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ZAGREB, 2016.

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EDITORIAL

Translational Medicine

Dear readers, it is with great pleasure that I present to you RAD 43 (2016), the thirteenth volume of our journal, which has been continually published annually or semi-annually since 2006.

In the present volume, we have published three excellent articles. Some of them were written by members of our Editorial Board (Iva Dekaris, Vida Demarin). I cordially thank our distinguished colleagues and all the others authors of the mentioned articles for their contribution.

The present volume, in addition to the three mentioned articles, introduces abstracts from the 3rd International Conference on Regenerative Orthopaedics and Tissue Engineering, entitled *New Insights in Cartilage Repair*. The Conference was organized by the Department of Medical Sciences of the Croatian Academy of Sciences and Arts and the University of Zagreb, in cooperation with the Croatian Orthopaedic Society – Section for Translational Medicine, and held on the 4 November 2015 at the Academy Palace. It was devoted to the review of recent progress achieved in the research of cartilage repair and regeneration in the view of the modern concept of translational medicine.

Tissue Engineering and Regenerative Medicine (TERM) and Tissue Engineering and Regenerative Orthopaedics (TERO) form a part of a new wave of scientific and biotechnological revolution in medicine, or rather orthopaedics, the final aim of which is complete regeneration of damaged tissues of the bone and joint system. This is a very dynamic and interdisciplinary area, in which expert from diverse domains take part and cooperate on an equal footing; it is hence most common for a team to include – for instance – a medical doctor, a biologist, a chemist and an engineer. The key moment in any research of this kind is the move from laboratory, experimental environment to clinical, human medicine. This step is referred to as translation; translational research in medicine is today the most important concept, through which basic scientific research is being transformed into practical, applicable knowledge.

Articular cartilage has an extraordinary biomechanical performance and simple structure. However, it is vulnerable to multifactorial damage and insufficient to self-repair, isolated in the articular capsule without nerves or blood vessels. Restoration of hyaline cartilage in

osteoarthritis (AO) is a clinical challenge to recreate normal functionality over a long period. Pre-clinical trials have achieved promising outcomes in cartilage regeneration using MSCs. The rationale and technologies of MSC-based hyaline cartilage repair involving tissue engineering, 3D biomaterials and growth factors is elaborated in translation and application of MSC-based chondrogenesis for OA treatment. In the articular cartilage repair process, it is imperative to consider the type of biologic and the method of delivery to achieve the desired effect in cartilage repair. A number of spatiotemporal strategies are discussed for the controlled delivery of bioactive factors in cartilage tissue engineering applications. Focal chondral and osteochondral knee lesions are common conditions which are hard to treat when involving young active patients with high expectations of symptoms relief. (Pecina M, Vukicevic S (2014) Tissue engineering and regenerative orthopaedics (TERO). Int Othop 38: 1757-1760).

In orthopaedics in the last two decades, the process of the so-called autologous transplantation of chondrocyte, by which a chondrocyte sample is taken from the patient and multiplied in the laboratory, followed by being returned to the place of the damaged articular cartilage, was developed. Today, it is already possible – thanks to 3D printing technology – to print live articular or nasal cartilage. The day when it will be possible to develop tailor-made whole joints and bones by using the same method is not far either.

Readers are informed of all the newest knowledge on current options for repair and regeneration of cartilage in a summarised form – published abstracts of articles from the 3rd International Conference on Regenerative Orthopaedics and Tissue Engineering.

The last article in this volume of RAD, entitled *Twenty-Five Years of the Functional Endonasal Endoscopic Sinus Surgery in Croatia*, shows that 25 years ago, endonasal endoscopic sinus surgery was rather avant-garde, similar to today's application of 3D printing technology in the repair of articular cartilage.

I gladly announce that in the upcoming volume of RAD, we plan to publish abstracts from a major international symposium, entitled *What's New in Refractive Eye Surgery* (Iva Dekaris). This symposium will take place on 10 June 2016 at the Academy Palace in Zagreb. We hope that RAD 43, now in front of you, will prove as interesting and informative as the previous volumes. Major interest for our journal has been confirmed by ca. 150,000 visits to our articles published on the website hrcak.srce.hr.

Marko Pećina

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Original scientific paper
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GENE EXPRESSION ANALYSIS OF MATRIX METALLOPROTEINASE 2, MATRIX METALLOPROTEINASE 13 AND TISSUE INHIBITOR OF MATRIX METALLOPROTEINASE 1 IN EPITHELIUM OF DISEASED HUMAN CORNEAS*

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Summary

It has been shown in animal models that matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are responsible for remodelling and neovascularization of the diseased cornea. Under normal conditions, MMPs are expressed at relatively low levels, which may increase in case of different corneal diseases. In this paper we have investigated gene expression of matrix metalloproteinase 2, matrix metalloproteinase 13 and their inhibitor TIMP-1, in keratoconus, bullous keratopathy and vascularized corneal scar; aiming to determine whether these molecules are differentially expressed in epithelium of diseased human corneas. Keratoconus, bullous keratopathy and vascularized corneal scar were chosen for two reasons: they represent low-, intermediate- and high-risk corneal disease regarding corneal graft rejection rate (respectively) and they are very common reason for corneal transplantation. Our results have show that in bullous keratopathy, expression of both MMP-13 and MMP-2 genes were significantly increased compared to vascularized corneal scar and keratoconus; however only MMP-13 reached significance in our relatively small human sample. These results are comparable to our previously published data on protein levels of MMPs in diseased human corneal epithelium, where we showed significant increase of MMP-13 and MMP-2 proteins in patients with bullous keratopathy compared to keratoconus. TIMP-1 gene expression remained constant across all groups of patients, impli-

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cating that this molecule does not play significant role in pathological epithelial changes during keratoconus, bullous keratopathy and vascularized corneal scar. Our results may be useful in development of new therapeutic agents both to treat pathological corneal changes and to prevent corneal graft rejection after corneal transplantation.

***Keywords*:** cornea; matrix metalloproteinase; keratoconus; bullous keratopathy; vascularized corneal scar.

INTRODUCTION

Healthy cornea is avascular and transparent in order to provide good vision. It consists mainly of extracellular matrix (ECM) predominantly built of different types of collagen [1]. In case of corneal disease, highly organized corneal architecture is disrupted, the extracellular matrix (ECM) is degraded, and new disorganized ECM is formed, resulting in the loss of corneal transparency [2].Matrix metalloproteinases (MMPs) are extracellular endopeptidases which have a key role in ECM remodelling; they are controlling intercellular interactions and interactions of cells with ECM [3]. More than 20 members of human MMP family has been identified and classified into five subgroups: gelatinases, collagenases, matrilysins, stromelysins and membrane-type MMPs. Under normal conditions, MMPs are expressed at relatively low levels, which may increase in case of different corneal diseases. MMP-2, MMP-9, MMP-13 and MMP-14, for example, have been shown to participate in corneal wound healing and in the pathogenesis of inflammatory corneal diseases [4-8]. Tissue inhibitors of matrix metalloproteinase (TIMPs) are specific inhibitors of MMPs which regulate and control the activity of MMPs in tissues [9-12].

In the advanced stage of many corneal diseases, corneal transplantation (CT) is needed to restore normal corneal transparency. Based on the inflammatory profile of their corneas at the time of CT patients are usually divided to be of low-risk (keratoconus - KC), intermediate risk (bullous keratopathy - BK) or high-risk (vascularized corneal scar - VCS) for postoperative graft rejection. Keratoconus is a non-inflammatory corneal thinning followed by anterior protrusion of the cornea, for which aetiology is not known and various genetic and environmental factors have been suggested [13,14]. Bullous keratopathy is a corneal disorder characterized by corneal stromal oedema with epithelial bullae formation due to endothelial cell loss decompensation; while vascularised corneal scar represents typical inflammatory corneal condition in which prolonged inflammation and neovascularisation lead to the loss of corneal transparency [15].

To aid in developing efficacious post-CT therapies for those patients, it is important to understand and further investigate the complex pathways related to MMP and TIMP signalling in particular human corneal disease [16,17]. Therefore, we have

investigated gene expression of MMPs and TIMPs in the corneal epithelium of patients suffering from KC, BK and VCS at the time of CT. Although it has been shown in animal model that those molecules promote pathological cellular interactions leading to remodelling and neovascularization of corneal tissue, it is still not known whether they are differentially expressed in high-, intermediate and low-risk human corneal diseases [18,19].

MATERIALS AND METHODS

Tissue preparation

With the proper informed consent and approval by the Ethical Committee of our Hospital human corneal epithelium was taken *ex vivo* from 18 recipient corneal buttons obtained during corneal transplantation (7 diagnosed with keratoconus, 6 with bullous keratopathy and 5 with vascularized corneal scar). Corneal epithelium was separated from the corneal stroma by fine forceps under the operating microscope, and homogenized using the TissueRuptor homogenizer (Qiagen, Germany). Control healthy epithelium (n=5) was collected from healthy corneas obtained from the eye bank.

RNA isolation

Total human corneal epithelial RNA was extracted from the individual corneal epithelium using Rneasy Mini Kit (Qiagen, Hilden, Germany); cDNA was prepared from 1 μg of purified total RNA in 20 μl reaction volume, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to manufacturer's instructions. Real-time PCR analysis was carried out on an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA) to quantify relative mRNA expression of matrix metallopeptidase 2 (MMP-2), matrix metallopeptidase 13 (MMP-13) and tissue inhibitor of MP-1 (TIMP-1) genes, respectively. Assay IDs used for the analysis are shown in Table 1. The housekeeping gene gapdh was used as an endogenous control to normalize the total amount of cDNA in each sample. Reactions were carried out in a total volume of 20 μl using TaqMan® Gene Expression Master Mix reagent (Applied Biosystems, Foster City, CY, USA) and 5 μl of cDNA for template with forward and reverse primers. Using the $2^{-\Delta\Delta Ct}$ method, data are presented as the fold-change in gene expression normalized to an endogenous reference gene and relative to the control. All reactions were carried out in triplicate [20].

Table 1. Primers used for quantitative real-time PCR analysis

Gene	Assay ID	Product size (bp)
GAPDH	Hs99999905_m1	122
MMP2	Hs01548733_m1	84
MMP13	Hs00233992_m1	91
TIMP1	Hs00171558_m1	104
VEGFA	Hs00900058_m1	81
VEGFR1	Hs01052936_m1	72

Statistical analysis

Statistical analyses of data were performed using R v2.15.3 (CRAN, http://cran.r-project.org) and RStudio for Windows, v0.97 (http://www.rstudio.com/). For the analysis of real-time PCR data, all groups were tested for normality of distribution using Shapiro-Wilk test. The differences between multiple groups were compared with Kruskall-Wallis non-parametric ANOVA, followed by Wilcoxon signed-rank test for testing differences between two related groups. For all tests significance level was set at $p < 0.05$.

RESULTS

MMP-2 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar

The expression of MMP-2 gene remained unchanged in patients with keratoconus, bullous keratopathy and vascularised corneal scar when compared to control patients, as revealed by Kruskal-Wallis non-parametric ANOVA (Kruskal-Wallis chi-squared = 6.8404, df = 3, p-value = 0.07716) (Fig. 1).

MMP-13 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar

The MMP-13 gene expression was not determined in patients with keratoconus because the expression of the MMP-13 gene was below detection limit of the method. Kruskal-Wallis non-parametric ANOVA has shown significant differences in MMP-13 gene expression between BK and VCS groups (Kruskal-Wallis chi-squared = 6, df = 2, p-value = 0.049). In patients with bullous keratopathy MMP-13 gene was significantly upregulated, as revealed by Wilcoxon rank-sum test (W=8, p = 0.012) (Fig. 2).

TIMP-1 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar

TIMP1 gene expression remained unchanged in any of groups, when compared to control samples (Kruskal-Wallis chi-squared = 5.5606, df = 3, p-value = 0.1351) (Fig. 3).

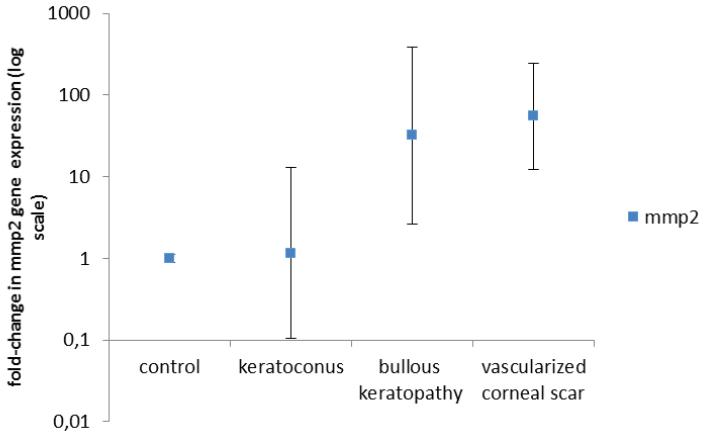

Figure 1. Real-time PCR analysis of MMP-2 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar. Fold-change in gene expression compared to control was calculated using the $2^{-\Delta\Delta CT}$ method as described in Materials and methods. Data are presented as fold-change ±SE on log scale.

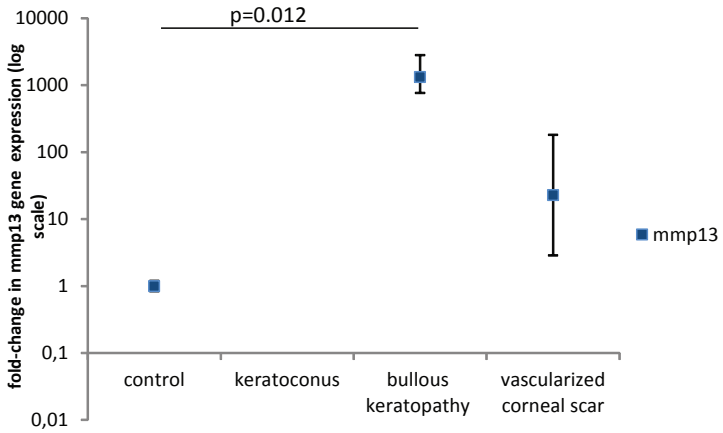

Figure 2. Real-time PCR analysis of MMP-13 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar. Fold-change in gene expression compared to control was calculated using the $2^{-\Delta\Delta CT}$ method as described in Materials and methods. Data are presented as fold-change ±SE on log scale.

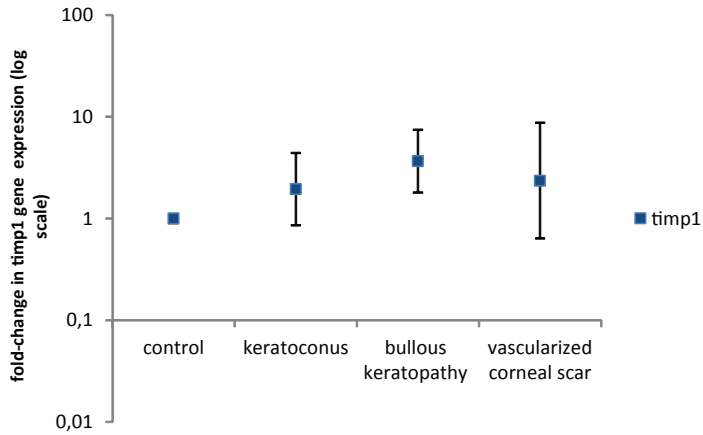

Figure 3. Real-time PCR analysis of TIMP-1 gene expression in corneal epithelium of patients with keratoconus, bullous keratopathy and vascularised corneal scar. Fold-change in gene expression compared to control was calculated using the $2^{-\Delta\Delta CT}$ method as described in Materials and methods. Data are presented as fold-change ±SE on log scale.

DISCUSSION

Corneal transplantation (CT) is one of the most frequently performed transplantations [21]. However, the molecular mechanisms leading to corneal opacification in different corneal diseases are still not well determined. From the clinical experience we know that the outcome of CT significantly varies according to the characteristics of the recipient cornea where the corneal graft is placed. Therefore, it is of great importance to understand which molecules are involved in pathological changes of corneal tissue in different diseases; both to invent new topical agents to treat those diseases and to improve the surgical prognosis of CT. Based on the experimental data in animal models it is clear that MMPs play an important role in changes of ECM and thus transparency of the cornea, and that their activity is regulated via TIMPs [8-12,22,23]. However, very little data on MMPs and TIMP role in changes of corneal transparency are available for human corneas [24-27]. For this reason we were interested whether any changes in gene expression of MMPs and TIMPs could be detected in human low-risk disease like keratoconus (KC) versus intermediate and high-risk ones like bullous keratopathy (BK) and vascularized corneal scars (VCS). In the study of Gordon et al., an comprehensive analysis for the entire MMP gene expression during mouse corneal abrasion injury was done, and it has been shown that mRNA levels for several MMPs and TIMP-1 were significantly upregulated during epithelial migration, while MMP-13 remained elevated even after

epithelialization suggesting that MMP-13 may be a marker of prolonged corneal epithelial injury [12]. In our study, we have shown that MMP-13 gene is significantly upregulated also in human epithelium of patients with BK, but not in KC and VCS, suggesting specific role of MMP-13 in epithelial corneal changes in patients with BK. In our previously published paper, we have also investigated protein levels of different MMPs and TIMPs in BK and KC corneas, and have similarly shown that epithelium of BK patients show higher level of MMP-13 protein compared to KC [27]. Moreover, we have shown that protein levels of epithelial TIMP-1 remained unchanged in both KC and BK [27]. This finding is also confirmed by gene expression analysis in this study, where no difference was found in TIMP-1 gene expression between BK, KC and VSC corneas. Although MMP-2 gene analysis in this study showed higher gene expression in the epithelium of BK and VCS patients compared to KC, this difference was not statistically significant. However, our previous protein analysis of MMP-2 showed increased MMP-2 levels in patients with BK compared to KC [27]. Current results may have been influenced by the relatively small sample in this study, so further investigations would be beneficial for confident conclusion whether MMP-2 has a role in corneal epithelial changes in BK patients. Overall, one may conclude that MMP-13 plays a role in pathological changes of corneal epithelium in BK, since both MMP-13 gene and protein are highly expressed in corneal epithelium of such patients.

CONCLUSION

Presented results revealed that the expression of MMP-13 gene is significantly increased in bullous keratopathy (BK), somewhat increased in vascularized corneal scar (VCS) and under levels of detection in keratoconus (KC). Although MMP-2 gene expression was increased in patients with BK and VCS compared to keratoconus, this difference did not reach significance in this relatively small sample. TIMP-1 gene expression remained constant across all groups of patients.

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Sažetak

Genska ekspresija matriks metaloproteinaze 2, matriks metaloproteinaze 13 te tkivnog inhibitora matriks metaloproteinaze 1 u epitelu oboljelih ljudskih rožnica

Matriks metaloproteinaze (MMP) i njihovi inhibitori (TIMP) smatraju se čimbenicima odgovornim za remodelaciju i neovaskularizaciju rožnice do koje dolazi kod raznih bolesti, što je pokazano na životinjskim modelima. U zdravim rožnicama matriks metaloproteinaze izražene su u relativno niskim koncentracijama te rastu u slučaju rožničnih bolesti. U ovome smo radu istražili gensku ekspresiju matriks metaloproteinaze 2 i 13 u ljudskim bolestima rožnice keratokonusu, buloznoj keratopatiji i vaskulariziranim ožiljcima rožnice kako bismo stekli uvid u to imaju li ti MMP-ovi značenje u razvitku humanih bolesti rožnice. Keratokonus, bulozna keratopatija i vaskularizirani ožiljci odabrani su iz dva razloga: zbog toga što klinički predstavljaju bolesti niskog, intermedijarnog i visokog rizika glede ishoda transplantacije rožnice te zbog toga što su to učestale indikacije zbog kojih se obavlja transplantacija rožnice. Naši rezultati pokazuju da u slučaju bulozne keratopatije dolazi do povišene ekspresije i MMP-13 i MMP-2 gena u usporedbi s keratokonusom i vaskulariziranim ožiljcima; međutim samo je povišena ekspresija MMP-13 bila signifikantna u našoj relativno maloj skupini bolesnika. Ti rezultati podudaraju se s našim prethodnim istraživanjima u kojima smo proteinskom analizom dokazali da su i MMP-13 i MMP-2 protein signifikantno više producirani u epitelu rožnica bolesnika s buloznom keratopatijom nego u onih s keratokonusom. Razina ekspresije TIMP-1 gena nije se razlikovala među ispitivanim uzorcima ukazujući na to da ta molekula nema bitnu ulogu u patološkim promjenama epitela u slučaju keratokonusa, bulozne keratopatije i vaskulariziranih ožiljaka. Dobiveni rezultati mogli bi biti korisni u razvitku novih terapija kako za liječenje patoloških rožničnih promjena, tako i za liječenje reakcije odbacivanja transplantata rožnice.

Ključne riječi: rožnica; matriks metaloproteinaze; keratokonus; bulozna keratopatija; vaskularizirani ožiljci rožnice.

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PSYCHOANALYSIS AND THEORETICAL BACKGROUNDS IN ATTACHEMENT THEORY RESEARCH

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Summary

In 1969, British psychoanalyst John Bowlby founded a scientific theory, which is now known as "an attachment theory" in which he proposed that keeping physical proximity with caregiving adults represents primary mechanism of child safety regulation and survival. As the theory evolved, due to the new pespectives gained by other fields of research such as biology, neuroscience and developmental psychology, everyday experience of working psychoanalysts showed also to be very useful in explaining how the psychologic attachment develops in humans. In this process, one of the most important concepts is mentalization that can be roughly defined as process of taking views that observe the intention, which means an approach to humans as beings guided by intrinsic intents. For this process to be effective, a person must have satisfactory capacity to mentalize, which finally leads to the development of mature "self". Ever-growing knowledge in this field of research should eventually establish theoretically sound and truly evidence-based clinical practices, working in harmony with a research literature that is sensitive to clinical discoveries and applications. In this paper, we review attachment theory in the light of this new research, with hope of applying it to psychoanalytical practice for the benefit of the patients.

Keywords: attachment theory; mentalization; intesubjectivity; "self".

BOWLBY'S ETHOLOGY – EVOLUTIONAL ATTACHMENT THEORY

Bowlby and Ainworth's attachment theory is one of the most successful psychological theories of the past half century [1,2]. It has generated thousands of published studies and scores of books.

Attachment theory as such is psychological, evolutional and ethological theory that researches inter-human relationships. Founder of this theoretical approach is psychiatrist and psychoanalyst John Bowlby. He improved multidisciplinary per-

spective in which psychoanalysis, ethology, sociobiology and psychobiology is integrated in cybernetic theory of the system control with modern structural cognitive development backed up by neuroscientific research.

In 1969 he proposed that keeping physical proximity with caregiving adults represents primary mechanism of child safety regulation and survival. In Bowlby's theory, behaviour patterns that have predictable outcome of increasing proximity between child and the caregiver (for instance cry, invocation, following, cuddling) represent an action of complex and instinctive but environmentally conditioned control system called "attachment behavioural system". It is presumed that this system developed to serve the biological function of protection of the younger and/or weaker members of the primate pack that lived on the ground. Once it chooses the person with whom it will be attached (generally, but not necessary child's biological mother), the child carefully observes where she is, keeping the proximity even under non-stressful conditions. If a threatening situation should occur, attachment behavioural system becomes highly active and immediately guides the baby towards behaviour that has a goal to establish direct physical nearness and contact [2].

Although Bowlby initially introduced an assumption that biological function of the attachment behavioural system was primarily protection from the predators, full knowledge of this system can best be obtained if we take in concern that it serves multiple survival functions. Besides from the protection of the predators, physical nearness also protects from the unfavourable temperature changes, natural disasters, attacks from the individuals of the same species, as well as the peril of the separation from the group [3].

After the introductory articles from 1958 on, Bowlby publishes thorough trilogy "Attachment And Loss" from 1969 to 1982 [2,4,5], which made attachment theory in upcoming decades a dominant approach in understanding early social development, and which initiated the wave of empirical research of model of close relationship forming in the childhood period. In the past, critic mostly came from the fields of psychoanalysis and ethology, and pointed out to perceived accent on mother. In recent times, critic mainly points out to genetical factors, temperament, complexity of the relationships inside the family, and to limitations of discrete classification patterns of the attachment behaviour styles.

Attachment behavioural concepts were incorporated inside the existing therapeutical interventions, and used to plan psychotherapeutic intervention based upon the attachment concept. Attachment theory concepts were also used to formulate various social laws, as well as laws in relation with child care.

ATTACHMENT PATTERNS IN CHILDREN

Next great pioneer in attachment theory research was Mary Ainsworth, developed well known laboratory based procedure for observation of child intrinsic working model in action. Children, when separated for a short time from their caregivers in, to them, unknown situations, show one of the four behaviour patterns. Children classified as "Secure", willingly explore in the presence of their primary caregivers, show anxiety in the presence of strangers, avoiding them, and express sadness because of the current absence of their caregivers, afterwards repeatedly seeking contact with caregivers that calms them. Those children have no problem with returning to the experiment. It is possible that some children, which show themselves to be less anxious because of the separation, will not seek nearness of parents or caregivers after the separation, and it is possible that they will not prefer parent or caregiver over stranger; these are classified as "Anxious-avoidant". Third category of children, "Anxious-resistant", show limitations in exploration and playing and are prone to express great sadness due to the separation, and have great problems in calming down after the separation, resist, become stiff, continue to cry or passively resist. Parent's or caregiver's presence or an attempt to comfort the child fails, and its anxiety and rage make alleviation and relief that can only be acquired through nearness, impossible [1].

Mary Main and Inge Bretherton independently draw attention to something that a philosopher Dennett would call "an intentional stance". In 1987, Dennett indicated that people try to understand each other in frames of the mental states: thoughts and feelings, beliefs and wishes, in the desire to give a meaning, or what is more important, to predict the actions of the others. If a child succeeds to explain mother's obvious rejecting behaviour as an emotion of sadness due to the loss, it will be protected from confusion and negative view of itself, and will not show helplessness because that situation happened [6].

The characteristic and acquisition of "the intentional stance" is child's recognition that behaviour can be based upon false beliefs. That happens around the age of three or four. Developmental theorists designed a great deal of tests for detecting the quality of false beliefs understanding and refer to these capacities as "the theory of mind".

MENTALIZATION AND THE DEVELOPMENT OF "SELF"

Mentalization is a term introduced by French psychosomatic states theorists, but the concept started to develop in different directions in the 1980s [7,8]. In the literature that deals with cognitive development, it is interchangeably used and in

the same context with more frequent and more expanded concept of "the theory of mind". This term is made by primatologists Premak and Woodruff in 1978 [9] and is applied in the developmental psychology, in terms of the child's capacity to ascribe thoughts, feelings, ideas and intentions to other persons and anticipate and influence their behaviour. Most of the developmental research that took place during the 1980s and 1990s were conducted in terms of the mind theory concept. Research that used false-belief task detection, used the results either for normative mapping of the development of the mind theory in pre-school children, or to demonstrate deficiencies of the same theory in persons with autistic disorder. As Baron-Cohen proposed in 2000, the test of detecting false-belief is the first-order test in the theory of mind, and includes concluding about mental status of other person ("She thinks that..."). Second-order test of the theory of mind includes concluding about the perspective of the second order that has been interposed in the mental state ("She thinks that he thinks that…") [10].

During the last decade, the construction of the theory of mind and its paradigm contained in the false-belief test, was criticized to be too narrow [11]. That guided some of the authors to change the term with "mentalizing", because it is somewhat more general and not limited to a specific task or an age group [12].

One of the most bitter attempts to expand the concept of the theory of mind beyond the false-belief paradigm, beyond autism, and finally beyond the pre-school children study comes from Peter Fonagy and his cooperates. In their definition of mentalizing, it can be used interchangeably with the theory of mind or social intelligence, although it refers to a lot more than the sole theory of mind [13,14].

Mentalizing refers to a process of taking views that observe the intention, which means an approach to humans as beings guided by intrinsic intents [15]. Allen points out that mentalization can be conscious or unconscious. When we talk about conscious (explicit mentalizing), it is closely related to language and therefore powered or limited by our verbal capabilities. The unconscious mentalizing (implicit mentalizing) can happen instantly, without our supervision and conscious interference, and can be perceived to be without our general intellectual development. For instance, without our conscious intention, through our facial expression or body talk, we are able to recognize in other people certain mental states that help us to explain their behaviour. Implicit mentalization is therefore procedural, and we are always acting upon it without even thinking about it [16].

INTERGENERATIONAL TRANSFERENCE OF MENTALIZATION CAPACITY

Phenomenon of capacity to imagine mental states as an explanation of the behaviour, both its own and of the others, is basic organizing construct of the approach to social development that has mentalization in its focus. In research studies the term that is often used to express measurable, quantifying index of mentalization in context of attachment concept is reflective function [13,14].

Capacity to mentalize, together with capacity to regulate the affect and also the control mechanisms of attention represent key designations of "self" organization. Capacity to mentalize is acquired in context of the early attachment relationship. Therefore, impairments in early attachment can disturb normal development of these essential socio-cognitive relationships and create a profound vulnerability in context of social relations.

The ability to understand oneself as a mental factor develops from the interpersonal experience, especially from the relationship with primary objects [17]. Child's understanding of itself as someone that has mind or personality is not given by genetics: it develops from the breastfeeding period, through childhood, and this development essentially depends on the interaction with more mature minds, presuming that they are well-intentioned, reflexive and well-coordinated. Mentalization also includes auto-reflexive and interpersonal component. It is backed up with great number of specific cognitive skills, including the understanding of emotional states, attention and directed effort control, capacity to make a judgement about subjective states, as well as explicit reasoning about states of the mind - all of which we call adequate mentalization. In combination, these functions enable the child to differentiate intrinsic from extrinsic reality, and inner mental and emotional process from the interpersonal happenings.

Evolutionary biologist Richard Alexander in 1989. proposed a model of human brain development that suggests that our unique intelligence evolved not for the reason of facing the aggressive forces of nature, but to face the competition of other humans. This more advanced evolution happened only after we reached relative dominance over our environment. At this stage of the development we were already "the aggressive force of the nature". In order to meet the challenge of the survival of our genes, those with common genetic material had to cooperate considerably more before complete development of the high intelligence took place [18].

Parents with more developed reflective function are more capable to initiate and build up secure attachment in child. In Fonagy's longitudinal research that comprised of 92 children, share of securely attached children was two times higher in

group that was successful in overcoming the false-belief task, in relation to group that did not. Reflective function of mothers was also connected to the success of children. 80% of children, whose mothers had above average reflective function, passed the false-belief task, in contrast with 56% of children of mothers that had below average reflective function. Reflective function of caregivers is predictive for trustworthy attachment, which we can confirm the existence of intergenerational transference of the capacity for reflective function, e.g. mentalization. Attachment with father has also proved to be important in this developmental achievement [19]. Reliable attachment in the relationship provides adequate context for child's exploration of the caregiver's mind, and also teaches it about mental function and processes of others and itself. Philosopher Georg Wilhelm Friedrich Hegel indicated: "Only via exploring the mind of someone-else, the child can build up the understanding of the nature of mental states". Process is intersubjective; the child cognizes the mind of its caregiver, while the caregiver tries to understand the state of mind or the child and to keep it in the process of the mentalization, and finally, to reflect this mentalized material to the child. Psychoanalysts Donald Winnicott and Wilfred Bion also recognized primary intersubjectiveness in their concepts of mirroring and containment

THE SOCIAL ACQUISITION OF SOCIAL COGNITION

Aforementioned evolutionary neurobiological considerations imply that social environment that takes care of the child, plays a crucial role in the development of social cognition. In its first months, the child starts to understand that it is a physical agent, whose actions can contribute to changes in bodies with which it has direct physical contact [20]. Parallelly, child's understanding of itself as a social agent also develops. Through interaction with the caregiver, since its birth, the newborn learns that its behaviour influences behaviour and emotions of the caregivers, e.g. that he is a social agent which can through communicating cause effects with distance in the social environment [21]. Both of these early forms of self-consciousness are probably develop through activity of the inborn mechanisms of compatibility detections that enable the newborn analysis of the possible connections between its actions and exogenous stimuli [22].

Child's initial preoccupation with perfectly response-contingent stimulation, assured with the help of proprioceptive sensory feedback which are always generated by its own actions, enable the child to differentiate its mediatory agentive self as a separate entity in the environment and construction of primary representation of the physical self.

In the age of 3 to 4 months, the newborn transits form the phase of preference of perfect contingency to the phase of high, but not complete contingency, which is the level of contingency that is characteristic to adapted empathic mirroring of the parents or caregivers onto the newborn's emotions presentation [23]. Repeated experiences of such affective-reflective reactions of their caregivers is essential for the beginning of the differentiation in order for the newborn to commence to differentiate its inner self states: process that has been called bio-social feedback [24].

Securing such state of the reflective environment and save relationship with the caregiver can significantly contribute to the creation of the early mentalization capacities, allowing the newborn exploration or finding its own psychological self in the social world. Discovering representational or psychological self, what can be considered to be complete mentalization, is most probably based upon the same mechanism [25].

Here, we will take an example of the development of understanding the affects. Let us presume that, in the beginning, newborns are not introspectively aware of the differences between the affective states. Their representations of these emotions are primarily based upon the stimuli from the outside world. Newborns learn how to differentiate inner patterns of physiological visceral stimuli that follow different emotions by observing facial or vocal mirroring reactions of their caregivers on those stimuli [26]

At the beginning, the newborn starts to associate its own control over mirroring that the parents offer, which results in the improvement of its own emotional status, and finally leads to experiencing self as regulating as well as mediating agent. Secondly, establishing second-order representation of the affective states creates basis for the affect regulation and control of the impulses: affects can be manipulated and released internally, as well as through action, and also be experienced as something recognizable, and therefore common and mutually shared. If parents do not express affects concurrently and compatible with the affect of the newborn, it can disrupt adequate signification of the inner states, which will therefore remain confusing, experienced as non-symbolized and hard to regulate.

If the ability to understand and regulate the expression of the emotions should evolve, two conditions must be fulfilled:

(a) acceptable compatibility of the mirroring by which the caregiver could correctly pair the newborn's mental status.

(b) signifying role of the mirroring, in which the caregiver can express affect and in the same time not to express its own feelings [27].

Two difficulties can arise from those two premises:

(I) in the case of incompatible mirroring, representation of the inner self of the newborn will not be compatible with constitutional status of the self and ten-

dency to narcissistic structure can be established, in analogy with Winnicott's concept of false self [28].

(II) in the case of undesignated mirroring, emotional expressions of parents or caregivers can be seen as an externalization of the newborn's experience and predisposition for experiencing emotions through other people can be established (just as it is the case in the borderline structure of personality, which uses massive projections as a basic defense model), (*Fig. 1*).

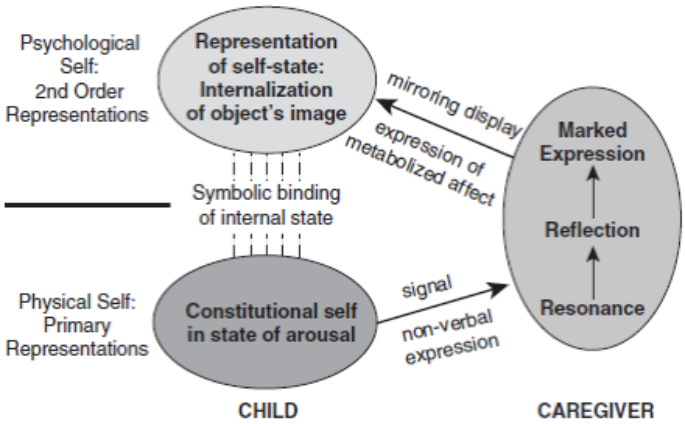

Figure 1. Model of the development of social acquisition and cognition according to Fonagy et al. [13]

Affective expression compatible with the child's status, but deprived of the signifying information with which the parent in the mirroring process establishes symbolical meaning can overwhelm the newborn. Without the signifying addition of the parent, the child feels that those are real emotions of the parents, thus creating an impression that the child's experience is transferable, which further, before regulating the child's state, leads to excitement. Reliable caregiver tranquilizes the child by combining mirroring with expression that is not compatible with child's feeling, thus combining affective contact and affective distance that settles the affect of the child and does the designatory move to symbolical. Such formulation of the sensitivity has much in common with Bion's point of view about the capacity of mother to mentally contain affective status that considers unbearable for the child, and reacts in the way that confirms the mental status of the child, but in the same time serves to adapt the unbearable feelings [29]. Well regulated affects in the parent-child pair, internalized in the child make a foundation for safe attachment and

internal working model [30]. Data resulted from the London Parent-Child Project about the quality of the reflective function of each parent during pregnancy, showed that reflective function is a predictor of later attachment of the child [31]. But, this knowledge is somewhat limited because only AAIRF has been investigated in relation to child's attachment [32,33]. Therefore, the capacity of the parents to mentalize measured in relation to their own childhood and their capacity to similarly influence their children is more presumed than apparent.

DEFENSES AND ATTACHMENT-RELATED SOURCES OF PAIN AND DISTRESS

The concept of psychological defense has been important to psychology ever since Breuer and Freud [34] first explained how repression works: "The basis for repression can only be a feeling of unpleasure, the incompatibility between the single idea that is repressed and the dominant mass of ideas constituting the ego" (p. 116).

Almost 110 years later, we [35] con- ducted two experiments in they shows that avoidant individuals used some combination of unconscious (repression) or conscious censoring (suppression) and self-image inflation to ward off painful thoughts of rejection and abandonment, but these defenses were defeated by an interfering cognitive load.

These studies, and hundreds of others, both classic and recent [36], indicate that:

1. Breuer and Freud were right in believing that the human mind is capable of repressing unpleasant thoughts (including thoughts about relationship losses and negative self-traits).
2. At least sometimes (e.g., when rejection experiences and negative self-traits are associatively linked in memory), repression involves "a psychical group" of related mental representations (or a neural network, as Freud, a neurologist, might also have conceptualized such psychical groups)
3. Repression requires mental effort (psychic energy, in Freud's terms)
4. The concept of defense is alive and well in present-day psychology, despite having been criticized over the decades.

The term defense was quite familiar and acceptable to Bowlby [5], a psychoanalyst and the founder of attachment theory. It has not, however, seemed completely acceptable to many research psychologists since Bowlby's time, so it is continually being resurrected under names such as coping strategies [37] and strategies of affect

regulation or emotion regulation [38]. In line with what all of these concepts have in common, defenses are mental mechanisms aimed at adaptation and self-regulation a view shared by psychoanalytic writers [39].

What is being defended against in a particular case may be as different as potentially uncontrollable impulses (which Freud viewed as instinctive, but which may arise from almost any motive, wish, or desire); a feeling of falling apart under stress (losing the coherence and solidity of the sense of self); negative emotions (e.g., anxiety, shame, guilt, fear, anger); and unacceptable thoughts about the self (e.g., about one's own mortality, mistakes, personal weaknesses, or social unacceptability) and others (e.g., about another person's indifference, rejecting behavior, or threats of abandonment). Although threatening experiences have been conceptualized in many ways—for example, as threatening one's life, one's self-control, one's social acceptance, one's self-image, or one's sense of coherence and consistency [40]—all of the conceptualizations are compatible with the claim that they involve "a feeling of unpleasure [attributable to] the incompatibility between the single idea that is repressed and the dominant mass of ideas constituting the ego" [34].

According to attachment theory [2,4,5], the unavailability or non-optimal responsiveness of a primary attachment figure in times of need, as well as the resulting disruption of one's sense of safety, security, and protection, is a major source of psychological suffering. When an attachment figure proves to be uninterested, unavailable, unempathic, unhelpful, or even frightening, a person in need fails to regulate the distress that has activated his or her attachment system (due, e.g., to an unexpected loud noise in infancy, an insulting comment by an elementary school teacher in childhood, the breakup of a romantic relationship in adolescence, or a miscarriage or serious physical illness in adulthood), and the distress is exacerbated by frustration of the person's attachment needs and the rejection of his or her efforts to seek love, understanding, comfort, and protection. As a result, the person not only feels vulnerable and anxious while trying to handle the threatening experience without the assistance of what Bowlby called a "stronger and wiser" attachment figure; the person also feels rejected and demeaned or humiliated. This complex of feelings, or state of mind, can take at least two organized forms [1,41,42] or be seriously disorganized.

In one organized state of mind, attention is focused mainly on the ways in which one's bids for proximity and support fail to achieve a positive interpersonal result (closeness, love, comfort), and instead typically result in a negative outcome (inattention, rejection, anger, disdain, abuse) for showing vulnerability or need. In such cases, reliance on an attachment figure, and involvement in a dependent or interdependent relationship more generally, is construed as frustrating, demeaning, and

painful. Under these conditions, a person may decide, consciously or unconsciously, to rely on him- or her- self—becoming what Bowlby [2] called compulsively self-reliant, and what others have called dismissive of attachment or simply avoidant (of intimacy, closeness, and interdependence).

This stance can be conceptualized in terms of deactivation of the attachment system. Anything that would normally activate the system, such as physical or psychological threats to the self, feelings of vulnerability, or wishes for an attachment figure's protection or support, is defended against, suppressed, countered with narcissistic self- enhancement, or denied [3,43-45].

The other organized state of mind that can develop in response to unreliable or unresponsive care includes ineffective regulation of distress and construing oneself as inadequately supported, insufficiently loved, vulnerable to uncontrollable threats, and improperly or unfairly treated. In this state of mind, attention is focused on one's own vulnerability and inadequacy in the threatening situation, which can be momentarily accepted ("I'm hopelessly alone," "I'm worthless") and/or raged against ("He's never there for me," "She promised me this would never happen"). This state of mind encourages anxiety, rumination, catastrophizing, jealousy, and envy, and can lead to increased efforts to capture a relationship partner's attention and loyalty (by acting seductive, being coy, or submitting to otherwise undesirable requests) and/or to attempts to coerce the partner into behaving more supportively (by making demands, expressing strong negative feelings, denigrating rivals, or spying on the partner). Ainsworth et al. [1] identified one group of infants who displayed both of these kinds of behavior (strong proximity seeking and angry contact resistance) following a brief laboratory separation from the parent; these infants were initially labeled anxious-ambivalent. In adults, this state of mind is what Main and her colleagues [46] called enmeshed and preoccupied with attachment, and what we call, more simply, anxious attachment. Theoretically, this state involves hyper-activation of the attachment system [47,48]. Although these two states of mind, avoidance and anxiety, are related, both being due to insecurity caused by an attachment figure's unavailability or unreliability, their relative strength may vary across situations, relation- ships, and individuals. Mikulincer et al. [42] summarized the broad array of internal and external factors that contribute to the relative strength of each of the states of mind.

The avoidant stance is encouraged by:

1. Consistent inattention, rejection, or angry disapproval on the part of an attachment figure in response to a person's bids for proximity and support.
2. Threats of punishment or rejection if the person makes a bid for proximity, intimacy, or protection.

3. Traumatic or abusive experiences during proximity-seeking efforts.
4. Explicit or implicit encouragement on the part of an attachment figure for one's greater self-reliance and suppression of expressions of need or vulnerability.

The anxious stance or state of mind is encouraged by:

1. Unreliable care, which is sometimes affectionate and at other times neglectful.
5. Intrusive care that is more related to the caregiver's own needs and anxieties than to the needs of the attached individual.
6. Care that discourages the acquisition of self-regulation skills and, directly or indirectly, punishes a person for attempting to function independently.
7. Comments that emphasize a person's helplessness, incompetence, or weakness when trying to operate autonomously.
8. Traumatic or abusive experiences endured when one is separated from attachment figures. This kind of treatment causes a person to feel ambivalent because, on the one hand, relying on the attachment figure is awkward, discomforting, and sometimes annoying, but on the other hand, trying to care for oneself can seem dangerous or hopeless and therefore daunting. This kind of care may encourage a person to verbalize neediness and protest temporary abandonment (real or imagined), because making noise has sometimes recaptured a neglectful or self-preoccupied caregiver's attention. This encourages inauthentic emotionality-a form of behavior that can be humorous or endearing, but that can also seem dishonest and become exhausting for anyone who tries to respond helpfully to it.

The most insecure state of mind, disorganized attachment, seems to stem from experiences with an attachment figure who was either seriously abusive, frightened, or frightening when a person sought proximity and safety from the attachment figure [41,49]. These experiences turn what should have been a safe haven and secure base into a source of threat, causing all organized attachment strategies to break down. Thus the individual's biologically based tendency to turn to an attachment figure in times of trouble is countered by the biologically based tendency to turn away from threat, and the individual is literally stuck. In childhood, these incompatible tendencies lead to unusual behavior—lying down on the floor in the midst of seeking proximity, veering off course when approaching the parent and going under a table, backing away from the parent while in obvious distress, or freezing. In adulthood, the equivalent state can involve odd beliefs, dissociative states, extreme lack of trust in others, and a lack of monitoring of discourse or reasoning.

The different insecure states of mind have implications for the kinds of defensive behavior a person is likely to exhibit. Individuals who score high on avoidant

attachment tend to be vigilant about becoming needy, intimate, dependent, or emotional; they deny vulnerability, emphasize their personal strengths, avoid threats, and resist becoming dependent on anyone. Individuals who score high on anxious attachment, in contrast, tend to be vigilant about possible neglect, rejection, or abandonment, and to be hypersensitive to signs of danger and lack of care. This can cause them either to act out noisily and intrusively, or to comply submissively with relationship partners' requests. If necessary to gain support, they will admit (and perhaps even exaggerate) their own weaknesses and vulnerabilities, criticize themselves, and attempt to remake themselves (e.g., by undergoing cosmetic surgery) [50]. That is, whereas avoidantly attached people are worried about intimacy, engulfment, and interdependence, anxiously attached people are worried about separation, abandonment, isolation, and interpersonal distance.

Although not all aspects of this theoretical analysis of defenses have been empirically tested, there is good evidence that avoidant people are intimacy-averse. Self-reported avoidant attachment is associated with higher scores on fear-of-intimacy scales [51,52], with placing relationship partners at a greater distance from one's "core self" [53], and with actually increasing physical distance from others, even in fairly simple settings. Kaitz et al. [54], for example, used a stop-distance research paradigm in which people rated their level of discomfort as an experimenter moved toward them. They also assessed the physical distance freely chosen by participants when seated facing an experimenter and talking about personal issues. More avoidant people were less comfortable with physical proximity, disliked having the experimenter move into their personal space, and sat significantly further away from the experimenter when given that option. Dismissingly avoidant attachment, as measured with the AAI [55], involves conversational tactics designed to maintain distance from the interviewer, such as not answering questions, engaging in long pauses, and failing to provide details necessary for the interviewer to understand the speaker's history.

Regarding anxious attachment, several studies have shown that anxious adults score higher than average on rejection sensitivity [56,57], are quicker to recognize rejection-related words in lexical decision tasks, and have difficulty inhibiting thoughts of rejection [58,59]. Baldwin and Kay [58] exposed people to sounds (pure tones) paired with rejecting (frowning) or accepting (smiling) faces, and then administered a lexical decision task in which rejection-related words were paired with each of the tones. Non-anxious (i.e., relatively secure) adults were slower to react to rejection-related words when they were paired with rejection tones (as compared to acceptance tones), but anxious people reacted faster to these words even in the presence of the acceptance tone. That is, anxious people were so sensitive to rejection-

related cues that they were unable to dispense with worries about rejection even in accepting contexts.

In another series of experiments, Mikulincer et al. [60] obtained indirect support for the idea that anxiously attached people construe an attachment figure's unavailability as a sign of their own helplessness and vulnerability. Participants were asked to imagine being separated from a loved one and then to perform a word completion task that measured the accessibility of death-related thoughts. Those who scored higher on attachment anxiety reacted to separation reminders with more death-related thoughts. This tendency was particularly strong when the imagined separation was long-lasting or final. In other words, for anxious individuals, social or psychological separation evoked thoughts of death. This finding is especially interesting in light of Bowlby's [2] theoretical speculation that the attachment system emerged during evolution because it protected primates (especially infants, but adults as well) from predation, dangerous accidents, and attacks by hostile others. In other words, seeking protection from attachment figures may have been motivated by fear of injury and death, and being rejected or abandoned by attachment figures might have accurately foreshadowed premature death.

This mental equating of separation and death was also noted by Hart et al. [61], who examined defensive reactions to separation and reminders of death. American undergraduates were asked to think about their own death, separation from a close relationship partner, or a neutral control theme, and then to report their attitudes toward the writer of a pro-American essay provided by the experimenter. People who scored relatively high on attachment-related anxiety or avoidance rated the pro-American writer more favorably in the death-primed condition than in the control condition—the typical defensive reaction to mortality salience [62]. However, anxious individuals, but not avoidant ones, also reacted more favorably to the pro-American writer in the separation-primed condition. In other words, anxious people showed the same defensive reaction (ethnocentric pro-Americanism) to reminders of death and separation.

Although anxiously attached adults are unusually afraid of losing a relationship partner, research has shown that they suffer from ambivalence or conflict between approach and avoidance relational tendencies [63]. On the one hand, they are highly motivated to gain a partner's attention and love. On the other hand, they suffer from intense fear of rejection and harbor serious doubts about their ability to gain a partner's love. These fears and insecurities can cause anxious individuals to restrain their approach tendencies when they sense a possibility of disapproval or rejection. Being caught in an approach–avoidance conflict, they are likely

to ruminate obsessively about how to react in social situations, thereby interfering with adaptive social behavior. Studies show that anxiously attached people make important mistakes in the social realm-at times failing to explain their needs and wishes clearly because they fear disapproval, which can make them vulnerable to unwanted sexual experiences and hurt feelings [64]; and at other times effusively or intrusively expressing their needs and fears to relationship partners [65], which can lead to rejection or partner withdrawal. Moreover, these individuals have difficulties both initiating new relationships that might be highly rewarding [66], and being able to end troubled or abusive relationships decisively [67].

Perhaps because of Bowlby's eclecticism, amazingly broad reading, and interest in a wide range of empirical studies (ranging from infant cognitive-developmental studies to community psychiatry studies of adult depression)-attachment research has been methodologically diverse and has benefited from the use of projective, observational, self-report, and physiological measures, as well as from cognitive and social-cognitive research paradigms. The research literature on attachment is exceptionally rich and conducive to diverse clinical applications. We hope that this book encourages such applications, which can then be rigorously assessed with appropriate research methods. Eventually we will have theoretically sound and truly evidence-based clinical practices, working in harmony with a research literature that is sensitive to clinical discoveries and applications. Ever-growing corpus of research results and practice based upon experiences of many Bowlby's psychoanalytic predecessors and contemporaries equipped psychoanalysts with theoretical instruments confirmed by verified methodological procedures and made them available to psychoanalytical practice for the benefit of the patients.

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Sažetak

Psihoanaliza i teorijska uporišta u istraživanjima na području teorije privrženosti

Godine 1969. britanski psihoanalitičar John Bowlby utemeljio je znanstvenu teoriju koja je danas poznata kao "teorija privrženosti" u kojoj tvrdi da je održavanje fizičke bliskosti s odraslima koji se brinu o djetetu primarni mehanizam regulacije sigurnosti i njegova preživljavanja. Kako se teorija razvijala, ponajviše zbog novih gledišta drugih znanstvenih disciplina kao što su biologija, neuroznanost i razvojna psihologija, i svakodnevna psihoanalitička praksa pokazala se veoma korisnom u objašnjavanju načina na koji se razvija psihološka privrženost u ljudi. U tom procesu jedan od najvažnijih koncepata jest mentalizacija, koja se ugrubo može definirati kao proces zauzimanja gledišta koja promatraju namjeru, što bi bio pristup ljudima kao onima koje vode unutarnje namjere. Da bi taj proces bio učinkovit, osoba mora imati zadovoljavajući kapacitet za mentalizaciju, što u konačnici dovodi do razvoja zrelog "selfa". Sve veće svakodnevno znanje o tom znanstvenom polju trebalo bi naposljetku uspostaviti teoretski čvrstu kliničku praksu zasnovanu na dokazima, koja će biti u skladu sa znanstvenom literaturom osjetljivom na klinička otkrića i kliničku uporabu. U ovom članku dajemo pregled istraživanja teorije privrženosti u svjetlu najnovijih otkrića, u nadi da će ih se moći upotrijebiti u psihoanalitičkoj praksi radi boljitka pacijenata.

Ključne riječi: teorija privrženosti; mentalizacija; intersubjektivnost; sepstvo.

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VALIDATION OF THE CROATIAN VERSION OF THE ASTHMA CONTROL TEST

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Summary

Validation of the Croatian version of the Asthma Control Test [ACT] in an adult persons with asthma was the objective of the study. A total of 90 consecutive asthmatic patients were recruited in a prospective observational study. A pulmonologist blinded to the ACT score assessed asthma control according to the Global Initiative for Asthma [GINA] guidelines. Lung function was measured and treatment prescribed until the control visit. Seventy-five patients attended the control visit after three months, according to the same protocol.

Two patients had intermittent asthma, 47 patients mild persistent, 31 moderate persistent and 10 severe persistent asthma. According to the pulmonologist's assessment during the first examination, 12 patients had uncontrolled asthma, 10 poorly controlled, 47 partially controlled, 16 well controlled, and 5 completely controlled under the current treatment. The ACT score was in the range from 5 points to 25 with mean (SD) score 16.3 [5] and Cronbach α of 0,79 [internal validity). ACT score had the best discriminating power for the pulmonologist's assessment of asthma control (F=17.9; $P<0.0001$), significantly discriminating uncontrolled, inadequately controlled and moderately controlled patients, from well controlled and completely controlled patients. In patients attending the control visit, significant correlation was found between the change in ACT score and the change in the level of asthma control assessed by the pulmonologist ($r^2=0.437$; $P<0.001$). The results of our study confirmed the validity of the Croatian version of ACT in the assessment of disease control in patients with asthma, aged 18-85 years.

Keywords: asthma; Asthma Control Test; questionnaire; validation.

INTRODUCTION

Due to a diminished impact of epidemics in our time and better watering with improved living standard, morbidity and mortality has been changed. Many chronic non-communicable diseases step on the health stage. Among them a big part takes chronic airways diseases, among which are asthma, chronic obstructive pulmonary disease (COPD), allergic rhinitis and sleep apnea, with around billion persons affected worldwide [1]. Asthma has been fascinating disease for millennia, yet still not enough recognized and treated. At the end of XX. Century the comprehensive strategy was establish Global Initiative for Asthma (GINA) [2], with the aim to increase awareness about asthma among physicians, health authorities and general population, with the purpose for better prevention, diagnostics and treatment of asthmatics. Each few years a revised document is publish, every time with emphasis on different part of disease management. After more than 10 years of using GINA strategy for classification of asthma severity, the revised GINA in year 2006. stress out asthma control concept. That concept means that the goal of asthma treatment is to achieve and maintain clinical control with least dosage medication [3]. In revised GINA 2012. the partnership doctor-patient was introduced, while in revised GINA strategy 2014. a focus was placed on modifiable risk factors, such as smoking, obesity and anxiety.

Asthma control is assessed by medical history data (incidence of asthma exacerbations), symptoms, lung function, but also assessment of inflammation in airways (exhaled nitric oxide, or induced sputum [4] and biomarkers, what makes asthma assessment complicated, expensive and less available. Physicians need simple clinical tool that measure asthma disease status. Several international and national guidelines have been developed, including the Croatian one [5], most of them recommended usage of questionnaires in the assessment of asthma control. Prevalence of asthma in Croatia, is middle high, around 6.9% in children [6]. Occasionally, a differentiation of inadequate asthma control from asthma exacerbation is complicated and consequently has occupied the attention of scientists for more than a decade [7]. Asthma questionnaires can facilitate decisions about the need for treatment modifications ("step up" or "step down"). Studies show that in adult patients questionnaires can identify patients who are at risk of asthma exacerbation, in need of emergency intervention and hospitalisation [8]. A similar study of a paediatric population was also published [9]. As a prerequisite for the application of a questionnaire in a specific language it is necessary to carry out a validation process. Emphasis has recently been placed on the control of asthma [10] and consequently we were interested in the extent of the correlation of the Asthma Control Test (ACT)

with diagnostic procedures in a specialised pulmonary out-patient department. The object of this study was the validation of the Croatian version of the Asthma Control Test (ACT) in adult persons with asthma.

MATERIAL AND METHODS

Study Design and Patients

An observational, prospective, single-centre study was conducted in the University Hospital Centre Zagreb, Clinical Department for Lung Diseases Jordanovac in Zagreb, Croatia. The study was carried out from September 1st, up until December 31st. Consecutive patients attending a routine check-up visit at the Outpatients department were recruited. Ninety asthmatic patients, aged from 18 to 85 years (mean 45.3, SD 17.4 years, 50 women (55.6%) were enrolled in the study. All patients were required to attend two (physician office) visits, baseline and a scheduled follow-up (control) visit after three months. During the first visit patients completed the ACT and were subject to an interview and clinical examination. In all patients the usual measurements of lung function [11] were conducted using forced spirometry testing with the flow-volume curve and reversibility testing with salbutamol [12]. Spirometry was conducted using computerized apparatus MasterLab Erich Jaeger, Wurzburg, Germany, and performed in accordance with the ATS/ERS Guidelines [13]. All spirometry measurements were compared with reference values published by the European Community for Coal and Steel [14] and expressed as a percentage of the predicted values. For reversibility testing, a standardized dose of salbutamol was used [4] puffs of 100 mcg with a total administered dose of 400 mcg [15]. Spirometry was repeated at a time when at least 75% of maximal bronchodilator activity is expected, after 15 to 30 minutes. A single pulmonologist, with long standing experience in asthma management, blinded for the ACT responses, rated asthma control on a scale from 1 to 5 and asthma severity according to GINA guidelines at both visits. Optimal therapy according to GINA guidelines was prescribed according to the rated level of control, using “step up” or “step down” approach. Seventy-five patients (83.0%) attended the control visit after three months and underwent a similar procedure as during the baseline visit, with the exclusion of reversibility testing (interview and clinical examination by the pulmonologist, lung function measurement and ACT).

Validation of the ACT was assessed according to internal consistency, test-retest reliability, discrimination validity and responsiveness as evidence of change in the ACT scores compared to changes in asthma control. The protocol was approved by the Local Ethic Committee of the University Hospital for Lung Diseases Jordanovac.

All patients provided their written informed consent before being included in the study.

Statistical analysis

Statistical analysis was performed using STATISTICA ver. 6 (StatSoft Inc., Tulsa, USA) and MedCalc ver. 9.2.0.1 (MedCalc Software, Mariakerke, Belgium). P=0.05 was used as statistically significant for all tests. Mean and SD were used to characterize continuous variables and number and percentage (%) for categorical ones. Internal consistency of the ACT was assessed using Cronbach α. Test-retest reliability was assessed in patients with no change in the level of asthma control .(according to the pulmonologist's assessment between the baseline and control visit, using intraclass correlation coefficient (ICC). ANOVA was used to compare continuous variables between subgroups according to asthma severity and asthma control. Chi-square test was used to compare categorical variables between subgroups. Linear regression analysis and Spearman rank order correlation were used to calculate the level of association between variables. Discriminant analysis and receiver operating characteristic (ROC) curve analysis was used to calculate the area under the ROC curve, cut-off value, sensitivity, specificity, positive and negative likelihood ratios (+LR, -LR) and diagnostic odds (DO) for ACT in predicting asthma control.

RESULTS

Table 1 shows a description of baseline values for the socio-demographic and clinical characteristics of patients recruited in the study. Two (2.2%) patients had intermittent, 47 (52.2%) mild persistent, 31 (34.4%) moderate persistent and 10 (11.1%) severe persistent asthma. At baseline visit, 12 (13.3%) patients were not controlled, 10.(11.1%) were poorly controlled, 47 (52.2%) were partially controlled, whila 16 (17.8%) were well controlled and five (5.5%) were completely controlled. Forty-nine (54.4%) patients used inhaled corticosteroids in monotherapy, 26 (28.9%) used inhaled corticosteroids + long-acting β_2-agonists; 18 (20.0%) used leukotriene receptor antagonists, 4 (4.4%) of them used leukotriene receptor antagonists + inhaled corticosteroids 5.(5.5%) leukotriene receptor antagonists + inhaled corticosteroids + long-acting β2-agonists and 1.(1.1%) leukotriene receptor antagonists + inhaled corticosteroids + teophyllin; 6 [6.7%) used just teophyllin; 1 (1.1%) used oral corticosteroid therapy and 58 (64.4%) short-acting β_2-agonists as needed. The average (SD) forced expiratory volume in the first second (FEV_1) as a percentage of the predicted value was 79.0% (21.1%) and the average reversibility to salbutamol was 16.0% (11.8%, range 1.4-64.0%) with a statistically significant inverse correlation to FEV_1(%) (r=-0.487;

P<0.001). ACT score rated from 5 points to 25, with a mean [SD) value of 16.3 (5.1) points with Cronbach α=0.796 (internal validity) showing high internal consistency. The only lung function parameter significantly associated with ACT score was peak expiratory flow (PEF) (Spearman R=0.224, P=0.037). ACT showed the best discriminant capacity for the pulmonologist's asthma control assessment (F=23.07; P<0.0001; Figure 1) significantly differentiating patients who were not controlled, poorly controlled and partially controlled (non-controlled asthma), from patients who were well and completely controlled (controlled asthma) with the area under the ROC curve of 0.921(95% CI, 0.868-0.957; P<0.001), sensitivity of 84.3% (95% CI, 74.7-91.4%), specificity of 85.0% (95% CI, 75.3-92.0%), +LR 5.62, –LR 0.18 and DO 31.22 for the ACT value ≤19 (Figure 2). The addition of FEV_1 (%) to ACT did not contribute to discriminant capacity for asthma control assessment (area under the ROC curve 0.895, 95% CI 0.818-0.971, P>0.05). Out of 75 patients who attended the control visit 7 (9.3%) were poorly controlled, 12 (16.0%) partially controlled, 32 (42.7%) well controlled and 24 (32.0%) completely controlled. Three patients (4.0%) showed two points poorer asthma control than on the baseline visit, five (6.7 %) one points poorer, fourteen (18.7%) showed the same level of control, twenty-two (29.3%) one points better, twenty-one (28.0%) two points better, eight (10.7%) three points better and two (2.7) even four points better control. During the control visit FEV_1 (%) was 85.5% (SD 19.9) and

Table 1. Demographic and clinical characteristics of the investigated asthma patients sample (N=90)

	Asthma severity according to GINA				
	Intermittent (N=2)	Mild persistent (N=47)	Moderate persistent (N=31)	Severe persistent (N=10)	Total
Gender (%) Male	1 (50.0)	20 (42.6)	15 (48.4)	4 (40.0)	40 (44.4)
Female	1 (50.0)	27 (57.4)	16 (51.6)	6 (60.0)	50 (55.6)
Age, mean (SD), yrs*	24.5 (9.2)	42.2 (16.3)	47.0 (17.1)	58.3 (17.7)	45.3 (17.7)
Duration of asthma, mean (SD), yrs	3.5 (2.1)	10.4 (10.7)	8.7 (10.1)	17.4 (20.0)	10.4 (11.9)
% FVC, mean (SD)*	101.3 (14.1)	98.6 (13.5)	80.1 (20.6)	64.6 (14.7)	88.5 (20.2)
% FEV_1, mean (SD)*	99.9 (24.2)	90.5 (14.4)	70.3 (15.4)	44.4 (15.5)	79.0 (21.1)
% PEF, mean (SD)*	93.1 (3.8)	85.6 (17.3)	69.5 (16.0)	42.9 (11.0)	75.8 (20.9)
% reversibility of FEV_1, mean (SD)	25.0 (12.8)	12.5 (9.8)	15.6 (9.7)	25.2 (22.1)	16.0 (11.8)
Concomitant conditions (%)	2 (100)	32 (68.1)	23 (74.2)	9 (90.0)	66 (73.3)
Exacerbation rate, mean (SD), n/yr	0.50 (0.71)	0.98 (1.04)	1.24 (1.01)	1.61 (0.79)	1.13 (1.01)

*p<0.01

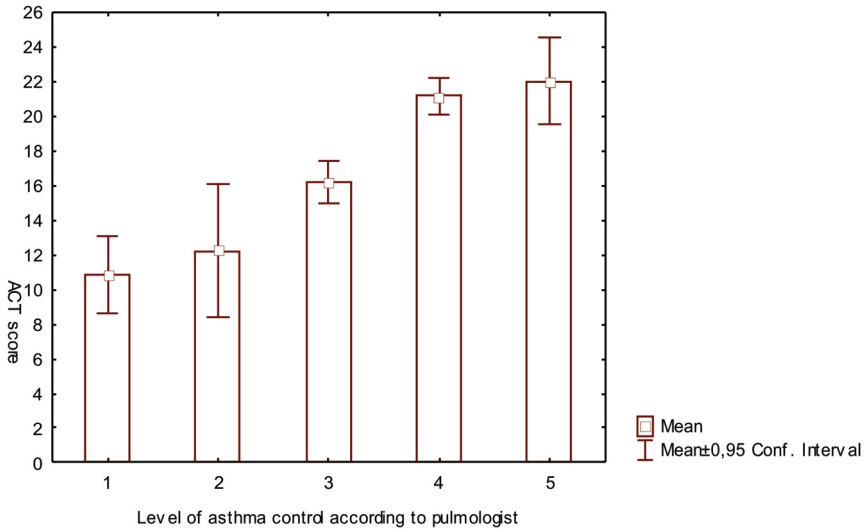

Figure 1. Distribution of ACT scores according to the level of asthma control according to pulmologist (N=90); 1 - uncontrolled (N=12), 2 - poorly controlled (N=10), 3 - somewhat controlled (N=47), 4 - well controlled (N=16), 5 - completely controlled (N=5).

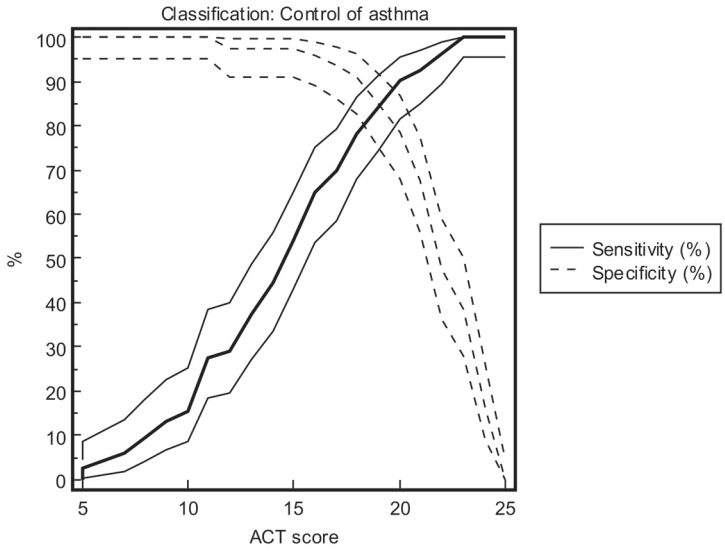

Figure 2. Plot versus criterion value for asthma control and ACT score (N=90); sensitivity and specificity plots with 95% CIs.

the average ACT score was 20.7 points (SD 4.2; range 10-25). In patients who attended the control visit, significant correlation was found between the change in ACT score and the change in asthma control assessed by the pulmonologist (Spearman R=0.729; P<0,001). In the subgroup of patients in which the same asthma control was assessed by the pulmonologist on both visits (N=14) a high level of correspondence of the ACT score (ICC=0.864; P<0.001) was found.

DISCUSSION

A person with asthma in good control has no limitation of productivity or physical activity and lead creative life, that why the asthma control is so important. Asthma control has been defined as the degree to which the symptoms and activity limitation have been prevented, and consequently the step of treatment is adjusted according to the level of asthma control achieved [3]. The results of the Asthma Insights and Reality in Europe study (AIRE), Asthma in America study (AIA) and European Community Respiratory Health survey (ECRHS) showed that despite national and international guidelines asthma management is suboptimal worldwide [18]. No gold standard measure for asthma control was universally accepted. A good indicator of asthma control in patients is required usage of rescue medications such as short-acting β_2-agonists. Patients are often well informed that the frequent usage of short-acting β_2-agonists is not recommended, although few of them are aware of the fact that the usage of short-acting β_2-agonists of more than once or twice weekly is a sign of loss of asthma control [3]. Asthma Control Test is a short patient-based 5-question survey which does not include pulmonary function tests and has been shown to accurately predict the degree of asthma control assessed by the pulmonologist on the basis of history, clinical examination and spirometry [10]. We were interested in the ability of the Croatian version of the ACT questionnaire to predict the level of asthma control assessed by the expierenced pulmonologist. In patient with astma whose astha in uncontrolled or oposite, completelly controlled, the physician interview is short, clinical examination typical, and an asthma specialist can easily make a clinical judgement. In our study less than half of patients included in this study, examined in the Outpatients Department, were in these categories: twenty-two (24.4%) were uncontrolled, and 21 (23.3%) patients were well and completely controlled on their regular asthma treatment. On the other hand, there is a great proportion (47/90) of partially controlled asthmatics in whom more time is needed for the clinical interview. It is important to ask the patient about the day- and nighttime symptom frequency, impact of asthma on usual daytime activities, such as everyday functining at school or work, interference with sleep, activity limitations,

usage of medicines, compliance, education, environment control, asthma emergency department visits, hospitalisations and comorbidities, particularly symptoms in the upper respiratory tract [19]. A comprehensive, lengthy and demanding interview is required with most patients in order to precisely estimate asthma control and its specific dimensions. This is time consuming even for an asthma specialist, not to mention less experienced physicians (general practitioners), and it is debated on how to assess control in such a way that it both supports management and simplicity of use. In contrast to this consuming time clinical approach, ACT offers brief, but still comprehensive, insight into asthma control which can help to establish the level of asthma control, together with its specific dimensions in conjunction with the results obtained from other clinical tests. This is also evident from the results of our validation study, showing that ACT has a high and significant association with the pulmonologist's evaluation of asthma control. It has been demonstrated that ACT predicts asthma control as determined by a specialist evaluation better than $FEV_{1,}$ which indicates that control cannot be measured by airway function alone [10, 20,21), and confirms that specialist assessments are based on more than lung function measurements. Asthma control is multidimensional in nature, and thus measures of pulmonary function only provide a score in time assessment. In our study the addition of FEV_1 did not contribute significantly to the discriminant ability of ACT, which was consistent with previous reports [21,22), but did contribute marginally in the validation study of the original version [10] showing that, although there was no significant correlation of ACT score with lung function parameters (apart from a very low level with PEF), FEV_1 value did not contribute any additional information about the level of asthma control that was not already present within the ACT score. Almost absent correlation of ACT score with lung function parameters most probably reflects the multifaceted dimension of asthma control which also depends on many other parameters such as underlying inflammation, which is thought to cause poor control [23]. The published validation of a Spanish version of ACT, contrary to our results, indicates the significance of spirometry in the assessment of asthma patients and also emphasises that FEV_1 should not be used on its own to assess asthma control, but in conjunction with clinical assessment [20]. A study in Japan also emphasised the need for multiple measures of control and the significance of measuring peek expiratory flow and fractional exhaled nitric oxide (FeNO) in the assessment of asthma control in patients in which the ACT score showed complete asthma control, because the disappearance of symptoms does not mean that the underlining inflammation is adequately controlled [21]. Our study shows that ACT correlates with complete assessment, including a comprehensive interview by an experienced pulmonologist, physical examination and lung function measurement. One of the

advantages of this quick and simple tool for the assessment of asthma control is the monitoring of patients, so that modulation of treatment is easily recognized.

Our study showed that the Croatian version of ACT was equally accurate in patients with a stable level of control between two consecutive visits and in patients in which control was improved or lost. In this respect it allows precise assessment of therapeutic interventions, compliance and indication of possible co-morbidities. American authors consider that the great value of ACT is in the monitoring of patients after asthma exacerbation [20], as well as predictors of exacerbation and changes in therapy [22].

After translation of the international questionnaires to other languages, validation procedure needs to be conducted. ACT has proved to be very useful in clinical practice, which is confirmed by validation of this questionnaire in Europe (Spain) [18,21] and in the world (Japan, Singapour) [19, 23].

CONCLUSION

After translation of ACT to the Croatian language we conducted validation of the Croatian version of ACT in adult patients with asthma and demonstrated that the Croatian version of ACT is useful in the assessment of disease control in adult patients with asthma.

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Sažetak

Validacija hrvatske inačice testa za kontrolu astme *(Asthma Control Test)*

U ovoj prospektivnoj opservacijskoj studiji provedena je validacija hrvatske inačice testa za kontrolu astme [Asthma Control Test (ACT)] u odraslih osoba s astmom. U istraživanje je uključeno 90 susljednih bolesnika s astmom. Pulmolog koji nije znao rezultat ACT testa procjenjivao je kontrolu astme u bolesnika prema globalnim smjernicama za astmu [Global Initiative for Asthma (GINA)]. Izvedeni su testovi plućne funkcije i preporučeno je liječenje do sljedećeg kontrolnog posjeta. U sljedeći kontrolni posjet za tri mjeseca došlo je 75 bolesnika, prema planiranom protokolu.

Dvoje bolesnika imalo je povremenu astmu, 47 bolesnika blagu trajnu astmu, 31 umjereno tešku trajnu astmu, a 10 bolesnika tešku trajnu astmu. Prema procjeni pulmologa na prvom pregledu, 12 bolesnika imalo je nekontroliranu astmu, 10 loše kontroliranu, 47 djelomično kontroliranu, 16 dobro kontroliranu, dok je njih 5 imalo astmu potpuno kontroliranu dotadašnjim načinom liječenja. ACT rezultat kretao se od 5 do 25 bodova sa srednjim [SD] zbrojem 16.3 [5] i Cronbach α od 0,79 [interna validacija]. ACT rezultat imao je najbolju diskriminirajuću snagu za pulmološku procjenu kontrole astme [$F = 17.9$; $P < 0.0001$] te značajno diskriminirajuću za nekontrolirane, djelomično kontrolirane i umjereno kontrolirane bolesnike u odnosu na dobro kontrolirane i potpuno kontrolirane bolesnike s astmom. U bolesnika koji su bili u drugome kontrolnom posjetu značajna korelacija nađena je između promjene ACT rezultata u odnosu na stupanj kontrole astme kako ju je procijenio pulmolog [$r2 = 0.437$; $P < 0.001$]. Rezultati ove studije potvrđuju valjanost hrvatske inačice testa za kontrolu astme ACT u procjeni stupnja kontrole bolesti u bolesnika s astmom u dobi između 18 i 85 godina.

Ključne riječi: astma; test za kontrolu astme (Asthma Control Test); upitnik; validacija.

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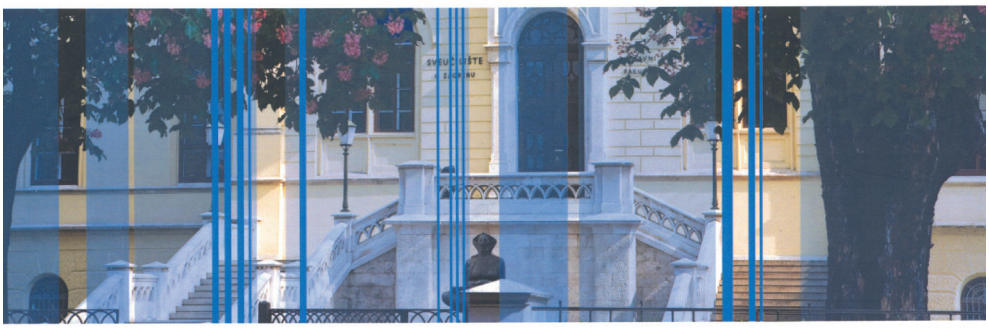
3rd International Conference on Regenerative Orthopaedics and Tissue Engineering

November 4th 2015, Zagreb, Croatia

NEW INSIGHTS IN CARTILAGE REPAIR



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PREFACE

Dear colleagues and friends,

The Department of Medical Sciences of The Croatian Academy of Sciences and Arts in cooperation with University of Zagreb and Croatian Othopaedics Society are honoured to have the privelege welcoming You in the 3rd edition of the "International Conference on Regenerative Orthopedics and Tissue Engineering", to be held in Zagreb, Croatia on November 4th 2015.

In line with the spirit of the successful first edition, that was held in 2010 in Zagreb and the second edition that was held in Opatija in 2012, this Conference's goal is to present the latest scientific and clinical innovations in the field of bone and cartilage repair and regeneration and the motto of this 3rd Conference is „New Insight in Cartilage Repair".

As we are all aware, the continuity of progress and achievement in orthopaedic surgery has been well-substantiated.Research in general, and research in our field in particular, requires intellectual power,imagination, enthusiasm, persistant and systematic work,and above all, friendly sharing and exchange of new ideas and views.

Once more, the 3rd International Conference on Regenerative Orthopaedics and Tissue Engineering will be a multidisciplinary program of research and practice, giving the opportunity to interact and share clinical and research experiences with colleagues from the orthopaedic and musculoskeletal community. Prominent domestic and international speakers will provide comprehensive, up-to-date, research-based answers to the most frequent questions that arise in this rapidly evolving field of medicine.

The Conference will take place in the heart of the 1300-year old Central-European and Mediterranean country of Croatia and its historical capital Zagreb.University of Zagreb was founded 347 years ago and Croatian Academy of Sciences and Arts celebrates 155 years of its foundation.

This 3rd Conference has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement n°278807 - Bioreactor based, clinically oriented manufacturing of engineered tissues (BIO-COMET). There is no participation fees, and the Conference is open to all those interested

in learning, sharing and networking (students, scientists, clinicians, pharma and biotech employees etc.).

Once again, on behalf of your Croatian hosts, we welcome you with the conviction that our Conference will prove to be the place to revive old and form new friendships, and with the hope that you will find it stimulating, informative and a pleasure.

Sincerely yours,

Marko Pećina, Ivan Martin
Presidents of the Conference

Alan Ivković
General Secretary of the Conference



Participants of the 3rd International Conference on Regenerative Orthopaedics and Tissue Engineering

ANIMAL MODELS IN CARTILAGE RESEARCH

Dražen Matičić

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Articular cartilage traumatic injuries and degenerative lesions are leading cause of disability in humans worldwide. Spontaneous healing of articular cartilage is poor and untreated defects predispose to osteoarthritis. The clinical and financial burden of osteoarthritis inspires clinicians and scientist to explore new therapies. Animal models in cartilage repair and regeneration research are widely used to evaluate novel concepts and improve current techniques. Animals commonly used in cartilage research include lapine, murine, canine, porcine, caprine and equine models. Direct injury to the articular cartilage of the used experimental animal, including creation of focal defect, joint destabilization or application of chondrotoxic agents is a common method to induce cartilage loss and osteoarthritis. Limitations related to the model and the translation of the results to the clinical practice should be carefully evaluated and recognized. Rodent and rabbit models are cost and space effective, useful for pilot and proof of concept studies but associated with spontaneous intrinsic healing of articular cartilage greater than in humans and large animal models. The small size of their joints especially thin articular cartilage, limit the types of treatment options and translational potential. Large animal models with thicker articular cartilage and larger joints, address many of the limitations associated with rodent and rabbit models. Healing potential of the cartilage in large animals is comparable with that seen in humans. Post-operative manipulation, particularly in dogs, like application of bandages, braces, limited weight-bearing and physical therapy can mimic various options used in human medicine. Besides, spontaneously occurring cartilage defects in client owned dogs and horses provide important data in assessments of different treatment strategies. No animal is ideal for every type of investigation in cartilage research. Anatomy, maturity and joint biomechanics and cost effectiveness must be taken into account. Comprehensive analysis of each species characteristics should be conducted in order to address the main research questions. Large animal models like dogs, sheep, goats and horses are necessary for translational research aimed at gaining regulatory approval for safe and effective clinical use in humans.

INDUCED PLURIPOTENT STEM CELLS IN CARTILAGE REPAIR

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The generation of functional cartilage tissue using stem cells is promising to be an important advance in regenerative medicine. Although progress has been made using adult mesenchymal stem cells, these cells suffer from limited accessibility, senesce upon expansion, donor and clonal variability and the generation of immature tissue. Investigating more potent stem cells such as human induced pluripotent stem cells (hiPSCs) could overcome a lot of those limitations but it also comes with its unique challenges. Whilst similar to embryonic stem cells they show differences in their epigenetic signature, suggesting different signalling requirements for differentiation. In-depth understanding of the signals that regulate cartilage development is paramount for harnessing the power of hiPSCs. We report a simple, chemically defined, efficient, scalable and reproducible protocol for the differentiation of hiPSCs toward chondrocytes that can be used to engineer mature cartilage in vitro and in vivo.

IMPROVED MR SCORING SYSTEM FOR OUTCOME ANALYSIS OF CARTILAGE REPAIR PROCEDURES

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Francesco Perdisa, Maurilio Marcacci

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Articular cartilage and its supporting bone are tightly coupled and should be viewed as a connected unit. New treatments are emerging aiming at the regeneration of the entire osteochondral structure, but MRI evaluation scores are mainly focused on the superficial cartilaginous layer. The aim of this study is to analyze the MRIs obtained after transplantation of an osteochondral scaffold in order to develop a score for the assessment of the imaging appearance of the regenerated tissue quality, both at the cartilage and bone level, and its correlation with the clinical outcome.

Ninety-one MRIs of patients treated with a cell-free collagen-hydroxyapatite osteochondral scaffold for lesions of the knee articular surface were considered (71 men, 20 women, age at surgery 31.2±11.5 years, defect size 2.8±1.6 cm2, 38 affected by degenerative and 14 by traumatic cartilage lesions, and 39 by OCD). All MRIs were performed at 2 years of follow-up. MOCART, the most commonly applied MRI scoring system for cartilage evaluation, was used for developing the osteochondral imaging evaluation tool, by expanding the focus also on the subchondral structure and adapting the scores attributed to each parameter according to their correlation with the clinical outcome.

The MRI analysis showed no correlation between MOCART score and the clinical outcome (IKDC subjective score 75.7±15.4). The results of the multivariate analysis, performed to assess the correlation of each osteochondral score parameter with the clinical outcome, were used to attribute the score to each parameter to develop a 0-100 MRI score. Statistical analysis confirmed the correlation between the MRI results and the clinical outcome, both for the overall group and the patellar lesions ($p=0.008$ and $p=0.038$, respectively). With a few exceptions, patients clinically successful with an IKDC > 80 also had an MRI osteochondral score of at least 70 ($p=0.002$).

In conclusion, after reducing the parameters for cartilage layer evaluation and expanding the focus on the subchondral layer, 7 parameters were selected: degree of filling and integration, surface appearance, signal intensity with DP FAT-SAT, subchondral bone appearance and edema, effusion. The new osteochondral score correlated with the clinical outcome, proving to be an useful tool for the study of tissue maturation after the implantation of scaffolds for osteochondral regeneration.

IMAGING TECHNOLOGIES IN TISSUE ENGINEERING

Gregor Liebsch

PreSens GmbH, Regensburg, Germany

Introduction

Monitoring the parameters oxygen, pH or CO2 is of great interest in many fields in cell biology and medical research. The knowledge on these parameters gives evidence on the metabolic status of cells, spheroids, engineered or native tissue. Ideally, a detection method should be non toxic and non invasive and at the same time the respective analyte should not be consumed during an online measurement. We

addressed these points and developed a new imaging device for online monitoring of metabolic activity.

Methods

The imaging system VisiSens consists of a compact fluorescence microscope and an optical sensor foil. The sensor foil is doped with fluorescent dyes which are sensitive to oxygen, pH or CO2. The foils are flexible and based on a transparent polyester support, thus they can be cut to any desired size to fit the experimental requirements. A non-transparent optical isolation layer above prevents cross-talk by optical interferences like sample auto-fluorescence or ambient light. The sensor translates concentrations of oxygen, pH or CO2 into specific light signals. Each dye molecule inside the sensor foil reacts independently and the light signals can be recorded with the digital camera inside the detector unit. One single image contains the information of a whole array of single sensor points. This way the oxygen distribution over a large 2D area can be visualized and analyzed with a high spatial resolution.

Results

We present application examples starting from monitoring cell monolayers over spheroids to engineered and native tissue. The method is simple, fast and reliable and even vasculated tissue may be examined in one step and monitored over time periods up to several days. The spatial resolution of the respective oxygen, pH or CO2 maps is shown to be as low as 25 μm in optimal case. The system was already applied for investigating microfluidic chips, native and engineered tissue.

Conclusion

The new imaging system VisiSens is compact, mobile, flexible and easy to use and allows imaging of pattern and gradients with high spatial resolution and without analyte consumption. VisiSens proofed to be a new and valuable tool for online monitoring of metabolic activity.

NOSE2KNEE – SCIENTIFIC RATIONALE FOR USE OF NASAL CHONDROCYTES IN ARTICULAR CARTILAGE REPAIR

Ivan Martin

University Hospital Basel, Basel, Switzerland

Articular cartilage injuries remain a clinical challenge and are known to double the incidence of degenerative joint disorders if left untreated. Current therapeutic options suffer from major drawbacks, such as defect-size limitations, long and complex rehabilitation times, donor-site morbidity and limited graft material. Even the more advanced cell-based therapies show highly variable clinical outcome and provide not entirely satisfactory treatment. Moreover, due to the use of articular chondrocytes (AC) their applicability for elder patients is limited, since the capacity of AC to redifferentiate and generate a functional matrix is limited, extremely donor dependent and is reduced with increasing donor age. The use of nasal chondrocytes (NC) could overcome these limitations.

Harvesting and donor site morbidity

In contrast to articular cartilage, harvesting of nasal cartilage, is less invasive and is performed under local anaesthesia leading to minimal donor site morbidity due to the fact that the donor site is easily accessible and not subjected to high levels of physical force.

Properties of Nasal Chondrocytes

The properties of NC are less age dependent than AC, they proliferate faster and have a higher and more reproducible capacity to generate functional cartilaginous tissues. In addition the contents of GAG and Collagen type II and, consequently, the mechanical properties are superior in tissues engineered from NC.

Compatibility of NC in a joint environment

In vitro experiments were conducted to study the response of NC to experimental conditions mimicking the mechanical and inflammatory joint environment. Although NC are not subjected to high mechanical forces in their native environment, they are capable to properly react to mechanical forces typical of joint loading. Furthermore, tissues generated from NC also showed a higher capacity to recover after a short exposure to IL-1 .Molecular compatibility of NC to an articular environment

has also been demonstrated both in vitro and in vivo in a large animal study.It was also shown that the quality of the repair tissue at 6 month achieved by NCs was statistically superior to AC controls.

In summary, the scientific knowledge generated in those pre-clinical research projects supports the compatibility and efficacy of NC for articular cartilage repair.

NANOCOMPOSITE SCAFFOLDS FOR NOSE2KNEE APPLICATION

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Due to the poor self-repair capacity, most severe chondral and osteochondral lesions are source of progressive pain and disability, limiting joint motion and quality of life. The major challenge in the regeneration of large osteochondral lesions is the rapid restoration of the joint function after treatment. The integration between the newly formed bone and cartilage at the interface and with the host tissues is paramount for the success of the regenerative treatment. Tissue engineering strategies involving perfusion bioreactors, offer the potential to improve the intimate integration between bone and cartilage layer during the culture, before implantation. Biphasic scaffolds with appropriate microstructure and gradient composition are crucial not only to physically and chemically guide cell response and tissue growth during the culture, but also to promote the formation of a well integrated osteochondral interface.

The developed material is a bi-layer scaffold comprising type I collagen isolated from the equine tendon for the cartilage component and type I collagen biomineralized with bioactive Magnesium-doped hydroxyapatite (Mg- HA) nano-crystals for the bone component. The use of natural materials was selected to enhance biological interaction with the host tissue. Crosslinking reaction was optimized to improve adhesion between layers and the resistance to the perfusion of culture medium in bioreactor. A peel test was performed to quantify the adhesion force between the two interconnected layers. The results obtained (4.1 ± 1.2N) showed better integration in comparison with other bilayer collagen hydroxyapatite scaffolds.

The morphological and microstructural analysis of the scaffold was performed by SEM. Cartilage layer possesses a sponge-like structure with well-defined, uniform, interconnected macropores. Freeze drying process was optimized to replicate

the vertical orientation of collagen fibers. The biomineralized layer shows a highly fibrous structure with well interconnected pores with diameters up to 600 micron. Chemical characterization was performed to verify the composition and quantify the percentage of the inorganic mineral content.

The biomaterial was shaped in disks of 2.5cm of diameter and appeared to be suitable for culture of nasal chondrocytes for 5 weeks in perfusion bioreactor. Preliminary experiments showed the potential of the engineered graft for mimimally invasive treatment of osteochondral lesions.

PRECLINICAL ANIMAL MODEL – NOVEL TECHNOLOGY FOR ARTICULAR CARTILAGE REPAIR

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Nasal chondrocytes could be viable alternative for ACI in human patients. Before using it in clinical settings, it is necessary to test this hypothesis on translational (large animal) model.

Designed model was chronic, full-thickness cartilage defect placed on lateral and medial femoral condyles in sheep. The protocol included two surgical procedures. First procedure was to create two partial-thickness defects, 4 mm in diameter on the lateral and medial femoral condyle with a standard mosaicplasty instruments used in human orthopaedic surgery. Biopsy of nasal septum cartilage was also performed with skin biopsy puncher, 8 mm in diameter. Cartilage samples from both origins were used for production of autologous tissue grafts. Chondrocytes were isolated, seeded on biphasic collagen-hydroxiapatite scaffolds, and cultured in automated bioreactor for 5 weeks. Engineered tissue was then implanted in condyle defects during second procedure. First, 4 mm partial-thickness defects were converted to osteochondral defects 6,5 mm in diameter and 5 mm deep. Then engineered cartilage tissue was implanted. There were four study groups. Autologous tissue grafts, engineered from scaffold and nasal septum chondrocytes were implanted

in the experimental group of animals. One group was implanted with autologous tissue grafts engineered from scaffold and articular chondrocytes. In one group cell free scaffolds were implanted while last group served as negative control in which only conversion of defect was performed, but it was left untreated. Animals were sacrificed and tissue analysis was performed at three different time points: 6 weeks, 3 months and 12 months after the implantation. Tissue analysis included macroscopic, microscopic and molecular evaluation. Microscopic analysis included different stains (hematoxylin-eosin, safranin O, picrosirius) and immunohistochemistry (collagen I, II and aggrecan). Semi-quantitative data of morphology were obtained with histological score ICRS II. ELISA and DMMB assays were performed to quantify the amount of collagen I, II and glycosaminoglycans in repair tissue.

Results confirmed the feasibility of production of autologous cartilage tissue grafts from nasal septum chondrocyte for treatment of condyle cartilage defects. Furthermore, nasal chondrocyte grafts showed promising results in restoration of damaged articular cartilage.

The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement n°278807 BIO-COMET.

CARTILAGE TISSUE ENGINEERING FROM NOSE2KNEE: 12-MONTHS RESULTS OF A PHASE 1 CLINICAL TRIAL

Marcus Mumme

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Purpose

As compared to commonly used cell based treatments for articular cartilage repair, grafting of cartilage tissues, engineered in vitro to reach a mature stage, could result in more durable repair. To reduce the variability in the quality of the engineered tissue grafts, nasal chondrocytes were used as a cell source with reproducible chondrogenic capacity. The purpose of this phase-1 study was to demonstrate safety and feasibility of the procedure. Preliminary indications on efficacy after 12 months are also presented.

Material and methods

Ten patients with symptomatic, post-traumatic full-thickness cartilage lesions (2-8cm2) on the femoral condyle/ trochlea were treated. Patients underwent nasal

septum cartilage biopsy in an outpatient procedure. Autologous nasal chondrocytes were isolated, expanded, seeded and cultured in collagen sponges (Chondrogide®, Geistlich) according to Good Manufacturing Practice (GMP) regulations. After four weeks production, the engineered nasal cartilage graft was implanted via mini-arthrotomy.

Patients were followed up radiologically by MRI (MOCART score), delayed Gadolinium Enhanced MRI (dGEMRIC, for assessment of glycosaminoglycans content) and clinically (IKDC score).

Results

No complications occurred by nasal cartilage biopsying or implantation of the engineered tissues,

10 patients with 12 cartilage defects reached 12 months follow up so far. One patient with 1 defect partially lost the graft due to new sports injury and was excluded from analysis. The MOCART score 12 months post-surgery was 41.3 (5-60). The dGEMRIC revealed a relative ΔR1 of 1.40 (0.78-2.17). The IKDC pre-surgery and 12 months post-surgery was 46.5 and 75.6 respectively. The KOOS pre-surgery and 12 months post-surgery was 63.9 and 81.3 (Symptoms), 70.4 and 88.9 (Pain), 76.1 and 93.2 (ADL), 36.1 and 69.4 (Sport), 41.7 and 57.0 (QoL).

Conclusion

These early results demonstrate safety and feasibility of the method and indicate that engineered nasal cartilage grafts can participate to the repair of articular cartilage defects in the knee. The mean relative ΔR1 of 1.40 (1.0 for native cartilage) suggests that hyaline repair tissue can be achieved, possibly to a higher extent than MACT (2.18, Trattnig+, 2008) or ACT (2.40, Trattnig+, 2007). Our results demonstrate safety and feasibility of the method and indicate that patients can benefit from this therapy.

This study opens a new approach in biological cartilage regeneration, based on engineering of mature cartilage tissues using autologous nasal chondrocytes.

Acknowledgment

Deutsche-Arthrose-Hilfe for financial support.

BIOPRINTING CARTILAGE GRAFTS

Marcy Zenobi-Wong

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Bioprinting is an emerging technology for the fabrication of patient-specific, anatomically-complex tissues and organs. A novel bioink was developed based on two unmodified regulatory-compliant polysaccharides, gellan and alginate, which undergoes cell friendly gelation in the presence of cations. Rheological properties of the bioink revealed optimal shear thinning and shear recovery properties for high fidelity bioprinting. Tensile testing of bioprinted dumbbell-shaped specimens revealed a strong, ductile material. To make the bioink tissue-specific and bioactive, extracellular matrix particles can be added, or the polymers can be sulfated for growth factor binding affinity. As proof of concept, clinical product BioCartilage® (cartilage extracellular matrix particles) and bovine chondrocytes were added to the bioink. 3D auricular, nasal, meniscal and vertebral disc grafts were printed based on computer tomography (CT) data or generic 3D models. The bioink containing BioCartilage supported proliferation of chondrocytes and, in the presence of transforming growth factor beta-3 (TGF-β3), supported strong deposition of cartilage matrix proteins. A clinically-compliant bioprinting method is presented which yields patient-specific cartilage grafts with good mechanical and biological properties. The versatile method can be used with any type of tissue particles to create tissue-specific and bioactive scaffolds.

3D CELL AND TISSUE CULTURING UNDER PERFUSION

David Wendt

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The in vitro culture of primary cells typically involves monolayer expansion on tissue culture plastic (e.g., on Petri dishes) and serial replating of the cells onto new dishes upon confluence. This technique fails to recapitulate a physiological environment and is associated with a loss of characteristics and functionality of the expanded cell progeny. Cell culture in a 3D setting (e.g., within the pores of 3D scaffolds), which in turn requires the use of perfusion-based bioreactor systems to maintain suitable mass transfer rates, has the potential to recapitulate features of a

physiological and pathological environment and thereby support cell functionality. This presentation will highlight the use of bioreactor-based 3D culture systems for applications including: (i) the expansion of progenitor cells while preserving their native properties ('niche' concept), (ii) the co-culture of different cell types, including those of the inflammatory/immune system, as well as (iii) the expansion of primary tumors and cell lines. These advanced 3D cell culture systems can represent organotypic tissue models based on human cells to investigate processes involved in tissue regeneration or disease modeling and treatment. The generated knowledge will be relevant to identify new strategies or compounds to instruct in situ regeneration, as well as to restore pathologic conditions.

BIOREACTOR-BASED CARTILAGE ENGINEERING

David Wendt

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Autologous Chondrocyte Implantation (ACI) and the second generation Matrix-assisted ACI (MACI) have long been established in the clinic for the repair of cartilage injuries. However, as these products are manufactured with minimal in vitro pre-cultivation times, they contain little to no extracellular matrix and therefore lack the complex biological and mechanical signals which can be delivered by a more physiological mature tissue graft. Our laboratory has recently conducted a clinical trial based on mature cartilaginous engineered tissues for treatment of cartilage lesions, demonstrating the safety and feasibility of the tissue grafts. However, the manufacturing processes used to produce the engineered grafts were based on traditional bench-top manual culture methods, requiring a large number of labor-intensive manipulations, which ultimately pose challenges towards regulatory compliance, process up-scaling, and long-term cost-effectiveness. As an alternative, bioreactor-based manufacturing systems, which automate and control the various bioprocesses, have the potential to overcome the limitations associated with conventional manufacturing methods. Robust and streamlined bioreactor-based processes, as described in this presentation, will be key for the future manufacturing of cartilage grafts for clinical applications, as they facilitate the establishment of simple, compact, and closed manufacturing systems, with minimal user intervention required, lower operating costs, and increased compliance to safety guidelines.

ADVANCED BIOREACTORS FOR APPLICATION IN CLINICAL MEDICINE

Tim Smith, Nuala Trainor

Octane Biotech Inc., Kingston, Canada

Cell therapy and tissue engineering hold great promise; however, current manufacturing methods constrain economic viability, especially for 'patient-scale' production

Despite significant production constraints in terms of clinical scale-up, autologous and matched allogeneic treatments continue to find considerable clinical interest due to the ability to avoid immunologic complications. In these clinical models, 'patient-scale' production is the term adopted for autologous or matched allogenic cell therapy procedures where the size of the cell production event is necessarily matched to the requirements of a single therapy.

We examine five critical issues in the successful clinical delivery of patient-scale cell therapy and evaluate potential solutions based on the use of advanced bioreactors. The high cost of cell therapy production protocols is considered in the context of automation where electronic instrumentation linked to disposable bioreactors creates production efficiencies. The adoption of such bioreactor-based platforms combined with flexible instrumentation programming allows cell production centers to produce cell therapy treatments for diverse clinical conditions. Furthermore, the incorporation of biosensors as essential monitoring aids in the bioreactor environment provides the opportunity for pre-validated software to effect discrete process changes to accommodate inherent variability in cell performance on a patient-by-patient basis. The use of such electronic protocols and biological feedback implicitly drives the availability of comprehensive electronic batch records, which enhance GMP traceability and support the complex clinical logistics inherent in autologous processes.

Through the use of automated processes delivered by bioreactor-based production platforms, we propose new clinical models for patient-scale cell production that address today's demanding expectations for reliable biological performance, effective GMP logistics, and economic viability.

GMP FACILITIES FOR BIOREACTOR-BASED TISSUE ENGINEERING

Michele Desogus

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GMP facilities have been traditionally conceived for standard sterile pharmaceutical production, based on chemical compounds concepts.

Production for cell-based active ingredients need a significant reinvention, that bioreactors for Biocomet project realized improving stability of products together with high potential compliance to current pharma products standards.

FROM HYDROGELS TO REINFORCED COMPOSITE STRUCTURES AS A POTENTIAL BONE SUBSTITUENTS

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There have been numerous investigations based on the synthesis of new biodegradable scaffolds for bone tissue replacement. To achieve required features of potential biomaterial substituent, wide range of synthetic and natural polymers along with calcium phosphate phases were used. The main goal is to produce a material that can mimic extracellular matrix of natural bone. Chitosan has shown to be a good candidate for tissue engineering materials due to its chemical similarity to biological molecules and short-time biodegradability in vivo through specific enzymatic reactions. Likewise, chitosan can be processed by different techniques for scaffold production (lyophilization, electrospinning, thermally induced phase separation, particulate leaching, microsphere sintering, etc). Scaffold's topography (surface roughness), charge and wettability, microstructure and interconnected porosity

are the main factors influencing the cell adhesion and activity, neovascularization and angiogenesis. Besides surface properties, the alteration of scaffold's composition during neotissue formation affects further cell proliferation and growth. Calcium phosphate salts have shown good resorption ability in physiological conditions, and depending on its composition, higher solubility. Even though chitosan/hydroxyapatite scaffolds show favourable properties for bone tissue engineering, biomechanical properties of those systems can not meet applications in vivo. To overcome the mechanical drawback, additional synthetic polymers have been used.

Up till now, we have demonstrated the importance of scaffold's composition and microstructure in cell proliferation and differentiation on chitosan/hydroxyapatite three-dimensional structures. In vitro cell cultures in static and dynamical conditions with different cell lines have indicated suitable amount of in situ formed HA within chitosan porous matrix. Since chitosan/hydroxyapatite porous structures form hydrogels in physiological conditions, their mechanical properties do not meet the conditions as bone implants. To overcome those limitations, 3D-printed PLA structure have been applied as a supporting construct for porous chitosan/hydroxyapatite porous hydrogel. Osteogenic evaluation with mesenchymal stem cells after 21 day of culture confirmed good osteogenic properties of three-component porous structure and subsequently indicated its potential application as bone substituent.

ARE TENDON-DERIVED STEM CELLS A BETTER SOURCE FOR BONE REGENERATION?

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Despite significant advancements in bone tissue engineering applications, the clinical impact of bone marrow stromal cells (BMSCs) for the treatment of large osseous defects remains limited. Therefore, other cell sources are under investigation for their osteogenic potential to repair bone. In this study tendon-derived stromal cells (TDSCs) were evaluated in comparison to BMSCs to support the functional repair of a 5mm critical-sized, segmental defect in the rat femur.

Analysis of the trilineage differentiation capacity of TDSCs and BMSCs cultured on collagen sponges revealed an impaired osteogenic differentiation and mineral deposition of TDSCs in vitro, whereas chondrogenic and adipogenic differentiation was evident for both cell types. Radiographic assessment demonstrated that neither cell type significantly improved the healing rate of a challenging 5mm segmental femoral defect in vivo. Both, transplanted TDSCs and BMSCs led to the formation of only small amounts of bone in the defect area and histological evaluation revealed non-mineralized, collagen- rich scar tissue to be present within the defect area. Newly formed lamellar bone was restricted to the defect margins resulting in closure of the medullary cavity. Interestingly, in comparison to BMSCs, significantly more TDSC-derived cells were present at the osteotomy gap up to 8 weeks after transplantation and were also found to be located wihin newly formed lamellar bone, suggesting their capacity to directly contribute to de novo bone formation. To our knowledge this is the first study investigating the in vivo capacity of TDSCs to regenerate a critical-sized defect in the rat femur.

GLOWBRAIN – AN IN VITRO AND IN VIVO PLATFORM FOR STUDYING THE REGENERATIVE POTENTIAL OF THE MURINE NEURAL STEM CELL APPLICATIONS IN REPAIR OF ISCHEMIC BRAIN INJURY

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Since the central nervous system shows very little capability for self-repair following ischemic injury, regenerative medicine approaches are increasingly interested in the use of neural stem cells (NSCs) for cell replacement strategies. Labelling NSCs with maghemite (γ-Fe2O3) nanoparticles allows direct in vivo tracking and imaging of NSCs during brain repair using medical imaging techniques such as magnetic resonance imaging. In addition, application of biomaterials, such as alginate, represents an interesting tool to carry out cell replacement therapies.

We reported in vitro NSCs viability and initial differentiation in alginate hydrogels. Furthermore, we investigated in vivo the potential of alginate hydrogels as support system for NSCs injection in the brain. Our preliminary in vivo results demonstrate the possibility to obtain injectable alginate hydrogels that crosslink once injected in the brain tissue. Inflammation profiles, obtained using TLR2-luc mouse reporter line after alginate injection, suggest that alginate presence is not harmful for the brain tissue. Taken together these findings show that alginate could be used as an efficient support for NSCs transplantation in the nervous system, able to increase cell survival and integration in an injury-affected brain.

Moreover, using GlowBrain platform we reported that coating γ-Fe2O3 nanoparticles with either poly(L-lysine) or D-mannose improves their biocompatibi-

lity with NSCs and their cell labeling efficiency in vitro when compared to both their uncoated γ-Fe2O3 counterparts and commercially available dextran-coated nanomag®-D-spio nanoparticles. Finally, we used poly(L-lysine)-γ-Fe2O3 and (D-mannose)-γ-Fe2O3 nanoparticles to label NSCs which were transplanted into mouse post-ischemic brain and tracked during brain repair using magnetic resonance. Both poly(L-lysine)-γ-Fe2O3 and (D-mannose)-γ-Fe2O3 could easily be visualized in ex vivo mouse post-ischemic brain, making them an attractive agent for future NSC in vivo tracking studies.

GlowBrain here presents a platform which allows in vitro and in vivo investigation of biocompatibility of biomaterials that can serve as support for NSCs, ultimately allowing their survival and differentiation in post-ischemic brain. Moreover, our platform can be used to assay in vitro the effects of labeling NSCs with differently coated γ-Fe2O3 nanoparticles, namely cell viability and proliferation, cellular uptake efficiency and labeling mechanism as well as potential to be visualized in vivo in mouse ischemic brain.

OPTIMIZATION OF CONDITIONS FOR IN VITRO THREE-DIMENSIONAL CARTILAGE GROWTH

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Articular cartilage is a poorly vascularized and innervated tissue that shows no capacity for effective spontaneous regeneration in cases of damage and injury, which represents a major health problem and unmet medical need. Common methods of the treatment and therapy have proved to be ineffective. Tissue engineering, as a new important field of regenerative medicine, emerges as potential effective alternative. Chondrogenesis, the process by which cartilage is formed, actually represents the consequence of several steps directed by signaling molecules, receptors, transcription factors, cells' interaction with ECM and other environmental factors. In this respect, the aim of our study was to optimize conditions for 3D in vitro chondrogenesis in order to produce functional cartilage implants that could be used in clinical practice to cure some specific cartilage defects.

The method of optimization includes: i) cell type; ii) source of dexamethasone; and iii) percentage of oxygen. In the experiment three different cell culture types were used: chondrocytes, human mesenchymal stem cells from bone marrow (hMSC) and combination of chondrocytes and hMSC in 2:1 ratio. Cells were grown in 3D culture, incorporated in a peptide hydrogel RADA (BD PuraMatrix Peptide Hydrogel). In order to induce chondrogenesis, cells were put in a differentiation medium containing ascorbic acid-2-phosphate, L-proline, ITS, TGFβ-1 and dexamethasone. Dexamethasone was added in a differentiation medium or it was incorporated with cells within a peptide hydrogel. In order to demonstrate the effect of oxygen level on the efficiency of chondrogenic differentiation, cells were grown in normoxic (20% O2) and hypoxic conditions (5%O2). Expression levels of two important cartilage marker genes, SOX9 and agreccan, were evaluated by quantitative PCR in samples on day10 and day21 after induction of chondrogenesis.

Our results provide important insights into effects of how different cell type, dexamethasone source and oxygen level affect efficiency of in vitro chondrogenesis.

PLATELET RICH PLASMA IN THE TREATMENT OF SPORTS INJURIES: INDICATATIONS AND TECHNIQUE

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KB Sveti Duh, Zagreb, Croatia

Platelet-rich plasma (PRP) due to its role in wound healing, has spanned various fields of orthopaedics and sports medicine . Among athletes muscle injuries are common and may be associated with impaired functional capacity. The vulnerability of athletes to strains and contusions represents a substantial problem for professional players and their clubs. Such injuries involve significant time lost from training and competition. Presently there are no drugs available to hasten restoration of muscle function after injury and the results of healing with conventional therapy including rest, ice, compression, and elevation (RICE) are often inadequate. Therefore platelet-rich plasma (PRP) therapies may help athletes by promoting muscle regeneration , enhancing the process of soft-tissue healing and to decrease time to recovery.PRP is generally considered an elective treatment for subacute and chronic conditions. In orthopaedics and sports medicine is used to treat tendinopathies, ligament sprains, muscle strains, degenerative joint conditions. PRP is obtained from a sample

of patients blood drawn at the time of treatment,and it is prepared in a process of differential centrifugation,where acceleration force is adjusted to sediment certain cellular constituents based on different specific gravity. Suspensions have different concentration of platelets and leucocytes depending on the method and time of its centrifugation.PRP injections may be performed unaided or under ultrasound guidance. Multiple injection techniques have been employed including intramuscular delivery of a singular bolus, multiple depots at the site of maximal injury, or a single injection into the muscle insertion site.

A NEURAL CREST ORIGIN OF TENDON CELLS?

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We have previously shown that tendon perivascular cells (TPCs) express markers associated with tendon cells and neural stem cells, such as Nestin and Musashi1. Findings that tendon cells express a variety of neuron associated markers such as acetylcholine, M2 acetylcholine receptors or substance P now lead us to hypothesize that tendons harbour a cell population of neural crest origin.

By immunohistochemistry using antibodies specific for tendon, neural crest and neuron associated markers on control- and Rosa26-YFP-Sox10-Cre mice as well as on Scleraxis-GFP mice and by in vitro differentiation assays on cultured murine tendon cells we characterized tendon cell in vitro and in vivo.

Murine Achilles tendon cells coexpress p75NTR, Tenomodulin, Doublecortin, Neurofilament and TUJ1.

Tendon cells from sox10cre mice express YFP together with tenomodulin the tail tendon anlagen of of E13.5 embryos. YFP is detectable in the Achilles tendons of adult animals. Tendon cells in tails of Scleraxis-GFP e13.5 embryos partly co-express p75 and GFP.

In vitro murine tendon cells differentiate into functional neurons with the capacity to generate spontaneous action potentials, detected by multi electrode array.

With this work we show that murine tendon cells express a variety of neuron associated markers. Analysis of Rosa26-YFP-Sox10-Cre and Scleraxis- GFP mouse tendons reveals a potential neural crest origin of a tendon cell population.

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From the history of medicine

TWENTY-FIVE YEARS OF THE FUNCTIONAL ENDONASAL ENDOSCOPIC SINUS SURGERY IN CROATIA

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Summary

The aim of this article is to give an overview through twenty-five years of endonasal endoscopic functional sinus surgery (FESS) in Croatia. The first operation of this type took place on December 6, 1990 at the Dept. of ENT Head & Neck Surgery, Clinical Hospital Center Zagreb, Croatia. The surgeon was Ranko Mladina. Later on Zagreb FESS School grew up and reached the high international level giving the rhinosinusologic community all over the knowledge on a new syndrome (Two Holes Syndrome) as a subclass of chronic rhinosinusitis, than the most popular and widely accepted method for teaching, learning and training the FESS surgical techniques developing the concept of the Lamb's Head FESS Dissection, and, finally a specific "5-steps technique" in performing the endonasal endoscopic orbital decompression. A retrograde elevator, crucial instrument for an non-traumatic orbital decompression, has its origin from Zagreb as well as the lamb's head holder for the training dissections. During these 25 years 17 CIRAS (Croatian International Rhinosurgical Advanced School) have been organized dispersing the most recent knowledge of this discipline to numerous participants coming from all over. One fundamental textbook on this technique has been published in 1994 and later on two manuals on lamb's head surgical anatomy and details how to dissect paranasal sinuses in this model (besides Croatian edition, it has been translated also in English, Italian, Russian and now in Chinese language) as well as a manual on 5-steps techniques for the orbital decompression.

Key words: Functional Endoscopic Sinus Surgery; orbital decompression; lamb's head dissection; CIRAS; retrograde elevator; Two Holes Syndrome.

HOW IT BEGAN AND WHAT HAPPENED DURING A QUARTER OF THE CENURY?

Twenty-five years have passed since Ranko Mladina performed the first endonasal endoscopic functional surgery on the patient suffering from chronic rhinosinusitis

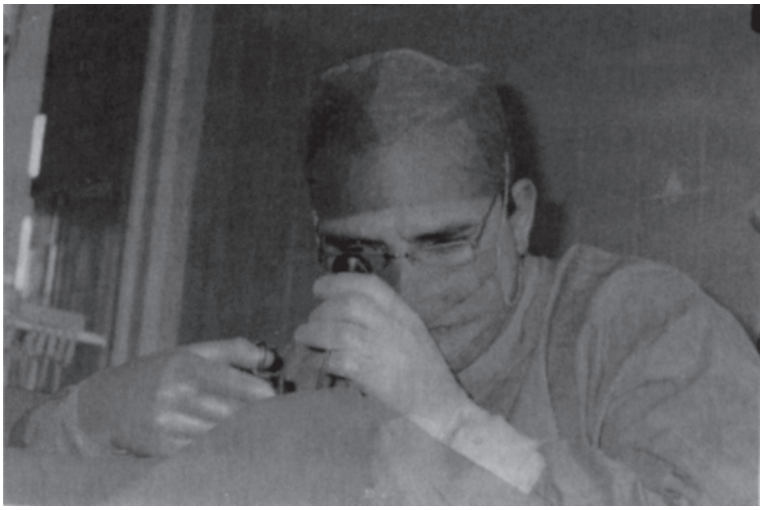

Figure 1. Mladina performing the first FESS ethmoidectomy on December 6, 1990



Figure 2. The 1st CIRAS in Zagreb, 1993. From left: Mladina as a speaker, Prof. Krajina, Prof. Subotić, Prof. Passali (Rome), Dr. Budrovich (Belluno), Dr. Gnjidić (neurosurgeon), Dr. Hat (radiologist)

(CRS). The left-sided classical uncinectomy, middle antrostomy, and anterior ethmoidectomy have been performed under the general anesthesia on the day December the 6th 1990 at the Dept. of ORL Head & Neck Surgery, Clinical Hospital Center Zagreb, Croatia (*Fig.1*). The nowadays advantages of hypotensive anesthesia were not yet developed at that time in general, neither we had the opportunity to have a preoperative CT scanning. The preoperative diagnosis has been established on anamnestical data, endoscopic examination of the nasal cavities and classical tomograms of the paranasal sinuses. Anesthesiologist was late Dr. Božica Trupković xcFotivec, whereas the instrumental nurse was a Saint Cross order's nun Judita (Lucija Baotić).

Shortly after the first procedure has been performed, few other colleagues from the same Clinic joined Mladina, and started to perform this type of sinus surgery as well. In this way, the concept of the endoscopic aspect of the CRS started to grow in Zagreb. Surprisingly, despite the Homeland War started in the year 1991, this technique continued to spread all over Croatia. So, in the year 1993, within the frame of the celebration of the 72th anniversary of the ENT Clinic Šalata, the first Croatian International Rhinosurgical Advanced School (CIRAS) has been organized (*Fig. 2*).



Figure 3. Mladina's textbook on Functional Endoscopic Sinus Surgery edited by „Školska knjiga" Zagreb in the year 1994

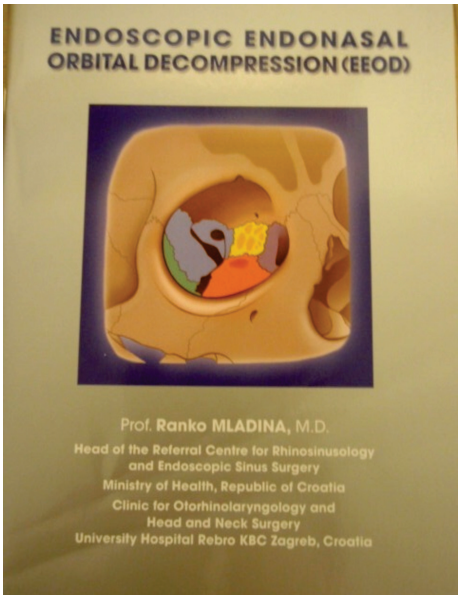

Figure 4. Mladina's „Doctor-to-Doctor" Karl Storz manual on his own technique named „5-Steps Endonasal Endoscopic Orbital Decompression (EndoPress, Tuttlingen, 2008)

The invited international speakers were Prof. Dr. Desiderio Passali and Dr. Romeo Budrovich, both from Italy. One of the invited speakers was Dr. Josip Hat, a radiologist from Zagreb who was a pioneer in the field of computed tomography imaging of the paranasal sinuses. That was the crucial time for us as we finally could start ordering preoperative diagnostic CT scans. Computed tomography produced the paradigm shift in understanding and therapeutic approaches to the paranasal sinuses. Those who did not believe us at that time, changed their opinion, and by time CT of the paranasal sinuses became a routine diagnostic step.

Because of our will to spread the idea and practice of functional endonasal endoscopic sinus surgery out of Zagreb, i.e. throughout Croatia, we continued with CIRAS courses and thus in the year 1994. The 2nd CIRAS has been successfully organized in Split, the capital of Dalmatia region, under the direction of Dr. Glunčić. The biggest editing house in Croatia, "Školska knjiga", has published the fundamental textbook on functional endonasal endoscopic sinus surgery written by Ranko Mladina [1], (*Fig. 3*). This textbook was intended to help other colleagues from other hospitals in Croatia to accept this technique and to serve as a real prop for all those who were enthusiastic and brave enough to start with this technique by themselves, far from the big centers. Next year (1995) the 3rd CIRAS has been organized in Osijek, the capital of Croatian region Slavonija, which at that time stood at the very borderline with the enemy army. This CIRAS passed, but nobody who was there will ever forget the terrible moments and sounds when bombs and grenades have been used by enemies during the live surgery sessions, lectures etc...

In the year 1996, the 4th CIRAS has been organized in Bjelovar, small town, some 50 kilometers northwest from Zagreb, under the direction of Dr. Piškorić. The fifth CIRAS took place in the year 1997 in Slavonski Brod, the town which General Hospital has achieved the greatest experience in the treatment of all kinds of war injuries. The local leader of the course organization was Dr. Đanić.

After the first five CIRAS meetings, we were very satisfied since it became obvious that the idea of the endonasal endoscopic diagnosis and surgical treatment became almost a routine in various places in Croatia.

At the same time, Croatian concepts in functional endonasal endoscopic surgery started to interfere with the world trends in the development of this item. In terms of that, after collecting a huge number of operated patients suffering from dysthyroid orbitopathy, Mladina wrote about his own way of performing orbital decompression, called 5-STEPS TECHNIQUE OF THE ENDONASAL ENDOSCOPIC ORBITAL DECOMPRESSION (2008), [2]. This has been published by EndoPress Tuttlingen, Germany, as a manual belonging to the well known chain of manuals of Karl Storz Company (Tuttlingen, Germany), so called "Doctor-to-Doctor" manuals,

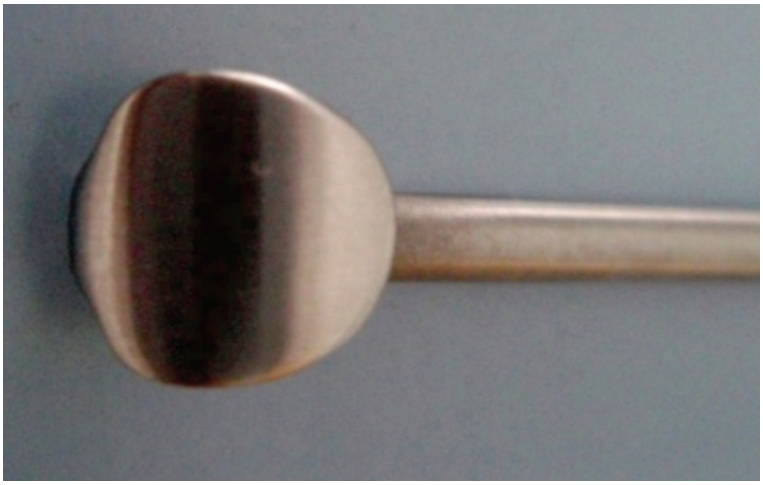

Figure 5. Mladina's retrograde elevator for endonasal endoscopic orbital decompression (the view from above), produced by Karl Storz GmbH, Germany

or popular "Silver Booklets" (*Fig. 4*). In this way the technique from Zagreb school became popular worldwide. Parallel with that event, a new endoscopic instrument, invented by Mladina, has been added to Karl Storz armamentarium for the endonasal endoscopic orbital decompression: a retrograde elevator *(Fig. 5)*. The purpose of this instrument was to allow the elevation of lamina papiracea of the medial orbital wall in a retrograde manner, without uncontrolled and undesired tearing of periorbital sac. After the non-traumatic elevation of the bony wall, it was finally possible to make parallel, horizontal and highly controlled incisions of the periorbit without making any damages to the surrounding tissues.

From the year 1998 all CIRAS meetings and courses have been organized in Zagreb exclusively.

A big shift in CIRAS practice happened in the year 2000 when the exercises on the lamb's head animal model have been introduced officially in the program. The special Head holder instrument has been developed by Bacc. Arch. Ivan Mladina, originally an architect, together with Karl Storz Tuttlingen, and all the problems on how to maintain the animal head in place and in an individually desired position during the endoscopic trainings and learning endoscopic endonasal techniques, was entirely solved for ever (*Fig. 6*).

The new "Doctor-to-Doctor" manual originated from Zagreb, dedicated to this kind of training, learning and teaching endoscopic endonasal surgical techniques has been published, again by Karl Storz EndoPress Tuttlingen, Germany [3], (*Fig. 7*).

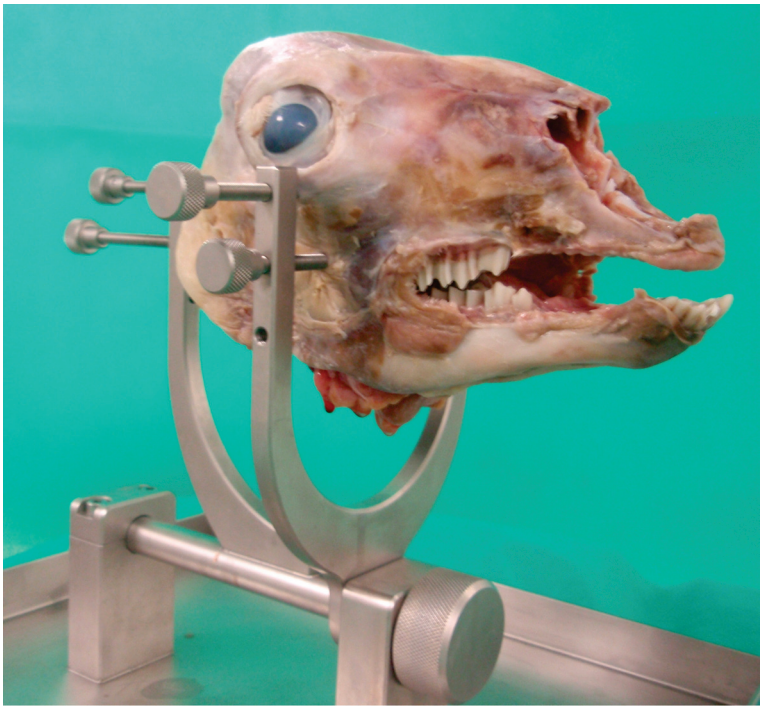

Figure 6. The lamb's Head Holder in use. The lateral and occipital screws fix the head in the desired position and can be changed during the procedure acorrding to surgeons comfort. The main screw (red arrow) allows controled rotation oft he main axis and adaptation of the lamb's head in an oblique position which additionally upgrades surgeon's comfort

From the very beginning, this textbook became very popular in several countries and because of their great interest it has been translated within the next 2-3 years in English, Russian and Italian languages. Today, in the year 2016, a Chinese edition is under preparation.

Finally, almost parallel to the growth of popularity of the Lamb's Head Dissection concept, Karl Storz also developed a navigational system with very user-friendly protocol, which enables all colleagues dealing or trying to deal with this kind of surgery to exercise also the dexterity in the use of navigational systems. In the year 2014 the video on all the steps of the Lamb's Head Dissection has been produced and placed on the domain www.ciras.eu, _thus becoming available for everybody in the world permanently.

In the year 2010 CIRAS was awarded for its great international echo and value by getting permanent auspices of the European Rhinologic Society (ERS)!

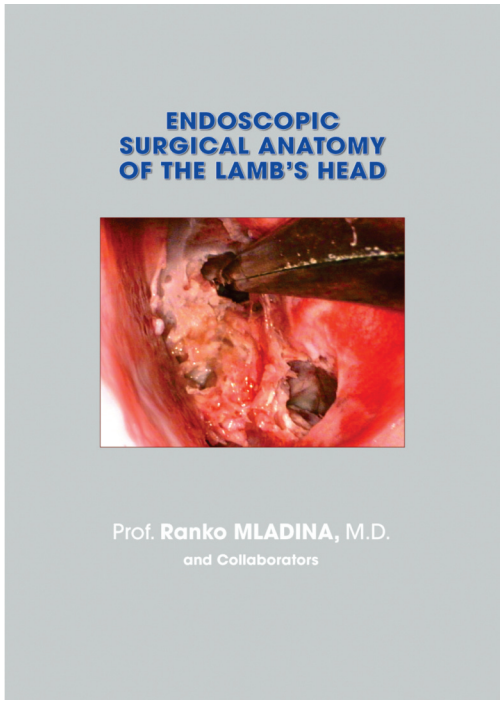

Figure 7. The „Doctor to Doctor" manual on Endonasal Endoscopic Lamb's Head Dissection which involves also the most precise anatomical analysis of the lamb's head and particularly paranasal sinuses ever published owing to a great help of colleagues from Veterinary Faculty in Zagreb

From the year 2011 the Dept. of ORL Head & Neck Surgery of the Clinical Hospital Center Zagreb has its own navigational system and a harmonic scalpel which made the progress in operating the most difficult cases of the tumors of the skull base, clivus, optic nerve and the pterygopalatinal fosse (Ass. Prof. Martin Jurlina and Dr. Gorazd Poje). The demanding pituitary gland surgery using the transnasal endoscopic trans-sphenoidal approach in very selective tumors of the pituitary gland also became relatively simplified for the endoscopic surgeon by means of the use of the navigational system (Dr. Marcel Marjanović Kavanagh). In this way our centre became one of the rare centres in Europe which is capable to fight against such cases at the highest level!

The CIRAS web-site offers also a Gallery. One click is enough to see how many of the world famous endonasal endoscopic sinus and skull base surgeons have been invited and participated to the CIRAS meeting over the last 15 years, and particularly how many of them have been dissecting the lamb's head affectionately and with a great passion, beginning with the icon in this surgical science (David Kennedy) and others.

Today, 25 years after the beginning, despite the 4-years lasting difficult Homeland war, Croatian endonasal endoscopic sinus surgery stays along with the biggest

centers in much larger countries in the world, be it from the aspect of technical level of the surgical procedures, be it regarding the own techniques which have been generally accepted all over the world, starting from endonasal endoscopic orbital 5-steps decompression technique, up to new diagnoses in rhinosinusology, like the THS (Two Holes Syndrome), [4], a Cob-web rhinitis [5], and the importance of two absolutely dominantly inherited types of nasal septal deformities, types 5 and 6 according to Mladina's international classification, 1987 [6,7]. The last perhaps will essentially contribute to both future of rhinosinusology and cardiology, and perhaps will open the door to the essential prolonging of the human life in general.

Acknowledgments

Ranko Mladina and his team (Martin Jurlina, Gorazd Poje and Marcel Marjanović Kavanagh) express their sincere gratitude to Karl Storz GmbH, Tuttlingen, Germany, for their great support through all the period of 25 years of the development of this kind of medical skills and philosophy regarding diagnosing and surgical treatment of sinus diseases in Croatia.

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Sažetak

25 godina endoskopske sinusne kirurgije u Hrvatskoj

U ORL klinici Kliničkoga bolničkog centra Zagreb 6. prosinca 1990. izvedena je prva endoskopska operacija sinusa – izveo ju je dr. Ranko Mladina. Time počinje era razvoja endoskopske misli u dijagnostici i kirurškom tretmanu nosnih bolesti, navlastito kroničnog rinosinuitisa. Razvijena je poznata zagrebačka škola FESS-a s međunarodnim odjekom koji se manifestirao objavljivanjem čak dva kirurška priručnika na hrvatskom i engleskom jeziku, a priručnik o vježbanju kirurške tehnike endoskopskih sinusnih operacija na janjećoj glavi preveden je još i na talijanski, ruski i kineski jezik. Iz te je škole proizašao i novi kirurški instrument za atraumatsku endoskopsku dekompresiju orbite i nezaobilazni držač za janjeću glavu pri kirurškim vježbama, oba proizvedena u njemačkoj tvrtki Karl Storz GmbH u Tuttlingenu.

Ključne riječi: funkcijska endoskopska sinusna kirurgija; orbitalna dekompresija; disekcija na janjećoj glavi; CIRAS; retrogradni elevator; Two Holes Syndrome (sindrom dvaju otvora).

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