Most of the MMPs are inducible either in a positive or negative way by cytokines (IL-1, -4, -6), growth factors, transforming growth factors (hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor -β (TGF-β)), tumor necrosis factor-α (TNF-α), GC and chemical agents, e.g. phorbole ester. MMPs have a feedback effect on some of these regulatory factors because they can either activate or inactivate them via cleaving. [124-126, 131, 132]

MMPs are synthesized as zymogen. Produced as pro-MMPs, they need extracellular activation. MMPs are restricted by the pro-domain that keeps the enzyme latent using the thiol group of a cysteine as fourth inactivating ligand for the catalytic zinc atom. Water cannot bind to the active site of the zinc atom, rendering the enzyme inactive. Activation of MMP depends on disruption of the pro-domain interaction with the catalytic site of the zinc atom. This disruption can take place either through proteolysis or conformational changes. After disrupting and replacing the thiol group by water, MMPs can hydrolyze the pro-peptide. Thus, they are able to cleave the peptide bonds of their substrates. This regulatory mechanism is called cysteine switch. [121, 124-126, 133, 134] Some active MMPs can also positively regulate other pro-MMPs, for instances MMP-3 activates MMP-1 and MMP-9, MMP-14 influences MMP-2. Moreover Interleukin-1 (IL) positively regulates the expression of messenger ribonucleic acid (mRNA) of MMP-2, -3, -9 and-13. An enhanced expression of these MMPs is associated with increased bone matrix degradation. IL-6 supports mRNA expression of MMP-2, -3, -9 and -13, as well, but its influence is much less than that of IL-1. [121, 124-126, 133, 134]

Once activated, MMPs are further influenced by autodegradation, selective endocytosis or endogenous inhibitors. These specific endogenous inhibitors are the four TIMPs, TIMP-1, -2, -3 and -4. Effectively, requirements for inactivating MMPs are the following:

a) a functional group that is able to bind the active site of the zinc atom

b) at least one functional group capable of providing a bond with water

c) at least one side chain that interacts with the enzyme substrates through van der Waals forces. [121, 124, 125, 128, 133-135]

2.5.5. MMPs and the Growth Plate

The human skeleton is rich in ECM and the remodeling of ECM is central to both development and repair. For a long time, the ECM of the GP has been seen as a passive structure, its only function in scaffolding cell attachment, giving mechanical stability and transmitting forces. Accordingly, MMPs were simply regarded as `remodeling molecules´ of the ECM. [69, 70, 71, 121, 126] Recently, results of mouse knockout models gave evidence that MMPs are not only the most important degraders of ECM proteins. They also control the activity of local growth factors in the ECM and take part in regulation of cell migration. Remodeling of the ECM is widely regulated by the same enzymes that determine rate and effectiveness of skeletal development and repair. In the following, the MMPs -2, -3, -9 and -13 are discussed in detail, since they are important for the GP as well as for bone repair after fractures. [69-71, 121, 126]

2.5.5.1. MMP-2

MMP-2, also known as collagenase type IV or gelatinase A, degrades collagen type IV, the major structure component of the basement membranes, and is crucial to maintain balance between bone synthesis and resorption. [136-138]

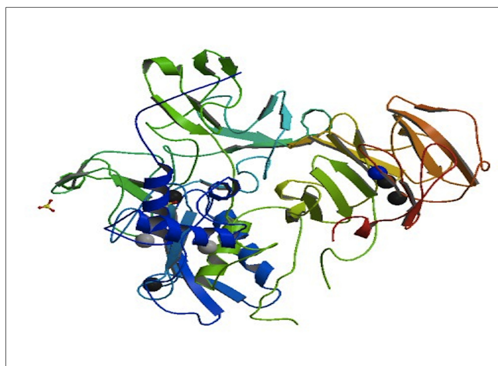


Fig. 5. MMP-2
(www.uscnk.us, accessed on 02/12/2011,
http://1.2.3.10/bmi/www.uscnk.us/img/Matrix-Metalloproteinase-2-%28MMP2%29-90100.jpg)

MMP-2 preferentially binds to TIMP-2 and TIMP-4. When MMP-2 binds with its carboxyl terminus to Integrin V3 or the MMP-14-TIMP-2 system, it translocates to the cell surface. Hereby, the catalytic site of MMP-2 becomes unoccupied. Thus, MMP-2 can be cleaved and activated. [107, 136-138] According to Sommerville et al. [121], MMP-2 is essential for processing an inductive factor required for both osteoblasts and osteoclasts. As a leading proteoglycan molecule of the ECM, Aggrecan provides osmotic properties needed to resist compressive loads. [107, 136-138] Martignetti et al. [139] showed in their study that in terms of the GP, mutations in the MMP-2 gene have been related to a multi-centric osteolysis and arthritis syndrome.

2.5.5.2. MMP-3

MMP-3 is the most potent proteoglycanase among the MMPs. This enzyme is also known as stromelysin-1 and has a rather broad substrate specificity, degrading the collagens type II, III, IV, IX and X as well as fibronectin, laminin, elastin and proteoglycans. [136-138]

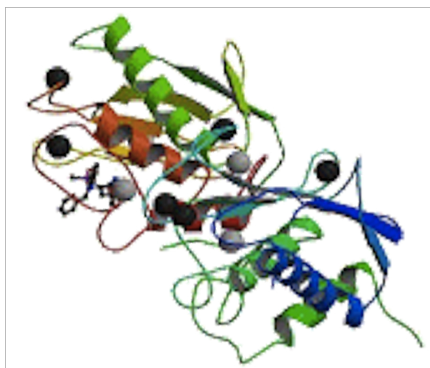


Fig. 6. MMP-3
(www.uscnk.us, accessed on 02/12/2011,
http://www.uscnk.us/img/Matrix-Metalloproteinase-3-%28MMP3%29-90101.jpg)

MMP-3 also activates other MMPs such as pro-MMP-1, -7 and -9. This quality makes MMP-3 important in the remodeling of connective tissue. MMP-3 regulates the connective tissue growth factor (CTGF) either by cleaving it into two parts or by binding to the CTGF promotor. CTGF is a key player of cartilage and bone regeneration. MMP-3 raises transcription and expression of CTGF that promotes proliferation, maturation, and hypertrophy of chondrocytes of the GP during endochondral ossification. [124, 140, 141] MMP-3 can also cleave E-cadherin and disrupts therefore the aggregation of cells and stimulates their invasion. Furthermore, stromelysin-1 can stimulate the release of a soluble form of the adhesion molecule L-selectin, can shed membrane-bound HB-EGF and can cleave Immunoglobuline G (IgG) proteins. The latter quality of MMP-3 is crucial since it inhibits the initiation of the complement cascade. Accordingly, MMP-3 helps to remove IgG from tissue that is inflamed or damaged. [69, 121, 134, 140, 146-148] According to Reich et al. [140], MMP-3 plays a crucial role in maintaining cartilage. However, a role in vascularization processes as seen in MMP-9 [66, 121, 134, 149-151] is not reported. MMP-3 is especially related to pathological conditions. According to Tchetverikov et al. [142], there is an elevated amount of synovial MMP-3 in acute knee injuries. Additionally, Lin et al. [143] showed increased levels of MMP-3 in the articular cartilage in the case of injuries that are

caused by mechanical stress. Asundi et al. [144] described that physiological mechanical stress impedes the expression of stromelysin-1 in the flexor tendons. Moreover Monfort et al. [145] discussed that mechanical loading also reduces the MMP-3 levels in healthy cartilage. Sasaki et al. [147] showed that MMP-3 in human osteoblasts becomes inducible by mechanical stimulation. This enzyme may therefore act as a central factor in remodeling human bone. The data of Sasaki et al. imply that stromelysin-1 is one of the key players that are responsible for collagenolysis of bone matrix surface after mechanical stimulation. [147]

2.5.5.3. MMP-9

MMP-9 is also known as collagenase type IV or as gelatinase B. Its substrates are the collagens type IV and V. Thrombospondins control activity levels of MMP-9 as well as MMP-2, TIMP-1 suppresses MMP-9 activity. Two chains of collagen type IV bind MMP-9, even if inactivated. This bond between MMP-9 and collagen type IV secures a high amount of gelatinase B, being quickly accessible for ECM remodeling. [66, 121, 134, 149-151]

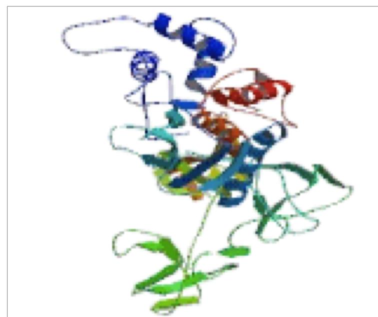


Fig. 7. MMP-9
(www.uscnk.us, accessed on 02/12/2011,
http://1.2.3.11/bmi/www.uscnk.com/img/Matrix-Metalloproteinase-9-%28MMP9%29-90553.jpg)

MMP-9 incites that unmineralized cartilage is dissolved by MMP-13. MMP-9 is especially needed to recruit osteoclasts into the intermittently mineralized cartilage of the HZ. The HZ expresses VEGF that connects itself to the ECM and becomes bioavailable through activation by MMP-9. In conclusion, MMP-9 is crucial in GP vascularization as well as ossification and is a key effector in remodeling of the ECM. [66, 121, 134, 149-151]

2.5.5.4. MMP-13

MMP-13 is also known as collagenase 3 and is the main degrader of collagen type II. It also mediates the degradation of collagens type III and X as well as Aggrecan and other cartilage proteoglycans. Like MMP-1, -8 and -14, this enzyme can efficiently cleave the fibrillar collagens types I, II and III in their triple-helical domains. [70, 121, 125, 152-157]

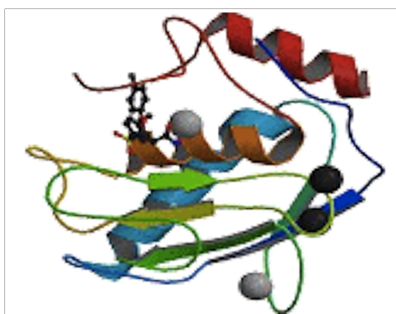


Fig. 8. MMP-13
(www.uscnk.us, accessed on 02/12/2011,
http://www.uscnk.com/img/Matrix-Metalloproteinase-13-%28MMP13%29-90099.jpg)

MMP-13 is involved in morphogenesis and pathophysiology of cartilage. MMP-13 also stimulates degradation of cartilage in the HZ and modifies endochondral ossification during long bone development. This enzyme is expressed by chondrocytes as well as osteoblasts and periosteal cells during fetal development. MMP-2, MT1-MMP and plasmin can activate proMMP-13. Expression of collagenase 3 is increased by TGF-β, but suppressed by BMP-2 and FGF-2 in hypertrophic chondrocytes. The suppression by FGF-2 leads to an inhibition of angiogenesis and enchondral ossification of the GP. [70, 121, 125, 152-157] Behonick et al. [82] demonstrate that MMP-13 plays an important role in normal remodeling of bone and cartilage during adult skeletal repair and that it may act directly in the initial stages of ECM degradation, prior to invasion of blood vessels and osteoclasts.

2.5.6. MMPs and Mechanical Loading

In animals it has already been proven that MMPs are mechanosensitive. However, there is no data available for mechanical loading influences on the expression of MMPs in chondrocytes of the human GP. Additionally, it is not known, which role MMPs play after a fracture occurred to the GP. [70, 74, 116, 117, 140, 144, 158]

3. AIM OF THE STUDY

Proper mechanical loading is essential for remodeling and repair of the human skeleton after a fracture occurred. The capability of postfracture remodeling is unique in the juvenile skeleton and thus, malpositioned fractures can be secondarily compensated. In growing bones corrections are depending on the growth potential of the affected GP. During remodeling, the ECM is of high functional importance. [70, 74, 116, 117, 140, 144, 158]

Several in vivo studies have examined the mechanobiology of the GP using animal models. Herein, it has already been demonstrated that the GP is highly responsive to mechanical stimuli and that MMPs are mechanosensitive. Moreover, these studies reported that under sustained compressive loading, the bone growth rate declines according to the Hueter-Volkmann principle. [116, 161, 162] However, there is no data available for the influences of mechanical loading on the expression of MMPs in the human GP. Additionally, it is not known, which role MMPs play after a fracture occurred. With more precise knowledge about how the GP and especially MMP-2, -3, -9 and -13 are influenced by mechanical stress, current treatment approaches could get improved. [70, 74, 116, 117, 140, 144, 158]

The aim of this study was to investigate for the first time the influence of mechanical loading on the expression of MMPs in chondrocytes of the human GP in vitro. We hypothesized that the capability of post-fracture remodeling strongly depends on the growth potential of the affected GP. Furthermore, we presumed that MMPs play an important role in conversing and degrading the ECM when chondrocytes mineralize and get restructured to bone. This study was focused on the MMPs-2, -3, -9 and -13, that play an important role in degrading the ECM. [69, 122-124, 159, 160]

4. MATERIALS AND METHODS

4.1. Cell Culture

4.1.1. Cell Source, Isolation and Culture

Supernumerary fingers and toes were obtained from children with polydaktylism at the time of surgical excision at the Department of Pediatric and Adolescent Surgery, Medical University of Graz. For the current study, four samples were taken and a two-pronged approach was applied. The study protocol was approved by the local Ethic Committee of the Medical University of Graz (file number: EK 20-344 ex 8/09).

Cell culturing was performed according to a well established local protocol. The GP samples were manually minced and dissected from soft tissue. Chondrocytes were isolated from the GPs of the supernumerary digits by sequential enzymatic digestion with 2 mg/ml Collagenase B (150 units/mg Collagenase B, Worthington Biochemical Corp., Lakewood, USA) and incubated at 37.5°C overnight. Following digestion, the cell suspension was filtered thoroughly through two layers of nylon grid with a pore diameter of 40 µm to remove undigested tissue fragments. Subsequently, the samples were washed twice with Phosphate buffered saline (PBS) (Invitrogen, Carlsbad, USA). Then, the suspension was centrifuged at 1300 rpm for 7 min, the cell pellet was re-suspended in PBS (Invitrogen) and the cell count was analyzed by Tryptan blue stain (Invitrogen). In 75 cm^2 flasks (Nunc Products, Denmark), $6x10^5$ cells were cultivated in 10 ml expansion medium (Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F-12), 1:1 supplemented with 5% Fetal Calf Serum (FCS), 1% Penicillin/Streptomycin and 0,1% Amphotericin B, all reagents Gibco/Invitrogen, Carlsbad, USA). After 24 h, the cells were rinsed with PBS (Invitrogen) to remove non-adherent cells and debris. During expansion of the cells, the culture flasks were kept at 37°C and 5% CO_2. Medium change was performed three times a week. If subconfluent to confluent, the cells were detached by TrypLE™ Express (Invitrogen). Following washing, the cells were re-seeded at a density of $8x10^3$ cells/cm^2.

4.1.2. Cell Seeding into Flexcell Plates

The following steps were performed for each sample separately.

The cell cultures were taken from the sterile cell culture incubator and the medium was aspirated. Then, the cultivated cells were rinsed with PBS (Invitrogen) to remove any medium residua that would interfere with the later added Trypsin (Invitrogen). After aspiration of the PBS (Invitrogen), TrypLE™ Express (all reagents Invitrogen) was added to dissolve the cell-cell-connections and the adhesion of the cells to the culture flask. Incubating at 37°C for approximately 5 min was applied, until the cells detached and floated. This was confirmed by visual inspection of the flasks through a microscope. Once the cells were detached, cell culture medium was added to stop the trypsin (Invitrogen) reaction. The cell solution was transferred to a falcon and centrifuged at 1300 rpm for 7 min, room temperature. After centrifugation, vital cells formed a pellet at the falcon bottom, dead chondrocytes were dispersed in the residual medium. It was aspirated and discarded. The pallets were suspended by means of a Vortex mixer and medium was added to the cell samples again. Subsequently, the cell suspension was mixed once more to ensure accurate cell counts.

For determining the number of cells per unit volume of the suspension, a hemocytometer, a microscope counting chamber, was used. Tryptan blue (Invitrogen) was mixed at equal parts with the cell probe. After vortexing, the suspension was introduced into one of the wells of the hemocytometer with a pasteur pipet. The charged counting chamber was then placed on the microscope stage and the counting grid was brought into focus at low power. To determine seeding density, the cell number counted in the hemocytometer was divided by four, then doubled and multiplied with the dilution volume. The probes were filled up with medium so that 300.000 cells could be seeded to the wells of the Pronectin-coated Flexcell plates (57.75 cm^2/plate; FlexCell International, Hillsborough, USA). Each Flexcell plate consisted of six wells. For each sample 4 wells were subjected to our loading pattern whereas 4 wells remained as control, as shown in Table 6. The Flexcell plates (FlexCell International) were maintained in the cell culture incubator at 37°C for 48 h to assure further growing and expansion of the cells.

Samples	Mechanical loading (ML)			Control (Ctrl)		
	1 h	4 h	24 h	1 h	4 h	24 h
E10	4 wells	4 wells	4 wells	4 wells	4 wells	4 wells
E12	4 wells	4 wells	4 wells	4 wells	4 wells	4 wells
E13	4 wells	4 wells	4 wells	4 wells	4 wells	4 wells
E14	4 wells	4 wells	4 wells	4 wells	4 wells	4 wells

Tab. 6. Flexcell plate subjected to mechanical loading

4.2. Mechanical Loading

After 48 h of incubation was performed. Then, the chondrocytes were subjected to repetitive mechanical loading, using a Flexcell Tension Plus System, FX-3000T (Flexcell International, Hillsborough, USA). The Flexell Tension Plus System applies a defined, controlled, cyclic deformation to growing cells in vitro. All for samples (E10, E12, E13, E14) underwent the same regime, as shown in Table 7.

For each sample (E10, E12, E13, E14)	Mechanical Loading 1 Hz, 10% elongation	Control
1 h	4 wells	4 wells
4 h	4 wells	4 wells
24 h	4 wells	4 wells

Tab. 7. Loading regime

The flexible bottom of the culture plates (FlexCell International) is deformed due to a vacuum. The experiment was carried out in the cell culture incubator at 37°C. Control samples were cultivated in the same culture plates but not mechanically loaded. Pictures were taken of each loaded and unloaded passage at each interval, from the margin and the center of the well with a magnification of four (Power High Load (PHL) filter) and ten times (Phase 1 (Ph1) filter).

4.3. RNA Isolation

Following the mechanical loading, total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions.

The medium was aspirated and the cells were washed twice with PBS (Invitrogen). According to RNeasy Kit protocol, Buffer RLT (Qiagen) was added to the monolayer cells for lysis. Then, the cell lysate was collected with a rubber policeman and pipetted into a 15 ml falcon, trying to avoid sudsing. The cell lysat was mixed by passing the lysate at least five times through a 20-G needle, fitted to a syringe - since incomplete homogenization would lead to significantly reduced yields and could cause clogging of the RNeasy mini spin colum. Afterwards, 70% ethanol was added to the homogenized lysate and mixed well by pipetting. The sample was applied to an RNeasy mini spin column sitting in a collection tube and centrifuged for 15 sec at 10.000 rmp. Discarding the flow-through, the collection tube was re-used. Buffer RW1 (Qiagen) was pipetted onto the RNeasy spin column and the sample was centrifuged for 15 sec at 10.000 rmp to wash. Again discarding the flow-through, Buffer RPE (Qiagen) was pipetted onto the RNeasy column and centrifuged for 15 sec at 10.000 rmp to wash. Discarding the flow-through, Buffer RPE (Qiagen) was added onto the RNeasy spin column and centrifuged at 10.000 rmp for 2 min to dry the RNeasy membrane. Then, the RNeasy spin column was transferred into a new collection tube and the old one was discarded together with the flow-through. This time, it was centrifuged at full speed, 15.000 rmp, for 1 min. The RNeasy spin column was inserted into a new collection tube, eluted with RNase-free water (Qiagen) and centrifuged for 1 min at 10.000 rmp. A second elution step was performed, using the flow through to elute into the same collection tube and thus, maximizing the RNA yield.

To measure the quantity of RNA in each sample, a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) was used. Hereby, the nucleic acid was quantified using UV absorption at 260 and 280 nm. The concentration of nucleic acid was determined using the Beer-Lambert law, predicting a linear change in absorbency with concentration. Therefore, an A260 reading of 1.0 is equivalent to about 40 µg/ml of RNA and

the optical density at 260 nm is used to determine the RNA concentration in a solution. The absorbency ratio at 260 and 280 nm was used to assess purity of an RNA preparation. Pure RNA has an A260/A280 value of 1.9-2.2. The samples were stored at -80°C until further use.

4.4. cDNA Synthesis

For further use as a template in qRT-PCR, first strand complementary deoxyribonucleic acid (cDNA) was synthesized by means of the RevertAid HMinus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) according to the manufacturers instructions.

To avoid DNA contamination, DNA was digested with 1 U DNase (Fermentas) per µg RNA. RNA was transcribed reversely using RevertAid cDNA Synthesis Kit (Fermentas). Designated RNA values were used to dilute RNA with MiliQWater. Afterwards, 10x DNase I reaction buffer (Fermentas) with $MgCl_2$ and RNase-free DNase I (Fermentas) were added and put into sterile RNase-free tubes. Incubation at 37°C for 30 min was performed. Further, 25 mM EDTA was added to each sample.

Positive and negative control reactions were used to verify the results of the first strand. Therefore, a No Template negative Control (NTC) and a Reverse Transcriptase minus (RT-) were prepared. The NTC is important to assess for reagent contamination. It contained every reagent for the reverse transcription reaction except for RNA template. RT- is important as negative control in qRT-PCR reactions to assess for genomic DNA contamination of the RNA sample. It contained every reagent for the reverse transcription reaction except for the RT enzyme.

Template RNA was added to Random Hexamer Primer (Fermentas), 5x Reaction Buffer (Fermentas), RiboLock RNase Inhibitor (Fermentas), 10 mM dNTP mix (Fermentas) and RevertAid H Minus (Fermentas). The samples were incubated at 25°C for 5 min, then at 42°C for 60 min and finally at 70°C for 5 min. Afterwards, the cDNA samples were stored at -20°C until further use.

4.5. qRT-PCR

The investigated chondrocytes were mechanically loaded for different periods of time: 1 h, 4 h and 24 h. To examine the effects of the various periods of mechanical loading on the expression behavior of MMPs, mRNA of MMP-2, -3, -9 and -13 was quantified by qRT-PCR. The examination by qRT-PCR was normalized to the expression levels of the house-keeping gene GAPDH. GAPDH is one of the most consistent and commonly used house-keeping genes for the normalization of target gene expression data. It is important that the housekeeping gene does not change in relative abundance due to the conditions of the experiment. Therefore, we used GAPDH, that plays an enzymatic role in glycolysis, since we assumed that mechanical loading has no influence on the process of glycolysis.

Preparing the reagent for the qRT-PCR, the SYBR Green Taq ReadyMix Kit (Invitrogen, Carlsbad, USA) was used according to the manufacturers instructions. Sequences of primers for the reference gene GAPDH and target genes (MMP-2, -3, -9 and -13) were all provided by MWG, Ebersberg, Germany. The PCR template source was first-strand cDNA (50ng/µl). A 1:12.5 dilution of the cDNA was performed by adding distilled water. The master mix was prepared by adding same amounts of forward and reverse primer as well as water to SYBR Green (Invitrogen). SYBR Green (Invitrogen) is a fluorescent dye that monitors DNA synthesis. cDNA was then added to the master mix and vortexing was conducted. The samples were analyzed in triplicates, as shown in Table 8 on the following page. After pipetting each sample in the corresponding well, the plate was sealed with a LC480 sealing foil and mounted to the Light Cycler LC480 instrument (Roche Diagnostics, Vienna, Austria).

	MMP-2	MMP-3	MMP-9	MMP-13
	1 - 2 - 3	1 - 2 - 3	1 - 2 - 3	1 - 2 - 3
A	X ML 1 h	X ML 1 h	X ML 1 h	X ML 1 h
B	X Ctrl 1 h	X Ctrl 1 h	X Ctrl 1 h	X Ctrl 1 h
C	X ML 4 h	X ML 4 h	X ML 4 h	X ML 4 h
D	X Ctrl 4 h	X Ctrl 4 h	X Ctrl 4 h	X Ctrl 4 h
E	X ML 24 h	X ML 24 h	X ML 24 h	X ML 24 h
F	X Ctrl 24 h	X Ctrl 24 h	X Ctrl 24 h	X Ctrl 24 h
G	NTC	NTC	NTC	NTC
H				

Tab. 8. Well layout for qRT-PCR – X stands for the corresponding sample.

4.6. Agarose Gel Electrophoresis

Prior to agarose gel electrophoresis 1x Tris-Acetate-EDTA (TAE) (Carl Roth GmbH, Karlsruhe, Germany) loading buffer was prepared by adding up TAE stock (Carl Roth) with MilliQ Water according to the manufacturers instructions. The loading buffer was poured into the casting gates and the combs were placed in the gel casting tray on level surface.

To pour a gel, agarose powder (Biozyme Scientific GmbH, Oldenburg, Germany) was mixed with 1x TAE buffer (Carl Roth) to the desired concentration of 2 %. Then, the solution was heated in a microwave oven for about 2 min on full power until completely melted. At this point, Gel Red Nucleic Acid Stain (Fermentas) was added to the solution. After cooling the solution to 60°C, it was poured into the casting tray containing a sample comb. All bubbles were removed and the gel was allowed to polymerize at room temperature for about 40 min until the gel was slightly opaque. After solidification of the gel, the comb was removed and the gel was inserted into the gel tank of the electrophoresis chamber, covered with 1x TAE buffer (Carl Roth) for not drying out. Further, 50 base pairs (bp)

DNA-ladder (Carl Roche, Karlsruhe, Germany) was used for marker mixture and was solubilized in 1x concentrated sample buffer (Tris-Chlor, pH 7.5, 10 mM, EDTA 10 mM, glycerol 10%, orange G 0.4 mg/ml). This gel loading buffer resembles a 6x diluted solution of gel loading buffer. Then, the PCR products were mixed with 5 µl of the 6x gel loading dye (Carl Roche) to visualize cDNA after electrophoresis and then pipetted into the sample wells of the gel. The lid and power leads were placed on the apparatus. A current of 70 V was applied until the blue dye had migrated as far as ⅔ the length of the gel. The gel was run for about 90 min from negative to positive. cDNA migrates towards the positive electrode. The distance the cDNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes. When adequate migration has occurred, cDNA fragments were visualized by placing the gel in a BioRad Quantity One (BioRad Laboratories, Hercules, USA).

4.7. Data Analysis

Samples were studied in triplicates. Data was analyzed with SPSS 19.0 software (SPSS Inc., Chicago, USA).

The comparative threshold cycle (CT) method, also known as $2^{-\Delta\Delta C}$ method, was used to calculate relative quantities (RQ) of expression level of the gene of interest relative to a reference sample for sample comparison. CT is reversely related to the amount of target molecules in the reaction. This yields a precise fold change value for each gene of interest in each sample. Purpose of normalization with the selected housekeeping gene GAPDH is to correct differences in the amount of nucleic acid input. The basic principle when using endogenous genes to normalize data is that they have to be expressed at constant levels across the samples and that their expression does not vary under the experimental conditions tested.

For a given well, CT represents the PCR cycle at which the software first detected a noticeable increase in reporter fluorescence above a baseline signal. ΔCT is the difference be-

tween CT of a sample assay (target gene) and CT of the corresponding endogenous reference (normalizing factor).

$\Delta CT = CT_{target\ gene} - CT_{housekeeping\ gene}$

Then, normalized ΔCT data is used for calculating the relative gene expression fold change using a selected reference sample (control group). This is, for a given cDNA target, the difference between the ΔCT value of a target sample and the average ΔCT for the control sample. Further, statistical analysis was performed to provide standard deviations (SD) for gene expression comparison between the samples.

$\Delta\Delta CT = \Delta CT_{target\ group} - \Delta CT_{control\ group}$

The ΔΔCT value is then used to calculate the expression fold change by equation.

Fold change (RQ) = $2^{-\Delta\Delta CT}$

Log10RQ = Log10 $2^{-\Delta\Delta CT}$

Log10RQ = 0 → no expression

Log10RQ = 1 → target group is expressed 10x greater than in the control group

Log10RQ = -1 → target group is expressed 10x less than in the control group

To address the question whether significance between the expression of MMP-2, -3, -9 and -13 was reached the non parametric Wilcoxon signed rank test was applied. This tests analyzes the median difference in paired data. P values less than 0.05 were considered statistically significant.

5. RESULTS

5.1. Cell Culture

GP cells from four different patients were selected for this study. Mean age ±standard deviation (SD) was 1.14±0.68 years (range from 0.25 to 2.3 years), independent of sex. A two-pronged approach was applied for evaluation and statistical analysis of the data.

The isolated chondrocytes showed good expansion ability and confluence of the cells in the culture flasks occurred in appropriate time. 300.000 cells per well were seeded into the wells of Pronectin-coated Flexcell plates and incubated for 48 h.

5.2. Mechanical Loading

Dynamic mechanical loading at 1 Hz frequency and 10% elongation was conducted. After 1 h of mechanical loading (Fig. 9a-b) the cells showed little changes in their orientation compared to control (Fig. 9c-d). Cell rarefaction and slight changes in shape were already visible. The cells in the centre of the well did not lie as close together as that of the control, whereas the cells at the margin showed enhanced networking.

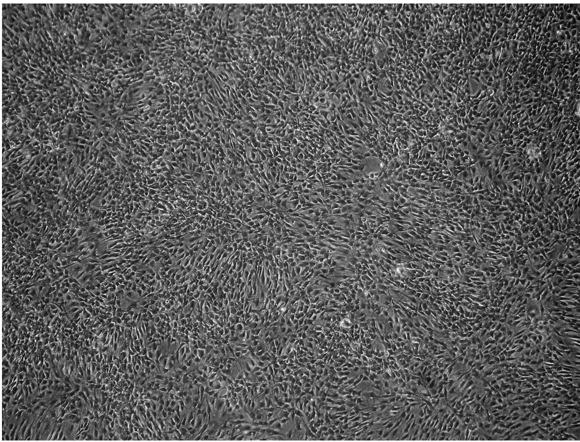


Fig. 9a. ML, 1 h, 4x magnification

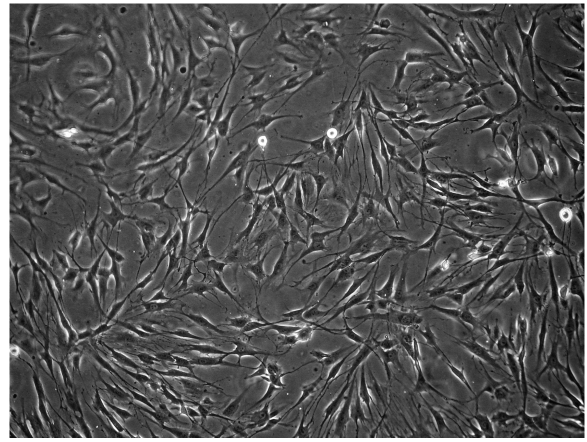


Fig. 9b. ML, 1 h, 10x magnification

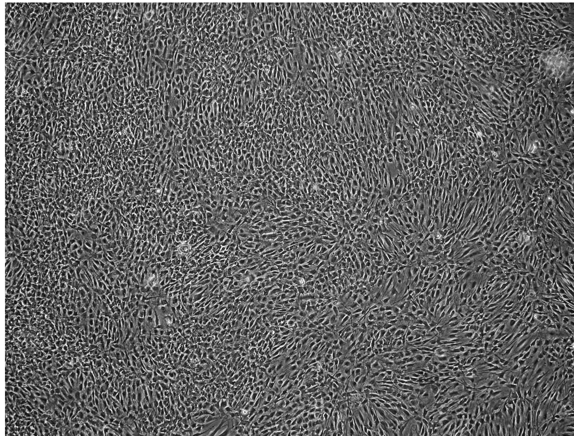


Fig. 9c. Ctrl, 1 h, 4x magnification

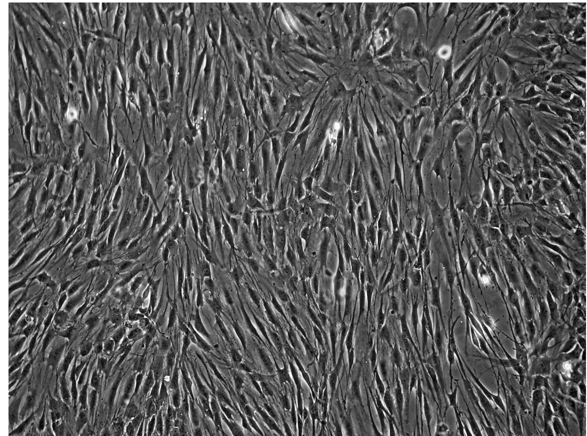


Fig. 9d. Ctrl, 1 h, 10x magnification

At 4 h of mechanical loading (Fig. 9e-f) it could be seen even more clearly that the cells were seeded thinner in the center of the well and that they showed even stronger networking at the margin of the well compared to the control (Fig. 9g-h). The cells started to align in clusters.

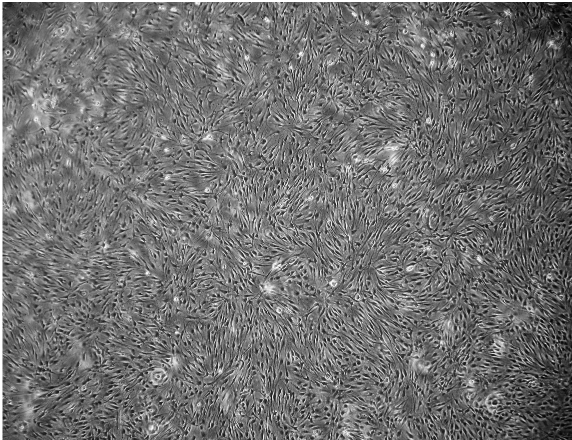


Fig. 9e. ML, 4 h, 4x magnification

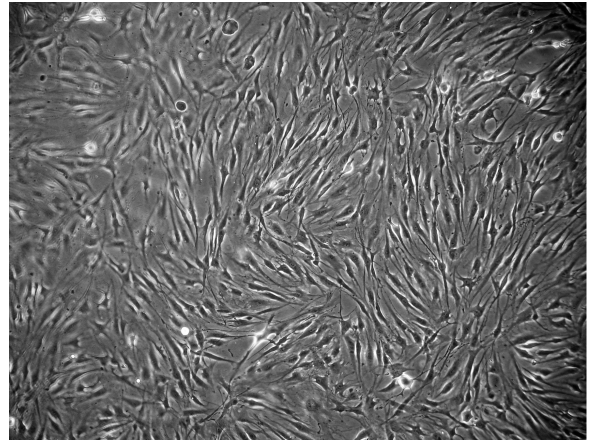


Fig. 9f. ML, 4 h, 10x magnification

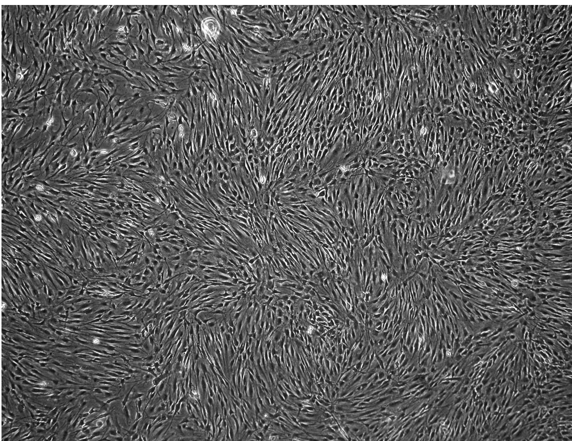


Fig. 9g. Ctrl, 4 h, 4x magnification

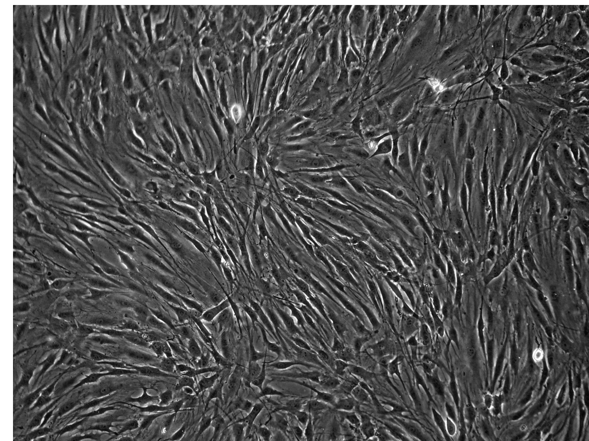


Fig. 9h. Ctrl, 4 h, 10x magnification

After 24 h of mechanical loading (Fig. 9i-k) the cells had exceedingly thinned out at the center of the well and were not clearly recognizable anymore. At the margin of the well the cells were highly linked compared to the control (Fig. 9l-m). The shape of the cells changed from a polygonal to spindle form.

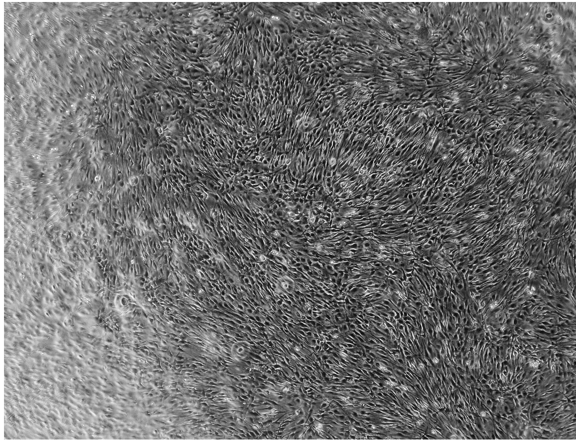


Fig. 9i. ML, 24 h, 4x magnification

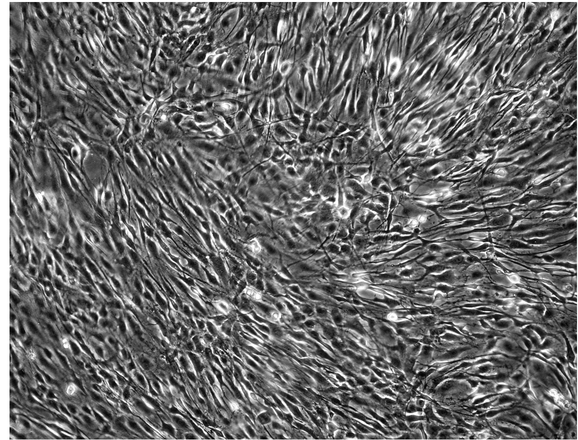


Fig. 9k. ML, 24 h, 10x magnification

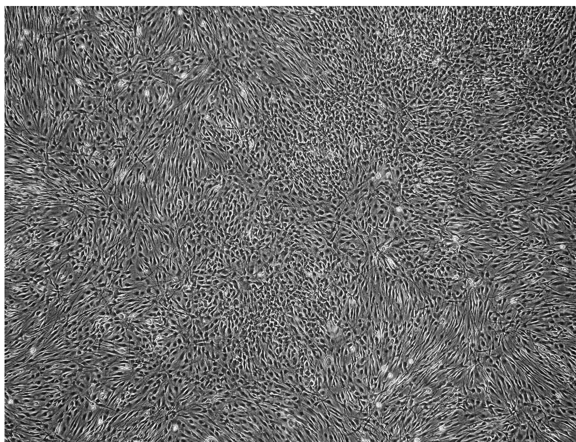


Fig. 9l. Ctrl, 24 h, 4x magnification

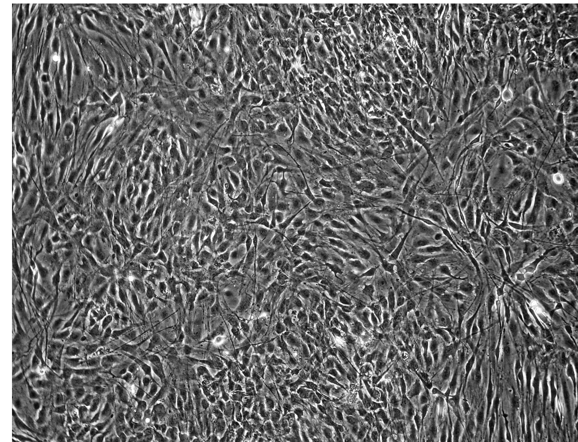


Fig. 9m. Ctrl, 24 h, 10x magnification

In contrast, non-loaded cells showed little changes in their orientation at the various time points. Additionally, less cells floated off in comparison to the corresponding sample.

5.3. qRT-PCR

Pure RNA has an A260/A280 value of 1.9-2.2. All of our samples were in the designated range. (Tab. 9, see chapter 9) MMP-2, -3, -9 and -13 mRNA expression was measured in the loaded groups and controls after 1 h, 4 h and 24 h of the experiment. Enzyme production was adequate. (Tab. 10 and 11, see chapter 9)

MMP-9 expression was detected in a range of high uncertainty (CP > 35). Therefore, no further calculations were performed. Analyzed by Wilcoxon signed rank test, qRT-PCR showed differing expression patterns of MMP-2, -3 and -13 after 1 h, 4 h and 24 h of mechanical loading with 1 Hz frequency and 10% elongation. Control group was normalized on the value 1. Mechanical loading significantly increased the expression of MMP-2 as well as MMP-3 and up-regulated that of MMP-13. For MMP-9 no specific mRNA signal was generated in qRT-PCR.

MMP-2 showed peak expression after 1 h and was at this time and after 24 h significantly (∗) up-regulated ($p<0.05$).

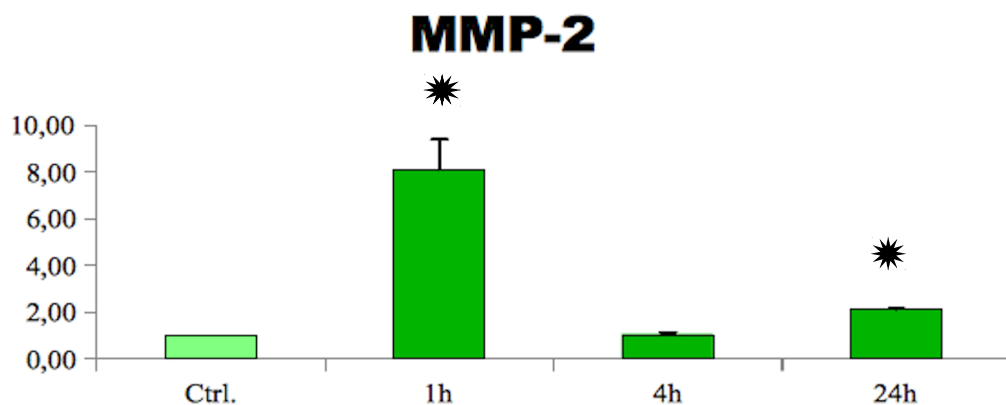


Fig. 10a. RQ results for MMP-2 according to Wilcoxon signed rank test (∗ indicates significant difference from control)

MMP-3 expression increased steadily over time and was significantly (∗) up-regulated at 24 h, with regard to the control group ($p<0.05$).

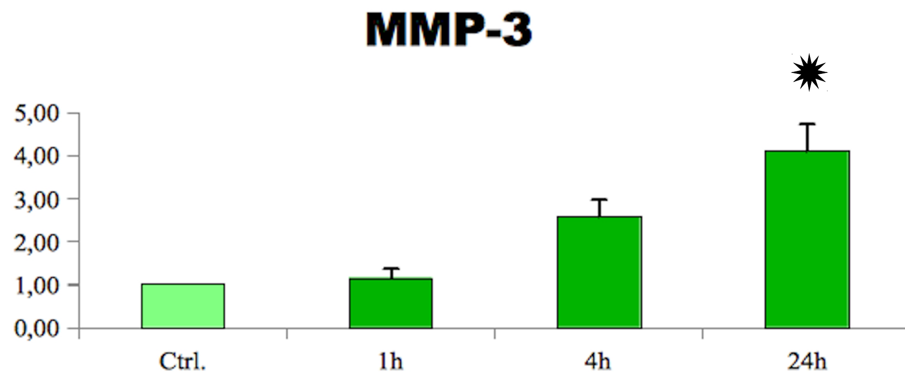


Fig. 10b. RQ results for MMP-3 according to Wilcoxon signed rank test (∗ indicates significant difference from control)

MMP-9 expression was detected in a range of high uncertainty (CP > 35) in loaded and control samples. Although melting curve of MMP-9 did not form a single peak, it formed base pairs in 2% agarose gel. Therefore, no further calculations were performed for MMP-9.

MMP-13, that also plays an important role in normal remodeling of bone, did not show significant changes. MMP-13 expression gradually increased over time but never reached significant values.

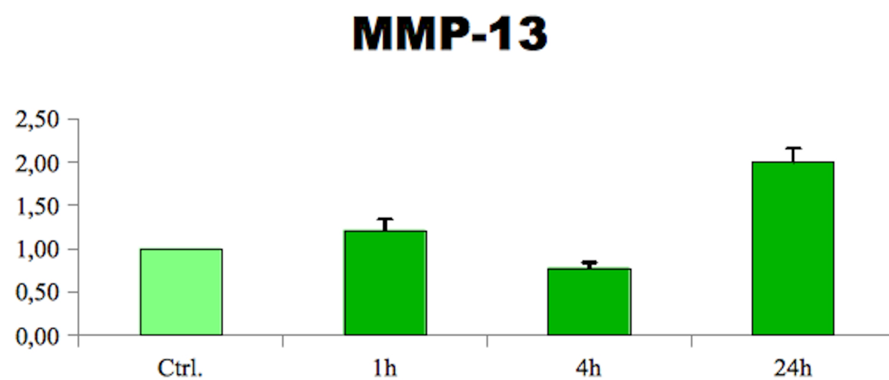


Fig. 10c. RQ for MMP-13 according to Wilcoxon signed rank test

5.4. Agarose Gel Electrophoresis

Below, the visualization of the DNA fragments can be seen. The most luminous bar of the marker shows 250 bps. The used DNA-ladder gives bars in steps of 50 bps.

Key:

GR = Marker
a = Mechanical Loading, 1 h
b = Control, 1 h
c = Mechanical Loading, 4 h
d = Control, 4 h
e = Mechanical Loading, 24 h
f = Control, 24 h

Since MMP-9 expression was detected in a range of high uncertainty (CP > 35), no further calculations were performed. However, it formed single base pair bands in agarose gel.

E14, GAPDH, 1 Hz, 10% elongation

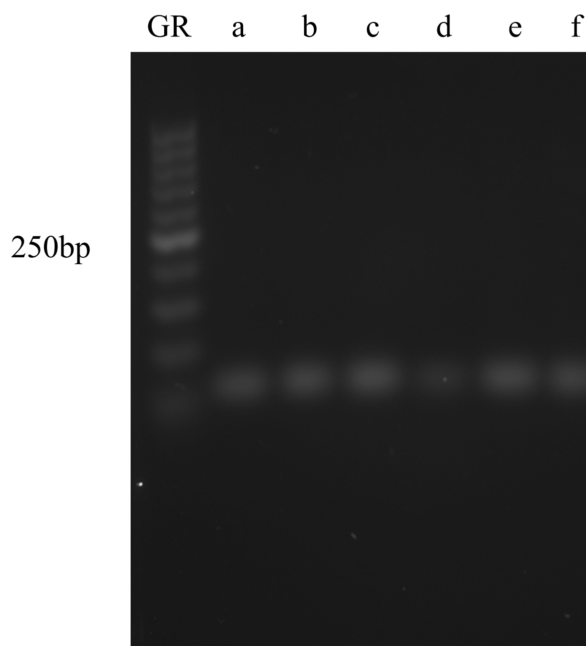


Fig. 11a. Agarose gel of GAPDH

E14, MMP-2, 1 Hz, 10% elongation

GR a b c d e f

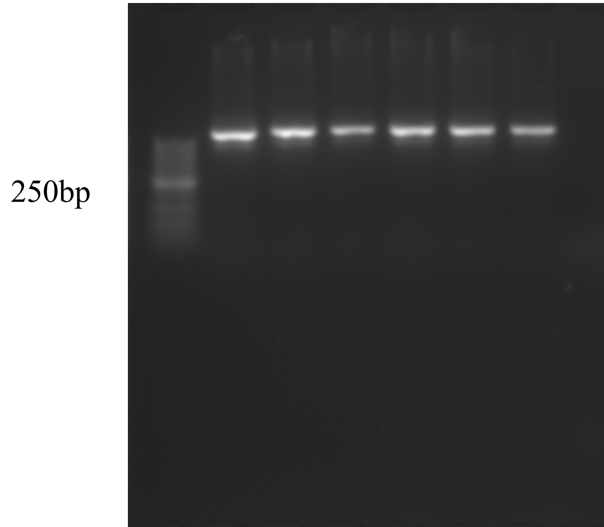


Fig. 11b. Agarose gel of MMP-2

E14, MMP-3, 1 Hz, 10% elongation

GR a b c d e f

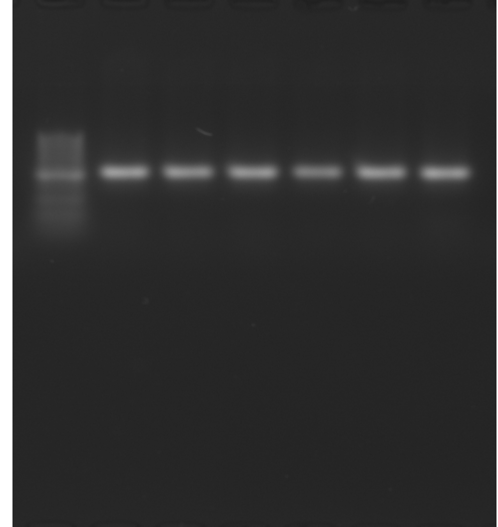


Fig. 11c. Agarose gel of MMP-3

E14, MMP-9, 1 Hz, 10% elongation

GR a b c d e f

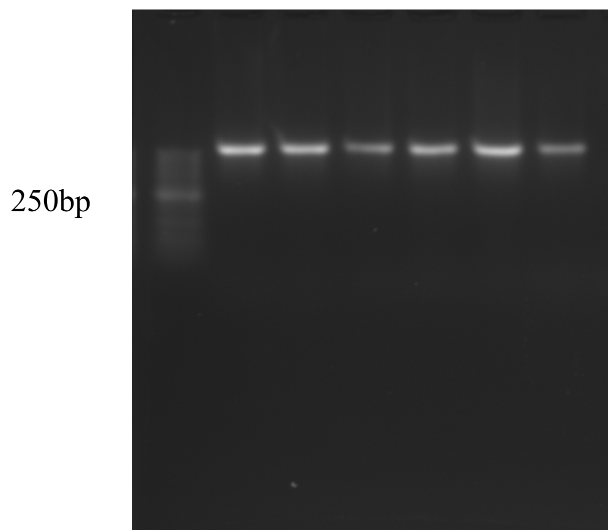


Fig. 11d. Agarose gel of MMP-9

E14, MMP-13, 1 Hz, 10% elongation

GR a b c d e f

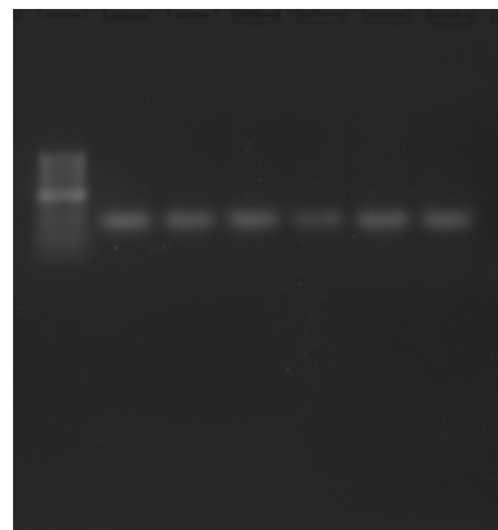


Fig. 11e. Agarose gel of MMP-13

6. DISCUSSION

The present study showed for the first time that mechanical loading has an influence on the expression of MMPs in chondrocytes of the human GP in vitro. Subjected to mechanical loading of 10% elongation and 1 Hz, the expression of MMP-2 was significantly ($p < 0.05$) up-regulated after 1 h and 24 h compared to control. The expression of MMP-3 was significantly ($p < 0.05$) up-regulated after 24 h. Although not statistically significant, expression rate of MMP-13 was increased after 1 h and 24 h. No changes were observed in the expression of MMP-9. To our best knowledge, there has been no previous study that investigated the influence of mechanical loading on the expression of MMPs-2, -3, -9 and -13 in cultured chondrocytes of the human GP. The present study addresses the essential role of MMPs in the post-fracture remodeling process and examines it by qRT-PCR after various periods of mechanical loading. The fact that human chondrocytes and not cells from animals are used, strengthens the value of this study. Since human chondrocytes act according to known biologic variability, the listed data are of better comparability to human conditions than data of animal models.

Mechanical loading of 1 Hz and 10% elongation for 1 h, 4 h and 24 h were chosen as loading parameters since their contiguity to slightly higher than physiological conditions in the human skeleton

In the present study we chose the above mentioned loading pattern since it represents slightly higher locomotion than under physiological conditions. [110, 140, 163-164] We aimed to mimic slightly higher than physiologic loading conditions of the human skeleton and the GP respectively during daily life as this might be the situation when bone remodeling occurs. The growth plate and its molecular mechanisms have been investigated under various loading conditions in former studies. [110-113, 116, 144, 162] To our best knowledge, Reich et al. [74, 140, 158] were so far the only research group that designed study models with MMPs and the GP. Reich et al. [74] conduct their experiments with loading of 10% body weight over 2 and 4 days. Asundi et al. [144] apply cyclic loads at 2, 3, 4 and 5 MPa for 18 h. Additionally, Cancel et al. [116] apply static compressive loads at 0.2 MPa for 2 weeks. Stokes et al. [111-113, 162] vary between 60% body weight of the studied ani-

mals and 0.1 to 0.2 MPa and apply mechanical loading over periods of 1, 3, 5, 7 and 8 weeks. However, Valteau at al. [161] use 0.2 MPa at 0.1 Hz over 15 days, Sun et al. [167] 2% axial strain for 1 h, Jin et al. [168] 1.6 Pa for 30 min to 24 h, Honda et al. [169] 17 kPa at 30 cycles/min for 12 h and Ueki et al. [163] 2 kPa at 2, 30 and 150 cycles/min. Ueki et al. describe these loading frequencies as low, moderate and high. This is consistent with our study design, that applies slightly higher than moderate loading parameters of 1 Hz. In vivo experiments with animals have already shown that the GP is highly responsive to mechanical loading. [110-117, 140, 144, 161-163] This is subjected to type and loading frequency. [70, 163] The result is an enhanced metabolism of GP chondrocytes with increasing intensity. [163] The latter is important due to the fact that compression inhibits and tension accelerates growth in accordance to the Hueter-Volkman principle. [111] However, these in vivo studies investigating the effects of mechanical loading show some shortcomings regarding normalizing or matching in terms of average stress. Cancel et al. [116] examined the expression of MMP-3 and -13 only, with one loading regime at 0.2 MPa for a period of 2 weeks. Additionally, Sergerie et al. [70] applied only one loading magnitude of 0.1 Hz and one loading period of 48 h. Stokes et al. [111] and Reich et al. [140, 158] conducted their experiments with loading schemes of 60% and 10% bodyweight of their study animals. The result of these studies are hardly comparable due to different loading design, differing animal species and age of animals. Furthermore, these in vivo studies [70, 111, 116, 140, 158] were conducted with mammalian chondrocytes. In contrast, our in vitro study demonstrates the central effect of mechanical loading on the expression of MMPs on chondrocytes of the human GP.

Mechanical loading of 1 Hz and 10% elongation up-regulates mRNA expression of MMP-2 significantly after 1 h and 24 h

Reich et al. [74] show that slightly increased mechanical strain of an additional 10% body weight increases MMP-2 expression in young chicken. In another in vivo avian study, Reich et al. [140] consolidate their prior findings while 4 days of mechanical loading up-regulate MMP-2 expression, particularly in the proliferative zone of the GP. Jin et al. [168] support these findings, showing that mechanical strain up-regulates various MMPs in mammalian chondrocytes in vitro. In the present study MMP-2 is significantly up-regulated after 1 h and 24 h ($p<0.05$). This result is consistent with the findings of Reich et al. [74,

140], since they show that the expression of MMP-2 is significantly up-regulated by mechanical loading. MMP-2 provides osmotic properties needed to resist compressive loads [136-138] and is known to be expressed by osteoclasts [69]. We assume that the significantly up-regulated expression of MMP-2 in our study is due to the fact that the chondrocytes try to resist these compressive loads and therefore, protect themselves.

Mechanical loading of 1 Hz and 10% elongation up-regulates mRNA expression of MMP-3 significantly after 24 h

MMP-3 has a rather broad substrate specificity. Thus, it is able to cleave basement membrane proteins including proteoglycans, laminin, fibronectin and collagen type II. [128] Accordingly, MMP-3 is a crucial factor in degrading the ECM. This leads to hypertrophying chondrocytes, the metabolism becomes catabolic. Effects of mechanical loading on the expression of MMP-3 have been shown in numerous studies. Monfort et al. [145] point out that physiological loading conditions reduces mRNA levels of MMP-3 in healthy human cartilage. Asundi et al. [144] describe the effects of 18 h of dynamic loading with physiologic parameters on the expression of MMP-3 mRNA in tendon explants of rabbits. In Asundi`s study MMP-3 is significantly reduced compared to stress shielded controls. Thus, Asundi concludes that MMP-3 may be very sensitive to mechanical loading. MacLean et al. [165, 166] study the influence of magnitude and frequency of mechanical loading on MMP-3 expression on rat vertebral disks. In contrast to Asundi, these authors show that increasing magnitude and frequency result in higher expression levels of MMP-3. Hereby, MacLean supports the findings of Honda et al. [169] that show the same expression pattern and up-regulation of MMP-3 in cultured rabbit chondrocytes under cyclic stretching. Reich et al. [74, 140, 158] state that mechanical load down-regulates the expression of MMP-3 in the GP of chicks in vivo and in vitro after 4 days, whereas release from load is followed by its up-regulation. In our study, the expression of the ECM enzyme MMP-3 is significantly up-regulated ($p<0.05$) after 24 h compared to control. This is in contrast with the findings of Reich et al. [74] and might be due to the fact that our study design applied shorter periods of mechanical loading. However, since MMP-3 is associated with cartilage injuries related to mechanical stress [142-145] and with functions to maintain cartilage itself [140] its continuously increasing expression rate in our experiment seems to be reasonable.

Mechanical loading of 1 Hz and 10% elongation shows no detectable change in mRNA expression of MMP-9

Reich et al. [74] show that an additional 10% body weight enhances the expression of MMP-9 – a key player in GP vascularization, apoptosis of hypertrophic chondrocytes [66] and activator of growth factors from the matrix [149] – by 50% in the avian model. In another study, Reich et al. [140] show that release from load up-regulates MMP-9 expression after 4 days. In the our study MMP-9 expression was detected in an unspecific range in qRT-PCR. However, the agarose gel confirmed specific qRT-PCR reaction. In contrast to Reich we could not show changes in levels of MMP-9 expression. MMP-9 is crucial in bone remodeling, GP ossification and also in ECM remodeling. [66, 121, 134, 149-151, 170] We therefore hypothesize that specifity in MMP-9 expression occurs only after longer periods of mechanical loading, as Reich et al. show in their study [74, 140].

Mechanical loading of 1 Hz and 10% elongation does not show any statistically significant mRNA expression rates of MMP-13

MMP-13 is an enzyme participating in cleavage of collagen type II and X, aggrecan and other proteoglycans. [153] Variation in expression of these proteins might lead to an alteration of the molecular structure of the GP and consequently, to modified mechanical features. Reich et al. [74] show an increase of 50% in MMP-13 mRNA expression due to mechanical loading when compared to control groups in avian GPs. This is consistent to a subsequent study [140], that shows a similar up-regulation in MMP-13 expression, especially in hypertrophic chondrocytes and the hypertrophic zone of the GP. In contrast, the in vivo study of Cancel et al. [116], in which the effects of compressive loading on molecular factors of the ECM in the GPs of rats are investigated, obtains no significant changes in MMP-13 mRNA expression. Interestingly, in our study MMP-13 expression does not show any significant changes neither, although its expression pattern is similar to that of MMP-2 with peaks after 1 h and 24 h of mechanical loading. We suppose that a period of more than 24 h of mechanical loading would raise MMP-13 expression and it might be possible that the results would show significance with a higher amount of samples. According to our results, we still hypothesize that MMP-13 plays a role in bone remodeling, nonetheless. This hypothesis is supported by the fact that MMP-13 works synergistically with MMP-9 in degrading ECM proteins. [149, 156]

Limitations of our study

The present study is limited by its in vitro approach and that mRNA expression is examined only. Further limitations include its focus on only one frequency level, one elongation level, limited periods of loading and a small sample size. Additionally, it is still not known what type or combination of stimuli in vivo reflects in vitro status best. Neither is known which loading pattern best resembles physiologic conditions in a post-fracture GP. Therefore, further research is needed and might be of interest regarding the following four aspects:

- further dynamic loading patterns (variation of load and frequency),
- variations of loading parameters not only in physiological but also under pathological conditions,
- effects of longer periods of dynamic loading, e.g. one week or two weeks and
- comparative effects of the loading parameters to evaluate related protein and gene expression involved in the biology of the GP.

There is a discrepancy between findings in animal models and human models regarding chondrocyte activation and bone remodeling by mechanical loading. Our study underlines the important role of MMPs in loading response in vitro and is designed to clarify their roles regarding the ECM in human GPs under mechanical loading. Usage of samples from human donors, as opposed to mammalian samples, provides results that are more relevant for understanding human physiopathology. Nevertheless, physiological variability can be regarded as limiting factor as well, since it makes it more difficult to obtain precise, reproducible results. Further experiments are needed to investigate the effects of mechanical loading on chondrocytes of the human GP and its effects on bone remodeling.

7. CONCLUSION

To sum up, this study shows a temporal differing expression of MMP-2, -3, -9 and -13 after various stages of mechanical loading:

- expression of MMP-2 was significantly up-regulated after 1 h and 24 h,
- expression of MMP-3 was significantly up-regulated after 24 h,
- expression of MMP-9 was detected in an unspecific range,
- expression of MMP-13 increased gradually over time but never reached significant values.

According to our results we hypothesize that these MMPs might also play an important role during post-fracture remodeling. Our findings provide a basis for future experiments focusing on the role of MMP-2, -3, -9 and -13 regarding the molecular mechanisms of remodeling in the human GP. According to our best knowledge our study is the first to investigate in vitro effects of dynamic mechanical loading on the expression of MMPs in chondrocytes of the human GP. In conclusion, our study gives new insights in the molecular aspects of human GPs to dynamic loading in vitro.

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9. APPENDIX

Tab. 9. A260/A280 values of pure RNA

Sample ID	A260/A280
E10 ML 1 h	1,97
E10 Ctrl 1 h	2
E12 ML 1 h	2,11
E12 Ctrl 1 h	2,09
E13 ML 1 h	2,01
E13 Ctrl 1 h	2,04
E14 ML 1 h	2,11
E14 Ctrl 1 h	2,14
E10 ML 4 h	2,04
E10 Ctrl 4 h	2,04
E12 ML 4 h	2,14
E12 Ctrl 4 h	2,11
E13 ML 4 h	2,05
E13 Ctrl 4 h	2,02
E14 ML 4 h	2,16
E14 Ctrl 4 h	2,06
E10 ML 24 h	2,02
E10 Ctrl 24 h	2,05
E12 ML 24 h	2,09
E12 Ctrl 24 h	2,09
E13 ML 24 h	2,04
E13 Ctrl 24 h	2,05
E14 ML 24 h	2,1
E14 Ctrl 24 h	2,09

Tab. 10. Values of mRNA expression – MMP-2, -3, -9 and -13

	MV GAPDH	MV MMP-2	MV MMP-3	MV MMP-9	MV MMP-13
E10 ML 1h	17,72	22,66	25,80	34,81	29,55
E10 Ctrl 1h	18,71	25,82	28,74	35,00	31,89
E10 ML 4h	17,82	24,71	27,05	35,00	31,47
E10 Ctrl 4h	17,79	23,59	26,24	35,00	30,58
E10 ML 24h	17,73	24,13	26,14	32,93	30,63
E10 Ctrl 24h	16,68	24,06	28,64	35,00	31,41
E12 ML 1h	15,89	25,74	28,68	35,00	27,30
E12 Ctrl 1h	20,60	35,00	31,26	35,00	32,69
E12 ML 4h	17,94	25,15	28,17	33,93	29,53
E12 Ctrl 4h	18,50	25,75	28,98	35,00	30,62
E12 ML 24h	18,00	24,40	29,67	34,44	29,98
E12 Ctrl 24h	17,79	25,19	29,56	34,39	30,87
E13 ML 1h	20,10	26,65	30,49	35,00	35,00
E13 Ctrl 1h	18,76	27,37	27,05	35,00	32,42
E13 ML 4h	18,96	26,63	26,10	33,09	31,76
E13 Ctrl 4h	18,93	27,57	28,97	33,72	29,55
E13 ML 24h	18,95	23,90	26,79	35,00	31,44
E13 Ctrl 24h	17,50	23,91	25,76	33,34	28,67
E14 ML 1h	16,26	24,29	26,89	31,90	25,63
E14 Ctrl 1h	17,81	24,12	26,59	31,87	25,25
E14 ML 4h	19,53	27,38	28,48	34,44	27,57
E14 Ctrl 4h	18,01	25,30	27,09	32,51	25,88
E14 ML 24h	17,15	23,85	26,47	31,99	25,97
E14 Ctrl 24h	18,34	25,76	28,83	35,00	28,07

Tab. 11a-c. Values of ΔCT, ΔΔCT and $2^{-\Delta\Delta CT}$ – MMP-2, -3, -9 and -13

DCT	DCT MMP-2	DCT MMP-3	DCT MMP-9	DCT MMP-13
E10 ML 1h	4,94	8,08	17,09	11,83
E10 Ctrl 1h	7,10	10,03	16,29	13,18
E10 ML 4h	6,89	9,22	17,18	13,65
E10 Ctrl 4h	5,79	8,45	17,21	12,79
E10 ML 24h	6,40	8,41	15,2	12,90
E10 Ctrl 24h	7,37	11,96	18,82	14,73
E12 ML 1h	9,85	12,79	19,11	11,41
E12 Ctrl 1h	14,40	10,66	14,4	12,09
E12 ML 4h	7,21	10,23	15,99	11,59
E12 Ctrl 4h	7,25	10,48	16,5	12,12
E12 ML 24h	6,40	11,66	16,44	11,98
E12 Ctrl 24h	7,41	11,77	16,6	13,09
E13 ML 1h	6,55	10,39	14,9	14,90
E13 Ctrl 1h	8,61	8,29	16,24	13,66
E13 ML 4h	7,67	7,14	14,13	12,80
E13 Ctrl 4h	8,64	10,04	14,79	10,62
E13 ML 24h	4,95	7,84	16,05	12,49
E13 Ctrl 24h	6,41	8,26	15,84	11,17
E14 ML 1h	8,04	10,64	15,64	9,37
E14 Ctrl 1h	6,31	8,78	14,06	7,43
E14 ML 4h	7,85	8,96	14,91	8,05
E14 Ctrl 4h	7,29	9,08	14,5	7,87
E14 ML 24h	6,70	9,32	14,84	8,82
E14 Ctrl 24h	7,43	10,50	16,66	9,73

DDCT	DDCT MMP-2	DDCT MMP-3	DDCT MMP-9	DDCT MMP-13
E10 ML 1h	-2,16	-1,95	0,80	-1,35
E10 ML 4h	1,10	0,78	-0,03	0,86
E10 ML 24h	-0,97	-3,55	-3,62	-1,83
E12 ML 1h	-4,55	2,13	4,71	-0,68
E12 ML 4h	-0,04	-0,25	-0,51	-0,53
E12 ML 24h	-1,01	-0,11	-0,16	-1,11
E13 ML 1h	-2,07	2,10	-1,34	1,24
E13 ML 4h	-0,97	-2,90	-0,66	2,19
E13 ML 24h	-1,47	-0,42	0,21	1,31
E14 ML 1h	1,73	1,86	1,58	1,94
E14 ML 4h	0,56	-0,12	0,41	0,18
E14 ML 24h	-0,72	-1,17	-1,82	-0,91

2-DDCTMMP-2	Ctrl.	1h	4h	24h
E10	1,00	4,48	0,47	1,96
E12	1,00	23,43	1,03	2,01
E13	1,00	4,19	1,96	2,76
E14	1,00	0,30	0,68	1,65
2-DDCTMMP-3	Ctrl.	1h	4h	24h
E10	1,00	3,86	0,58	11,71
E12	1,00	0,23	1,19	1,08
E13	1,00	0,23	7,45	1,34
E14	1,00	0,28	1,09	2,26
2-DDCTMMP-9	Ctrl.	1h	4h	24h
E10	1,00	0,57	1,02	12,3
E12	1,00	0,04	1,42	1,12
E13	1,00	2,53	1,58	0,86
E14	1,00	0,33	0,75	3,53
2-DDCTMMP-13	Ctrl.	1h	4h	24h
E10	1,00	2,54	0,55	3,55
E12	1,00	1,61	1,44	2,16
E13	1,00	0,42	0,22	0,4
E14	1,00	0,26	0,88	1,88