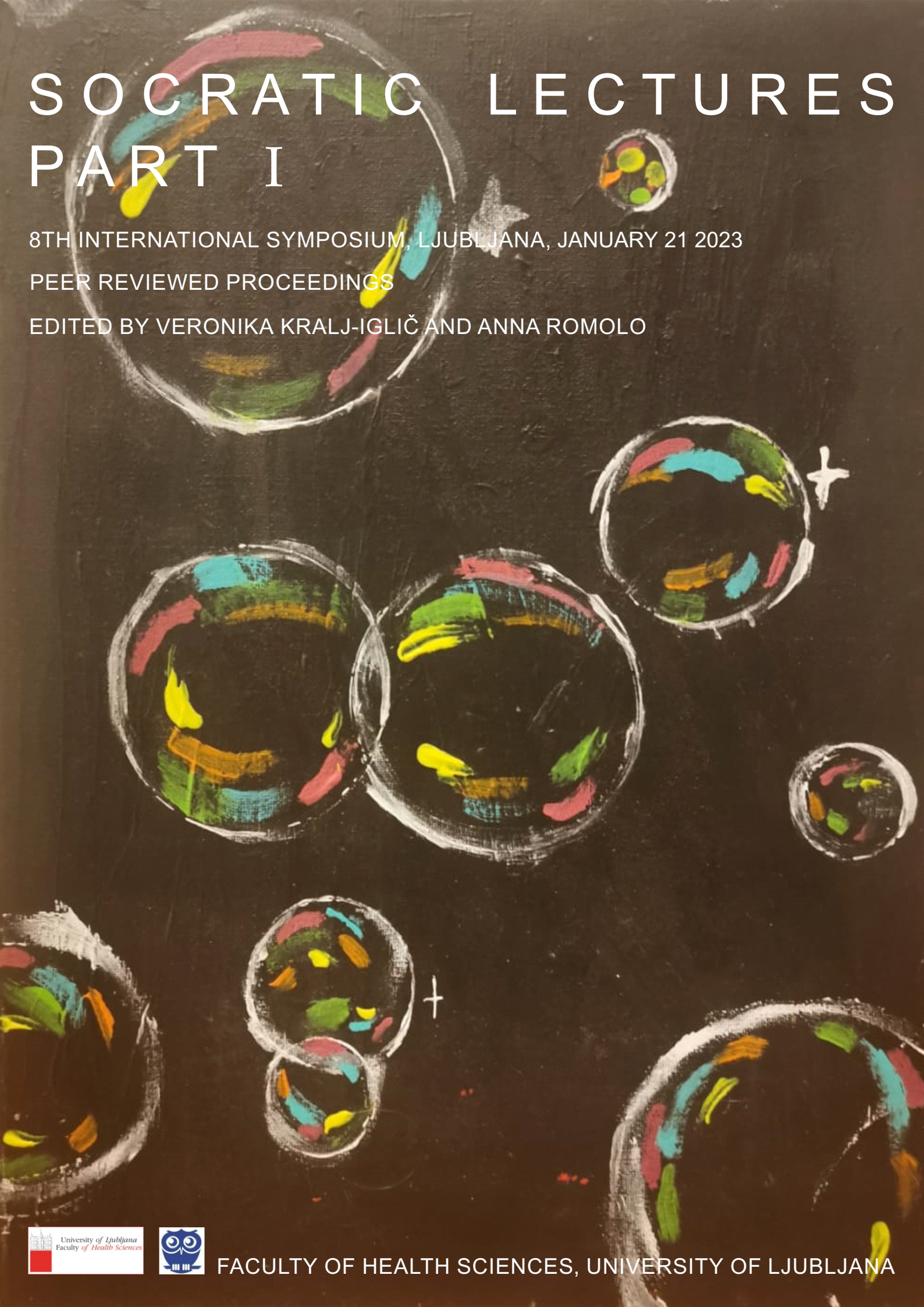
SOCRATIC LECTURES PART I

8TH INTERNATIONAL SYMPOSIUM, LJUBLJANA, JANUARY 21 2023

PEER REVIEWED PROCEEDINGS

EDITED BY VERONIKA KRALJ-IGLIČ AND ANNA ROMOLO



University of Ljubljana Faculty of Health Sciences

FACULTY OF HEALTH SCIENCES, UNIVERSITY OF LJUBLJANA

Socratic lectures
8th International Minisymposium, Part I, Ljubljana, January 21, 2023
Peer Reviewed Proceedings
Edited by Prof. Veronika Kralj-Iglič, Ph.D. and Anna Romolo
Reviewers: Yelena Istileulova, Vesna Spasovski
Published by: University of Ljubljana Press
For the publisher: Gregor Majdič, The Rector
Issued by: University of Ljubljana, Faculty of Health Sciences
For the issuer: Andrej Starc, The Dean
Design: Anna Romolo
Gallery Saint Champs: Judit Nyirkos
Image on the front page: Fia DeNardis
First digital edition.
Publication is available online in PDF format at: https://www.zf.uni-lj.si/images/stories/datoteke/Zalozba/Sokratska_8_I.pdf
DOI: 10.55295/PSL.2023.I
Publication is free of charge.
Ljubljana, 2023

Kataložni zapis o publikaciji (CIP) pripravili v

Narodni in univerzitetni knjižnici v Ljubljani

COBISS.SI-ID 147228163

ISBN 978-961-297-109-0 (PDF)

The members of the Organizing Committee of 8th Socratic Lectures: Angelo Beletić, Matej Daniel, Tatia Dolidze, Drago Dolinar, Vladimira Erjavec, Haque M, Aleš Iglič , Yelena Istileulova, Boštjan Kocjančič, Veronika Kralj-Iglič, Blaž Mavčič , Gabriella Pocsfalvi , Anita Prelovšek, Anna Romolo, Gitta Schloser , Renata Vauhnik.

Program of the Socratic Symposium January 21, 2023, 10:00 – 13:00(Ljubljana time)

10:00 – 10:45 Plenary lecture: Sergej Tomić; Belgrade, Serbia: Immune response in COVID-19: Biomarkers and therapeutic targets

Section 1: Human Medicine organized and moderated by **Boštjan Kocjančič** and **Yelena Istileulova**

11:00 - 11:15 **David Malidze, Rishu Bansal; Tbilisi, Georgia:** Cardiac arrhythmias in patients with myocarditis in the post-COVID period

11:15 - 11:30 **Marika Zhamutashvili, Rishu Bansal, Shweta Tilante, Nino Badridze, Ekaterina Dolmazashvili, Natia Jojua, Tinatin Gognadze; Tbilisi, Georgia:** Diagnostic and prognostic analysis of serological and biochemical markers in patients with COVID-19: A retrospective study

11:30 - 11:45 **Eka Kokhreidze, Zaza Avaliani, Maia Zhamutashvili, Nino Gabashvili; Tbilisi, Georgia**: Observation on tuberculosis preventive treatment in Georgia

11:45 - 12:00 **Špela Tadel Kocjančič; Ljubljana, Slovenia:** Neuroprognisitcs in determination of brain death after reanimation

12:00 - 12:15 **Boštjan Kocjančič; Ljubljana, Slovenia:** Use of tranexamic acid in orthopaedic surgery

12:15 - 12:30 **Darja Pestotnik; Ljubljana, Slovenia:** Effects of aerobic exercise with blood flow restriction on cardiorespiratory system with emphasis on respiratory parameters in healthy young men

12:30 - 12:45 **Sara Bitenc Zore; Ljubljana, Slovenia:** Narrow-band imaging - Clinical application in otorhinolaryngology

12:45 - 13:00 **Yelena Istileulova; Ljubljana, Slovenia:** International accreditation and rankings

Section 2: Veterinary Medicine organized and moderated by **Vladimira Erjavec** and **Angelo Beletić**

11:00 - 11:30 **Angelo Beletić; Zagreb, Croatia:** Serum, saliva, and liver proteome indices associated with platelet biology during inflammatory conditions in the different animal species

11:30 - 11:45 **Vida Eraghi; Zagreb, Croatia:** Vaccine development against paratuberculosis

11:45 - 12:00 **Barbara Lukanc, Vladimira Erjavec; Ljubljana, Slovenia:** Portosystemic shunts in cats

12:00 - 12:15 **Levan Tsitskishvili, Levan Makaradze, Tengiz Kurashvili, Nino Milashvili, Zura Makaradze, Ekaterina Sanaia; Tbilisi, Georgia:** Canine sirofilariasis in the South Caucasus and its pathomorphology

12:15 - 12:30 **Naida Kapo; Sarajevo, Bosnia:** Phenotypic and genotypic analysis of anthelmintic resistance

12:30 - 12:45 **Paul Kail; Canada:** Successful second intention healing of a large skin wound in a cat's cheek using Manuka honey

12:45 - 13:00 **Manca Novak; Ljubljana, Slovenia:** Tracheal stents in dogs

Section 3: Musculo-Skeletal Health organized and moderated by **Renata Vauhnik**

11:00 - 11:30 **Arcangelo Russo; Enna, Italy:** Untreated associated lesions as predictors of failure of anterior cruciate ligaments

11:30 - 12:00 **Duško Spasovski; Belgrade, Serbia:** Recent results in treatment with mesenchymal stem cells

12:00 - 12:15 **Maja Petrič; Ljubljana, Slovenia:** New approach in trunk muscle endurance testing

12:15 - 12:30 **Sergeja Bec, Ljubljana, Slovenia:** Effects of capacitive and resistive electric transfer therapy on skin temperature

12:30 - 12:45 **Fabio Valenti; Ljubljana, Slovenia:** Short- and long-term relaxation effects of soft tissue structures of the posterior myofascial line in patients with non-specific low back pain

12:45 - 13:00 **Jana Hočevar; Ljubljana, Slovenia:** Arthrogenic muscle inhibition and ankle instability

Section 4 : Prosthetics organized and moderated by **Blaž Mavčič** and **Drago Dolinar**

11:00 - 11:14 **Blaž Mavčič; Ljubljana, Slovenia:** Periprosthetic fractures of proximal femur

11:15 - 11:30 **Borut Pompe; Ljubljana, Slovenia:** Total knee prosthesis in hemophilics

11:30 - 11:45 **Doroteja Okorn, Puh U; Ljubljana, Slovenia:** Knee injury and osteoarthritis outcome score (KOOS) in total knee replacement: Systematic review of measurement properties

11:45 - 12:00 **Oskar Zupanc; Ljubljana, Slovenia:** Advances in shoulder endoprosthetics

12:00 - 12:15 **Drago Dolinar; Ljubljana, Slovenia:** Mechanisms of endoprosthesis wear and loosening

12:15 - 12:30 **Aljaž Merčun; Ljubljana, Slovenia:** Case of revision of Copf hip endoprosthesis

12:30 - 12:45 **Sophio Samkharadze, Marika Zurmukhtashvili, Eka Kokhreidze, Elene Kharashvili, Sesili Beriashvili; Tbilisi, Georgia:** Availability of dental services for medical students in Georgia

12:45 - 13:00 **Sophio Samkharadze, Marika Zurmukhtashvili, Eka Kokhreidze, Elene Kharashvili, Sesili Beriashvili; Tbilisi, Georgia:** Assessment of oral health status among medical students in Georgia

Section 5: Physics: organized and moderated by **Aleš Iglič** and **Matej Daniel**

11:00 - 11:30 **Samo Kralj, Luka Mesarec, Aleš Iglič; Maribor and Ljubljana, Slovenia:** Topological excitations in nematic liquid crystals and particle physics

11:30 - 11:50 **Szymon Starzonek, Sylwester Rzoska , Aleš Iglič; Ljubljana, Slovenia and Warsaw, Poland:** Dielectric study of induced phase transitions in lyotropic liquid crystals

11:50 - 12:10 **Mitja Drab, Katarina Mendova, Matej Daniel; Prague, Czech Republic and Ljubljana, Slovenia:** Observation of a second-order thermoporetic effect in lipid vesicle populations depends on phase transition temperature

12:10 - 12:30 **Luka Mesarec, Aleš Iglič, Samo Kralj; Ljubljana, Slovenia and Maribor, Slovenia:** Altering the position of topological defects in nematic shells

12:30 - 12:50 **Matej Gartner, Matej Perovnik, Martin Horvat; Ljubljana, Slovenia:** Bioelectricity – from ion transfer to anatomy formation

Section 6 : Nanostructurome organized and moderated by **Gabriella Pocsfalvi** and **Gitta Schloser**

11:00 - 11:15 **Gabriella Pocsfalvi; Naples, Italy:** Proteomics of extracellular vesicles

11:15 - 11:30 **Gitta Schlosser; Budapest, Hungary:** Advances in mass spectrometry for characterization of biological samples

11:30 - 11:45 **MD. Mozzammel Haque; Gono, Bangladesh:** Protein misfolding and aggregation

11:45 - 12:00 **Maneea Mabrouk AbdElkhalk Mubarak; Alexandria, Egypt:** Green EV. The production of extracellular vesicles using plant cell suspension culture

12:00 - 12:15 **Shota Nebieridze, Maia Kereselidze, Maia Beruashvili, Vazha Kvachrelishvili, Marine Matskepladze; Tbilisi, Georgia:** Clinical and hematological examination of cattle affected by theileriosis

12:15 - 12:30 **Matevž Arko, Anna Romolo; Ljubljana, Slovenia:** Characterization of plasma preparations by interferometric light microscopy and flow cytometry

12:30 - 12:45 **Kaja Troha; Ljubljana, Slovenia:** Storage of plasma rich with extracellular vesicles and platelets

12:45 - 13:00 **Matej Hočevar; Ljubljana, Slovenia:** Imaging of plasma by scanning electron microscopy

Section 7 : Universal Science, Art and Education organized and moderated by **Anita Prelovšek** and **Tatia Dolidze**

11:00 - 11:15 **Tatia Dolidze, Ioseb Kelenjeridze, Levan Meskhoradze; Tbilisi, Georgia:** Modern and innovative teaching methods of legal education

11:15 - 11:30 **Natia Jojua, Tinatin Gognadze, Tsisana Giorgadze, Ana Lolishvili; Tbilisi, Georgia:** Implementation of modern technologies in Medical Education

11:30 - 11:45 **Petra Pergar; Ljubljana, Slovenia:** Evaluation of circular management with water in towns with models and visualization of data

11:45 - 12:00 **Ema Kocjančič, Špela Tadel Kocjančič; Ljubljana, SLovenia:** Clinic Charitee, Berlin, Germany

12:00 - 12:15 **Roberta Schmid, Giancarlo Lamberti; Naples, Italy:** Beethoven in Heiligenstadt in the museum dedicated to him: the Man and the Artist

12:15 - 12:30 **Nelfi Paliska; Koper, Slovenia:** Amour, sors pour jamais – Armide by Gluck

12:30 - 12:45 **Ana Ligia Mastruzzo; Buenos Aires, Argentina:** The Mesoamerican sound heritage in present Argentine music for flute

Editorial

Dear colleagues and friends,

The International Symposium 8 th Socratic lectures marked 15 th anniversary of the meetings that started in 2008 with a single lecture by prof. Bernd Engelmann from Munich on blood microparticles (now called extracellular vesicles or extracellular particles or small cellular particles). The lecture took place at the lecturing room of the Department of Orthpopaedic Surgery, University Medical Centre Ljubljana. At that time we could not imagine that 15 years later the event would evolve into an online meeting covering 7 sections (Human Medicine, Veterinary Medicine, Musculo - Skeletal Health, Prosthetics, Physics, Nanostructurome and Crossroads of Science, Medicine, Art and Education) with about 100 lecture contributions and about 130 attendants of the symposium from 14 countries. This year, symposium included 56 undergraduate students from University of Ljubljana, Faculty of Health Sciences and Faculty of Medicine, 10 students from Doctoral School of University of Ljubljana and 18 members of organizing committee. We were amply supported by the colleagues from European University in Tbilisi, Georgia to which we are especially thankful to dr. Yelena Istileulova who mediated our collaboration. As we were happy to receive more than 40 contributions to the Proceedings of 8 th Socratic Lectures, they will be composed into two parts, each of them having its own gallery. The Gallery “Sacred Fields” of the Part I is exposing 20 images of a painter Judit Nyirkos from Budapest, Hungary, and the cover by Fia de Nardis from Zagreb, Croatia. Our special thanks are to the artists who contribute essentially to the contents of the Proceedings.

Looking back, we can evidence an enormous gift of all those who came and donated. It means a proof that science and education have a meaning. In the name of the students who have been a spiritus movens of the Socratic lectures, and in my name, with gratitude, and hope to see you again,

Veronika Kralj-Iglic

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Invited lecture/Review

Serum, Saliva, and Liver Proteome Indices Associated with Platelet Biology during Inflammatory Conditions in Different Animal Species

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Citation: Beletić A, Kuleš J, Rubić I, Kovačević Filipović M, Mrljak V. Serum, saliva, and liver proteome indices associated with platelet biology during inflammatory conditions in the different animal species. Proceedings of Socratic Lectures. **2023**, 8; 1-9. https://doi.org/10.55295/PSL.2023.I1

Publisher's Note: UL ZF stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract:
The understanding of platelet biology stepped out of the (patho)physiology of hemostasis long ago. Currently, platelets are acknowledged as effective sentinels against pathogens and powerful regulators of inflammatory processes. While accomplishing these tasks, their structural and physiological features undergo constant changes, often associated with the proteomics indices in the tissues and biofluids. Assessing these associations in different animal species provides a substantial comparative benefit. Nevertheless, the *sine qua non* for the reliable interpretation of the obtained data is a comprehensive understanding of the applied analytical and bioinformatics methods.

Keywords: Tissue; Body fluids; Proteomics; Platelet biology; Animals; Infections

1. Introduction

In general, two main perceptions provide the rationale for the multidisciplinary research interest in the platelet biology features as part of the immunity-related network. The first is the evolutionary perspective, as the platelet immunocompetency in vertebrates occurs as a relict from the predecessor in the invertebrates–hemocyte, the cell in control of the hemolymph volume homeostasis and the immune response. The second concept emerged some ten years ago, with the description of immunothrombosis, a powerful intravascular innate immunity phenomenon, conjoining coagulation with the immune mechanisms, both cellular and humoral (Carestia et al., 2022).

An extensive repository of experimental results allows consideration of platelets as sentinels against pathogens and acknowledges their involvement in shaping the immune response against them. Platelets accomplish these tasks either alone or via crosstalk with immunocompetent cells, such as monocytes, neutrophils, and lymphocytes (Magraf and Zarbock, 2019; Carestia et al., 2022). An analogy is evident regarding the platelets' roles in hemostasis and immunity. First, their involvement requires a trigger signal. During hemostasis, vascular injury initiates the platelet aggregation cascade. In terms of immune properties, the presence of pathogen- or damage-associated molecular patterns, recognized via their corresponding receptors, generates their activation. The analogy further expands to effector molecules secreted from platelet granules. Depending on the trigger responsible for platelet activation, the individual components of the platelet secretome converge their activities towards hemostatic outcomes or pathogen elimination. Finally, both hemostatic and immune/inflammatory activities require tight control mechanisms to prevent their transformation from protective to severely detrimental phenomena such as thrombosis, systemic inflammatory response syndrome, or chronic inflammation (Margraf and Zarbock, 2019). Plasticity characterizes the platelet functional phenotype, thus facilitating adjustment to real-time (micro) environmental requirements. Among the numerous factors, reliable sensing of these requirements depends on a cluster of host-originating proteins (Margraf and Pareti, 2022). Therefore, a comprehensive analysis of the different proteome compartments, such as tissue(s) and body fluids, is an approach of choice for deciphering the interactions between platelets and their environment.

The interspecies differences in the morphological and functional features of platelets are among the essential factors guiding the interpretation of experimental data about their biology (Weiss, 1999; Margraf and Zarbock, 2019). In this regard, gathering the evaluation of these features in different animal species yields a substantial comparative advantage. As the best outcome, this generated research knowledge allows for reliable translation between animal and human biology models.

The article will start with a brief outline of the biology of platelets, with an emphasis on the features that make them immunocompetent. This review will continue with a compendious overview of the proteomics workflow. After that, four examples will present recent results illustrating the association between host proteome indices and platelet biology within the inflammatory scope in different animal species. The first two examples provide results on experimental inflammation and natural infection in pigs. In the third example, the focus will be on natural parasitic infestation in deer, while the fourth will bring the data obtained in the study on dairy cows with postpartum infection. A summary of the applicability of the current results and directions for further research are presented.

2. The biology of platelets-a brief outline

The first description of platelets, the small cytoplasmic fragments of megakaryocytes, dates back to the second half of the 19th century. The bone marrow hubs for platelet production; the lungs also represent a suitable environment for their maturation. Thrombopoietin monitors the number of circulating platelets. The concentration of this protein in the blood increases in parallel with a reduction in platelet count. The spleen and liver are the main organs that remove platelets from circulation, while the lungs, brain, and macrophages can also contribute. Numerous factors influence platelet count and functionality, such as hemorrhage or inflammatory challenges, age, sex, and circadian variation (Margraf and Zarbock, 2019).

When an endothelial injury occurs, the adhesion of platelets to the subendothelial structures initiates the hemostatic cascade. It continues via platelet activation and aggregation until the establishment of a thrombus as a barrier against the further extravasation of blood. The underlying mechanisms are receptor-mediated and involve changes in platelet morphology coupled with conformational changes. As a result, platelets release granular content, thus increasing the local levels of secondary hemostatic mediators such as von Willebrand factor, adenosine diphosphate, calcium ions, biogenic amines, platelet factor 4, and chemokine ligand 5 (Sandmann and Köster, 2016; Estevez and Du, 2017; Margraf and Zarbock, 2019).

The circulating platelets are also constantly patrolling with a "mission" of protecting from pathogens. Their molecular "equipment" for this purpose is versatile, with a plethora of pattern recognition receptors reactive against the diverse pathogen-associated molecular patterns (such as lipoproteins, lipopolysaccharides, or nucleic acids) or the damage-associated molecular patterns (Shevchuk et al., 2021). When sensing the bacterial presence, the platelet membrane receptors interact with the molecules on the bacterial surface or secreted from the bacterial cell, directly or indirectly, using the host-originating proteins for the "bridging". The platelet glycoprotein Ibα, Fc, complement, or toll-like receptors are the representative structures involved in these interactions (Kerrigan, 2015; Shannon, 2015). The resulting effects on the platelet activity depend on the numerous bacterium- and host-related factors (McNicol, 2015). The mechanisms underlying the platelet-virus interactions largely resemble those occurring in the case of bacteria. The type of platelet receptors "in charge" (like toll-like receptors, Fc receptors, or DC-SIGN) can vary between the viruses. The net effect of these interactions is the "adsorption" of the virus cells on the layer formed of platelet, leading to their activation and joint removal with the adsorbed virus cells. A deleterious side effect of this otherwise protective mechanism is protruding thrombocytopenia, which can be multicausal. Besides the overactivation, it can originate also from the viral penetration into the megakaryocytes or the overconsumption if the infection of endothelial occurs. Malaria represents a suitable model to study platelet-parasite interactions. Generally, during Plasmodium spp. Infection platelets function in the "Yin and Yang" mode. On one side, they elicit the initial immune response or even act cytotoxically, thereby contributing to parasite clearance. Opposite to these beneficial effects, platelets can also mediate the binding of the infected erythrocytes to the endothelium, resulting in the formation of heterogenous multi-structural aggregates and consequentially to the cerebral sequel (Alonso and Cox, 2015).

In the next step, platelets join the cross-talk with the other pathogen-recognizing cells - neutrophils and monocytes, and the traditional innate immunity mechanisms are initiated (Shevchuk et al., 2021). Analogous to other immune cells, the role conferred to platelets is tightly balanced between pro- and anti-inflammatory effects. The cross-talk with neutrophils, mediated via the membrane glycoproteins and so-called kinocidins (like platelet factor 4 or antimicrobial peptides), strengthens the recruitment and transmigration of the immune cells, formation of neutrophil extracellular traps and immune-thrombosis. As the counterbalance, the activated platelets prevent the inflammation-associated hemorrhage or cell death, while via the CLEC2-mediated mechanisms potentiate the anti-inflammatory phenotyping changes of the macrophages (Nicoali and Massberg, 2020; Carestia et al., 2022). Finally, the platelet research field in the Immunobiology area is expanding towards new aspects like interferon signaling, T-cell response, or host homeostasis restoration (Nicolai and Massberg, 2020).

The classification of platelets into the different functional phenotypes can rely on the combinations of their morphological, hemostatic, inflammatory, and immunomodulatory characteristics. During platelet senescence, besides the decrease in their volume, the impairment in the response upon stimulation occurs, together with the proteomic changes indicating apoptosis. On the contrary, immature platelets tend to show more pronounced pro-inflammatory properties (Margraf and Pareti, 2022). Also, the functional effects depend on the site of maturation. For example, the megakaryocytes in bone marrow supply the platelets for hemostatic purposes. Megakaryocyte populations in the lungs express an "immune phenotype"-those located on the blood vessel releases platelets, and the extravascularly positioned ones share features with the lung dendritic cells. The continued migration of megakaryocytes between these two structure-functional compartments is the

basis of platelet phenotype plasticity, an emerging exclusive research area (Boilard and Machlus, 2021).

3. A compendious overview of the proteomics workflow

Proteomic research in animal and veterinary science offers advantages in experimental design, sample selection, and preparation because of the broad availability of diverse biological samples, like tissues, cells, and fluids, such as serum, plasma, urine, saliva, milk, or semen (Bilić et al., 2018). Mass spectrometry (MS) -based proteomics includes two broad groups of techniques: "top-down" proteomics, measuring the intact proteins, and "bottom-up" proteomics, which analyzes peptides derived from proteolytic digestion. Tandem MS/MS, in combination with nanoflow liquid chromatography (LC), has become the analytical technique of choice for comprehensive analysis of complex samples.

An optimized sample preparation protocol is a prerequisite for any robust and sensitive bottom-up proteomics workflow. Drafting of these standardized protocols involves a detailed explanation of the sampling, handling, and storage conditions. To ensure comparable and reproducible results, the protocols also need to address the selection of collection tubes and additives and specific issues such as the influence of variations in clotting time, allowable lag time before centrifugation, hemolysis, and repeated freeze/thaw cycles. Significant attention has also been paid to strategies to minimize sample heterogeneity and disruption (Rai et al., 2005; Hsieh et al., 2006).

Sample preparation for protein profiling using MS requires multiple steps. The workflow includes the extraction of proteins and their denaturation, reduction, and alkylation, after which proteins are digested into peptides with a site-specific protease (Bodzon-Kulakowska et al., 2007; Switzar et al., 2013; Vandermarliere et al., 2013). Optionally, the protocols include the depletion of the highly abundant or enrichment of target proteins (Marco-Ramell and Bassols, 2010; da Costa et al., 2017) or clean-up procedures, removal of salts, denaturing agents, and other interfering substances with filtered assisted sample preparation (FASP) (Wiśniewski et al., 2009).

To quantify abundance changes in proteome's, labeling approaches, such as those based on isobaric mass tags, have been used for over a decade. The tandem mass tag (TMT) labeling approach utilizes chemical derivatization (Rauniyar and Yates, 2014). Isobaric mass tags are isotope-coded molecules with the same chemical structure and molecular weight that are used to differentially label peptides without introducing mass differences and sample complexity. The isotopically derivatized peptides displayed a single peak in the MS spectrum and yielded a series of low-mass reporter ions for quantification upon fragmentation in tandem mass spectrometry. Quantitative results are obtained from the direct correlation between the relative intensities of the reporter ions and peptides selected for MS/MS fragmentation (Rauniyar and Yates, 2014).

After protein identification and quantification, characterization and bioinformatics analysis follow to determine gene ontology. In addition, the pathway enrichment analysis is indisposed in identifying the biological pathways associated with the observed proteome changes. Unique challenges appear when the available bioinformatic repositories do not include data on the studied animal species (Heck and Neely et al., 2020). In such a case, the usual first step is assigning protein sequences to their model species' equivalent using the "Basic Local Alignment Search Tool" (BLAST) analysis.

4. The examples illustrate the association between host proteome indices and platelet biology within the inflammatory scope in different animal species

4.1. Case No. 1: Pigs with septic and non-septic inflammation

The interconnection between the proteomic features and platelet biology during an infection can depend on the intensity of the triggered inflammation, as evidenced by López-Martínez et al., 2022). Serum and salivary proteome were compared, using pigs as the experimental model, during the time course of the septic (induced in five pigs via intramuscular administration of lipopolysaccharide (LPS) from *Escherichia coli*) and non-septic inflammation (triggered in five pigs after subcutaneous injection of turpentine oil). Table 1 brings the platelet-associated proteome changes which occurred in the saliva and serum proteome after the first six hours of the experiment.

Protein	Sample/Inflammation			
	Saliva/Nonseptic	Saliva/Septic	Serum/Nonseptic	Serum/Septic
Fructose-biphosphate aldolase	ø change	↑	ø change	ø change
Alpha-2-macroglobulin	↑	↑	ø change	ø change
Fibrinogen alpha chain	ø change	ø change	ø change	↓
Fibrinogen beta chain	ø change	ø change	ø change	↓
Fibrinogen C-terminal domain-containing	ø change	ø change	ø change	↓
Fibronectin	ø change	ø change	ø change	↓
Transferrin	↑	ø change	ø change	ø change
Albumin	↑	ø change	ø change	ø change
Histidine-rich glycoprotein	↑	ø change	ø change	ø change

Table 1. The platelet-associated proteome changes which occurred in the saliva and serum proteome after the first six hours of the experiment. Changes in the relative abundance basal conditions vs. After 6 hours of experiment: ↑-increase, ↓-decrease.

Under the non-septic conditions, several platelet-associated changes appeared in the salivary proteome, while none emerged in serum. In the septic environment, the alterations occurred in serum; nonetheless, their decreased nature limited their practical applicability. On the other side, saliva offered a biomarker candidate for early recognition of sepsis–the increased relative abundance of fructose-biphosphate aldolase, further verified by the increase in the enzyme activity. This glycolytic enzyme shows adhesin-like and immunostimulatory properties (Elhaik Goldman et al., 2016; Pirovich et al., 2021). Notwithstanding, aldolase participates in the platelet cytoskeletal (re) organization, accompanying their activation (Arias-Salgado et al., 2008).

4.2. Case No. 2: Pigs with meningitis caused by Streptococcus suis

The proteomic-based model for assessing the interplay between the serum and saliva proteomic indices related to platelet biology, established during the experimental infection in pigs (López-Martínez et al., 2022), required verification in natural infection settings. In this context, pigs with meningitis caused by *S. suis* were an attractive study cohort (López-Martínez et al., 2022). The authors compared the differences in salivary and serum proteome compositions between diseased and healthy pigs, with each group containing 10 animals. The relative abundance of 21 salivary proteins differed between the two groups, of

Which 7 were associated with platelet biology (Table 2). Similarly, the pathway enrichment analysis allocated to platelet biology showed six out of 20 proteins identified to have different serum relative abundances between the infected and healthy pigs (Table 2).

Table 2. Proteins differing between healthy and pigs with meningitis by the relative abundance in serum and saliva which were allocated to the platelets' biology.

Protein	Relative abundance meningitis vs. healthy	
	Saliva	Serum
Transferring	lower	lower
Apolipoprotein A-I	lower	lower
Histidine-rich glycoprotein	lower	lower
Extracellular matrix protein 1	higher	ø difference
Alpha-1B-glycoprotein	lower	ø difference
Vinculin	higher	ø difference
Fructose-biphosphate aldolase	higher	ø difference
Albumin	ø difference	lower
Clusterin	ø difference	lower
SERPIN domain-containing protein (LOC100156325)	ø difference	higher

Both the serum and salivary pattern were the comprehensive "puzzle", consisting of the proteins showing potentially opposing effects on the platelet activity. Therefore, it might be very challenging to stand out whether they were "platelet activating". Regardless, two very important features were evident. The first was that the salivary and serum patterns had only three proteins in common, which might imply that the indices of these two proteome compartments provide somewhat different but complementary contexts regarding platelet biology.

The second feature brings a practical upgrade. Namely, the proteomic investigation within the natural infection environment verified the higher relative abundance of the fructose-biphosphate aldolase in the infected pigs exclusively in saliva. In this manner, the salivary biomarkers' potential for the effective management of swine infections further increases (Cerón et al., 2022).

4.3. Case No. 3: Infestation of red deer with the giant liver fluke

The analysis of the proteome of the pathogen's target organ represents an intriguing research task. The study of Šimonji et al. (2022) brought insights into the liver proteome qualitative and quantitative traits associated with the giant liver fluke (*Fascioloides magna*) infestation in the red deer (*Cervus elaphus*). Relative quantification analysis revealed the differences in the abundance of 234 proteins between the infected and healthy deer. Further pathway enrichment analysis linked 12 (Table 3) with the molecular response to elevated calcium ion levels in the platelet cytosol and platelet activation, signaling, aggregation, and degranulation.

Table 3. Proteins differing between the liver of the red deer with *F. magna* infestation and healthy deer.

Protein	Infested vs. healthy
Fibrinogen alpha chain	higher
Fibrinogen beta chain	higher
Transferrin	higher
Apolipoprotein A-I	lower
Superoxide dismutase	lower
Calmodulin	lower
Alpha-1-acid glycoprotein	lower
Saccharopine dehydrogenase-like oxidoreductase	higher
Lysosome-associated membrane glycoprotein 1	lower
Annexin	higher
Alpha-actinin-4 isoform X3	higher
Acyl-CoA synthetase medium chain family member 3	lower

The integrative interpretation of the observed differences needs caution and merits additional studies by cause of at least two reasons. The first is that the proteins which showed the platelet-associated differences were also allocated to the other (patho) biological pathways occurring in the liver. Another one appeared from the bioinformatics algorithm, which, in cases of the identified proteins without gene ID for *Cervus elaphus* or the uncharacterized proteins, had to include BLAST analysis and the replacements with the bovine orthologue (Šimonji et al., 2022).

4.4. Case No. 4: Cows with the retained placenta

Extensive research has been conducted to determine the structural and functional characteristics of bovine platelets. Their diameter under non-activated conditions is 1–5 μm, which is approximately two times less than that of companion animals and pigs. The most prominent element in their functional morphology is the absence of the open canalicular system, which probably causes a lower collagen adhesion potential when compared to other animals. Based on these features, an opinion appeared, suggesting that bovine platelets evolved from thrombogenic to inflammatory functions due to the expulsion of granule content (Weiss, 1999).
A recent study on serum proteome alterations associated with the retained placenta (RP) in dairy cows (Beletić et al., 2023) provided experimental evidence of the proinflammatory features of bovine platelets. RP is common and has a significantly negative impact on dairy management. RP risk factors are associated with diverse immunometabolic, obstetric, inherited, and environmental conditions. The pathophysiological hallmark is uncontrolled amplification of low-grade inflammation present in cows with physiological puerperium (Bradford et al., 2015; Dervishi and Ametaj, 2017).

According to Beletić et al. (2023), the extent of serum proteomic changes is positively correlated with the intensity of the acute phase reaction in RP. Using a bioinformatics enrichment tool, they allocated the three proteins with altered relative abundance (fibrinogen alpha chain (FGA), inter-α-trypsin inhibitor heavy chain 4 (ITIH4), and tetranectin) to the following pathways: platelet degranulation, response to elevated calcium ion levels in the platelet cytosol, and platelet activation, signaling, and aggregation. Establishing the corresponding pathobiological correlates was an easy task, as the literature supports an association between an increase in platelet activation with a combination of higher fibrinogen (Frojmović et al., 1996), increased ITIH4 levels (Koch et al., 2021), and decreased tetranectin concentration (Chen et al., 2020; McDonald et al., 2020). Unexpectedly, the validation results for FGA and TNCT, obtained using bovine-specific immunometric methods, did not confirm the LC-MS/MS results. These findings were presumably due to the presence of

FGA and TNCT isoforms, differing in pathophysiological implications and immune reactivity (Dardé et al., 2010; Shang et al., 2019), which merits further investigation.

5. Conclusions

Reliable data support the associations between the indices in various proteome compartments and the main platelet functions in animals with various infections. Quite expectedly, these associations were dependent on the nature of the pathogen, infection severity, and the type of specimen analyzed. In creating molecular correlates, caution is necessary owing to analytical and bioinformatic specificities. Sample complexity, the presence of isoforms, or the discrepancy between LC-MS/MS and validation results are just some examples of potential analytical issues. In addition, bioinformatic assessment bears challenges, such as the choice of the background genome, database coverage, or the requirement for BLAST-ing. Nevertheless, current achievements provide a reliable and encouraging rationale for further research targeting methodological upgrades, (patho)biological significance, and translational potential.

Funding: This research was supported by a grant from the European Regional Development Fund (Grant Agreement KK.01.1.1.04.0086) and by Ministry of Education, Science and Technological Development of the Republic of Serbia (Contract number: 451-03-68/2022-14/200143).

Conflicts of Interest: The authors declare no conflict of interest.

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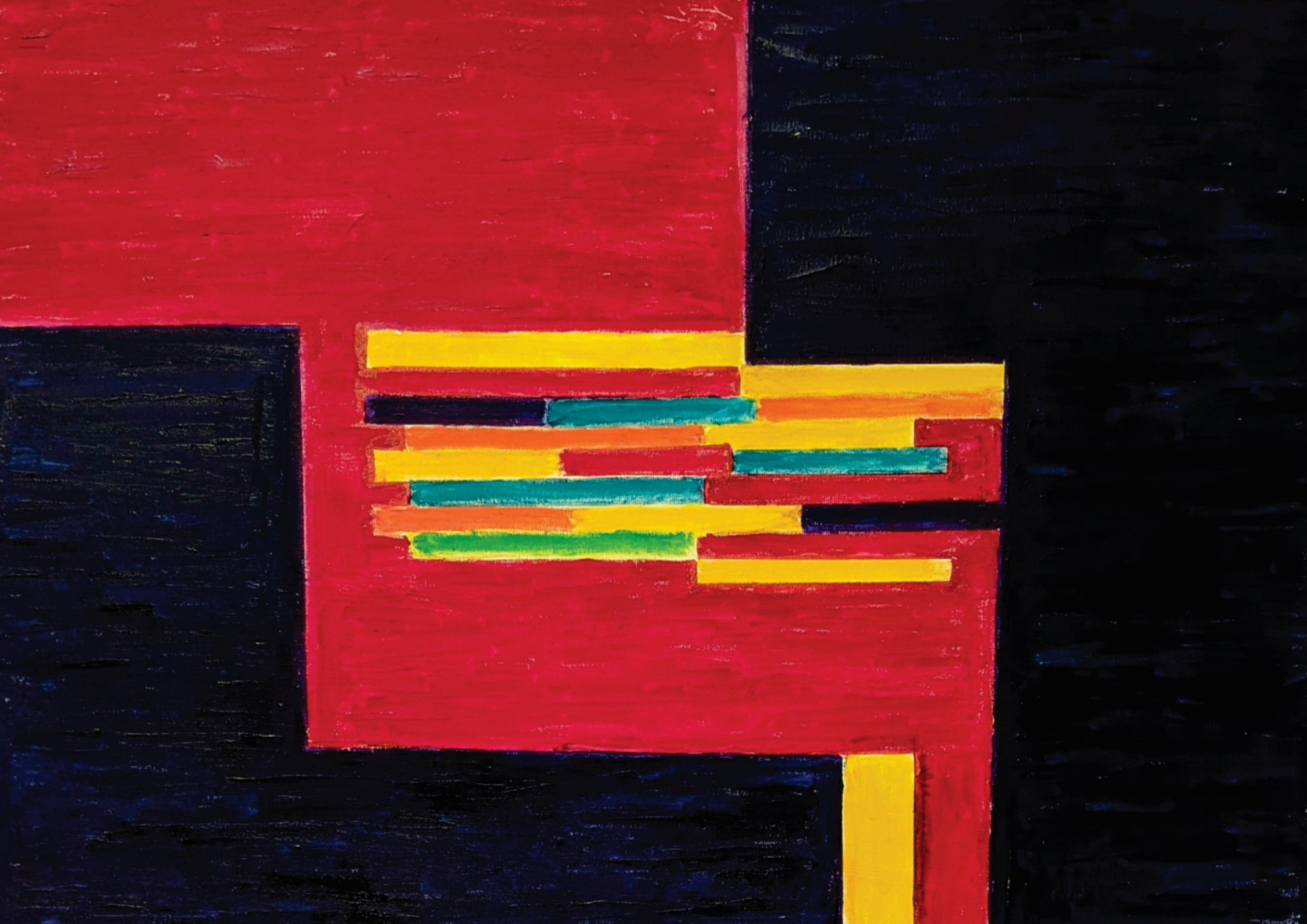

Invited lecture/Review

Vaccine Development against Paratuberculosis

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Abstract:
Paratuberculosis or Johne's disease (JD) is a chronic granulomatous enteritis affecting ruminants worldwide. It is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and the rate of prevalence is increasing. Based on high economic impacts and public health concern, vaccine development against paratuberculosis is very essential. There is a lot of research articles about finding the best management approach for eradicating MAP, and also finding an ideal vaccine against the disease. But unfortunately, until now, there is no ideal management approach against the disease because we don't have any ideal vaccine against it. This mini review discuses about management strategies with the focus on researches about various types of vaccines against JD.

Citation: Eraghi V. Vaccine Development against paratuberculosis. Proceedings of Socratic Lectures. **2023**, 8; 11-15. https://doi.org/10.55295/PSL.2023.I2

Publisher's Note: UL ZF stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis* (MAP); Vaccine; Johne's disease; Paratuberculosis; Control

1. Spread status of JD

1.1. Transmission

The bacteria infecting animals via faeco-oral route and also by bioaerosols. The infected animals with no clinical signs, even in early stages of paratuberculosis, shed MAP in their faeces and spread JD in the field silently (Scanu et al., 2007; Faisal et al., 2013; Gurung et al., 2012; More S. et al.,2017).

The pathogenesis of JD is similar to other mycobacterial infections and the clinical signs of infected animals in the end stage of paratuberculosis are including weight loss, decreasing milk production, diarrhoea, and death.

1.2. Epidemiology

MAP can infect all domestic and wild ruminants worldwide, even some infected monogastric species are reported (Hutchings et al., 2010; More et al., 2017). The disease is not eradicated in any country until now; but Sweden and Norway claim that they did not observed any infected animals by MAP until 2008 and 2015, respectively (Whittington et al., 2019).

2. MAP characteristics

Mycobacterium avium subsp. *paratuberculosis* is an intracellular pathogen and it uses macrophages inside the body as a shelter for surviving and multiplying (Arteche-Villasol et al., 2022). There are some evidences about the potential role of neutrophils in preventing of development of MAP and protection against paratuberculosis (Khare et al., 2009; Dotta et al., 1991; Ladero-Auñon et al., 2021; Martineau et al., 2007). The bacteria are very persistent to acidic soils, sunlight, low temperature like freezing, heat like pasteurization and dry climate. Then it can survive in the environment for a long time (Whittington et al., 2004; Eppleston et al., 2014).

3. Treatment and Diagnosis

Because the disease is asymptomatic until the end stage, there is no treatment for JD. As a routine strategy for control of JD, it needs to be diagnosed and all infected animals should be culled (Park and Yoo, 2016). Culturing of bacteria from faecal samples, detection of MAP DNA from faecal and intestine samples, and detection of antibodies from serum and milk samples are routine ways for diagnosis of paratuberculosis.

4. Control

The global spread of MAP is high and based on its high economic impacts and public health concern, paratuberculosis should be controlled worldwide. There are some strategies for decreasing of MAP prevalence like testing and culling, preventing the exposure of calves to adult faeces, and vaccination. Seven countries are using vaccination as a control strategy including Iceland, Spain, New Zealand, Australia, India, Netherlands, and Canada (**Table 1**). The other countries are using test and cull strategy.

Table 1. Countries using vaccination as a part of control strategies

Country name	Control Strategy	Vaccine type	Output of control strategy	Reference
Australia	Testing and Culling; Testing of environmental faecal samples; Vaccination	Killed vaccine	Significantly reduced JD incidence; Reduced risk of MAP infection entering the human food chain	Whittington et al., 2019; Windsor et al., 2020.
New Zealand	Testing and Culling; Testing of environmental faecal samples; Vaccination	Killed vaccine	Significantly reduced JD incidence	Gautam et al., 2018
Spain	Testing the animals; Culling; Vaccination	LAV and killed vaccines	Significantly reduced JD incidence	Juste et al., 2011
Iceland	Testing the animals; Culling; Vaccination	Killed vaccine	Significantly reduced JD incidence	Sigurdsson., 1960; Whittington et al., 2019
Canada	Testing and Culling; Testing of environmental faecal samples; Vaccination	Killed vaccine	Significantly reduced JD incidence	Whittington et al., 2019
India	Testing the animals; Culling; Vaccination	LAV and killed vaccines	Significantly reduced JD incidence	Singh et al., 2009
Netherlands	Testing the animals; Culling; Vaccination	Killed vaccine	Significantly reduced JD incidence	Groenendaal et al., 2003

5. Vaccination

There are some commercially available vaccines against paratuberculosis, but all of them can interfere with tuberculosis or paratuberculosis test. A lot of researchers are working on finding an ideal vaccine against JD especially in the field of Live Attenuated Vaccine (LAV), and inactivated vaccines including vector-based vaccines, and subunit vaccines.

4.1. LAV

Knock-out and deletion of known virulence genes of MAP and creating mutant, is a strategy to create LAV against paratuberculosis (Phanse et al., 2020; Shanmugasundaram et al., 2018; Ghosh et al., 2014; Ghosh et al., 2015; Rathnaiah et al., 2014). This kind of vaccine, unfortunately, can not eradicate the disease and also, it can interfere with the test of paratuberculosis.

4.2. Inactivated vaccines

Researchers used Viruses, Bacteria and Nanoparticles as vectors to create vector-based vaccines against MAP. Using nanoparticles is quiet new strategy with promising results (Abdellrazeq et al., 2019; Thukral et al., 2020). As a virally vectored vaccine against JD, Lentivirus (Franceschi et al., 2019) and Adenoviruses (Bull et al., 2007; Bull et al., 2014) were used until now. Bacterial vectors like *Escherichia coli* (Qiu Xu et al., 2021) and *Salmonella* (Faisal et al., 2013; Motamedi boroojeni et al., 2019) were used in some researchs and based on their results, the protective immunity against paratuberculosis was reported by using *E. coli*. The most of researches about creating vaccine against paratuberculosis are in the field of subunit vaccines. There are a lot of research on engineered plasmids that can produce antigens in bacterial and mammalian hosts (Eraghi et al., 2017; Monreal-Escalante et al., 2021). Also, several purified proteins were investigated as vaccine candidates against MAP (Gupta et al., 2021; Eraghi et al., 2019; Fernández et al., 2022).

6. Conclusion

Regarding huge spread of paratuberculosis worldwide, public health concern, the outcome of using control strategy including vaccination, also, the need to ideal vaccines against MAP, doing research in the field of finding new control strategies and vaccine development are very important and helpful.

Conflicts of Interest: The author declares no conflict of interest.

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Invited lecture/Review

Phenotypic and Genotypic Analysis of Anthelmintic Resistance

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Abstract:
A growing issue on a global scale is the emergence of helminth species and populations that are resistant to one or more anthelmintics. The majority of currently available anthelmintics used to control parasitic nematodes of cattle and sheep belong to only three main groups, benzimidazoles, imidazothiazoles and avermectins/milbemycins. The availability of reliable and precise techniques for its identification and monitoring is a critical component of the success of helminth control programs intended to prevent the spread of resistance in nematode populations. *In vivo* method like fecal egg count reduction test and *in vitro* methods such as egg hatch assays, larval motility test, larval development test and polymerase chain reaction (PCR) can be used for the detection of anthelmintic resistance although each has some reliability, repeatability, sensitivity, and ease of interpretation issues. The genetic basis of resistance to the majority of anthelmintics are still not well understood. Thanks to recent developments in high-throughput sequencing, it is now possible to define features such as drug resistance using genome-wide techniques.

Citation: Kapo N, Omeragić J, Goletić Š, Softić A, Šabić E, Goletić T. Phenotypic and genotypic analysis of anthelmintic resistance. Proceedings of Socratic Lectures. **2023**, 8; 17-21. https://doi.org/10.55295/PSL.2023.I3

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Keywords: Anthelmintics; Helminths; Resistance; Detection assays; Molecular diagnostics; Parasite control

1. Introduction

Helminths are a group of worms which cause a major health problem to humans and animals worldwide. These health impacts include significant production losses and death in livestock, as well as weight loss, anaemia and death in companion animals, and morbidity in humans.
Even though controlling pastures for domestic animals could reduce the impact of parasites, these techniques are not adequate to get rid of these parasites (Shalaby, 2013). Currently, anthelmintic drugs are the basis for management of infection caused by helminths, and will probably remain so in the future due to the general lack of antiparasitic vaccines (Workye et al., 2021). Due to its extraordinary effectiveness—which has resulted in a parasite reduction of over 95%—generally acceptable safety margins, broad-spectrum nature, and affordable pricing of anthelmintics, anthelmintics has had great success over the past 50 years (Potârniche et al., 2021). Unfortunately, the extensive administration of anthelmintic medications has resulted in a major and dramatic development of anthelmintic resistance (AR), primarily in the gastrointestinal nematodes (GIN) of cattle, sheep, goats, and horses. Resistance to anthelmintics is also increasingly reported in canines (Kopp et al., 2007).

At least hundreds of millions of sheep, goats, and cattle receive anthelmintic treatments every year to treat or prevent illness. Chemicals from the same major drug classes are utilised across all three areas; benzimidazole drugs (e.g. albendazole) are widely used for the control of GIN parasites of livestock, hookworms in companion animals, and human soil-transmitted helminths (STH). Macrocyclic lactones (avermectins, e.g. ivermectin, and milbemycins, e.g. moxidectin) are used for GIN and ectoparasite control in livestock, as well as heartworm prevention in companion animals, and onchocerciasis, lymphatic filariasis, strongyloides and scabies in humans. Pyrimidines (e.g. pyrantel) are commonly used for the control of GIN in companion animals and occasionally in humans for control of STH. Levamisole (an imidazothiazole) is used for GIN control in livestock. For almost all anthelmintic classes, AR has been reported.

The host, the parasite, animal management, and climatic characteristics all play a role in the highly complex process of AR development. The development of efficient alternative strategies to control helminth infection has not yet been discovered, despite the fact that AR is occasionally developing and becoming a significant concern due to the widespread use of anthelmintics. Therefore, it is crucial to regularly detect AR and comprehend its risk factors and mechanisms to stop the spread of resistant parasites.

2. Factors Contributing for Development of Anthelmintic Resistance

Several factors determine how quickly AR develops, the frequency of treatments is the most important (Jabbar et al., 2006). It has been indicated that providing mass prophylactic treatment contributed to the development of AR in helminths. However, it is possible to delay the development of resistance by treating about 80% of the flock (Jabbar et al., 2006).

Anthelmintic dose rates is one of the main factors that may lead to the development of AR and refers to the administration of an improper and inappropriate anthelmintic dose. The most popular approach in veterinary medicine to calculate the dosage rate of a drug, specifically an anthelmintic, is visual weight estimation. In turn, this underdosing allows heterozygous resistant worms' survival and contributes to selection of resistant strains (Nielsen et al., 2010). The introduction and continuous use of an anthelmintic give resistant worms a survival advantage. This allows them to reproduce faster than susceptible worms, resulting in an increase in the frequency of worms with a resistance phenotype within the population.

3. Methods for Detection of Anthelmintic Resistance

The detection of AR in helminths of veterinary importance has been reviewed on several occasions for each of the different species by the World Association for the Advancement of Veterinary Parasitology (WAAVP). The majority of approaches for AR detection have limitations, either in terms of cost, application and interpretation, or repeatability of results.

Tests for AR fall into three general categories: *In vivo*, *in vitro* and molecular diagnostic tests.

3.1. In vivo methods

In vivo tests measure the impact of drug treatment on the parasite population within the animal or human host. These tests generally rely on indirect measures of parasite burden before and after drug treatment in order to quantify the impact of the drug, and hence, determine if its effectiveness is reduced by drug resistance in the worm population.

3.1.1. Fecal egg count reduction test (FECRT)

This test is widely used across GIN parasites of livestock and is currently used for assessment of drug efficacy in terms of egg reduction rate (ERR) for human STH. FECRT has undergone extensive standardization, enabling its widespread usage.

Because nematode egg output does not usually correspond well with real worm populations and the test mainly evaluates effects on egg production by adult worms, test findings may not appropriately represent anthelmintic efficacy. For *Haemonchus contortus*, there has been a strong link between faecal egg counts and worm counts, but not for *Trichostrongylus colubriformis* or *Ostertagia circumcincta*. Egg production may be inhibited if there is less than a 10-day break between treatments, which might lead to an overestimation of the effectiveness of benzimidazole anthelmintics. For this reason, it is recommended to collect feces samples 10 to 14 days after therapy. It was demonstrated in a study using goats infected with *O. circumcincta* that, when treated with ivermectin, egg production suppression may occur during the 10–14 day interval, resulting in a false negative result (Papadopoulos et al., 2012). Due to the development of immature nematode stages, the FECRT, when used to assess the presence or absence of levamisole resistance, produced false positive results when based on faecal egg counts obtained 11 or more days after treatment (Papadopoulos et al., 2012).

FECRT is very insensitive, and hence, is not suitable for detecting low levels of resistance (<25%).

3.2. In vitro methods

Measure the sensitivity of helminth eggs, larvae or occasionally adult worms, to drug exposure in laboratory-based assays. Such assays detect the phenotypic effects of drugs on various aspects of worm development, activity or viability.

3.2.1. Egg hatch assay (EHA)

Benzimidazoles prevent embryonation and hatching of the eggs of nematode parasites. Resistance to this class of anthelmintics has been identified using EHA. Tetrahydropyrimidines, imidazothiazoles, and macrocyclic lactones cannot be used in the test since they are not ovicidal. After 48 hours of incubation at 27°C, fresh eggs are inserted in each well of a 24-well plate. Several concentrations (0.5, 1, 2, 3, 5 ppm) of the benzimidazoles are then added. The remaining eggs and hatched larvae are then counted, and the LD50 values are calculated (Robles-Pérez et al., 2014).

3.2.2. Larval development test (LDT)

LDT is used for benzimidazole and levamisole. This test is determined by how long larvae can live and grow in different anthelmintic drug concentrations. Using this method, AR against the main anthelmintic families is found. The timing of infection has been shown to affect the LD50 in this assay, particularly when macrocyclic lactones (ML) are used (Fissiha and Kinde, 2021).

3.2.3. Larval Motility Test (LMT)

Larvae are incubated at 25°C for 24 h in various concentrations of drugs while in the dark. Then they are exposed to light for 20 min to stimulate those not paralyzed. The number of nonmotile larvae is then estimated as a percentage of all larvae present at each drug concentration (Kohler, 2001).

3.3. Molecular diagnostic tests

Molecular diagnostic test may aim to detect "causal" genetic differences within genes coding for drug receptors or various processes within the nematode that act to regulate the amount of drug that reaches the receptor (for example, genes involved in drug detoxification, drug efflux, or amphidial drug uptake) (Kotze et al., 2014; Kotze et al., 2020). Alternatively, a molecular diagnostic test may aim to characterise sequence polymorphisms that are genetically "linked" to the causal variants within functionally relevant genes, and so act as genetic markers for resistance (Kotze et al., 2020).
Molecular tests can generally be performed within 2 days, and can be automated, allowing for examination of many samples in a short period of time, also, can provide accurate measurements of resistance alleles even at low frequencies.

3.3.1. Polymerase Chain Reaction (PCR)

The genotyping of resistant (rr) or susceptible (rS and SS) adult worms or larvae is possible using PCR. Worms can be genotyped for the mutation on β-tubulin residue 200 (phenylalanine to tyrosine), which is implicated in BZ resistance, by employing four primers in the same reaction mixture (Elard et al., 1999; Álvarez-Sánchez et al., 2005).

4. Conclusion

The development of AR, is a highly complex process and it is a result of the intensive use of anthelmintics for the control of helminths in livestock. The FECRT is the only diagnostic currently used in the field, however, it suffers from a lack of sensitivity, high costs, and labour-intensive sampling procedures, and hence is not used widely. *In vitro* phenotypic tests remain as laboratory tools only and currently lack utility across different drug classes and parasite species. Although molecular tests are currently used as research tools, they offer significant advantages in terms of sensitivity, cost, sampling procedures and speed that make them ideal for use in diagnosing resistance in field settings.

Conflicts of Interest: The authors declare no conflict of interest.

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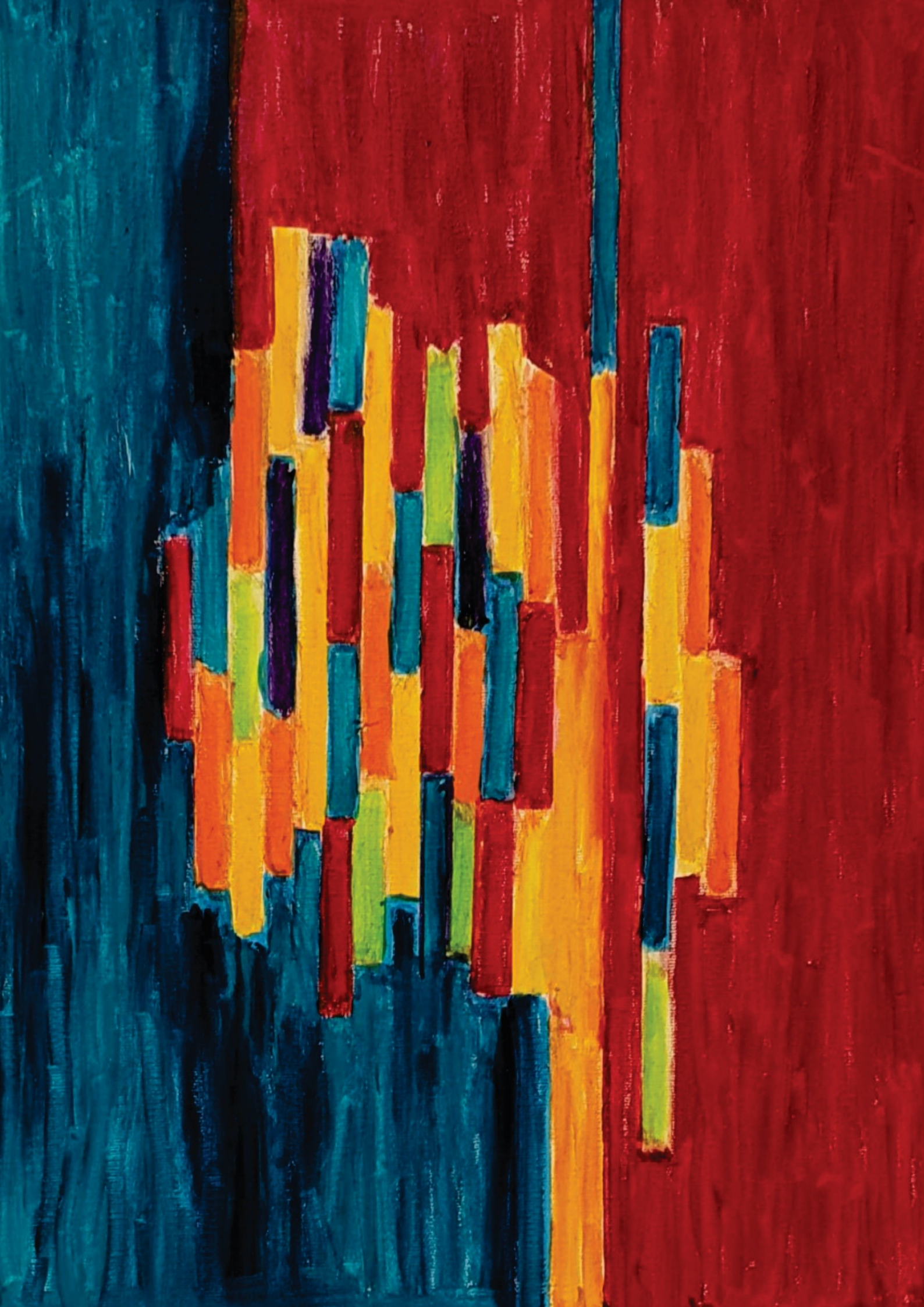

Invited lecture/Review

Materials of Absorbable Tracheal Stents and their Potential use for Treatment of Canine Tracheal Collapse

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Abstract:
Tracheal collapse in dogs can be treated by inserting tracheal stents. Currently used methods are placement of silicone or metallic intratracheal stent or extraluminal prosthetic rings, which have many post-operative complications and relatively short median survival times. The recent research is oriented in development of absorbable tracheal stents. Absorbable materials are showing great potential in preventing granulation formation, furthermore, the possibility of slow release of drugs incorporated into the stent could add to prevention of restenosis and even affect the tracheal cartilage properties. However, complications such as stent migration and fragmentation during degradation lead to many losses in in-vivo research. The aim of this article is to provide an overview of the current research on absorbable materials for tracheal stents, their complications, and advantages.

Keywords: Canine tracheal collapse; Absorbable materials; Copolymers; Drug release; Granulation prevention

Citation: Novak M, Erjavec V. Materials of absorbable tracheal stents and their potential use for treatment of canine tracheal collapse. Proceedings of Socratic Lectures. 2023, 8; 23-28. https://doi.org/10.55295/PSL.2023.I4

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1. Introduction

1.1. Canine tracheal collapse

Canine tracheal collapse is a progressive disease in middle-aged small and toy breed dogs. It is characterized by focal or generalized dorsoventral flattening of the trachea. 71-93% cases respond to medical treatment, and in half of cases the medication can be gradually withdrawn. In severe cases, structural support of the trachea may be required (Tappin, 2016). Surgical correction is recommended in patients who have no other medical conditions, do not respond to medical treatment, and have a grade II or higher disease with severe clinical signs (Payne et. al., 2006).

1.2. Non-absorbable tracheal stents and complications after placement

The currently used stents are intraluminal stents (ILS) and extraluminal prosthetic rings (ELR). In a study of 74 dogs, major complications occurred in both ELR (42%) and ILS (43%). The median survival time of ELR of 1460 days was significantly higher than of ILS (365 days) (Tinga et al., 2015). Widely used silicone stents can disrupt mucociliary function of the epithelium due to their thickness and tubular geometry. Commonly observed complications include prosthesis migration, granulation formation, and sputum retention (Liu et al., 2011). In a review of 75 cases by Weisse et al. (2022) 7% of cases did not survive to hospital discharge, and 44 % of cases required more than 1 stent placements due to complications, most commonly stent fracture and tissue ingrowth.

1.3. Absorbable tracheal stents and complications in in-vivo studies

A bioabsorbable stent that is self-supporting and dissolves after competition of the remodeling process has advantages over metallic and silicone stents (Liu et al., 2011). An ideal airway stent should (1) be easy to place and remove, (2) provide effective airway expansion, (3) maintain position by being appropriately sized and adhering to the tracheobronchial wall, (4) be flexible enough to mimic airway physiology but have sufficient radial force to resist airway compression, (5) be biodegradable, (6) not cause tissue irritation during material degradation, (7) avoid granulation tissue reactions, (8) not impair mucociliary clearance, and/or (9) provide effective pharmaceuticals for a sustained period of time (Chao et al., 2013; Li et al., 2020)

Complications after placing the tracheal stent include airway obstruction due to degradation fractions leading to patient death (Liu et al., 2011) and stent migration, which is one of the most common reasons for stent failure. Complications occur with high incidence in clinical practice, especially in the initial period after stent placement (Jin et al., 2018).

2. Materials of absorbable tracheal stents

2.1. Polycaprolactone (PCL)

PCL showed good flexibility, fully regaining shape after loading, and had 90% of mechanical strength compared with metallic stents. There was no evidence of fracture or fragmentation. Complications included moderate excretion and intermittent stridor. Marked infiltration of lymphocytes, plasma cells, and eosinophils in the submucosa, although the cilia were preserved (Chao et al., 2013).

2.2. Poly-lactic acid (PLA)

Robey et al. (2000) state that adding PLA to PLGA makes the stent stronger under compressional stress. However, it also increases the degradation time and thus the potential for inflammation or foreign body reaction to the stent.

2.3. Poly-l-lactide acid (PLLA)

PLLA stents showed good mechanical properties (Zhu et al., 2011). However, PLLA has degradation time of 2-4 years. Saito et al. (2007) researched their implication in the

treatment of benign stenosis of the gastrointestinal tract. The radial force was 117 gf, comparable to commercially available metallic stents.

2.4. Polydioxanone (PDO)

12 patients (52%) had significant benefit from treatment, early failure (migration and inadequate radial force) occurred in 2 patients, inadequate or questionable effects were recorded in 7 patients, and 2 patients died of comorbidities before the stent was fully degraded (Stehlík et al., 2016). Novotny et al. (2012) concluded that the highest tracheal damage occurred 5 weeks after implantation, which included immediate necrosis. Stent degradation was complete after 10 weeks, and trachea was completely healed after 15 weeks. Morante-Valverde et al. (2022) found that PDO stenting caused mild inflammatory changes and no increase in collagen matrix in the rabbit trachea. Grolich et al (2015) used PDO for biliary stent research. It degraded in 13 weeks and showed no cholangitis, necrosis, abscess, or excessive fibro-plasia.

2.5. Poly(lactic-co-glycolic acid) (PLGA)

Robey et al. (2000) concluded that stents in buffer solution almost completely degraded at 14 weeks. In vitro data showed that the stents tended to break after only 4 weeks in buffer solution.

2.6. PLLA-PCL copolymer 70/30

In the experiment with tubular stents by Zhu et al. (2011), mucous plugging occurred in 5/5 rabbits, and one died. Tracheal stenosis was more severe compared to silicone stents. To increase water absorption into the copolymer and shorten the degradation time from 6 weeks to 3 months (Zhu et al., 2011). Duvvuri et al. (2019) found that the PLLA-PCL construct demonstrated superior mechanical strength and greater drug elution compared to PLGA stents.

2.7. Poly(lactic-co-glycolic acid) – polyisoprene (PLGA-PI) copolymer

Schopf et al. (2018) compared a complete stent with a stent fragment, although the fragment group had fewer complications, both groups showed stridor, agitation, and had inflammatory damage.

2.8. Biodegradable magnesium alloy stent

After complete degradation of the LZ61-KBMS stent, the tracheal tis-sue was normal compared with the healthy rabbit tracheal tissue as shown by both endoscopic and histological analysis. The tracheal mucosa was also fully restored in the LZ61-KBMS stent after 8 weeks. This is criti-cal because scarring of the mucosa can promote the formation of stenotic tissue (Wu et al., 2020). Magnesium alloys are attracting interest due to their mechanical properties, excellent biocompatibility and unique biodegradability. High performance Mg alloys are mainly Mg-Zn-based alloys, Mg-Ca-based alloys, Mg-Li-based alloys, Mg-Cu-based alloys and Mg-RE-based alloys.
Zn has a strengthening effect in the Mg matrix, and the Mg-Zn alloy has three times the yield strength and Young's modulus of pure Mg. Mg-Ca alloy has the best corrosion resistance. Mg-Ca-Zn-Ag had even better bio-compatibility, osteogenic activity, and corrosion resistance. Mg-Li en-hances the plasticity of the alloy (Chen et al., 2022).

3. Drug-impregnated stent options

The incorporation and controlled release of various drugs gives bio-absorbable stants great potential for various clinical applications (Liu et al., 2011). The combination of a drug-loaded film and a stent can provide high drug loading and meet various drug release requirements to maintain effective drug concentration. Reportedly the drug loading of the film on the stent can reach up to 50% and the drug release can last for more than 3 months (Jin et al., 2018). Tatekawa et al. (2010) reported the use of biodegradable gelatin hydrogel sheet for controlled release of drugs, which degrades

by hydrolysis in about 2–4 weeks. These authors also reported the use of PLGA-collagen hybrid mesh for drug release. The mesh degraded in 2-3 months.

3.1. Dexamethasone

Results from PLLA stents showed near-linear release profiles for dexamethasone (Zhu et al., 2011).

Neutrophils and macrophages ingest pathogens, debris, and damaged tissue, allowing for protection and healing (Alhajj and Goyal, 2022). However, many authors report severe inflammatory reactions in the tracheal submucosa after stent placement. Chao et al. (2013) found marked infiltration of lymphocytes, plasma cells and eosinophils in the submucosa. Jin et al. (2018) reported severe inflammation and exudation of inflammatory cells on day 10 after insertion, and mild inflammation without exudation 30 days after insertion.Anti-inflammatory drugs could alleviate inflammation in the early days after stent insertion.

3.2. Mitomycin C (MMC)

MMC is an antimitotic drug that is widely used to treat various cancers. After 12 weeks, the tracheal stenosis in bioabsorbable tubular PLLA stents with MMC was half of that in silicone stents, taking into account that the stenosis in silicone stents was even smaller than in PLLA stents without MMC (Zhu et al., 2011).

3.3. Cisplatin

Polycaprolactone stents coated with cisplatin released cisplatin in vivo for 5 weeks, with minimal concentration detected in blood. High concentrations could be released for more than 30 days. The ciliated epithelium remained intact, but marked submucosal leukocyte infiltration occurred (Chao et al., 2013).

3.4. Polysaccharides, derivatives of cellulose and poly(acrylic acid)

The mucoadhesive delivery of drugs can prolong the residence time of the drug, leading to an improvement of the bioavailability of the drug and a reduction in the frequency of administration. In addition, it is pos-sible to achieve targeted delivery to specific site or tissues using specific mucoadhesive polymers. The mucoadhesion of carbomer can be used to prevent stent migration and achieve local drug release (Jin et al., 2018).

3.5. Carbopol

The nitinol tracheal stent was combined with a bilayer film containing Carbopol 974P to maintain mucoadhesion, to prevent migration and to achieve effective local drug delivery. The mechanical performance of the stent was not affected by the combination with the mucoadhesive bilayer. However, upon degradation, the fractions caused airway obstruction, leading to patient death (Liu et al., 2011).

3.6. Rapamycin

The addition of rapamycin to nitinol stents significantly decreased airway inflammation and granulation tissue formation compared with bare metallic stents (Chen et al., 2022).

3.7. Paclitaxel

The use of paclitaxel coated tracheal stents in a canine model significantly reduced granulation tissue formation after stent implantation, but granulation tissue still grew through the stent mesh (Wang et al., 2016).

3.8. Arsenic trioxide (ATO)

In cardiovascular stents, exposure of ATO reduced porcine coronary artery smooth muscle cell viability and promoted endothelial cell proliferation and re-endothelialization faster than rapamycin (Zhao et al., 2018). ATO also prevented the

growth of granulation tissue through the stent mesh into the lumen of the trachea (Li et al., 2021).

3.9. Collagenous gel seeded with cells

Nomoto et al. (2006) reported that epithelialization of trachea is accelerated when covered with a collagenous gel seeded with isolated autologous tracheal epithelial cells, adipose-derived stem cells or multipotential bone marrow-derived cells (mesenchymal stem cells).

3.10 bFGF and BMP-2

Controlled and sustained release of growth factors could promote accelerated cartilage growth across the reconstructed segment. The growth of new cartilage across the reconstructed segment would ultimately provide the greatest stability for a newly reconstructed airway (Robey et al., 2000). Tetekawa et al. (2010) concluded that although the use of bFGF in stents did not develop complete cartilage the regeneration of cartilage was evident. bFGF also significantly improved the elastic modulus of the stent, but still did not reach the levels of the native trachea. Stable release of bFGF was achieved by impregnation of a gelatin hydrogel.

Igai et al. (2006) also used bFGF and bone morphogenetic protein (BMP)-2 collagenous sponge as scaffold to promote the regeneration of the tracheal cartilage. The use of BMP-2 is also reported by Yasumichi et al. (2003).

3.11. Antibacterial drugs

Granulation tissue formation results from repetitive motion trauma and infection. Lower respiratory tract infections were associated with lower survival (Ost et al., 2012). Shuai et al. (2018) reported that Mg-Cu- based alloys during degradation enhance the antibacterial ability and de-stroy bacterial cells.

4. Conclusion

Absorbable tracheal stents have great potential to reduce complica-tions in the treatment of canine tracheal collapse. Granulation formation can also be reduced by incorporating drugs into the stents. Further re-search should focus on developing thinner stents without fragmentation or migration and with high biocompatibility

Conflicts of Interest: The authors declare no conflict of interest.

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Scientific contribution

User Experience of Canine Assistive Mobility Aids

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Abstract: Assistive mobility aids play an important role in the overall well-being, mobility, and activity of animals with neurological and/or orthopaedic impairments. The user experience relates not only to the pet owner, but also to the animal using it. Animals cannot express how they feel when using an assistive mobility aid, so the owner's observation of the animal is of great importance in this case. In this study the experiences of users of mobility aids for dogs were investigated using a questionnaire. All pet owners invited to participate had received a mobility aid (orthosis, prosthesis, or wheelchair) made by us for their pets in the past year. Of fifteen pet owners invited, eight dog owners responded to the questionnaire. The aids used were orthosis (25%), prosthesis (50%) and wheelchair (25%). Our survey shows that most owners (5/8) believe that assistive devices have a very positive impact on their dog's life. To improve the use of mobility aids in animals, future research should focus on making them more accessible. To restore normal limb function after injury, chronic disease or amputation, good collaboration between veterinarians, technicians (orthotists/prosthetists) and owners based on scientific evidence should be encouraged.

Citation: Pavlovic M, Mursec A. User experience of canine assistive mobility aids. Proceedings of Socratic Lectures. **2023**, 8; 30-37. https://doi.org/10.55295/PSL.2023.I5

Publisher's Note: UL ZF stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Keywords: Orthotics; Prosthetics; Wheelchair; Animal; Pet; Dog

1. Introduction

Assistive mobility aids play an important role in the overall well-being, mobility, and activity of animals with neurological and/or orthopaedic impairments. They help animals become independent, support a weak, non-functional or amputated limb, and aid in rehabilitation and movement (Adamson et al., 2005). Their use improves the biomechanics of movement, leading to a more active life and preventing obesity and other related diseases. They significantly reduce pain due to compensatory movement reducing earlier health problems or the need for euthanasia (Borghese et al., 2013). Assistive mobility aids for animals include orthoses, prostheses, and wheelchairs for the front or rear limbs or other injured body parts (Mich PM, 2014).

The user experience refers not only to the animal owner, but also to the animal using it. Animals cannot express how they feel when using an assistive mobility aid, so the owner's observation of the animal is of great importance in this case. The inadequacy of the device is reflected in the animal's (altered) behaviour, such as unusual barking and whining, and skin lesions such as blisters, abrasions, etc. The owner is also responsible for the care of the device and must be consistent in fitting (**Figure 1**). It is also important that he takes enough time to get the animal used to aid itself. Regardless of how good the fitting process is, some animals do not become accustomed to the device (Lee S et al. 2021).

Figure 1: Canine orthosis fitting.

1.1. Assistive mobility aids for pets

Orthotics and prosthetics are medical specialties that are rapidly developing in veterinary medicine as well. The main goal in orthotics is to improve the functionality of the limbs (usually injured), while the goal of prosthetics is to replace body parts after amputation (Mich, 2014). In clinical practice, prosthetic care for pets and other animals is not a routine procedure, but it improves the lives of animals that require such care (Kleinschmidt, 2018). Prosthetic limbs are considered permanent devices that increase mobility and ensure safe use throughout life (Mich, 2014)(**Figure 2**). They are attached to the residual limb and allow physiological movements (Marcellin-Little et al., 2015). With the rapid development and use of new materials, the process of manufacturing assistive mobility aids has changed, and the quality and strength of the products have improved (Keszler et al., 2019). The main function of orthoses is to stabilize joints and provide support to potentially weakened muscle groups (Adamiak et al. 2022). They can be used as a solution before or after surgery, or when surgery is not possible. The orthosis can also be used to immobilize the limb, preventing or reducing the severity of some injuries (Goldberg, 2018). Their use helps in pain management, improves the biomechanics of movement, and thus contributes to a better quality of life for animals (Mich, 2014).

Sometimes due to mobility limitations and medical needs, the age of the animal age and its environment, neither orthosis nor prosthesis can help. In this case a subgroup of the assistive mobility aids mentioned above – wheelchairs - can be of great help (McNutt , 2020) (**Figure 2**). Wheelchairs are also receiving more attention recently and are often used by animals (Charbonneau et al., 2016). They are most often used for chronic or degenerative conditions that worsen over time (McNutt J, 2020).

Figure 2: Canine assistive mobility aids including orthosis (left), wheelchair (middle) and prosthesis (right).

1.2. The need for assistive mobility aids in animal world

It has already been shown that the need for assistive mobility aids for animals is high in some European countries. Veterinarians believe that mobility aids affect the quality of life of animals and would recommend such rehabilitation to pet owners. It turns out that there are not enough specialists in orthotics and prosthetics for animals, compared to the number of amputations performed annually. Veterinarians also state that they would be willing to adapt surgical techniques to the purpose of using a mobility aid (Mursec, 2020).

The purpose of this study was to investigate user experiences of custom-made mobility aids for dogs. A questionnaire was used to collect feedback from pet owners on their experiences.

2. Materials and methods

Fifteen pet owners (including dogs, cats, and a doe) who received custom-made assistive mobility aids (orthosis, prosthesis or wheelchair) were invited to participate in this study. A total of eight dog owners responded to our questionnaire (Appendix 1). It consists of 21 questions. First basic information such as animal breed, sex, and injured body part was collected. That was followed by questions about the reason and frequency of using assistive mobility aids, satisfaction when the animal was first examined, occurrence of possible difficulties during use, visit to the veterinarian or physical therapist before or after receiving the aid, appearance, price, cleaning of the aid, overall satisfaction, and willingness to recommend a mobility aid to other owners. The rating scale ranged from 1 to 10, with 1 being the lowest rating and 10 being the highest score. An open-ended question was available at the end for any additional comments or suggestions.
All pet owners invited to participate had received our custom-made assistive mobility aids (orthosis, prosthesis, or wheelchair) for their pets in the past year. Prior to treatment of their pets and participation in this study, all pet owners provided written informed consent. Consents were gathered and mobility aids were manufactured between 19th January and 11th October 2022. The study protocol was compliant with the guidelines of the Declaration of Helsinki. The questionnaire was conducted in Slovenian and Croatian, and it was available for participants on 1ka oneclick survey (www.1ka.si) – an open-source applica-

tion that enables online survey services. The invitation to participate was sent to participants via e-mail on October 21, 2022. A reminder to those who have not yet participated was sent on October 24, 2022.

3. Results

A total of eight owners completed the questionnaire; there were five female and three male dogs, four of which were mixed breeds, one Doberman, one medium Poodle, one Rottweiler and one Yorkshire terrier. The most frequently injured body parts were the front (50%) and rear (50%) extremities. In addition, one dog (13%) had injured spine. Half of the pets (50%) wore prostheses, other two quarters (25% each) used orthoses and wheelchairs. The average satisfaction with the pet's initial examination was 9.4/10, and five/eight dogs had no problems caused by the aids they received. The other three dogs had experienced blisters, unfavourable extremity position and refused to walk with the assistive device. The same three dogs required additional corrections to the aid itself. Only three of eight dogs visited veterinarian or physical therapist before or after receiving the mobility aid.

Assistive mobility aids are used between 30 and 120 minutes per day. Only 13% of our participants walk between 100 and 500 metres per day, 37% manage to walk between one and two kilometres with the mobility aid, 25% take longer walks of more than two kilometres. Display of activity using assistive mobility aids is presented in **Figure 3.** Some owners indicated that they do not use assistive aids for their pets every day. Most owners (38%) clean the mobility aid once a day, others (13% each) clean it more than once a day, once a week, once a month, less than once a month, and some (13%) do not clean it at all.

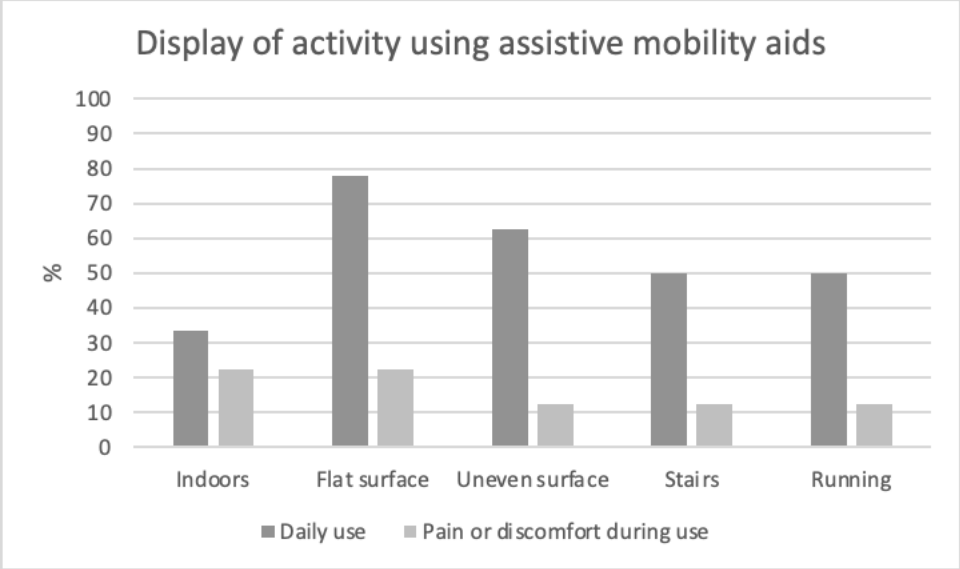

Figure 3: Activities performed using assistive mobility aids.

According to the dog owners' observation, the aids had a very positive impact on five/eight dogs' lives. Two rated the influence as somewhat positive and one owner rated the influence as very negative. The average satisfaction with the appearance of the aid was 7.8/10. In general, our respondents seem very satisfied. They indicated that they would most likely (8.7/10) recommend the mobility aid to other owners if their pet needed one. Comments/suggestions on the open-ended question at the end of the questionnaire were positive and were to continue the good work, to develop further, and praise for kindness. It was also suggested to organise a control examination of the animal after a certain period of using the aid.

4. Discussion and conclusion

This study investigated the experiences of users of assistive mobility aids for dogs using a questionnaire. Of fifteen pet owners invited to participate, eight dog owners responded to the questionnaire. The aids used were orthosis (25%), prosthesis (50%) and wheelchair (25%). Daily use was estimated to be 30 to 120 minutes per day.

The ability to provide animals with mobility aids is important for their health and well-being. Mursec A (2020) found that 35.9% of owners choose to euthanize their dog when the only solution for complete recovery is limb amputation. Prosthetic devices can reduce the percentage of unnecessary euthanasia and improve quality of life when overall health state and other key factors allow. Our survey shows that most owners (5/8) believe that assistive devices have a very positive impact on their dog's life. Similar results have been reported in previous studies. Philips A, et al. (2017) demonstrated that 83.3% of owners reported good to excellent quality of life after receiving a prosthesis. In addition, 89.3 % of owners felt that their pet was fully functional with the prosthesis (Wenland et al. 2019). Similar results exist for orthotics. Lee S, et al. (2021) found that 44/56 dog owners perceived positive effects of the orthotic devices on their pet's quality of life. In the same study similar complications were noted after the aid fitting, such as skin sores, pain/sensitivity and swelling. However, most of these complications could be resolved with corrections to the orthosis.

Assistive mobility aids have recently become more common in animals. They do not speak our language, so the biggest difference in satisfaction with assistive devices for humans and animals is that their feedback is mostly based on owner perception. However, the results of previous research, as well as ours, show the assistive aids have a positive impact on the quality of life of dogs and their owners. To improve the use of assistive mobility aids in animals, future research should focus on making them more accessible. To restore normal limb function after injury, chronic disease, or amputation, good collaboration between veterinarians, technicians (orthotists/prosthetists) and owners based on scientific evidence should be encouraged.

Funding: No funding was received for this research.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A - User experience of animal assistive mobility aids questionnaire

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Appendix A - User experience of animal assistive mobility aids - Questionnaire

Short name of the survey: User experience of animal assistive mobility aids
Number of questions: 21
Number of variables: 54
Status: Active from: 17/10/2022 Active until: 30/10/2022

Introduction

Dear participants, the aim of this survey is to find out how satisfied pet owners are with assistive mobility aids for animals. It will take you about 5 minutes to complete the questionnaire. The collected data will be used for scientific contribution. We kindly thank you for your cooperation.

Q1 - **Animal breed:**

_____

Q2 - **Sex of the animal:**

o Female
o Male

Q3 - **Your pet's name** (optional):

_____

Q4 - **Which part of the body is injured?**

Several answers are possible

o Front legs
o Hind legs
o Spine
o Other:

Q5 - **Reason for injury:**

_____

Q6 - **What type of assistive mobility aid does your animal currently use?**

o Orthosis
o Prosthesis
o Wheelchair

Q7 - **How satisfied were you as the owner with the first examination and measurement procedure?** Rating scale 1 – 10

Q7\2 - If you were dissatisfied, please describe what bothered you:

_____

Q8 - **How much time per day does your pet use the aid?**

(If they do not use the assistive mobility aid every day, write the number 0 below for the minutes and hours)

_____ minutes
_____ hrs

Q9 - **What activities does your pet perform with the device?**

Please mark below

Mark yes, if the device causes pain or discomfort during mentioned activity.

o Walking around the apartment Yes no
o Walking on flat surface Yes no
o Walking on uneven surface Yes no
o Walking up the stairs Yes no
o Run Yes no

Q10 - **How much does your pet walk with the aid on average per day?**

o Up to 100 meters (walking around the apartment)
o From 100 to 500 meters
o From 500 meters to 1 kilometer
o From 1 to 2 kilometers (shorter walk)
o more than 2 kilometers
o He doesn't use the gadget every day

Q11 - **Did your animal have any difficulties when starting to use the assistive mobility aid?**

o Yes

What difficulties were present: _____

o No

Q12 - **How has the assistive mobility aid affected your pet's life?**

o A very positive influence
o A positive influence to some extent
o No impact
o A somewhat negative influence
o Very negative impact

Q13 - **Was rehabilitation or physiotherapy part of your pet's veterinary care before or after receiving the device?**

o Before
o After
o Before and after
o We did not receive physiotherapy
o

Q14 - **Did you visit a veterinarian with your pet after receiving the assistive mobility aid?**

o Yes

What was the reason for visiting veterinarian: _____

o No

Q15 – **Were there any additional corrections needed after first receiving the aid?**

o Yes

What corrections were needed? _____

o No

Q16 - **How satisfied are you with the appearance of the aid itself?** Rating scale 1 – 10

Q17 - **Did you pay the expected amount of money for the aid?**

o Yes

Have you paid more or less than the estimated amount for the gadget? _____

o No

Q18 - **How often do you clean the device?**

o Multiple times a day
o Once a day
o Once a week
o Once a month
o Less than once a month
o I do not clean the device

Q19 – **How likely is it that you would recommend the manufacture of an assistive mobility aid for pets (if needed) to your friends or acquaintances?** Rating scale 1 – 10

Q20 - **Is there anything else you would like to tell us?** Do you suggest any improvements or ideas?

Please write your answer.

_____

Scientific contribution

Thermography as an Aid in the Performance Testing of Lipizzan Horses

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Abstract

The athletic results or training performance of Lipizzan horses are hardly known in equestrian sports, because they rarely participate in equestrian sports. The aim of this study was to determine the values of physiological parameters of horses with emphasis on the temperature changes of the body skin areas by thermography and to investigate their acclimatization to different training loads. The study included 6 purebreed Lipizzaners with a mean age of 9 years and consisted of two work tests (spring, autumn) that included lunging at walk, trot and canter. Measurements were taken before and after the work tests and were within normal limits for warm-blooded horses in both experiments. In both experiments, body skin temperatures at rest were different between different areas or body parts. After the work test, temperatures in all body skin areas were significantly elevated compared to those measured at rest and then decreased during the rest period after the work test (recovery period) in the fall or remained nearly unchanged in the spring. This study contributes to the knowledge of thermoregulation and the use of thermography in horses, and the results not only demonstrate the physiological responses to graded exercise in Lipizzan horses, but also contribute to the knowledge of equine physiology and sports medicine. The results of our research also contribute to the establishment of standards and protocols for monitoring readiness and progress in training Lipizzan horses and provide relevant data for monitoring health status, athletic ability, and assessing welfare of horses.

Keywords: Horses; Lipizzaner breed; Exercise test; Thermoregulation; Thermography

Citation: Kruljc P. Thermography as an aid in the performance testing of Lipizzan horses. Proceedings of Socratic Lectures. **2023**, 8, 39-44. https://doi.org/10.55295/PSL.2023.I6

1. Introduction

Equine performance during exercise can be affected by disruptions in thermoregulation, especially in hot and humid environmental conditions (Hargreaves et al., 1999; Mckeever et al., 2010) or in horses that are not adapted to ambient temperatures (Marlin et al., 1996; Mckeever et al., 2010). Therefore, body skin temperature (BST) can be an important indicator of changes in acute thermoregulatory acclimation (Jodkowska et al., 2011), which can be successfully measured using infrared thermography (Jodkowska et al., 2011; Simon et al., 2006; Redaelli et al., 2014). Thermography is an imaging, non-invasive remote diagnostic technique based on determining the surface temperature of an object and measuring the heat emitted (Turner, 1991; Kastberger and Stach, 2003). The body surface emits mid-infrared and infrared radiation, which is recorded in the form of a temperature distribution map and is the result of the movement of electrons transmitted from the body surface as electromagnetic radiation of different wavelengths. Since the wavelength of infrared radiation within the electromagnetic spectrum is not perceptible to humans, it can normally be perceived by heat (Čebulj Kadunc et al., 2020). A thermogram (image of the temperature field, **Figure 1**) is created by converting infrared signals into a pseudocolored image of visible light. Different shades of the colour palette correspond to specific temperatures and produce a map of the temperature distribution in the areas under study. Most often, thermograms show the warmest areas in white or red, areas with an average temperature in green and yellow, and the coldest areas in blue and black (Soroko et al., 2015).

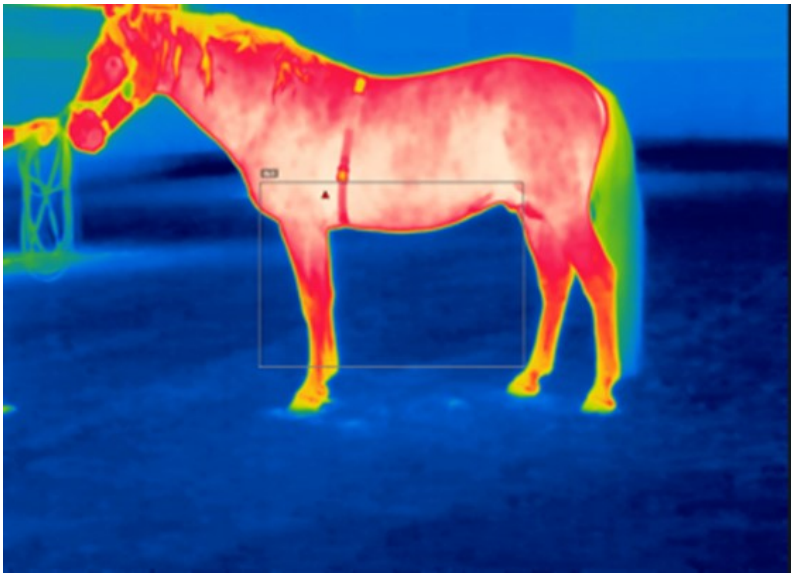

Figure 1: Thermogram of the Lipizzan horse.

Heat exchange between the horse's body surface and the environment through infrared radiation plays an important role in the animal's heat balance, and the animal's skin and hair play an important role in the heat exchange between the animal's body and the environment. Therefore, the body surface temperature measured by a thermal imaging camera is the total result of the body heat generated and the influence of the environment (temperature and velocity of air movement, humidity) (Turner et al, 2001; Soroko et al, 2014; Westermann et al, 2013). The aim of this study was to determine the values of physiological parameters with emphasis on the temperature changes of the body skin areas by thermography and to investigate their acclimatization to different training loads.

The results of our research contribute to the establishment of standards and protocols for monitoring readiness and progress in training Lipizzan horses and provide relevant data for monitoring health status, athletic ability, and assessing welfare of horses.

2. Materials and Methods

2.1. Animals

The study was performed with 6 pure-bred Lipizzans with a mean (± standard deviation) age of 9.0 ± 0.8 years and a mean (± standard deviation) body mass of 455 ± 36 kg.

2.2. Test protocol and physical activity

The study consisted of two exercise tests, performed by lunging. The first test was conducted in May in an open riding arena (20x60 m with sand footing) and the second one in October of the same year in an indoor riding arena (13x29 m with sand footing).
The exercise test protocol consisted of 8 phases with specific activities (lunging at the walk, trot and canter or resting) and measurements of physiological values (BSTs of various body regions) and environmental parameters (air temperature and humidity). Each test was preceded by a 5-minute walk from the stable to the riding arena (Phase 1; P-1) and a 10-minute rest that was devoted to the measurement of the basal values of the physiological parameters (Phase 2; P-2). Each horse was then lunged for 30 minutes at the walk, trot, and canter (for 10 minutes at each gait), and left and right reins were exchanged every 5 minutes (Phase 3 to Phase 5; P-3 to P-5). This was followed by a 10-minute break (Phase 6; P-6) intended for the repetition of the measurements and then by 10 minutes of lunging at the walk (Phase 7; P-7). Thereafter, each horse was returned to its stall (Phase 8; P-8), and the measurements were repeated.

2.3. Measurements and equipment

Body skin temperatures were measured by an infrared thermal imaging camera FLIR (model E40bx, FLIR Systems, Wilsonville, OR, USA) at a distance between 0.75 and 1.0 m from the left and right side of each body region (neck (R. coli lateralis), breast (R. pectoralis), back (R. lumbalis), croup (R. sacralis), and buttock (R. femoralis)) before lunging (P-3), immediately after lunging (P-6), and during recovery (P-8). Ambient temperature and humidity were measured using a digital humidity metre, Testo 635 (Testo AG, Lenzkirch, Germany).

2.4. Data analysis

Data were analysed using commercial SPSS 20.0 software IBM (Chicago, USA). One-way repeated-measures ANOVAs (RM ANOVA) were used to compare results between phases for each trial and between trials for each phase. Normality was assessed with a Shapiro-Wilk test, and significance was determined with all pairwise multiple comparisons (Tukey test). Pearson product-moment correlation was used to examine correlations between the results. To compare BSTs between the selected regions on the left and right sides of the horses, a paired Student t test was performed. To compare the interactions between BST and the phases of the study, a one-way ANOVA followed by Holm-Sidak test for multiple comparisons was performed. Values are expressed as mean ± standard deviation. Differences are considered significant at $P \leq 0.05$.

3. Results

The mean distances covered during the exercise tests in May and October were 794 ± 25 m and 785 ± 78 m at the walk and 1851 ± 38 m and 1828 ± 48 m at the trot, respectively, and 3089 ± 10 m and 2432 ± 81 m at the canter. Temperature and air humidity are presented in **Table 1**.

Table 1: Air temperatures and air humidity (mean ± SD) before exercise (P-2), immediately after exercise (P-6) and during recovery (P-8) of the May and October tests

Parameter	Trial	Phase		
Air temperature		P-2	P-6	P-8
Air temperature [°C]	May	18.42 ± 0.77[a]	21.10 ± 0.77[a]	21.27 ± 0.68[a]
	October	11.38 ± 0.70[a]	11.95 ± 0.73[a]	12.48 ± 0.71[a]
Air humidity [%]	May	62.48 ± 4.32[b]	55.33 ±3 .94[c]	50.63 ± 4.20[b]
	October	81.47 ± 4.62[b]	85.80 ± 4.78[c]	84.15 ± 5.09[b]

[a,b]P<0.001; [c]P<0.01 for values in the same column

The body skin temperatures (BST) of different body regions before the two exercise tests (P-2), immediately after the exercise test (P-6), and during recovery (P-8) are shown in **Table 2** and thermograms of selected body parts are shown in **Figure 2**. The differences between the left and right sides of all body regions were insignificant in both months. Therefore, the temperatures measured on both sides of each region were used for further calculations. The website ANOVA showed significant differences (P<0.0001) in the mean BSTs between the different body regions in all phases. In October, the mean basal BSTs of all body regions were lower than in May (P=0.05). BSTs in P-2 ranged from 26.5°C at the croup to 30.4°C at the breast during the May test (P<0.0001) and from 22.8°C at the croup to 28.8°C at the back during the October test (P=0.0004).

A significant increase in BSTs was noted immediately after the exercise test (P-6) compared with basal values (P-2) in May (P<0.001 for buttocks, chest, and neck, P=0.003 for croup, and P=0.005 for back) and in October (P<0.0001 for all regions). In May, an insignificant increase (P>0.05) in BSTs was observed at the end of the study (P-8) compared with P-2 for buttocks, croup, back, and neck, while the chest temperature decreased slightly (P>0.05). In October, a significant decrease in BSTs was observed when comparing P-6 and P-8 (P=0.05 for buttocks, P=0.016 for neck, P=0.001 for back, P=0.003 for chest), but the difference for back was insignificant (P=0.095).

Table 2: Changes in the body skin temperatures (mean ± SD) of various body regions before the exercise test (Phase 2; P-2), immediately after the exercise test (Phase 6; P-6) and during the recovery (Phase 8; P-8) in both periods (May and October)

		Body skin temperature (BST) [°C]		
Body region	Trial	Phase		
		P-2	P-6	P-8
Buttocks	May	30.2 ± 0.6 [A,B]	33.4 ± 1.6 [A,B]	33.6 ± 1.1 [A,B]
	October	26.8 ± 1.8 [a]	32.6 ± 0.7 [a]	30.7 ± 0.7 [a,b]
Croup	May	26.5 ± 1.5	29.6 ± 2.1	30.5 ± 1.8
	October	22.7 ± 2.2	27.5 ± 2.2	26.1 ± 2.3
Back	May	27.7 ± 1.3	30.6 ± 2.3	31.4 ± 1.3
	October	24.1 ± 1.5	29.1 ± 1.3	27.5 ± 1.4 [c]
Chest	May	30.4 ± 1.8 [A]	34.0 ± 2.7 [A,B]	33.6 ± 1.4 [A,B]
	October	28.7 ± 1.1 [b]	34.9 ± 1.3	32.0 ± 1.5 [a]
Neck	May	29.8 ± 1.6 [A]	33.1 ± 2.0 [A,B]	33.1 ± 1.3 [B]
	October	28.0 ± 1.7 [a,b]	32.5 ± 2.0 [a]	30.2 ± 1.5 [b]

[A,B,C]P>0.05 for values in a row with the same labels; other combinations of values for May in the same column are significantly different (P<0.05). [a,b,c]P>0.05 for values in a column with the same labels; other combinations of values for October in the same column are significantly different (P<0.05).

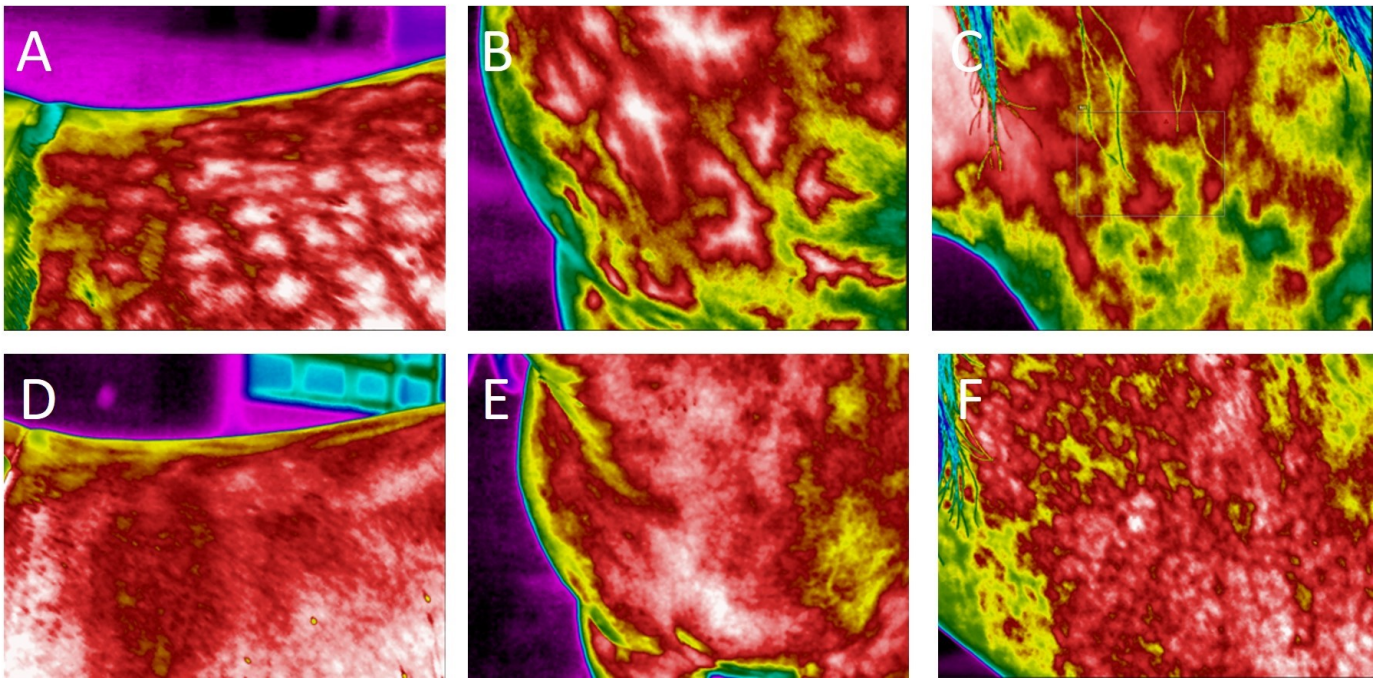

Figure 2. Thermograms of selected body regions before (A-C) and immediately after (D-F) the exercise test. A,D: back, B,E; chest and C,F: neck.

4. Discussion

In this study we investigated the BST responses of Lipizzan horses to graded exercise tests in May and October with lunging at the walk, trot, and canter. To our knowledge, this is the first study with exercise testing conducted in this breed of horse.

Metabolic activity in resting muscles generates a constant amount of heat that increases in proportion to workload during exercise. In order to maintain body temperature within the physiological range, various thermoregulatory mechanisms are activated, leading to an increase in BST (Jodkowska et al., 2011; Redaelli et al., 2014). As for BST, horses are bilaterally symmetrical (Jodkowska et al., 2011; Simon et al., 2006), which was also confirmed in this study in Lipizzan horses, indicating balanced muscle work and appropriate running track surface. Resting BSTs of the horses studied ranged from 26.5 °C to 30.4 °C in May and from 22.7 °C to 28.7 °C in October, similar to other studies (Simon et al., 2006; Jodkowska et al., 2011; Wallsten et al., 2012). Resting BSTs of the same regions were significantly lower in October than in May, and the differences between BSTs of different regions were significant in both months, indicating a different blood supply to different parts of the skin and a different contribution of these regions to the thermoregulatory functions of the horse (Jodkowska et al., 2011).

After graded exercise, BST values of all regions increased significantly compared to basal values (Table 1); this increase was more pronounced in October than in May. The absolute BST values and the differences between pre- and post-load BST values were comparable to those measured in horses after jumping tests (Jodkowska et al., 2011) or treadmill tests (Simon et al., 2006). Body skin temperature values for all body regions of Lipizzan horses, except for the chest, increased slightly during the recovery period in May but decreased in October. As mentioned earlier, we attribute this to a higher ambient temperature in May than in October, which reduces the efficiency of convection (Redaelli et al., 2014]. These results also suggest that the high relative humidity measured in October, which exceeded the critical value for sweat evaporation of 80-85%, did not hinder the skin cooling reported at high air temperatures (Redaelli et al., 2014).

Differences between the resting BSTs of various body regions were determined in both trials. Following exercise tests, the BSTs of all regions were increased when compared to the resting values, and decreased thereafter during the recovery time in October or

remained almost unchanged in May. The results of our study present the physiological response of Lipizzans to graded exercise and can be accepted as an important contribution to sports physiology and medicine pertaining to the Lipizzan breed.

5. Conclusion

The results of our study represent the physiological response of Lipizzan horses to graded exercise and can be considered an important contribution to sports physiology and medicine in relation to the Lipizzan breed. This study also contributes to the knowledge of equine thermography in different seasons and to the recognition of the complex physiological processes during exercise, which provide a basis for further research in the field of equine exercise testing and sports medicine.

Funding: This research was supported by the Slovenian Research Agency (research program P4-0053).

Conflict of Interest: The author declares no conflict of interest.

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Invited lecture/Review

The role of the Concomitant Lesions in Determining Failure of Anterior Cruciate Ligament Reconstruction

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Abstract:
Anterior cruciate ligament (ACL) tear is one of the most common sport-related injuries and the request for ACL reconstructions is increasing nowadays. Unfortunately, ACL graft failures may occur in about 5.2% of cases. Unrecognized concomitant meniscus and ligamentous lesions are estimated to be responsive of about 15% of ACL reconstruction failures. Isolated ACL reconstruction in this setting may not be enough to properly restore knee stability. If not properly treated, such lesions may expose ACL graft to excessive stress, thus predisposing to failure. This article aims at highlighting the role of associated lesion in determining failure of ACL reconstruction, while also providing an evidence-based algorithm about proper management.

Citation: Russo A, Costa GG. The role of the concomitant lesions in determining failure of anterior cruciate ligament reconstruction. Proceedings of Socratic Lectures. **2023**, 8; 46-51.
https://doi.org/10.55295/PSL.2023.I7

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Keywords: Anterior cruciate ligament reconstruction; Failure; Concomitant lesions; Meniscus; Medial collateral ligament; Posterolateral corner

1. List of abbreviations

ACL anterior cruciate ligament; ALL anterolateral ligament; MCL medial collateral ligament; PLC posterolateral corner; LMPR lateral meniscus posterior root.

2. Introduction

Anterior cruciate ligament (ACL) tear is one of the most common injuries in sports active population, involving about 3% of amateur athletes every year, and up to 15% of elite athletes per year (Mayer et al., 2015). Surgical reconstruction has always been supported by the international literature since conservative treatment was proved not to be capable of properly restoring knee kinematics and preventing osteoarthritis development (Noyes et al., 1983; Kessler et al., 2008; Hurd et al., 2008).

Despite the recent advances in surgical techniques, knee biomechanics knowledge and injury prevention programs, 10-to-15% of patients undergoing ACL reconstruction report unsatisfactory outcomes (Samitier et al., 2015). Two systematic reviews reported only 60% of amateur athletes (Ardern et al., 2014) and 83% of elite athletes (Lai et al., 2018) returned to their preinjury sport level after ACL reconstruction. Graft failure is claimed as the main determinants of outcomes. In a meta-analysis involving 1,272 elite athletes, the pooled failure rate was estimated in 5.2% (range 2.8% - 19.3%) (Lai et al., 2018), but this rate has been shown to grow up to 34.2% when including high-risk cohorts like younger athletes (Wiggins et al., 2016).

Graft failure after ACL reconstruction may be secondary to technical errors, biologic causes, or traumatic events (Vermeijden et al., 2020; Kamath et al., 2011). Unrecognized concomitant meniscus and ligamentous lesions are estimated to be responsive of about 15% of ACL reconstruction failures (Samitier et al., 2015). Isolated ACL reconstruction in this setting may not be enough to properly restore knee stability. If not properly treated, such lesions may expose ACL graft to excessive stress, thus predisposing to failure.

This article aims at highlighting the role of associated lesion in determining failure of ACL reconstruction, while also providing an evidence-based algorithm about proper management.

3. Anterolateral Ligament

The anterolateral ligament (ALL) is one of the most debated issues about this topic. High interest is fueled by the common finding of residual pivot-shift phenomenon after ACL reconstruction, which is estimated in up to 25% of cases regardless of the chosen graft (Sonnery-Cottet et al.,2017). Persisting rotational instability was shown to predispose to recurrent injuries and ACL failure (Kunze et al., 2021). Several biomechanical studies demonstrated a better restoration of anteroposterior and rotatory stability when an ALL reconstruction is combined to an ACL reconstruction, rather than performing an ACL reconstruction alone (Na et al., 2021). Such biomechanical findings also result in clinical evidence of reduced risk of graft failure.

A recent meta-analysis of 20 randomized and nonrandomized controlled trials found that the rate of graft failure was two-to-four times lower in the ACL/ALL group than in the isolated ACL reconstruction group, regardless the adopted technique or the surgical timing (Na et al., 2021). Therefore, international literature supports the ALL reconstruction in high-risk patients. Indications include patients with high-grade pivot shift, patients with concomitant Segond fractures and high-level athletes participating in pivoting sports and in ACL revision settings (Na et al., 2021).

4. Medial Collateral Ligament

Medial collateral ligament (MCL) injury is quite often associated to ACL tears (Grant et al., 2012), as a result of the typical valgus stress trauma determining ACL lesion. ACL and MCL play a synergistic role in maintaining anteromedial knee stability (Wierer et al., 2021). Several cadaveric studies demonstrated that ACL strain is increased after sectioning MCL, when applying a valgus stress or an intra-rotation movement of the tibia (Wierer et al.,2021; Battaglia et al., 2009). In addition, combined MCL and ACL sectioning increases anterior knee laxity greater than isolated ACL sectioning (Mains et al., 1977). Despite these findings, the treatment of combined ACL and MCL tears is still controversial. Most authors support the conservative management of the MCL injury, especially in acute settings and low-grade injuries (Grant et al., 2012; Bollier and Smith, 2014). A "wait and see" approach is recommended by some authors also in high-grade MCL tears (Grant et al., 2012). However, a recent study from the Swedish National Knee Ligament Registry highlighted a higher risk of ACL revision in patients with ACL reconstruction and non-surgically treated MCL injuries compared to isolated ACL reconstructions. When a repair or reconstruction of concomitant MCL injuries was performed, this risk was comparable to isolated ACL reconstructions (Svantesson et al., 2019). These findings encourage the authors supporting early MCL repair or reconstruction (DeLong and Watermann, 2015) because ACL insufficiency might adversely affect the MCL process healing (Woo et al., 1990). On the other hand, delayed ACL reconstructions have been related to better functional outcomes with earlier motion recovery (Mook et al., 2009). MCL surgical treatment should be considered in patients with severe valgus alignment, entrapment over the pes anserinus tendon (Stener-like lesion), large bony avulsions and persistent instability after ACL reconstruction (DeLong and Watermann, 2015; Mook et al., 2009)

5. Posterolateral Corner

The posterolateral corner (PLC) of the knee is another important issue of academic interest, because of an evolving appreciation for its biomechanical relationship with the ACL function. PLC injuries are commonly associated to cruciate ligaments tears, occurring in isolation in only 28% of cases (Dean and LaPrade, 2020). Specifically, 7.4% - 13.9% of patients with ACL injury have a concomitant PLC injury (LaPrade et al., 2007). Biomechanical data demonstrated a significant increase in force on the ACL in PLC-deficient knee, when applying a varus moment or a combined varus-internal rotation moment to the knee joint (LaPrade et al., 1999; Plaweski et al., 2005), as well as during simulated gait and squatting (Kang et al., 2019). In addition, Plaweski et al. (2005) found that an ACL reconstruction was not enough to prevent varus and external rotation displacement in the setting of ACL-PLC deficient knee; a return to native kinematics was achieved only after adding a reconstruction of PLC static structures. Despite such promises, the role of PLC on the risk of ACL failure has not been adequately investigated. In one registry study, a concomitant PLC injury would appear to not affect the risk of ACL failure, whatever the treatment is (Svantesson et al., 2019). However, this analysis was impaired by the small size of the study groups, which limits the relevance of such findings.

6. Menisci

The biomechanical role of the menisci on knee stability must not be overlooked. The medial and lateral menisci act as secondary restraints for anterior and rotatory tibial displacement (Musahl et al., 2010; Grassi et al., 2019; Hoshino et al., 2020) Meniscus repair would seem to restore knee stability comparable to ACL-reconstructed knees with intact menisci (Hoshino et al., 2020). These findings also apply to meniscus posterior root lesions (MPRL) (Zheng et al., 2020; Samuelsen et al., 2020) Lateral MPRLs (Figure 2) were reported to increase anterior tibial subluxation of the lateral compartment in patients with ACL injuries (Zheng et al., 2020). Similarly, medial MPRLs were found to significantly increase ACL graft loads over the intact state, while root repair restored the function of the medial meniscus as a secondary stabilizer (Samuelsen et al., 2020). Finally, a ramp lesion in an ACL-deficient knee has also been shown to increase anterior tibial translation and external rotational laxities (Stephen et al., 2016; Naendrup et al., 2019). This aberrant laxity cannot be

completely restored after ACL reconstruction alone but with combined posterior meniscocapsular repair (Naendrup et al., 2019). Nevertheless, there is poor clinical evidence regarding increased risk of graft failure following meniscal loss. Only one study identified medial or lateral meniscus deficiency as significant factor for predicting graft failure (Parkinson et al., 2017), since several other studies did not detect significant difference between isolated ACL reconstruction and ACL reconstruction combined with medial and/or lateral meniscectomy (Young et al., 2021; Akada et al., 2019). However, the fundamental role of the meniscus in preserving joint function and preventing osteoarthritis development is well known. Furthermore, meniscectomy has been clearly recognized as a risk factor for delayed return to sport (Akada et al., 2019) and career shortening in athletes (Akada et al., 2019; Neyret et al., 1993; Brophy et al., 2009). As a result, meniscus repair should be considered even in athletes.

7. Conclusion

Associated lesions to ACL tear play a non-secondary role in determining graft failure after ACL reconstruction. Careful preoperative evaluation as well as proper management of such lesions is fundamental to not expose ACL graft to excessive stress, thus minimizing the risk of failure.

Conflicts of Interest: The authors declare no conflict of interest.

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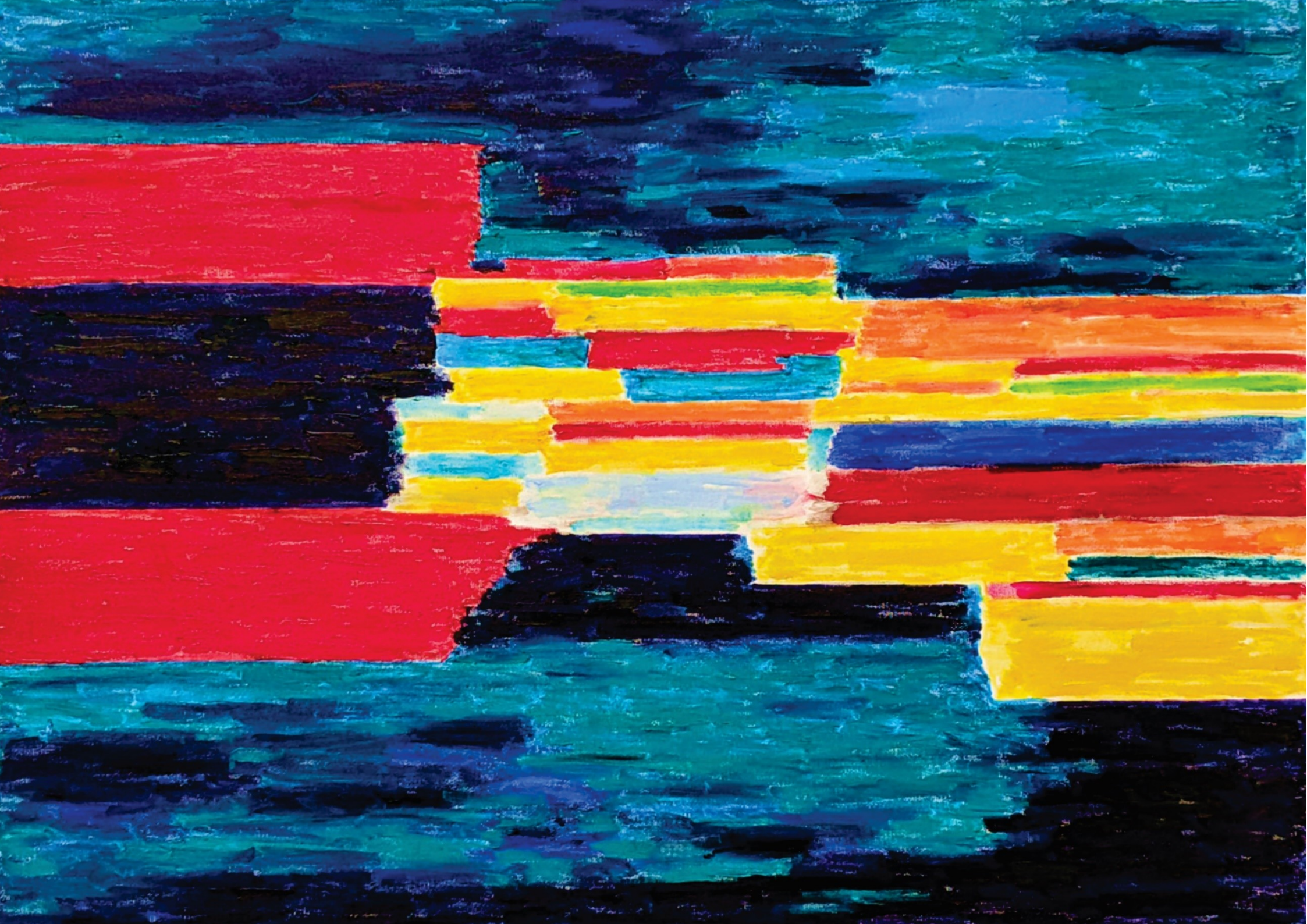

Invited lecture/Review

Femoroacetabular Impingement

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Abstract:
Femoroacetabular impingement (FAI) is an anatomical hip condition caused by malformations on femoral head and acetabular rim resulting in abnormal contact across the joint. FAI can cause the labral, cartilaginous, and tissue damage that leads to early osteoarthritis. FAI can be divided into three groups: cam (bump on femoral head- neck junction), pincer (acetabular over coverage), or mixed (most common) by the characteristic morphological changes of the bony structures. The exact ethology of FAI is still unclear, mostly considered as idiopathic. Cam lesions demonstrate a near 3:1 male predominance and are more often seen in the younger population. Pincer is typically seen in middle-aged women. A plain radiography of the pelvis and hips is considered as the primary imaging modality for diagnosing FAI, which can be used to quantify the severity. MRI and direct MRI arthrography allow assessment of concomitant labral and chondral injuries. Conservative treatment is typically considered first-line treatment for mild to moderate FAI syndrome, but usually not to successful. However, the outcomes following postoperative surgical intervention have demonstrated excellent results. The most common surgical treatment option for FAI is done arthroscopically, other procedures such as a reverse periacetabular osteotomy or surgical dislocation of the hip are rarely indicated.

Citation: Kovačić B, Zore LA, Stražar K. Femoroacetabular impingement. Proceedings of Socratic Lectures. **2023**, 8; 53-57. https://doi.org/10.55295/PSL.2023.I8

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Keywords: Femoroacetabular impingement; cam, pincer; hip arthroscopy

1. Introduction

Twenty years ago, first papers were presented describing anatomical hip condition caused by malformations on femoral head and acetabular rim resulting in abnormal contact across the joint (Beck et al., 2005). At the beginning in the focus were young adults with early onset osteoarthrosis because it was later shown that femoroacetabular impingement (FAI) can cause the labral, cartilaginous, and tissue damage that leads to early osteoarthritis (Ganz et al., 2003; Ganz et al., 2008).

FAI can be divided into three groups: cam (bump on femoral head- neck junction), pincer (acetabular over coverage) (**Figure 1**), or mixed (most common) by the characteristic morphological changes of the bony structures (Kassarjian et al., 2007). In the case of cam FAI abnormally shaped femoral head repeatedly impinging upon an acetabulum that cannot accommodate the increased radius of the femoral head.

It typically occurs with flexion of the hip joint as this lesion is usually situated in the anterior aspect of the head-neck junction of the femur. In pincer FAI over-coverage could be global as in coxa profunda and acetabular retroversion or localised as in an anterior osteophyte. The repeated intersection of this abnormally shaped femoral head and/or acetabular rim on the labro-chondral junction generates shear forces in this region which in turn may lead to a labral tear, labro-chondral separation, articular cartilage peeling off the bone and in the longer term osteoarthritis (Khanduja et al., 2007).

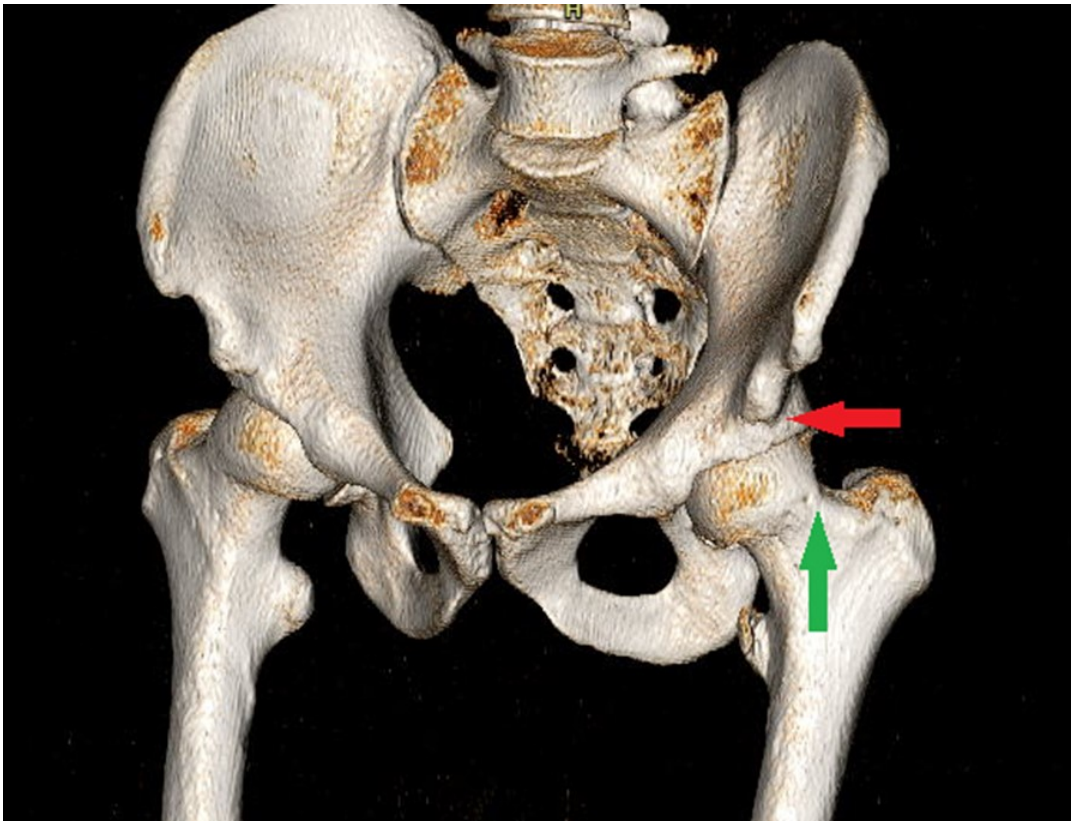

Figure 1. 3D computer reconstruction of CT scan of pelvis and hips shoving mixed type of FA impingement: cam (green arrow) and pincer (red arrow).

The exact ethology of FAI is still unclear: genetic predisposition, trauma, paediatric hip disorders such as slipped capital femoral epiphysis (SCFE), and Legg–Calve–Perthes disease (LCPD) have been shown to predispose to the development of cam impingement in adulthood. Despite that, it is most often idiopathic, and particularly common in the athletic population. Cam lesions demonstrate a near 3:1 male predominance and are more often seen in the younger population. Pincer is typically seen in middle-aged women (Gosvig et al., 2010; Leunig et al., 2000).

Studies show that athletes with excessive participation in high-impact sports (soccer, basketball and ice hockey during adolescence when the skeleton matures, have a higher prevalence of FAI when compared to non-athletes. (Agricola et al., 2012). Proposed mechanism for development of the cam deformity in adolescent athletes is thought to be either new bone formation at the anterosuperior head-neck junction or changes in the shape of the growth plate as a reaction on high shear forces at the growing hip during these athletic activities (Siebenrock et al., 2011).
In the clinical presentation, chronic, persistent groin pain is the most frequent initial symptom. On physical examination, patients will typically have a positive FADIR test (flexion, adduction, internal rotation), described as a positive impingement sign (Jager et al., 2004; Jaberi et al., 2007).

2. Radiology

Radiographic findings suggestive of a cam FAI: a pistol grip deformity on a standard AP pelvic view and increased alpha angle and decreased femoral head-neck offset on a lateral view (cross-table, frog-leg, or 45° Dunn view) (**Figure 2**).

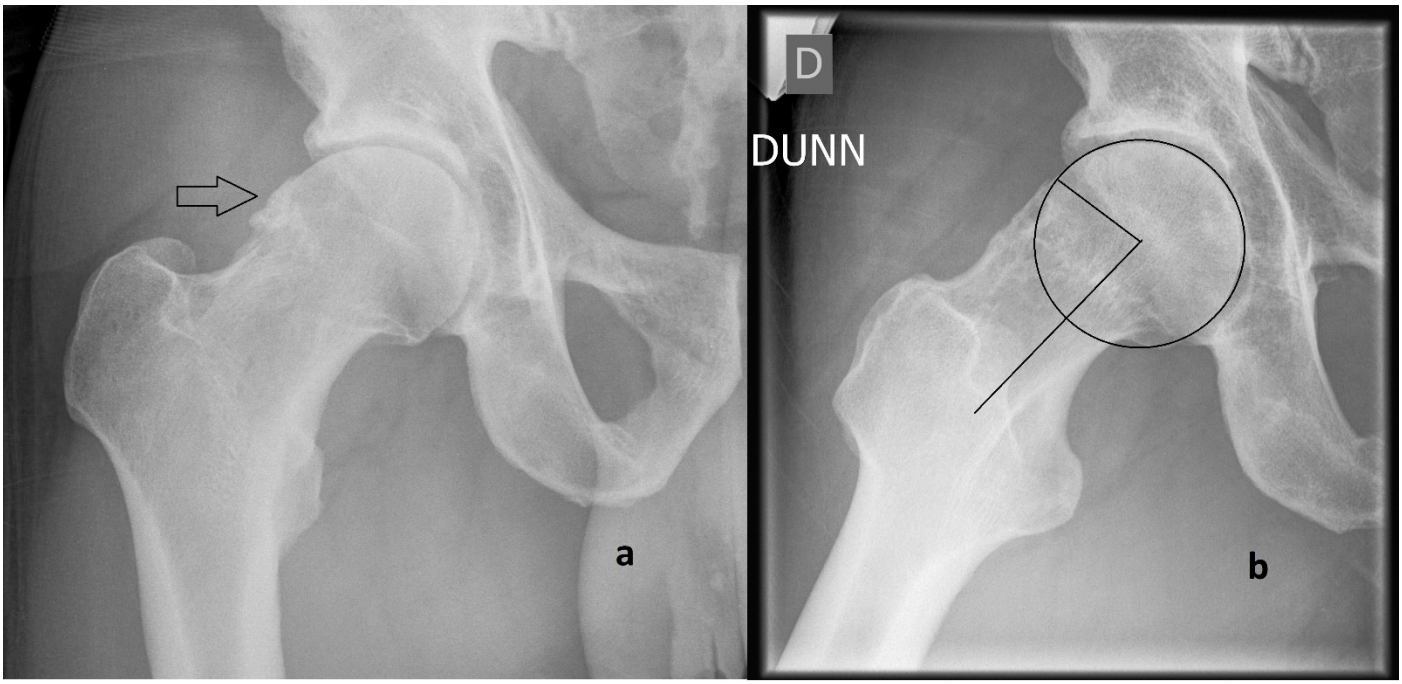

Figure 2. Standard x- ray AP (a): typical pistol grip deformity (arrow) and Dunn view (b): increased α angle; radiological signs of cam impingement.

On standard AP view, radiographic findings for a pincer impingement include: acetabular over coverage (increased lateral center-edge angle and acetabular inclination angle, retroversion and/or protrusion (crossover sign, posterior wall sign, and ischial spine sign) and coxa profunda (Zhou et al., 2020; Diesel et al., 2015).
Both CT and MRI allow the assessment of cam and/or pincer morphology trough objectifying alpha angle and femoral head-neck offset for cam and acetabular retroversion for pincer FAI. A 3D sequence of the hip can be made for more accurate visualization of the femoral neck changes and proper evaluation of the acetabulum and operative planning. MRI and direct MRI arthrography allow assessment of concomitant labral and chondral injuries like chondrolabral separation or carpet lesion and changes associate to FAI such as paralabral cysts, head- neck junction cyst and bone edema. (Bredella et al., 2013)

3. Treatment

Conservative treatment is typically considered first-line treatment for mild to moderate FAI syndrome as it can provide marked symptomatic relief, but it is not significantly helpful in most patients with FAI syndrome.
The goal of FAI surgery is to re-establish the normal relationships between the femoral and acetabular part of the hip joint to restore normal function. The most common surgical treatment option for FAI is hip arthroscopy: femoral osteochondroplasty to resect a cam deformity (**Figure 3**), debridement and selective acetabular rim resection and arthroscopic labral repair. (Hartmann et al., 2009; Philippon et al., 2009; Philippon et al., 2010).
In case of global acetabular overcoverage and cam deformity that is not accessible by arthroscopy, a surgical dislocation of the hip, in which the femoral head is surgically dislocated from the acetabulum is indicated. An acetabular retroversion causing pincer FAI, is addressed by reverse periacetabular osteotomy (PAO), a surgical method of completely reorienting the acetabulum (Clohisy et al., 2008; Mardones et al., 2005).

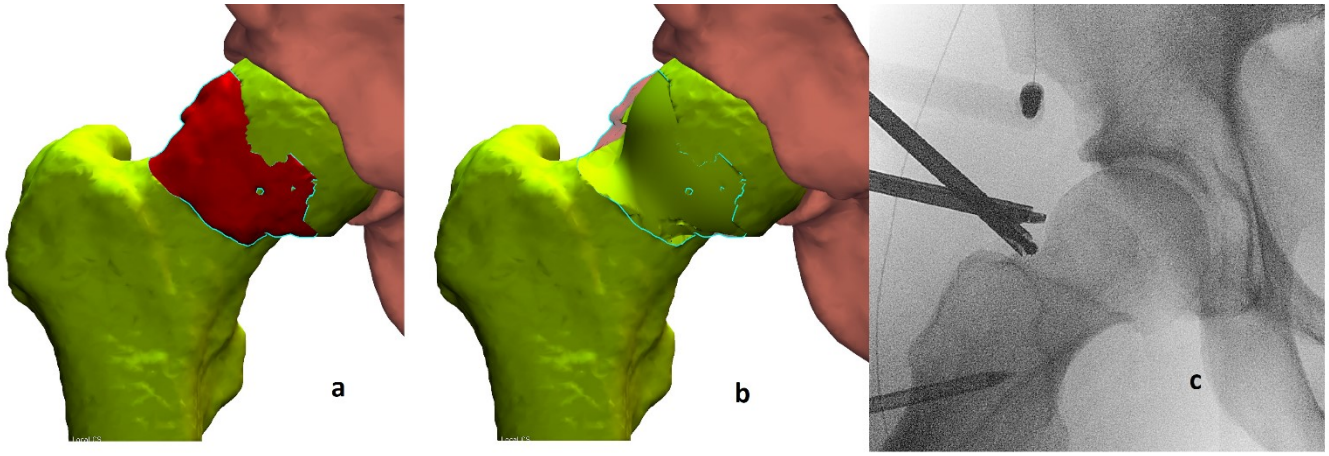

Figure 3. 3 D reconstruction model of right hip from CT scan with a cam lesion shown in red (a), preoperative projection of the needed resection of the lesion (b) and an intraoperative x-ray during the arthroscopic osteochondroplasty in the same patient.

In Ljubljana Medical Centre at Orthopedic Clinic standard for the treatement of the FAI is the use of a computer assisted hip arthroscopy. With the help of currently available software that provides preoperative identification of hip deformity on a CT-based 3-D model and planning of the surgical correction using kinematic protocols. This protocol provides a real-time intraoperative 3-D orientation, and exact execution of surgical correction either with navigation of surgical tools (Figure 3.) or with printed templates. First clinical experiences of its use in treatment of femoroacetabular impingement are promising (Stražar et al., 2021).

Conflicts of Interest: The authors declare no conflict of interest.

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Invited lecture/Review

The Use of Tranexamic Acid in Orthopaedic Surgery

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Abstract:
New surgical techniques (minimally invasive surgery – (MIS), laparoscopic surgery), meticulous consideration of haemostasis, use of robots, Cell Saver and tranexamic acid, new transfusion criteria and single red blood cell (RBC) unit ordering have greatly changed clinical practices. Implementation of these therapeutic options along with other practices has significantly contributed to the effectiveness of the patient blood management approach to surgical patients. In recent years use of anti-fibrinolytic agent tranexamic acid (TXA) has been introduced at our department and intravenous administration as well as topical TXA administration were successfully implemented. Use of topical TXA was effective at reducing both post-operative red blood cell loss and transfusion rates with good tolerance and no clinically relevant adverse events. Within 6 years of Patient Blood Management (PBM) protocol implementation in our institution, the total number of transfusions was reduced by 76 % and the percentage of patients requiring transfusion fell from 38 % to 9 %.

Keywords: Tranexamic acid; Blood loss; Orthopaedic surgery; Endoprosthesis; Joint arthroplasty

Citation: Kocjančič B. Kocjančič E. The use of tranexamic acid in orthopaedic surgery. Proceedings of Socratic Lectures. **2023**, 8; 59-62. https://doi.org/10.55295/PSL.2023.I9

1. Tranexamic acid

Tranexamic acid (also referred to as TXA) is a drug commonly prescribed in order to control bleeding in patients. As it helps blood to clot it is most commonly used for nosebleeds and heavy periods. Tranexamic acid has also proven to be beneficial for patients who suffer from excessive bleeding and are about to undergo surgical or dental procedures (NHS, 2020).

Tranexamic acid is a synthetic derivative of the amino acid lysine with a chemical formula $C_8H_{15}NO_2$, as shown in **Figure 1** (DrugBank, 2023).

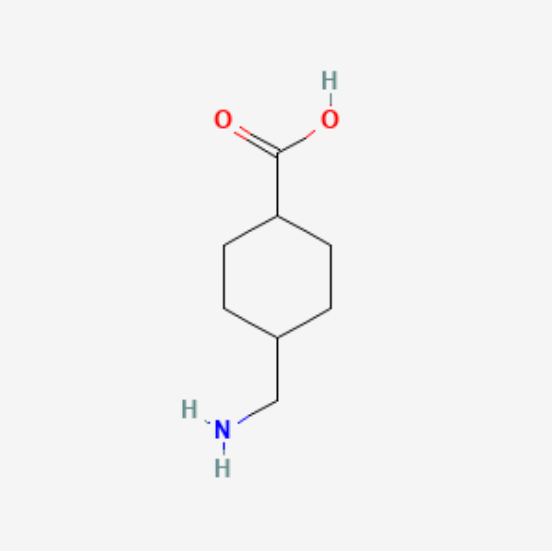

Figure 1: Chemical structure of tranexamic acid (National Center for Biotechnology Information, 2023).

Tranexamic acid is an antifibrinolytic which competitively and reversibly inhibits the activation of plasminogen to plasmin. It does so by binding to several sites of plasminogen, one of which is a high-affinity site which is involved in binding fibrin. The binding of fibrin and plasminogen induces fibrinolysis, that is, the breakdown of fibrin in the blood. Bound tranexamic acid occupies the necessary sites on plasminogen, thus preventing fibrinolysis and stabilising the blood clot (Drugbank, 2023).

Tranexamic acid may be administered to patients either orally in the form of tablets, or intravenously via injection. It is only available with a doctor's prescription and is only available for patients over 12 years of age (Mayo Clinic, 2023). Tranexamic acid is provided as 500mg tablets. The normal dose prescribed for adult patients is 2-3 tablets, taken 3 times per day. The time intervals should be spaced out as evenly as possible. For patients with kidney problems, prescribed doses are normally lower (NHS, 2020).

2. Clinical use of tranexamic acid

A beneficial finding regarding tranexamic acid has been that there are little to no common side effects with tranexamic acid tablets, however, with tranexamic acid injections, patients might experience nausea, diarrhoea and itchy skin.

Additionally, tranexamic acid can be taken with most other medicines (NHS, 2020). All this makes for a useful medication used in surgical procedures, such as orthopaedic ones.

Orthopaedic surgery, especially total joint arthroplasty (**Figure 2**), is commonly associated with major blood loss, and patients require blood transfusion to avoid postoperative anaemia. It has been reported that up to almost 40% of patients undergoing primary total hip

arthroplasty and almost 25% of patients undergoing total knee arthroplasty require such transfusion, as the average blood loss during said surgeries ranges between 1000 and 2000 ml of blood (Kim et al., 2015).

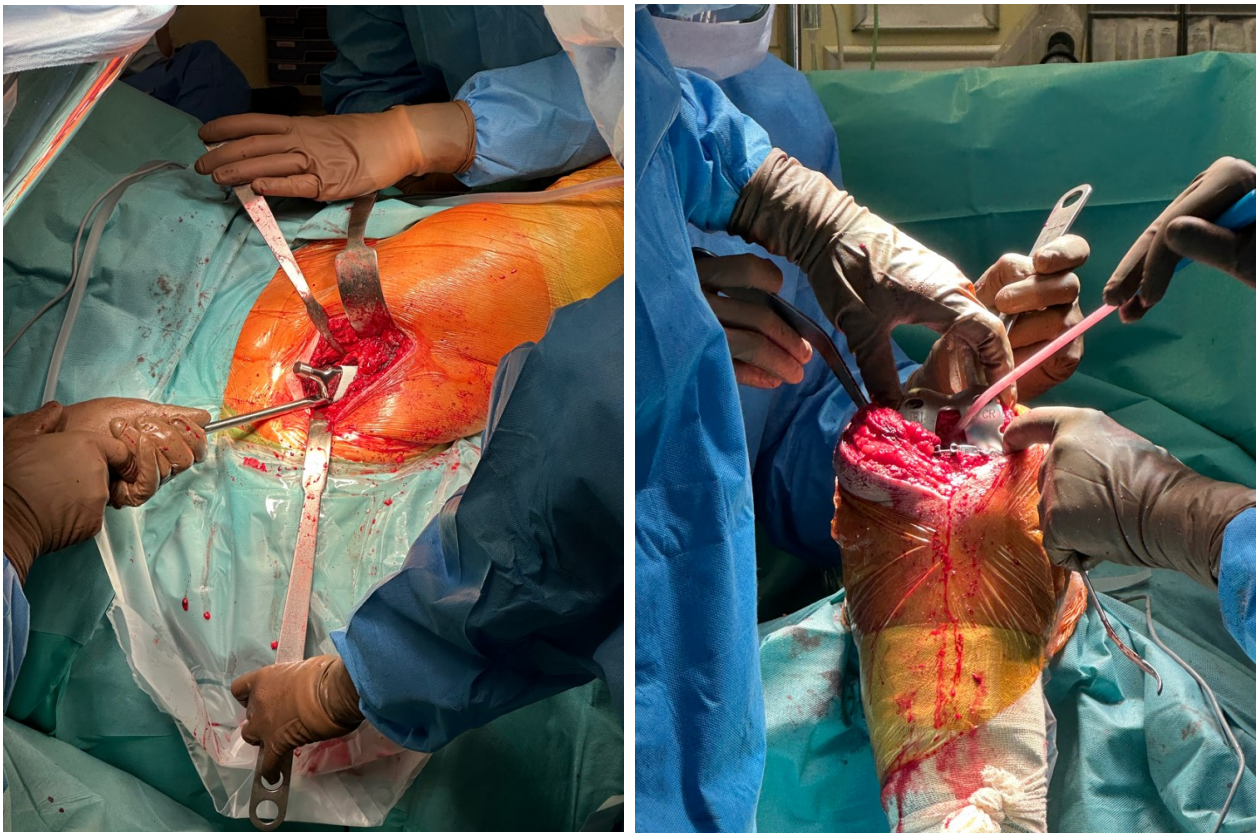

Figure 2: Left: Primary total hip arthroplasty surgery. Right: Primary total knee arthroplasty surgery (Author's own photographs).

Attempts to minimize blood loss during surgery, as well as the need for patient blood transfusions, have been made. The use of haemostatic agents, such as tranexamic acid and the use of Cell Saver, have considerably increased in orthopaedic lower limb surgery (Reale et al., 2021).

Clinical studies show that multiple administrations of tranexamic acid have proven to be useful in reducing patient blood loss, postoperative pain, and markers of inflammation, all without a significant increase in the incidence of thromboembolic events compared to placebo and single-dose tranexamic acid groups. Findings also show a more favourable hospital stay length and a lesser need for expensive blood transfusions which might, in rare cases, lead to unnecessary secondary patient infections (Haratian et al., 2021).

3. Conclusion and the use of tranexamic acid at the Department for Orthopaedic Surgery, University Medical Centre Ljubljana

With the help of minimally invasive surgical methods, updated transfusion threshold guidelines, and recently rising tranexamic acid usage, transfusion rates at the Department for Orthopaedic surgery of Ljubljana have been dropped by over 80% during the past ten years. The overall number of transfusions was decreased by 76% within 6 years of the Patient Blood Management protocol's implementation, and the proportion of patients needing transfusions decreased from 38% to 9%. Use of topical TXA was effective at reducing both post-operative red blood cell loss and transfusion rates with good tolerance and no clinically relevant adverse events.

It is certain that a multidisciplinary approach is required for the implementation of Patient Blood Management program and reduction of blood loss in surgery. Surgeons, general practitioners, as well as anaesthesiologists, play a pivotal role.

Clinical practices for red blood cell transfusion have significantly changed as a result of new surgical techniques (including MIS, laparoscopic surgery, robots), rigorous consideration of haemostasis, use of Cell-Saver, and most recently, use of tranexamic acid, new transfusion criteria, and single red blood cell unit ordering. The effectiveness of the patient blood management approach to surgical patients will be greatly enhanced by the implementation of this therapeutic option along with other procedures (early detection and treatment of pre-operative and post-operative anaemia, a restrictive transfusion strategy, policy of transfusing single units of red blood cells, etc.).

Our experience and statistical data demonstrate a significant decline in the number of transfusions and the proportion of patients who required transfusions in recent years. The causes for this include new surgical procedures, increased public awareness of the drawbacks of blood transfusions, and the introduction of innovative techniques and agents, such as tranexamic acid.

Conflicts of Interest: The authors declare no conflict of interest.

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Invited lecture/Reflection

Narrow-Band Imaging – Clinical Application in Otorhinolaryngology

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Abstract:

Narrow-Band Imaging (NBI) is an optical endoscopic technique using optic filters to select two wavebands from a white light source and revealing mucosal and submucosal vascular patterns. NBI endoscopy is implemented to detect various lesions of the nasal and oral cavity, oropharynx and hypopharynx, and larynx; for finding bleeding vessel in recurrent epistaxis, for more precise tissue biopsy, in the diagnostics of synchronous cancers, for tumours of unknown origin, in defining surgical margins, inflammation and for follow-up of oncologic patients. According to lesion area in otorhinolaryngology, three classifications are known for observation of suspicious lesions: the "IPCL classification" for oral mucosa, the Ni's classification, and the classification recommended by the European Laryngological Society, for vocal cords. The correct recognition of vascular patterns by physician is strongly influenced by the learning curve of the clinician. In line with limits of NBI, a tissue biopsy remains the gold standard for definitive proof of malignancy. However, NBI endoscopy is especially useful tool for early detection of malignant and precursor lesions when the lesions are invisible during classical otorhinolaryngological examination.

Citation: Bitenc Zore S, Šifrer R. Narrow-band imaging – clinical application in otorhinolaryngology. Proceedings of Socratic Lectures. **2023**, 8; 64-68 https://doi.org/10.55295/PSL.2023.I10

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Keywords: Narrow-band Imaging; Endoscope; Blue and green light; Vascular pattern; Detecting carcinoma

1. Introduction

Narrow-Band Imaging (NBI) as one of biological enodoscopies was developed in Japan in 1999 and was initially used for observing vascular patterns of intestinal mucosa. Later, it was implemented in various fields of medicine, including otorhinolaryngology (Gono, 2015). NBI is an optical image enhancement technology that enhances the visibility of vessels in the surface of the (sub)mucosa. The article presents the NBI endoscopic technique, its characteristics, its use in clinical practice, characteristics of the vascular patterns and its limits .

2. Narrow-Band Imaging characteristics and equipment

NBI is an endoscopic diagnostic method where the examined mucosa is illuminated with only two narrow bands of blue and green light spectra through optical filters instead of the entire spectrum of white light, ranging from 400 to 700 nm. A different wavelength is visually perceived as a different color. Hemoglobin in the vessels contains chromophores and absorbs blue and green light. The peak of blue light has a wavelength of 415 nm and shows us the superficial capillaries in the mucous membrane, while the green light with the peak of 540 nm reveals the submucosal vessels.
NBI takes advantages of selected light spectrum and some physics behind it. Firstly, the longer the wavelength, the deeper the light penetrates into the tissue (Ni et al., 2015). Secondly, since the energy of the blue and green spectrum is lower than the energy of white light, the light penetrates less deep than normal light, so we can observe only the surface of the mucous membranes, i.e., NBI is not appropriate method for observing deeper tissues. Thirdly, when the depth penetration is shorter, there is also less light scattering that sharpens the image. Finally, unlike the rest of the surrounding tissues, blue and green light is well absorbed by (sub)mucosa and consequently the contrast is improved (Šifrer, 2017a; Piazza et al., 2008; Piazza et al., 2010).
For NBI examination we need a light source and NBI filter placed between the xenon light source and a red-green-blue filter. Moreover, a rigid or flexible endoscope is needed, connected to a standard definition television (SDTV) camera or a high definition television (HDTV) camera or even an ultra high definition camera (ultra high definition, ultra HDTV), which uses 4K technology for the highest resolution needed. A light source and a monitor for observing vascular patterns are also indispensable accessories (Šifrer, 2017a; Lukes et al., 2013; Piazza et al., 2008).

3. Narrow-Band Imaging in clinical practice

NBI endoscopy is based on recognition of vascular patterns during formation of new blood vessels in neovascularization (as a phisiological process) or during neoangiogenesis (as a vessel growth proccess in malignant lesions) (Piazza et al., 2010).
NBI in otorhinolaryngology might be used for detecting pathological lesions in the oral and nasal cavity, oropharynx and lower hypopharynx and larynx (Lukes et al., 2013). In the nasal cavity, NBI can be appropriate for finding the bleeding vessel in the recurrent epistaxis (Šifrer et al., 2013). Furthermore, NBI is used for screening and early diagnostics of superficial mucosal cancer and pre-cancerous lesions; to distinguish malignant and benign lesions; for precise detection of the spread of mucosal lesions; for preoperative decision-making; for searching for the cancers of unknown origin; for intraoperative assistance and for follow-up of all oncologic patients after primary oncologic and/or surgical therapy. It improves visualization of the cancer pre- and intraoperatively; in revealing synchronous cancers due to the cancerization field; in the diagnosis of tumors of unknown origin; in the diagnosis of leukoplakias, erythroplakias and oral ulcers; in determination of safety surgical margins during the intervention and in inflammation (Srivastava, 2019; Šifrer, 2017a; Šifrer et al., 2017b; Piazza et al., 2010). Some authors also use NBI as an aid in more accurate intraoperative tissue biopsy (Piazza et al., 2010).

4. Vessel pattern presentation

During NBI endoscopy we can detect possible tumor lesions that are smaller than 5 mm or even invisible during classical ENT examination (with white light observing) (Lukes et al., 2013; Piazza et al.; 2010; Muto et al.; 2005).

The tip of the endoscope is firstly placed far from the mucous membrane under observation. Under the NBI filter, healthy capillaries in the mucosa are stained brown, and healthy veins in the submucosal area are stained blue (Srivastava et al., 2019). Then the mucous membrane is slowly approached. In the case of suspicious oncological lesion, a well demarcated brown area is observed first. It follows the description of dimensions and the structures contained in it. Further, the endoscope is gradually brought closer to the lesion, thereby the image becomes apparently larger and the examination becomes more accurate. After the magnification, the previously observed sharply demarcated brown lesion becomes the image of thick brown spots which may be scattered over the area of the suspicious epithelium and represent pathologically changed intraepithelial papillary capillary loops (IPCL) (Sano et al., 2016; Watanabe et al.; 2009). During neoangiogenesis, IPCLs can be transformed by expanding in diameter, branching, making elongations, meandering, etc (Lukes et al., 2013). Regarding anatomical site, there are many classifications for describing the vascular patterns: the IPCL classification for oral mucosa, the Ni's classification for vocal cords and the classification recommended by the European Laryngological Society, for vocal cords. However, as opposed to normal vocal cord vessel patterns, healthy oral mucosa is presented as regularly scattered thin brown dots (if they are in a perpendicular position in relation to the surface of mucosa), or as waved lines (if they are in the parallel position in relation to the surface of mucosa). IPCL elongated, meandering IPCL or IPCL in the form of the tangled lines represent malignant alteration of the oral mucosa. Healthy vocal cord vessels under NBI endoscopy are seen as tiny parallel vessels. But when vessels are presented like dilated, "worm shaped" or brown dots, this might be a sign of malignancy of vocal cords (Bitenc Zore and Šifrer , 2022; Šifrer et al., 2018; Šifrer et al., 2020). According to pathological vascular samples discovered as part of NBI endoscopy, this indicates further action - tissue biopsy, imaging diagnostics and regular check-ups of the current condition. NBI endoscopy serves as an aid in the detection of early (pre)malignant lesions. Tissue biopsy for histopathological examinations still remains the gold standard for confirming malignancy (Šifrer, 2017a; Piazza et al., 2011).

5. Limitation of Narrow-Band Imaging

The success of correct identification of vascular patterns with NBI endoscopy in benign and malignant mucosal lesions is dependent on the number of treated patients and thus the steep learning curve is typical (Srivastava, 2019; Piazza et al., 2010). During NBI examination, saliva and bleeding may severely limit the procedure. The latter leads to vascular patterns being completely obscure. Moreover, a strong pharyngeal reflex prevents the examiner to come in contact or at least close enough to mucosa of interest (Srivastava, 2019). With the NBI method, microvascular patterns can be misinterpreted, leading to a higher number of false-negative and false-positive results. We can successfully identify vascular patterns, especially of squamous cell carcinoma. False-negative cases often include patients with submucosal, non-squamous cell tumors (e.g. sarcomas, non-Hodgkin's lymphoma, neuroendocrine tumors,...) and hyperkeratoses, where whitish plaques obscure vascular patterns. False-positive cases are common in patients after radiotherapy, ulcers and infections (e.g. in granulomatous tuberculosis, histoplasmosis), in the oral cavity due to the complexity of the epithelium in various places and in patients with numerous scars after surgical procedures (Chabrillac et al., 2021; Gale et al., 2017; Odell et al., 2021; Vilaseca et al., 2017; Valls-Mateus et al., 2018).

6. Conclusion

NBI is a useful, fast and patient-safe endoscopic diagnostic method which can be used to identify many pathologies of the head and neck based on distribution of vascular patterns of the mucosa. However, tissue biopsy still remains the gold standard for confirming malignant lesions. A key advantage of NBI endoscopy is early identification of cancer-suspicious lesions based on the distribution of vascular patterns that would be missed using just white light endoscopy.

Conflicts of Interest: The authors declare no conflict of interest.

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Invited lecture/Reflection

Neuroprognostication after Cardiac Arrest

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Abstract:

Survival of patients with out-of-hospital cardiac arrest (OHCA) is still very low. After the return of spontaneous circulation (ROSC), survivors are admitted to the intensive care unit. They can be conscious or comatose. Conscious survivors of cardiac arrest generally have a good prognosis. In comatose patients, prognosis is better in patients with shockable rhythm (ventricular tachycradia or ventricular fibrillation) as the initial rhythm at the arrival of Emergency medical team.

In comatose patients we try to predict the neurological outcome with everyday clinical examination, a neuron specific enolase (NSE), comuter tomography (CT) scan or magnetic resonance imaging (MRI) of the brain, electroencephalogram (EEG) and somoatosensoric evoked potentials (SSEP). Neurological outcome is presented according to Glasgow-Pittsburgh Cerebral Performance Category Scale. Certain proportion of comatose patients may regain consciousness even after their discharge from the intensive care unit (ICU).

Citation: Tadel Kocjančič S. Neuroprognostication after cardiac arrest. Proceedings of Socratic Lectures. **2023**, 8; 70-73. https://doi.org/10.55295/PSL.2023.I11

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Keywords: Out-of-hospital cardiac arrest; Comatose survivors; Postresuscitation brain damage; Neuroprognostication

1. Introduction

Sudden out-of-hospital cardiac arrest (OHCA) remains the leading cause of death in developed countries. Incidence varies from 50 - 100 per 100.000 inhabitants all over the world (Tadel et al, 1998). Survival is still very low, 1-22% . It is better in small towns with low traffic and no skyscrapers, where the access to the patient is quick. Prognosis is also better in patients with shockable initial rhythm, ventricular tachycardia (VT) or ventricular fibrillation (VF), and worse with non-shockable initial rhythm - pulseless electrical activity (PEA) or asystole. In many cases asystole is a secondary rhythm after non-resuscitated ventriculer fibrillation, after a few minutes due to acidosis and hypoxia.

Following initial cardiopulmonary resuscitation, reestablishment of spontaneous circulation (ROSC) is typically achieved in 40 to 60% of patients who are subsequently transported to the hospital. There is an increasing number of patients admitted to our ICU each year, starting from 25-35 per year until 2002, to maximum 90 patients per year until now (**Figure 1**). From 1995 until 2021 we admitted 1352 patients after primary OHCA (Tadel-Kocjancic et al., 2022).

Because of typical delays in prehospital "chain of survival", a great majority of patients remain comatose despite ROSC and require intensive post-resuscitation care (Nolan et al., 2021) Introduction of hypothermia after the publication of landmark clinical trials in 2002 undoubtedly revolutionized post-resuscitation treatment (Hypothermia after Cardiac Arrest Study Group, 2002). Such comprehensive post-resuscitation care has been shown to significantly improve survival with good neurological outcome compared to historical controls. Conscious patients (the ones who re-gained consciousness after ROSC) have excellent prognosis.

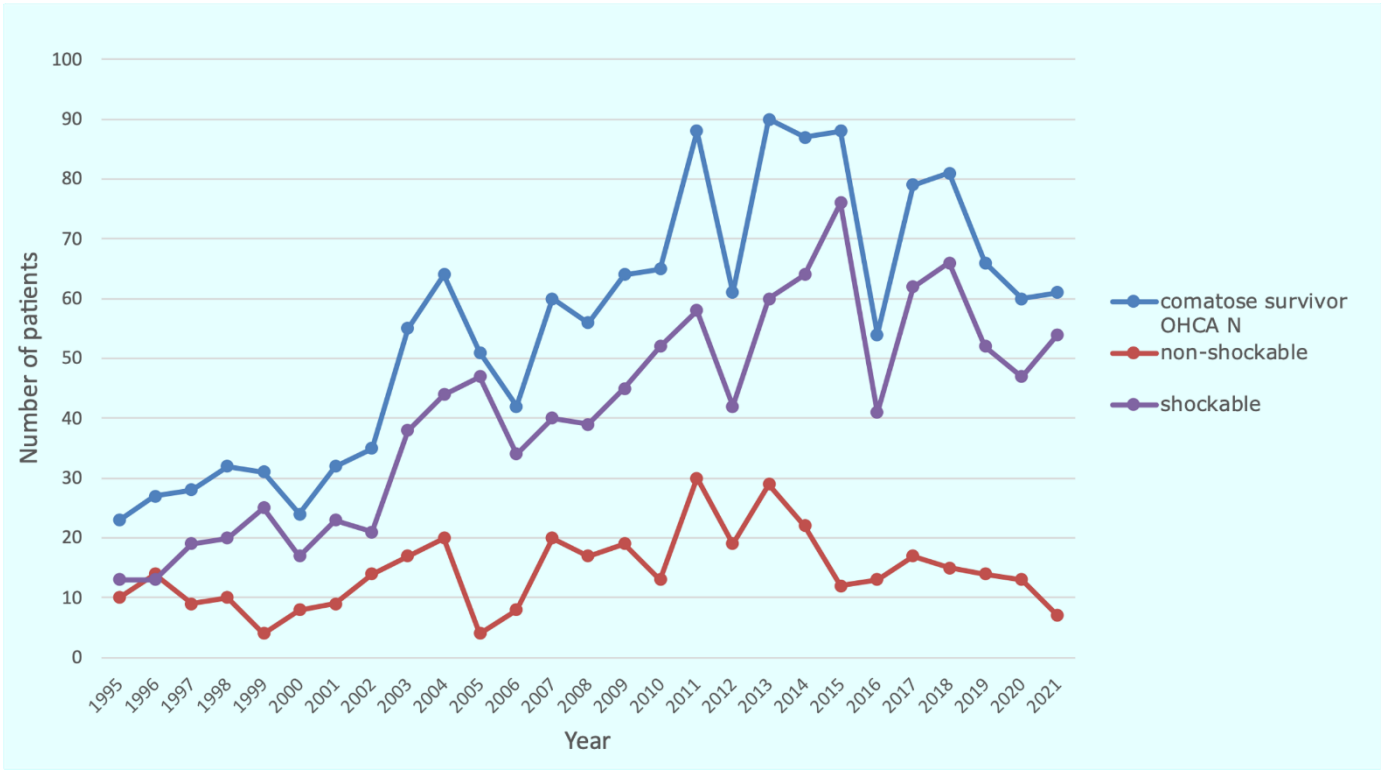

Figure 1. Number of comatose survivors of OHCA admitted to ICU after ROSC.

2. Brain damage after cardiac arrest

During cardiac arrest there is no blood flow through the body resulting in ischemic damage to the organs. Brain seems to be the most vulnerable, and patients often suffer an irreparable brain damage after cardiac arrest. If the patient regains consciousness after ROSC, there is no or very little brain damage. About 70% of patients remain comatose, which means that brain damage is very likely. After ROSC there is also post-resuscitation brain damage which we try to limit with therapeutic hypothermia or normothermia for 48 hours.

At first, it is hard to assess consciousness due to the sedatives, analgesics and/or muscle relaxants the patients receive during cardiopulmonary resuscitation. Neuroprognostication comes later. After all that we classify patients into 5 groups according to Glasgow-Pittsburgh Cerebral Performance Category Scale (CPC) (**Table 1**). Standard definitions are: CPC 1 to 2 - favorable and 3 to 5 - poor neurologic outcome.

Table 1. Glasgow-Pittsburgh Cerebral Performance Category Scale (Safar et. al.,1986).

Note: If patient is anesthetized, paralyzed, or intubated, use "as is" clinical condition to calculate scores.
CPC 1. Good cerebral performance: conscious, alert, able to work, might have mild neurologic or psychologic deficit.
CPC 2. Moderate cerebral disability: conscious, sufficient cerebral function for independent activities of daily life. Able to work in sheltered environment.
CPC 3. Severe cerebral disability: conscious, dependent on others for daily support because of impaired brain function. Ranges from ambulatory state to severe dementia or paralysis.
CPC 4. Coma or vegetative state: any degree of coma without the presence of all brain death criteria. Unawareness, even if appears awake (vegetative state) without interaction with environment; may have spontaneous eye opening and sleep/awake cycles. Cerebral unresponsiveness.
CPC 5. Brain death: apnea, areflexia, EEG silence, etc.

For evaluation of comatose patients we use everyday clinical neurological examination, levels of NSE 72 hours after ROSC, EEG recordings on day 3, SSEP and CT of the brain (Henson et al., 2022). Daily clinical neurological examination is performed. We must be careful to exclude the influence of sedatives, muscle relaxants on consciousness and reflexes. Signs of poor neurological outcome are absent or extensor motor response to pain at 72 h or later after ROSC, bilaterally absent pupillary light reflex at ≥72 h from ROSC, bilaterally absent corneal reflex at 72 h after ROSC, presence of an early (≤48 h) post-anoxic status myoclonus. NSE levels are measured 72 hours after ROSC. High levels (mostly more then 60) mean a bad prognosis. On day 3-5 we record EEG. We then divide EEG recordings into 3 groups (discretion of the neurophysiologist who interpret them): very malignant, malignant, or benign recording. The presence or absence of SSEP is noted. Absence of somatosensoric evoked cortical N20 potentials means poor neurological outcome, but to correctly interpret these findings, injuries to the cervical spinal cord must be excluded.

Neuroimaging is also used for neuroprognostication. MRI or CT of the head are performed, mostly CT in our hospital. The findings are then interpreted by a radiologist. Generalized brain edema, where we cannot distinguish between white and grey matter, extensive diffusic restrition on MRI or extensive lesions mean bad prognosis, but focal lesions have no significant clinical importance.

3. Delayed awakening of patients after OHCA

Despite advanced neuroprognostication using NSE, EEG and brain imaging, prediction of neurological outcome in comatose survivors of OHCA remains challenging and early discontinuation of post-resuscitation treatment may be harmful for patients with delayed awakening. In our study (Tadel-Kocjancic et al., 2022) we find that in about 20% of patients in CPC 3 or 4 there is neurological improvement later, so it is important that we do not stop treatment too early.

4. Conclusion

Survival of patiens after OHCA is still very low. Conscious survivors have a good prognosis, but shockable initial rhythm (VT/VF) means a better prognosis for comatose survivors. Intensive hospital treatment improves prognosis in patients with shockable rhythm, but not in patients with non-shockable rhythm.

In the last years we have been trying to predict neurological outcome in comatose patients with neuroprognostication. We have been using everyday clinical examination, NSE, neuroimaging and neurophysiologic tests. We hope that with all this data our decision whether to continue or to stop treatment will be easier.

Conflicts of Interest: The author declares no conflict of interest.

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Invited lecture/Scientific contribution

Critical Appraisal of a Systematic Review on Effectiveness of Trunk, Hip and Knee Exercise Programs in Patellofemoral Pain

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Abstract:
Although patellofemoral pain (PFP) is one of the most common musculoskeletal disorders, the clear aetiology behind it remains unknown. One of possible factors could be increased hip adduction and internal rotation caused by weak hip abductors and external rotators. A recent systematic review assessed the effectiveness of trunk, hip and knee exercise programs for pain relief, functional performance and muscle strength in PFP. The aim of our study was to critically evaluate this systematic review using the updated PRISMA checklist. The authors adequately described the relationship between hip and knee muscles and PFP, but insufficiently linked the condition to trunk muscles throughout the review. Overall the methods used were satisfactory, however the methods used to assess risk of bias due to missing results and certainty in the body of evidence for outcomes were not reported and therefore not presented in the results or debated in the discussion. Few discrepancies were found between the text and presented tables. The results of conducted meta-analysis were sufficiently presented in included forest plots or can be accessed through links in the review as publicly available supplementary figures. Possible extraction of data on description of exercises used in programs could further improve the synthesis. The discussion on effectiveness of hip and/or knee exercise programs on pain relief and functional performance was adequate, meanwhile the discussion was insufficient for effect on muscle strength. The review was satisfactorily conducted with few items not reported or reported insufficiently due to discrepancies between the former and updated PRISMA statement.

Keywords: Patellofemoral pain; Exercise programs; Critical appraisal; PRISMA checklist

Citation: Okorn D, Vauhnik R. Critical appraisal of a systematic review on effectiveness of trunk, hip and knee exercise programs in patellofemoral pain. **2023**, 8; 75-81. https://doi.org/10.55295/PSL.2023.I12

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1. Introduction

Patellofemoral pain (PFP), characterized by diffuse pain around or behind the patella (Crossley et al., 2016), is one of the most common musculoskeletal disorders (van Middelkoop et al., 2008), accounting for 25–40 % of all cases of anterior knee pain (Décary et al., 2018). The prevalence of PFP is reported to be 23 % in general population and 29 % in adolescents (Smith et al., 2018) and is two times higher in women and athletes than males (Dolak et al., 2011). PFP is aggravated by activities overloading the patellofemoral joint during weight bearing on a flexed knee such as squatting, stair ambulation, jogging/running, hopping/jumping or even prolonged sitting with knees flexed over 90° (Crossley et al., 2016).

Although the clear aetiology behind PFP remains unknown, the condition is thought to be multifactorial (Lankhorst et al., 2012). Both local and nonlocal factors could be included (Lankhorst et al., 2012) in causing the maltracking/altered movement of the patella, which may lead to overload of the patellofemoral joint (Powers et al., 2017). Local factors are associated with imbalances between the vastus medialis oblique and the vastus lateralis as well as impaired quadriceps strength (Cowan et al., 2002; Khayambashi et al., 2014). Nonlocal factors are related to the mechanics of the proximal and distal segments (Powers et al., 2017), including increased hip adduction and internal rotation during weight bearing tasks (Souza & Powers, 2009).

Hip abductors and external rotators are crucial for knee and pelvic stabilization as well as eccentric control of the hip adduction and internal rotation movements during ambulation (Lankhorst et al., 2012; Robinson & Nee, 2007). Weak hip abductors and external rotators supposedly lead to excessive hip adduction and internal rotation, which contributes to altered tibiofemoral and patellofemoral joint kinematics and patellofemoral joint stress (Lee et al., 2003). The reduction in PFP following hip muscle strengthening in allegedly directly related to the improvement of biomechanical changes in the knee area (Fukada et al., 2012).

A systematic review and meta-analysis (Manojlović et al., 2021) on effectiveness of trunk, hip and knee exercise programs for pain relief, functional performance and muscle strength in PFP was recently conducted.

The aim of the review was to assess the effects of exercise programs focusing on training of muscle groups proximal to the knee in patients with PFP. The authors concluded that hip&knee and hip-only exercise programs are most effective in decreasing pain levels and improving functional performance, along with increasing hip abduction and external rotation strength.

The aim of our study was to critically evaluate the aforementioned systematic review.

2. Methods

The critical appraisal of a selected systematic review (Manojlović et al., 2021) was conducted according to the updated Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement (Page et al., 2021) using the PRISMA 27-item checklist. We removed the section "abstract" from the checklist. Additional observations were included in the discussion.

3. Results

Table 1 represents the PRISMA 27-item checklist. In the uttermost right column we filled in the location where certain item from the checklist is reported (the number of the page, figure and/or table) and whether an item is not reported or is reported insufficiently.

Table 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist red – new/changed items in the updated PRISMA statement as compared to the former PRISMA statement; green – adequately reported; orange – insufficiently reported; blue – not reported

Section and Topic	Item	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Page 1431
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Pages 1431–2
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Page 1432, insufficient
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Pages 1432–3; insufficient
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Page 1432; insufficient
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Page 1432; insufficient
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Pages 1432–3; insufficient
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Page 1433; insufficient
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Pages 1433–4; insufficient
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Page 1433
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Page 1433; insufficient
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Pages 1433–4
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Pages 1433–4; table 3; insufficient
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Pages 1433–4
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Page 1434
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Pages 1433–4
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Page 1434
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Pages 1434

Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Not reported
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Not reported
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Pages 1434–5
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Not reported
Study characteristics	17	Cite each included study and present its characteristics.	Table 2 and 3
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Table 1
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Table 2; Figures2–4; Supplementary Figures 1–2
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Pages 1436, 1443–4; insufficient
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Pages 1436, 1443–4; Figures 2–4; Supplementary Figures 1–2
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Same as item 20b
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Same as item 20b
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Not reported
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Not reported
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Pages 1444 and 1446
	23b	Discuss any limitations of the evidence included in the review.	Page 1447
	23c	Discuss any limitations of the review processes used.	Page 1447
	23d	Discuss implications of the results for practice, policy, and future research.	Pages 1446–7
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Not reported
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Not reported
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	Not reported
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Not reported
Competing interests	26	Declare any competing interests of review authors.	Page 1447
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Not reported

4. Discussion

We used the updated PRISMA 27-item checklist to conduct the critical appraisal of the selected systematic review by Manojlović et al. (2021). Since the updated PRISMA statement was published in the same year as the mentioned systematic review, the authors could not had followed the new guidelines. Therefore, some differences were expected as they used the former PRISMA statement (Liberati et al., 2009).

The authors adequately presented the existing knowledge on PFP, however they did not sufficiently explain it in the context of their title. The review sets an intention of presenting the effectiveness of training trunk, hip and knee muscles, which they encompassed in a term "muscle groups proximal to the knee". The trunk muscles were mentioned only once in the introduction and the relationship between the trunk muscles and PFP was not explained. The authors, however, clearly stated the objective of the review, yet in further explanation they again did not explicitly include the trunk muscles apart from the broad term "muscle groups proximal to the knee".

The inclusion and exclusion criteria were presented systematically and adequately using the PICOS (population, intervention, comparison, outcomes, study design**)** technique. The outline for the grouping of the studies was presented in the objective of the systematic review. The authors specified all databases and additional sources but not the exact date each source was searched. A common search strategy was presented without mentioning of any filters or limits used.

The authors additionally used several keywords combinations, yet did not specify where and how were they integrated into the search for relevant articles. Information on whether the reviewers worked independently was not included in the description of the selection and data collection processes, although it represents a possible bias of a systematic review.

The outcomes were listed but not sufficiently defined as the authors did not specify which methods of measuring maximal voluntary isometric contraction (MVIC) were eligible for inclusion or on the other hand which methods were used in the included studies. They did, however, mention that MVIC was a cause of some heterogeneity since it was reported in different measurement units. Since only randomised controlled trials (RCT) were included in the review, the choice of using PEDro scale for assessment of risk of bias was reasonable.

The PEDro scale was adequately justified and described, but with a missing information that one item of the selected tool does not contribute to the overall score and whether the reviewers worked independently. Additionally, in the methods section, the authors explained the characterization of the study quality as high and low depending on the overall PEDro score. However, in the results section the studies were rated as either of poor, fair, moderate and excellent quality. Because the studies weren't explicitly grouped for each planned synthesis during the eligibility criteria, the comparison against the executed grouping could not had been carried out.

The process for deciding which studies were eligible for each synthesis was not described but could be made out of the Table 3 presenting the description of the exercise programs in each study. The authors sufficiently described and/or presented the synthesis methods but did not report methods used to assess risk of bias due to missing results and methods used to assess certainty (or confidence) in the body of evidence for each outcome.

Item 16b in the section results is newly added to the updated PRISMA checklist, which explains why authors did not list and explain why studies that might had appeared to meet the inclusion criteria were excluded. The outcomes were presented in three synthesis – pain, function and strength.

The characteristics (other variables) of included studies were summarised together and not separately for each synthesis, while the risk of bias was summarised for synthesis on pain and function, but not strength. Because the authors did not assess the risk of bias due to missing results and certainty in the body of evidence for each outcome, they consequently did not present corresponding results. Further investigation into results yielded some additional observations that are not included in the PRISMA checklist.

The authors stated that the information about exercise supervision should be evident from Table 3, however it was true for only one study in Table 2. There was also discrepancy between the text and Table 3 for the information on exercise frequency and progression. When listing the number of studies, which reported other variables such as duration of a

single exercise session and its progression, exercise intensity and breaks between series or blocks, the authors did not cite the studies, while this information was also not evident in tables. Consequently, the reader does not know which studies were implicated. The Tables 2 and 3 that continue through pages 1437 and 1443 do not include the header row in their third part, which impacts their clarity.

If the authors extracted the description of the interventions (the exercises used in each study) both them and the reader could evaluate if the program consists of correct exercises. The fact that a study defines their program as "knee only exercise program" does not mean that the exercises adequately present the program.

The systematic review successfully covered items 23a–d in the discussion section. However, the authors did not comment on the quality of included studies and how that affects the main findings of the review. To support a certain claim in the discussion, the authors mentioned that an included study came to the same conclusion, yet they did not proclaim the study was of poor quality (the lowest in the whole review). The discussion on effectiveness of hip and/or knee exercise programs on pain relief and functional performance was satisfactory, while on the other hand, the discussion was insufficient for effect on muscle strength. The conclusions about muscle strength therefore seemed vague. Despite including trunk muscles in the title, this was not debated in the discussion or mentioned in the conclusions.

Section "other information" is a new section in the updated PRISMA statement and therefore the authors Manojlović et al. (2021) could not add this in to their systematic review and meta-analysis.

5. Conclusions

The systematic review by Manojlović et al. (2021) adequately assessed the effectiveness of hip and/or knee exercise programs for pain relief and functional performance in PFP, however their conclusions on effectiveness for muscle strength were not based on sufficient discussion. The review was satisfactorily conducted according to the PRISMA checklist with few items not reported or reported insufficiently due to discrepancies between the former and updated PRISMA statement.

Funding: No funding was reported.

Conflicts of Interest: The authors declare no conflict of interest.

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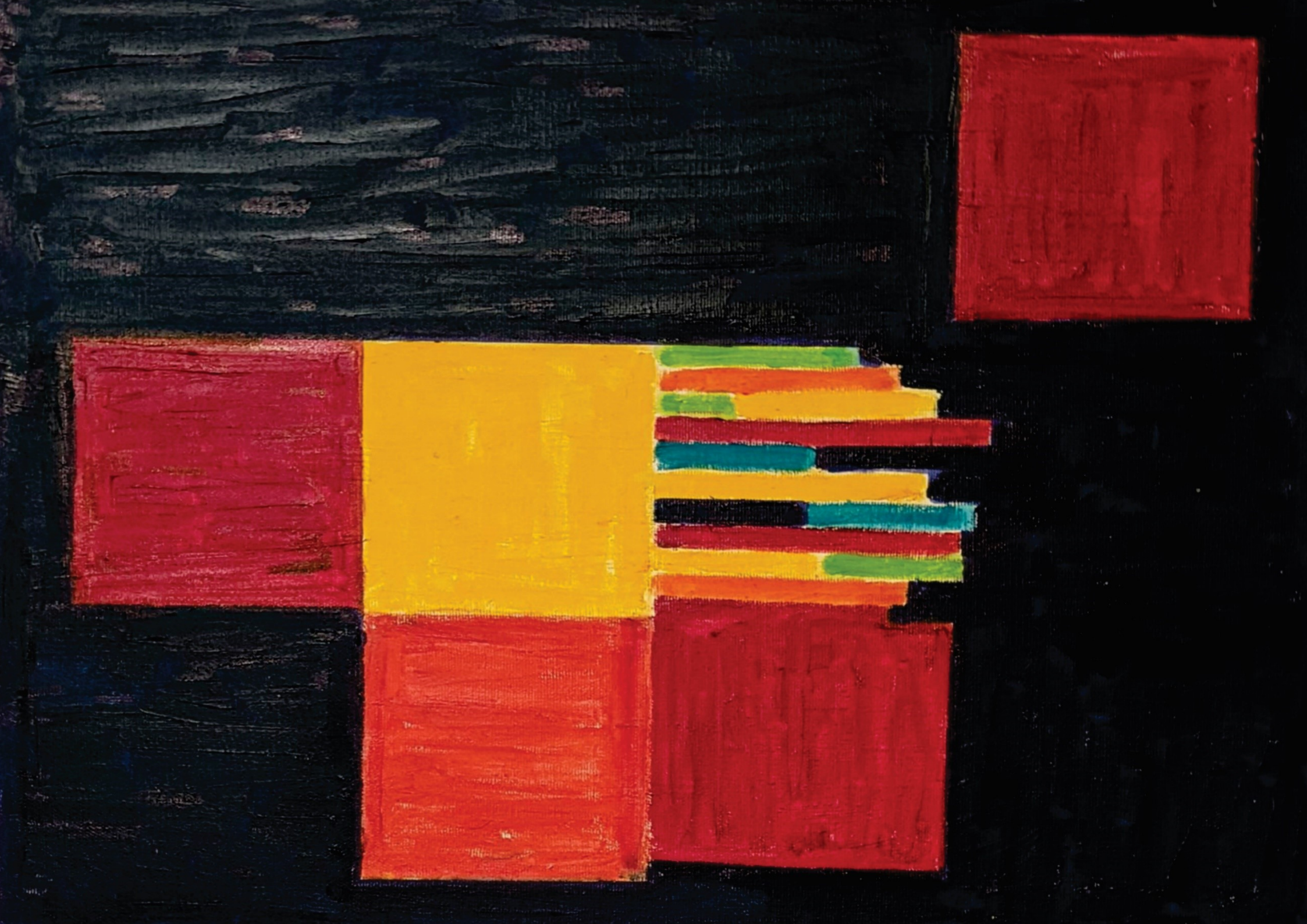

Invited lecture/Review

The Fascial System

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Abstract:
The word Fascia has long been used by gross anatomists to embrace a spectrum of undifferentiated mesenchymal tissues that wrap organs and tissues of the body, or form a packing material between them. The inherent implication of this traditional view is that fasciae are inconsequential residues that are less important than the tissues with which they are associated. The errors of this assumption are being exposed and undoubtedly fascia is becoming more and more of considerable importance to many professionals working in health-related disciplines. Encouragingly, there has been a strong resurgence of interest into both basic and applied research in fasciae in recent years, also thanks to new fascia related findings. Knowledge of the fascial system's characteristics and functions is spreading from primary medical researchers to professionals in many health fields throughout the world. Nowadays is well known that the Fascia is a mechanically active tissue with a proprioceptive and nociceptive properties. The Fascial continuum complexity is the result of perfect synergy evolution among different tissues made up of solid and fluid portions, which interpenetrate and interact with each other, forming a polymorphic three-dimensional network. Normal movement of the body is allowed because of the presence of the fascial tissues and their inseparable interconnection, one of the fundamental characteristics of the fascia is the ability to adapt to mechanical stress, remodeling the cellular/tissue structure and mirroring the functional necessity of the environment where the tissue lays. So, Fascia can transmit tension and in view of its proprioceptive and nociceptive functions could be responsible for disorders and pain radiating to remote anatomical structures. Dysfunction of the fascial system that is perpetuated in everyday movements can also cause an emotional alteration of the person. So, the fascial unity could influence not only movement but also emotions. Because the importance of fascia in human movement (both motion and emotion), shock absorption, metabolic and physiological processes, proprioception, healing and repair, the fascia in a broadest sense may be the literal representation of our inner being. Theoretically, Fascia probably hold many of the keys for understanding muscle action and musculoskeletal pain, and maybe it is of pivotal importance in understanding the basis of the body functioning. Further intensive research is essential to understand the function of the Fascia. The proposed article is a reflection to better understand the anatomy and main characteristics of the human fascial system.

Keywords: Fascia; Facial system; Myofascial chains

Citation: Valenti F. The fascial system. Proceedings of Socratic Lectures. 2022, 8; 83-88. https://doi.org/10.55295/PSL.2023.I13

1. Fascia

The word Fascia has long been used by gross anatomistsis to embrace a spectrum of undifferentiated mesenchymal tissues that wrap around what are sometimes regarded as being the more 'specialized' organs and tissues of the body, or form a packing material between them. The inherent implication of this traditional view is that fasciae are inconsequential residues that are less important than the tissues with which they are associated (Benjamin, 2009). Increasingly, the errors of this assumption are being exposed and undoubtedly fascia is becoming more and more of considerable importance to many professionals working in health-related disciplines. Encouragingly, there has been a strong resurgence of interest into both basic and applied research in fasciae in recent times, but researchers do not agree on one comprehensive "fascia" definition (Benjamin, 2009). Despite the scientific uncertainty, there is an agreement with medical text that the fascia covers every structure of the body, creating a structural continuity that gives form and function to every tissue and organ.

The fascial tissue has a ubiquitous distribution in the body system; it is able to wrap, interpenetrate, support, and form the bloodstream, bone tissue, meningeal tissue, organs, and skeletal muscles. The fascia creates different interdependent layers with several depths, from the skin to the periosteum, forming a three-dimensional mechano-metabolic structure (Bordoni et al., 2017). The fascia includes everything that presumes the presence of collagen/connective tissue or from which it is derived. All the tissue considered as "specialized connective tissue" of mesodermal derivation is inserted into the fascial system. These include blood, bone, cartilage, adipose tissue, hematopoietic tissue, and lymphatic tissue. The fascial system has no discontinuity in its path, with layers of different characteristics and properties overlapping (Bordoni et al., 2022). Bordoni et al., (2019 and , 2021) more accurately explained that fascia is any tissue that contains features capable of responding to mechanical stimuli.

The fascial continuum is the result of the evolution of the perfect synergy among different tissues, liquids, and solids, capable of supporting, dividing, penetrating, feeding, and connecting all the districts of the body: epidermis, dermis, fat, blood, lymph, blood and lymphatic vessels, tissue covering the nervous filaments (endoneurium, perineurium, epineurium), voluntary striated muscle fibers and the tissue covering and permeating it (epimysium, perimysium, endomysium), ligaments, tendons, aponeurosis, cartilage, bones, meninges, involuntary striated musculature and involuntary smooth muscle (all viscera derived from the mesoderm), visceral ligaments, epiploon (small and large), peritoneum, and tongue. The continuum constantly transmits and receives mechano-metabolic information that can influence the shape and function of the entire body.

These scientific definitions allow healthcare practitioners to see the functioning of the body in a holistic way and make some deductions about fascia. Normal movement of the body is allowed because of the presence of the fascial tissues and their inseparable interconnection, which allow the proper distribution of tension information produced by different tissues covered or supported by the fascia so that the entire body system can interact in real-time, including the epidermis. One of the fundamental characteristics of the fascia is the ability to adapt to mechanical stress, remodeling the cellular/tissue structure and mirroring the functional necessity of the environment where the tissue lays (Bourne et al., 2022). This further indicates the importance of fascia as a sensory system. Fascia probably hold many of the keys for understanding muscle action and musculoskeletal pain, and maybe of pivotal importance in understanding the basis of the body functioning (Langevin et al. 2001; 2002; 2006; Langevin & Yandow, 2002; Iatridis et al. 2003). Dysfunction of the fascial system that is perpetuated in everyday movements can also cause an emotional alteration of the person. So the fascial unity could influences not only movement but also emotions (Bordoni et al., 2017).

2. Embryology

During embryonic development, the connective tissue influences the form (morphogenesis) of the structures that it will contain and connect. The embryonic mesenchyme or connective embryonic or undifferentiated mesenchyme is formed by star-branched cells with a high mitotic rate (high reproductive capacity). They are considered to be pluripotent cells, as they can differentiate into different tissues. The mesenchyme is found and is derived from all three embryonic layers (ectoderm, mesoderm, endoderm), especially mesoderm and ectoderm (Bordoni et al., 2020). The fascial system is classically understood to originate from the mesoderm layer divided during week 2 of development during the embryo's gastrulation phase. There is also some evidence that certain fascial layers, particularly in the cervical and cranial neck, derive from the ectoderm (Van der Wal, 2009).

3. Structure and function

To understand the function and architecture of the fascial system, it is important to understand its composition. Fascia must be understood first and foremost as connective tissue (textus connectivus) (FCAT, 1998). Fascia is made up of sheets of connective tissue that is found below the skin. Each fascial layer is distinct in important ways from each other. Each layer has its own orientation and composition. It can be classified as superficial, deep, visceral, or parietal and further classified according to anatomical location. Superficial fascia is loose and irregular, whereas deep fascia is a well-organized fibrous layer (Stecco et al., 2011).

3.1. Superficial fascia

The superficial fascia (tela subcutanea) is a membranous layer of connective tissue (thicker in the trunk and thinner in the limbs), formed by loosely packed interwoven collagen fibers mixed with elastic fibers. It is absent in the face, palm of hand and sole of the feet (Varghese and Priya, 2017). Its arrangement and thickness vary according to body structure, gender and region. It is connected to the skin by the retinaculum cutis superficialis, which presents vertical and thick collagen septa and with the deep fascia through the retinaculum cutis profundi, which presents loose oblique, very elastic collagen septa. The region between the skin and superficial fascia that includes the superficial retinacula cutis is called superficial adipose tissue, while the one between superficial fascia and deep fascia is called deep adipose tissue. Both impart the subcutis with specific mechanical proprieties (Lesondak et al., 2020). At some bony prominence the superficial layer adheres to the deep fascia (Varghese and Priya. 2017). Functionally, the superficial fascia may play a role in the integrity of the skin and support for subcutaneous structures, particularly veins, by ensuring their patency and also for the integrity of the lymphatic vessels. Within the superficial fascia the subcutaneous plexus that function for thermoregulation is also found. Some muscular fibers found in superficial fascia are platysma muscle in the neck and musculo aponeurotic system in the face (Varghese and Priya. 2017).

3.2. Deep fascia

The deep fascia is a well-organized connective membrane. It surrounds all the muscles, ligaments, bones, nerves, blood vessels, envelopes, various glands and organs and binds all these structures together. The deep fascia duplicates itself to form deep lamina in some regions of the trunk and limbs. For example, specialized structures of the deep fascia are termed as periosteum over the bone, paratendon over the tendon, capsule and tendons over the joint and neuromuscular sheath over the nerves and blood vessels. The deep fascia can be divided into the aponeurotic and epimysial fascia according to orientation, composition, and architecture. Aponeurotic fasciae are formed by two to three layers of parallel bundles of collagen fibers. Each layer is separated from the adjacent one by a thin layer of loose connective tissue (Lesondak et al., 2020). Aponeurotic fasciae envelope and connect whole groups of muscles. It covers the extremity muscles and includes both the thoracolumbar fascia (TLF) and the rectus abdominal sheath (RAS) in the torso. Under the aponeurotic deep fascia, the muscles are free to slide because of their epimysium. Loose connective tissue rich in hyaluronic acid lies between the epimysium and the deep fasciae responsible

for free gliding of the deep fascia with epimysium (Mc Combe et al., 2001). Epimysial fasciae covers and adheres to the whole muscle and can be used to refer to all the intramuscular connective tissue, which includes the epimysium, perimysium, and endomysium. It is not possible to separate the epymysial fascia from the muscle because it is so intertwined with the muscle tissue, and the function of one is strongly dependent on the other (Lesondak et al., 2020).

4. Innervation

The main sensory receptors integrated into the fascial system are the proprioceptors, usually referred to as mechanoreceptors. Collagen fibers surround and are attached to the capsules of corpuscles and free nerve endings. The tissue function and the type of mechanical force transmission that is necessary in different parts of our body determines the number of mechanoreceptors that will be available. The most innervated tissue are the superficial layers of the deep fascia (Schleip and Muller, 2013; Stecco et al., 2007; Tesarz et al., 2011). Ruffini corpuscles, or free nerve endings, and Merkel discs are the slowly adapting touch receptors. They are responsive to prolonged stimuli. Merkel discs are abundant in the fingertips, hands, lips, and external genitals. Ruffini endings lie deep in the dermis, ligaments, tendons, and fasciae are most sensitive to stretch resulting from muscle movement, particularly movement in the limbs or digits. Ruffini and Meissner corpuscles are rapidly adapting touch receptors mostly located in hairless skin that react at the onset of a stimuli. Pacinian corpuscles are rapidly adapting receptors located in the dermis and subcutaneous tissue, tendons, and joints. They react to pressure against a broad area as opposed to a localized touch area. The Ruffini and Pacinian corpuscles are also present in deep fascia and retinacula (Vesalius, 1543). Together with the retinacula, the superficial and intermediate layers of the deep fascia are the most highly innervated structures of the fascia system (Schilder et al., 2018). The superficial tissues are rich in free nerve endings. The amount of free nerve endings, which can also sense temperature, mechanical stimuli, and nociception, may be up to seven times more numerous than other mechanoreceptors. These nerve endings are aligned perpendicularly to the collagen fibers, so stretching the muscle and fascia stimulates these receptors easily. Free nerve endings act as sensory receptors and a percentage of them also transmit pain. The pain caused by the fascia also called Myofascial pain syndrome can be even more aggravating than pain from the muscles. The pain may appear anywhere along the fascia even in an area more distal from the cause of the pain. Fascial pain is usually described as a stabbing, irritating, stinging, or beating sensation, whereas muscle pain is described as a more dull and aching type of pain (Schilder et al., 2014).

5. Myofascial chains

Understanding the fascial tissue mechanisms have become increasingly popular also thanks to new recent histological findings. The discovery of contractile cells, free nerve endings and mechanoreceptors in the fascia suggests that fascia in contrast to prior assumptions plays a proprioceptive and mechanically active role in the body (Bhattacharya et al., 2010; Yahia et al., 1992). We can conclude that the muscles do not function as independent units. Instead, they are regarded as part of a tensegrity-like, bodywide network with fascial structures acting as linking components. As fascia can transmit tension (Barker et al., 2004; Norton et al., 2013) and in view of its proprioceptive and nociceptive functions, existence of myofascial meridians could be responsive for disorders and pain radiating to remote anatomical structures. Myers (2014) defined from cadaveric dissections eleven myofascial meridians connecting distant parts of the body by means of muscles and fascial tissues. The central rule for the selection of a meridian's components is a direct linear connection between two muscles. Evidence for the existence of myofascial chains is growing, and the capability of force transmission via myofascial chains has been hypothesized. However, there is still a lack of evidence concerning the functional significance and capability for force transfer. Wilke et al. (2016) showed that there is good evidence for the existence of three myofascial chains proposed by Myers (2014): the superficial backline (SBL: plantar fascia, gastrocnemius, hamstrings, erector spinae); the back functional line (BFL: latissimus dorsi, contralateral glutueus maximus, vastus lateralis); and the front

functional line (FFL: adductor longus, contralateral rectus abdominis, pectoralis major). In contrast to the solid evidence for these five meridians, doubts have to be raised about the existence of the superficial front line SFL. There is no structural connection between the rectus femoris muscle and M. rectus abdominis. Also, M. sternalis, which is suggested to be the cranial continuation of rectus abdominis, exists only in a small percentage of the population. Even if present, it does not fuse consistently with the rectus abdominis (Barlow, 1935; Saeed et al., 2002). Though the available evidence points towards existence of tensile transmission via myofascial pathways, most experimental research was carried out in vitro using cadavers. Randomized, controlled in-vivo studies are warranted in order to draw more precise assumptions on the significance of myofasical chains for the movement system (Wilke et al., 2016).

6. Conclusion

The fascia's nomenclature is the subject of debate in the academic world, as it is classified starting from different scientific perspectives. This disagreement is not a brake but is, in reality, the real wealth of research, the multidisciplinarity of thought and knowledge that leads to a deeper understanding of the topic. Since clinicians and anatomists show increasing interest in fascia, it is well possible that in the future, more focused research will verify the remaining myofascial missing links to understand the role and functioning of the fascia (Bordoni and Myers, 2020). Another topic of discussion is how the fascial model fits into the reality. Biotensegrity is a mechanical model, which takes into consideration the solid aspect of the fascia (structure) and fascintegrity considers the solid and the liquid aspect of the fascia (structure and gliding). Myofascial chains converge attention on the movement and transmission of force in the muscle continuum (Bordoni and Myers, 2020). Another aspect that is fundamental to understand the functioning of the fascia is the relationship between fascia and emotions. Chronic altered information from conditions such as pain, depression, lack of movement will negatively affect the cognitive aspect (memory, problem-solving, elaboration of ideas) and viceversa. From these concepts, the need arises to frame the fascial system in a model that can represent the living being and understand, prevent and possibly cure the dysfunctions that can result from the fascia (Malfliet et al., 2017). It was indicated that the importance of fascia in human movement (both motion and emotion), shock absorption, metabolic and physiological processes, proprioception, healing and repair the fascia in a broadest sense may be the literal representation of our inner being (Lesondak, 2017).

Conflicts of Interest: The author declares no conflict of interest.

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Invited lecture/Scientific contribution

Arthrogenic Muscle Inhibition in Ankle Instability

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Abstract:
Ankle sprain is the most common injury among athletes and in the general population. A previous ankle sprain is a major risk factor for re-injury or the development of instability. Instability may be related to the neuromuscular changes after the injury. These include arthrogenic muscle inhibition (AMI), which is likely to be influenced by central regulatory mechanisms that lead to reduced muscle activation after injury. Our aim was to determine whether AMI is present in subjects after acute ankle sprain or in subjects with ankle instability. The literature search were performed in PubMed, Cochrane Library, EMBASE (Ovid) and Medline databases. We used a combination of English keywords. In addition, the literature lists of included studies were reviewed. Studies were screened regarding the inclusion and exclusion criteria. We included five studies investigating the presence of AMI in subjects with ankle sprain or /and instability. Statistically significant reduced activation of m. soleus was reported in four studies. In two studies, reduced activation of m. peroneus longus was reported, but only in subjects with ankle instability. Conclusions: We found that AMI, manifested as reduced activation of m. soleus and m. peroneus longus, is present in subjects with ankle sprain or instability. Inhibition is present bilaterally only in the acute phase. The mechanisms of AMI are most likely not only under local control, but also under central control.

Keywords: Ankle sprain; Ankle instability; Arthrogenic muscle inhibition

Citation: Hočevar J, Vaunik R. Arthrogenic muscle inhibition in ankle instability. Proceedings of Socratic Lectures. **2023**, 8; 90-95. https://doi.org/10.55295/PSL.2023.I14

1. Introduction

1.1. Ankle sprain and chronic ankle instability

Ankle injuries are the most common injuries in both athletes and general population (Doherty et al., 2014). A previous ankle sprain is an important risk factor for a re-injury (Hertel, 2002; McKay et al., 2001). Between 15 and 64% of people develop chronic or functional instability after an ankle sprain (van Rijn et al., 2008). This could be due to the mechanical or functional insufficiency of the structures of the injured joint, but research has shown that mechanical laxity of the joint is not necessarily present in people with chronic instability (Gribble et al., 2016; Gribble et al., 2014). Therefore, neuromuscular changes occurring at the time of or after the injury are thought to play an important role in the development of chronic ankle instability (Kim et al., 2019). These include, in particular, altered afferent sensory information from the joint to the central nervous system, which occurs due to damage to the ligaments in the joint and joint capsule (Freeman et al., 1965).

1.2. Arthrogenic muscle inhibition (AMI)

Arthrogenic muscle inhibition (AMI) is often an overlooked consequence of joint injury and is defined as the reflex inhibition of the intact muscles around the injured joint. AMI is thought to be a protective mechanism that protects the injured joint from increased stresses on the joint after injury, but the presence of AMI also makes rehabilitation more difficult (Hopkins & Ingersoll, 2000). AMI is not the same as atrophy and muscle weakness, but it means impairment of muscle activation or inability to develop maximal voluntary contraction. In the knee joint, AMI after injury or surgery has been shown to result in a reduced (voluntary) activation of the m. quadriceps femoris (Urbach et al., 1999; Urbach & Awiszus, 2002; Sonnery-Cottet et al., 2022), and a previous review of the literature has showed that muscle inhibition at the spinal cord level is also present in people with chronic ankle instability (Kim et al., 2019).

1.3. Muscle activity measurements

EMG measurements are used to determine the presence or absence of AMI. Among them, the H-reflex is observed, which assesses α-motor neuron's excitability (in response to the stimulation of sensory nerve). A change in the maximum H-reflex value (H_{max}) represents a change in the ability to activate the motoneuron (Palmieri et al., 2004). Lower H_{max} represents less muscle activation and therefore indicates the presence of AMI. Higher H_{max} represents increased activation or excitation. Usually, the H_{max} value is normalized by the M_{max} value, which represents the maximum possible activation of the whole motor neuron (direct stimulation of the α-motor neuron). Thus, the H_{max}/M_{max} ratio is reported in the results (McVey et al., 2005).

1.4. Purpose

The aim of this literature review is to determine whether arthrogenic muscle inhibition is present in subjects after acute ankle sprain or in subjects with ankle instability.

2. Methods

Literature was searched in PubMed, Cochrane Library, EMBASE (Ovid) and Medline databases. The last review was carried out on 7 December 2022. We used the following English keywords: ankle AND arthrogenic muscle inhibition. In addition, we have reviewed the reference lists of the included articles. Inclusion criteria was articles in English, population of subjects with ankle sprain or ankle instability, and EMG-measured muscle activity had to be reported during the outcomes. Studies that were not fully accessible, studies that simulated joint swelling and studies that did not observe the muscles around the ankle were excluded.

3. Results

After excluding duplicates, a total of 16 different studies were found. After screening and eligibility assessment, five studies published between 2004 and 2022 were included in the review (McVey et al., 2005; Palmieri-Smith et al., 2009; Klykken et al., 2013; Bowker et al., 2016; Kim et al., 2022).

The characteristics of the participants in the included studies are presented in **Table 1**. In all studies, the subjects were young adults.

Table 1. Characteristics of the participants in the included studies.

Study	Pathologies	Samples
Kim et al (2022)	Acute ankle sprain	n = 60, majority M
Klykken et al (2013)	Acute ankle sprain	n = 20, majortiy F
McVey et al (2005)	Chronic (functional) instability	n = 29, majortiy F
Palmieri-Smith et al (2009)	Chronic (functional) instability	n = 42, majortiy F
Bowker et al (2016)	Chronic instability	n = 93, majortiy F

M: males, F: females.

In four included studies (Kim et al., 2022; Klykken et al., 2013; McVey et al., 2005; Palmieri-Smith et al., 2009) the subjects were divided into two groups: subjects with present ankle pathology (experimental group – EG) and subjects without present ankle pathology (control group – CG). In those studies, H_{max} and M_{max} were measured in both lower limbs and in both groups. In the study by Bowker et al (2016) there were three groups: subjects with a history of ankle injury with instability (EG1), subjects with a history of ankle injury without instability (EG2) and subjects with no history of ankle injury (CG). In this study, measurements were taken only on the injured limb. Within group comparison has been done in four studies, comparing injured and uninjured side (Kim et al., 2022; Klykken et al., 2013; McVey et al., 2005; Palmieri-Smith et al., 2009) and in four studies, comparison between groups has been done (Kim et al., 2022; Klykken et al., 2013; Bowker et al., 2016). In three included studies activation of m. soleus, m. peroneus longus and m. tibialis anterior was observed (Kim et al., 2022; Klykken et al., 2013; McVey et al., 2005), in only one study (Palmieri-Smith et al., 2009) m. peroneus longus activity was observed and in only one study (Bowker et al., 2016) m. soleus activity was observed.

In all four studies reporting differences between the injured and uninjured leg within the control groups, there was no statistically significant difference in the H_{max} / M_{max} ratio (Kim et al., 2022; Klykken et al., 2013; McVey et al., 2005; Palmieri-Smith et al., 2009). In two studies (Klykken et al., 2013; McVey et al., 2005), the H_{max} / M_{max} ratio of m. soleus in the experimental group was statistically significantly lower in the injured leg as compared to the uninjured side, while Kim et al (2022) have not found any differences. In the experimental group, the H_{max} / M_{max} ratio was higher in m. tibialis anterior at the injured side than at the uninjured side in only one study (Klykken et al., 2013). In two studies (McVey et al., 2005; Palmieri-Smith et al., 2009), a statistically significantly lower H_{max} / M_{max} ratio was observed in the experimental groups for m. peroneus longus at the injured side compared to the uninjured side. The results of the comparison between sides within each group are summarized below in the **Table 2**.

Table 2. Within group comparison – injured and uninjured leg.

STUDY	m. soleus	m. peroneus longus	m. tibialis ant.
Kim et al (2022)	EG: / CG: /	EG: / CG: /	EG: / CG: /
Klykken et al (2013)	EG: SS ↓ H_{max}/M_{max} ratio on the injured side than on the uninjured side CG: /	EG: / CG: /	EG: SS ↑ H_{max}/M_{max} ratio on the injured side than on the uninjured side CG: /
McVey et al (2005)	EG: SS ↓ H_{max}/M_{max} ratio on the injured side than on the uninjured side CG: /	EG: SS ↓ H_{max}/M_{max} ratio on the injured side than on the uninjured side CG: /	EG: / CG: /
Palmieri-Smith et al (2009)	N/A	EG: SS ↓ H_{max}/M_{max} ratio on the injured side than on the uninjured side CG: /	N/A

EG: experimental group, CG: control group, SS: statistically significant, ↓: lower, ↑: higher, N/A: not available, /: not statistically significant changes.

The difference between groups has been observed in three studies (Kim et al., 2022; Klykken et al., 2013; Bowker et al., 2016). A statistically significant reduction in the H_{max}/M_{max} ratio of m. soleus in the EG compared to the CG has been reported in two studies (Kim et al., 2022; Bowker et al., 2016). In the study by Bowker et al (2016), there was a statistically significant difference when comparing the group of subjects with a history of injury and present instability (“non-copers”) with the group of subjects with a history of injury and no instability ("copers") as well as when comparing “non-copers” group to the control group (no history of injury).

Table 3. Comparison between groups.

STUDY	m. soleus	m. peroneus longus	m. tibialis ant.
Kim et al (2022)	SS ↓ H_{max}/M_{max} ratio in the EG than in the CG (both ankles compared to both ankles)	/	/
Klykken et al (2013)	/	/	SS ↑ difference between both ankles in the EG than in the CG.
Bowker et al (2016)	SS ↓ H_{max}/M_{max} ratio in EG1 compared to EG2 and CG. No SS difference between EG2 and CG.	N/A	N/A

SS: statistically significant, ↓: lower, ↑: higher EG: experimental group, CG: control group, /: not statistically significant changes, EG1: non copers, EG2: copers.

Additionally, there was no difference between the "copers" and control groups. However, in the study by Klykken et al (2013), there was no difference between EG and CG in m. soleus. Similarly, in neither of the studies that observed the activation of m. peroneus longus and compared the results between the two groups, there was a difference between EG and CG (Kim et al., 2022; Klykken et al., 2013). In the study by Klykken et al (2013) where they have compared the Hmax/Mmax ratio of the m. tibialis anterior between the two groups, the difference between the sides within the EG was statistically more significant than the difference within the CG. On the other hand, study by Kim et al (2022) did not report such difference. The results of the comparison between groups are summarized in **Table 3**.

4. Discussion

The results of the included studies suggest that the EMG-measured H_{max}/M_{max} ratio is reduced after an ankle sprain or in ankle instability in m. soleus and m. peroneus longus, indicating the presence of arthrogenic muscle inhibition.

It is assumed that the occurrence of AMI is not related to acute symptoms or specific changes at the local level, but to central mechanisms at the spinal cord level or even supraspinal level (Kim et al., 2022). Furthermore, findings from Palimieri et al (2004) indicated that all muscles around the joint showed facilitation rather than inhibition after simulated ankle swelling. In addition, the presence of bilateral inhibition of m. soleus, which was reported by Kim et al (2022), also suggests that central mechanisms are involved in the occurrence of AMI.

Bilateral inhibition seems to be present only after an acute ankle sprain, while in chronic ankle instability only unilateral inhibition of m. peroneus longus and m. soleus is present (McVey et al., 2005; Palmieri-Smith et al., 2009; Bowker et al., 2016). These results are consistent with the results of a previous meta-analysis by Kim et al (2019) that also confirmed the presence of unilateral muscle inhibition in subjects with chronic instability. In fact, it has been reported that m. soleus activation was reduced only in the subjects with a history of ankle sprain and presenting instability, but not in the subjects with a history of ankle sprain and no presenting instability (Bowker et al., 2016). This is consistent with the findings that ankle instability is not necessarily related to mechanical instability or increased joint laxity, but rather to neuromuscular changes that persist over time after injury (Gribble et al., 2016; Gribble et al., 2014, Kim et al., 2019). The presence of central mechanisms is also suggested in the study by Sedory et al (2007), where authors have reported ipsilateral inhibition of the quadriceps femoris and the knee flexors in subjects with chronic ankle instability.

The results of our review show the importance of appropriate management of ankle sprain and AMI after injury or in chronic ankle instability. By preventing and eliminating AMI, non-mechanical ankle instability could be prevented or reduced, which could lead to a lower incidence of re-injury. The impact of disinhibitory techniques such as cryotherapy and therapeutic exercise, which have been shown to reduce AMI after anterior cruciate ligament reconstruction in the study by Sonnery-Cottet et al (2019), should be tested in the future in the subjects after ankle sprain and in the subjects with chronic instability.

A limitation of our literature review is the small number of included studies and heterogeneity of population and methodologies. There are further studies needed to draw firm conclusions.

Based on the literature review, we found that AMI, manifested as reduced activation of m. soleus and m. peroneus longus, is present in subjects with ankle sprain or ankle instability. Inhibition is present bilaterally in the acute phase, and the mechanisms of AMI are more likely to be centrally controlled rather than locally controlled. Further research is needed to draw firm conclusions about the presence of AMI in ankle pathologies, including focusing on the potential for AMI reduction after injury or ankle instability.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Cannabinoid Molecules from *Cannabis Sativa L.* as a Promissing Solution for Methicillin-Resistant *Staphylococcus Aureus* (MRSA)

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Abstract:
Scientists are working to develop new types of antibiotics to combat the growing problem of antibiotic resistance in bacteria. One potential source of these new drugs is the plant *Cannabis sativa* L., which has been used for medical purposes for centuries. The beneficial properties of this plant are mainly due to the presence of compounds called cannabinoids. Researchers are currently exploring the use of cannabinoids to treat various infections, although they are mainly known for their psychoactive effects. Some studies have shown that certain cannabinoids can be effective against harmful bacteria including those that are resistant to common antibiotics. In addition, a combination of different antibiotics has been shown to be more effective than a single antibiotic.

Keywords: *Cannabis sativa L.*; Cannabinoids; Methicillin-resistant *S. aureus* (MRSA); Antibiotics; Bacteria; Biological activity

Citation: Pečan LI, Barrios Francisco R, Jeran M. Cannabinoid molecules from *Cannabis sativa* L. as a promising solution for methicillin-resistant *Staphylococcus aureus* (MRSA). Proceedings of Socratic Lectures. **2023**, 8; 97-105. https://doi.org/10.55295/PSL.2023.I15

1. Introduction

Since Alexander Fleming discovered penicillin in 1928, antibiotics have been a lifesaving treatment for bacterial infections around the world (Zaman et al., 2017; Saleemi et al., 2022). However, the overuse and misuse of these drugs have led to a growing problem of antibiotic resistance, which is a significant threat to global health. The problem is caused by the increasing number of multi-drug resistant organisms (MDRs) that are resistant to most antibiotics, taking place due to the lack of action and insufficient efforts to address the issue of antimicrobial resistance (Zaman et al., 2017; Saleemi et al., 2022). As more and more bacteria become resistant to antibiotics, the options available to treat these infections are becoming increasingly limited. In this regard, researchers are investigating alternative methods for combating these resistant pathogens. One promising area of study is the use of helper molecules, such as resistant breakers or antibiotic potentiators. These molecules work in combination with antibiotics to enhance their effectiveness and make them more efficient against resistant bacteria (Saleemi et al., 2022; Tyers and Wright, 2019). Helper molecules are non-antibiotic compounds that can be used in combination with antibiotics to improve their effectiveness. These molecules work by various mechanisms, such as altering the permeability of bacterial cell membranes, inhibiting enzymes, and blocking the pump that bacteria use to resist antibiotics. These mechanisms can increase the effectiveness of antibiotics against resistant bacteria (Saleemi et al., 2022; Douafer et al., 2019).

The misuse of antibiotics is the leading cause of the emergence of antibiotic-resistant bacteria. The World Health Organization (WHO) has identified this phenomenon as one of the most significant threats to global health. People with weakened immune systems, such as those undergoing chemotherapy, are particularly at risk of becoming infected with these resistant bacteria (Saleemi et al., 2022; World Health Organization, 2017). The emergence of antibiotic-resistant bacteria is a major concern for the development of new medical procedures and treatments. To tackle this problem, it is necessary to create new classes of antimicrobial agents that can effectively fight these organisms. One approach is to use helper molecules in combination with antibiotics, which may reduce the risk of antibiotic resistance. Evaluating these helper molecules is crucial to identify the most effective ones. The WHO has also released a list of the 12 most dangerous families of bacteria that are resistant to antibiotics, highlighting the urgency of finding solutions to this problem (Farha et al., 2020; World Health Organization, 2017).

A major concern is, for example, that certain strains of MRSA have developed resistance to antibiotics commonly used to treat them, such as vancomycin, linezolid, and daptomycin. This limits the treatment options available for serious methicillin-resistant *Staphylococcus aureus* (MRSA) infections (Farha et al., 2020; Nannini et al., 2010). Antibiotic resistance in Gram-negative bacteria is a growing concern in the medical field. The World Health Organization notes that many of the most dangerous bacteria are Gram-negative, and new classes of antibiotics to combat these infections have not been discovered in over 30 years. The lack of effective antibiotics and the limited number of new drugs under development highlights the need for alternative treatment options (Farha et al., 2020).

2. Cannabis

Cannabis is a type of plant that belongs to the family Cannabaceae. Since it can adapt to different environmental conditions it can grow in different climates. It is an annual herb that produces male and female inflorescences on different plants. The leaves are arranged in a palm-like shape or in a line, with toothed edges. Usually, the first set of leaves has one leaf, but the number can increase up to thirteen, depending on the specific variety and how it is grown. The way that the plant determines its sex is complex (Tavčar Beković et al., 2019; Pečan et al., 2021; Appendino et al., 2008).

2.1 *Biologically active compounds in Cannabis sativa*

Cannabis sativa (*C. sativa*) is a plant that has been extensively studied and more than 420 chemical compounds have been found (Tavčar Beković et al., 2019; Pečan et al., 2021). The most significant ones are present in the trichomes, which are small glands on the surface of the plant. These trichomes are found on both male and female plants but are especially concentrated on certain parts of the female plant. The resin produced by the trichomes contains various ingredients including cannabinoids, terpenes, and flavonoids, which are all secondary metabolism products. Cannabinoids are the most active biological components of cannabis, and over 90 different cannabinoids have been identified so far. They are divided into two categories: endogenous cannabinoids produced by the human body, and exogenous cannabinoids that can be made synthetically or by the *C. sativa* plant. They work by binding to cannabinoid receptors and thus causing specific effects (Pečan et al., 2021; Appendino et al., 2008).

3. Main cannabinoids

3.1 *Δ⁹-Tetrahydrocannabinol (THC)*

Δ⁹-Tetrahydrocannabinol (THC) is a solid substance that can be dissolved in alcohols, hydrocarbons and oils but not in water. It has a boiling point of 165 °C, which is the lowest temperature required for it to be inhaled (Tavčar Beković et al., 2019). As a controlled substance, it is classified as a Schedule II drug and is only allowed for medical and research purposes. THC is an active ingredient approved by the US Food and Drug Administration and the European Medicines Agency and is used in authorized medicines. It works by partially activating the cannabinoid receptors CB1 and CB2, primarily located in the central nervous system and in the immune system. This decreases the concentration of the second messenger molecule *c*AMP and results in psychoactive effects. The discovery of these receptors in the brain also led to the discovery of endocannabinoids such as anandamide and 2-arachidonoyl glyceride (2-AG). THC is a lipophilic molecule that can bind to various entities in the brain and body, such as fat. It also has mild antioxidant properties that can protect neurons against oxidative stress caused by excessive glutamate (Pečan et al., 2021).

3.2 *Cannabidiol (CBD)*

Cannabidiol (CBD) is a compound that was first isolated from cannabis in 1940, its structure was identified in 1963 (Tavčar Beković et al., 2019). It can be obtained through various extraction methods and is also semi-synthetically derived from limonene. At room temperature, it is a solid substance that is colourless and boils at 175 °C (Tavčar Beković et al., 2019). When exposed to certain acids or high temperatures during smoking, it can convert to THC in small amounts. CBD is widely used in dietary supplements and cosmetics. It is a phytocannabinoid that is derived from cannabis and has pain-relieving, anti-inflammatory, anti-tumoral and chemo-preventive properties but it doesn't have psychoactive effects. CBD activates the endoplasmic reticulum stress and suppresses AKT/*m*TOR signalling, promoting autophagy and apoptosis. It also raises the production of reactive oxygen species (ROS) which further enhances apoptosis. CBD also regulates the expression of intercellular adhesion molecule 1 (ICAM-1) and tissue matrix metalloproteinase-1 (TIMP1) inhibitors and reduces the expression of DNA 1 binding inhibitor (ID-1) which in turn inhibits cancer cell invasiveness and metastasis. CBD also activates the transient receptor potential of vanilloid type 2 (TRPV2) which may increase the uptake of various cytotoxic agents in cancer cells. CBD's pain-relieving effect is brought about by its binding to CB1 receptors (Pečan et al., 2021).

4. Effect of cannabinoids on methicillin-resistant *Staphylococcus aureus* (MRSA)

4.1 Methicillin-resistant Staphylococcus aureus (MRSA)

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most prevalent modern pathogens (Otto, 2013). *Staphylococcus aureus* (*S. aureus*) is known to cause a wide range of pyogenic infections, involving several organs, and both hospital-acquired and community-acquired infections are well recognized. In nature, *S. aureus* infections can be fatal. Some strains have developed resistance to several β-lactam antibiotics used in hospitals. MRSA is a significant opportunistic pathogen that causes both nosocomial and community-acquired infections (community-associated MRSA, CA-MRSA) (Chakraborty et al., 2018; Deurenberg et al., 2007). *S. aureus* is a Gram-positive, coagulase-positive coccus of the family of Staphylococcaceae. Since it was first identified in 1961, MRSA has spread throughout the world and become one of the most frequent pathogenic bacteria causing healthcare-associated infections. MRSA can cause various types of life-threatening infections, such as septic shock, endocarditis and severe pneumonia (Chakraborty et al., 2018).

Different MRSA strains have emerged worldwide, and they have become resistant to a variety of antibiotics, including penicillin, tetracycline, methicillin and vancomycin. Recently, in several countries, MRSA has been found to infect livestock and humans exposed to those infected animals. This type of MRSA has been named livestock-associated MRSA (LA-MRSA) (Chakraborty et al., 2018; Nemati et al., 2008). The interactions among these different types of MRSA reservoirs have been reported, including nosocomial infections by CA-MRSA and importation of LA-MRSA in hospitals, leading to hospital-acquired infections (Chakraborty et al., 2018; Moore et al., 2009; Skov and Jensen, 2009). Due to a modification in penicillin-binding protein 2a, MRSA has a decreased affinity to β-lactam.

The *mec*A gene encodes this protein and is located on a mobile SCC*mec* cassette chromosome. This genetic element resists most currently available β-lactam antibiotics. Unfortunately, though several agents and protocols have been proposed, no prophylactic strategies have been yet proven useful. Therefore, new alternative therapies must be developed to kill extended-spectrum β-lactamase (ESBL) strains of MRSA. Herbal medicine can solve this problem, and many plant extracts have been reported to possess inhibitory activity towards *S. aureus* as well as MRSA and ESBL-MRSA (Chakraborty et al., 2018).

4.2 Antimicrobial activity of different cannabinoids from Cannabis sativa on MRSA

The idea that cannabinoids from Cannabis sativa can have antibacterial properties was first reported in the 1950s (Rabinovich et al., 1959). At the time, the bactericidal properties of cannabis were studied before the full chemical makeup of the plant was understood. This means that the antibacterial effects were not attributed to a specific component. In 1976, it was discovered that Δ^9-THC and CBD can be used as bacteriostatic agents and were able to kill a panel of human pathogenic strains (Turner et al.,2019; Klingeren and Ham, 1976).

There has been significant interest in the antibacterial properties of various *C. sativa* plant extracts, such as the oil and extract from the plant. Different methods have been used to isolate these extracts, with cold-pressing and solvent extraction techniques being commonly used to produce products such as cosmetics and food. However, new technologies are being developed to improve the efficiency of these methods, such as pressurized liquid extractions and ultrasonic extractions, which use less solvent and have shorter processing times than traditional methods (Fathordoobady et al., 2019).

According to a study by Farha et al. (2020), cannabinoids present in *C. sativa* have been found to have antibacterial properties against MRSA. Selected known cannabinoid analogues that are active against MRSA USA300 are depicted in **Figure 1.** They can inhibit the formation of biofilms and also eradicate pre-existing ones. Research results indicate that cannabigerol specifically targets the cytoplasmic membrane of Gram-positive bacteria and has been shown to be effective against MRSA in a mouse model *in vivo*. Additionally, cannabinoids have also been found to be effective against certain gram-negative organisms by

targeting their inner membrane. The study also shows that these compounds can be used in combination with polymyxin B against multi-drug resistant gram-negative pathogens, indicating the broad-spectrum therapeutic potential of cannabinoids. The study discovered that of the five major cannabinoids and some of their derivatives, seven molecules are potent antibiotics with minimum inhibitory concentrations of 2 μg/mL. This group includes cannabichromene (CBG), CBD, cannabinol (CBN), cannabichromene acid (CBCA), and THC along with its Δ^8- and exolefin regioisomers. However, it was also found that these compounds lose potency when the benzoic acid moiety is present or when the *n*-pentyl substituent is replaced with *n*-propyl. Additionally, the two most common human metabolites of THC, (±)-11-nor-9-carboxy-Δ^9-THC and (±)-11-hydroxy-Δ^9-THC, as well as cannabicyclol were inactive at the highest concentrations screened (minimum inhibitory concentration (MIC): >32 μg/mL) (Farha et al., 2020).

MRSA's ability to form biofilms on necrotic tissues and medical devices is considered a major factor in its ability to persist in both the environment and host organism, as stated by Otto (2013). These biofilms, which are highly structured communities of MRSA on surfaces, are known to be resistant to many antimicrobial compounds and are less susceptible to host immune responses. Studies have shown that certain cannabinoids such as CBG have the ability to inhibit MRSA biofilm formation. The research used static solid surface abiotic assays to test the effects of increasing concentrations of cannabinoids on MRSA biofilm formation under conditions that favour biofilm growth.

The results showed that the effectiveness of the cannabinoids in inhibiting biofilm formation correlated with their antibacterial activity against MRSA. The five major cannabinoids tested were found to be effective in repressing MRSA biofilm formation, with CBG having the most potent antibiofilm activity. In fact, just 0.5 μg/mL (1/4 MIC) of CBG was able to inhibit biofilm formation by about 50%. Overall, this experiment highlights the potent ability of cannabinoids to inhibit MRSA biofilm formation (Farha et al., 2020).

Martinenghi et al. (2020) found that purified CBDA and CBD extracted from *C. sativa* L. had exhibited potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA USA300) when compared to four conventional antibiotics clindamycin, ofloxacin, meropenem, and tobramycin. CBD was found to be particularly effective against Gram-positive bacteria with a minimal inhibitory concentration between 1 and 2 μg/mL, while CBDA had two-fold lower activity.

Blaskovich et al. (2021) also reported that CBD was effective against MRSA biofilms with a minimum biofilm eradication concentration (MBEC) of 1-2 or 2-4 μg/mL, similar to its minimum inhibitory concentration (MIC). These MBEC values were found to be much better than daptomycin and clindamycin against MRSA. Confocal microscopy showed that CBD was able to penetrate the biofilm and kill the bacteria.

Overall, CBD is reported to have useful antimicrobial activity against a broad spectrum of more than 20 species of Gram-positive bacteria, including several strains of MRSA, with potential clinical utility for nasal decolonization due to its consistent MIC of 1-4 μg/mL (Blaskovich et al., 2021).

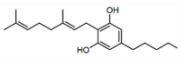
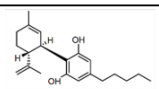
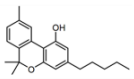
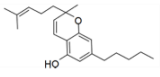
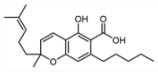
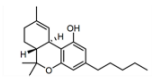
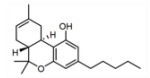
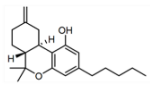
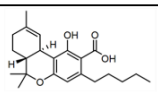
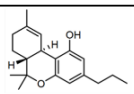
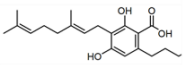
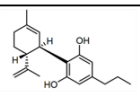
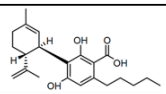
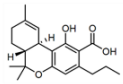
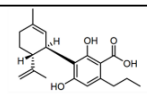
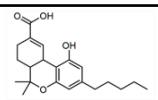
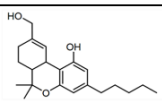
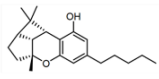
Entry	Name	(Abbreviation)	Structure	MIC (μg/mL)
(1)	Cannabigerol	(CBG)		2
(2)	Cannabidiol	(CBD)		2
(3)	Cannabinol	(CBN)		2
(4)	Cannabichromene	(CBC)		8
(5)	Cannabichromenic acid	(CBCA)		2
(6)	(-)Δ⁹-Tetrahydrocannabinol	(THC)		2
(7)	(-)Δ⁸-Tetrahydrocannabinol	(Δ⁸-THC)		2
(8)	*exo*-Tetrahydrocannabinol	(*exo*-THC)		2
(9)	Δ⁹-Tetrahydrocannabinolic acid A	(THCAA)		4
(10)	Δ⁹-Tetrahydrocannabivarin	(THCV)		4
(11)	Cannabigerolic acid	(CBGA)		4
(12)	Cannabidivarin	(CBDV)		8
(13)	Cannabidiolic acid	(CBDA)		16
(14)	Tetrahydrocannabivarinic acid	(THCVA)		16
(15)	Cannabidivarinic acid	(CBDVA)		32
(16)	(±) 11-Nor-9-carboxy-Δ⁹-THC			>32
(17)	(±) 11-Hydroxy-Δ⁹-THC			>32
(18)	Cannabicyclol	(CBL)		>32

Figure 1. Selected known cannabinoid analogues that are active against MRSA USA300. MIC: minimum inhibitory concentration. From Farha et al (2020).

5. Current challenges and future perspectives

As Vickers (2017) notes, the complexity of cannabis-related laws is a major barrier to the development of effective cannabinoid research. The difficulty of complying with legal requirements for cannabis research can prevent researchers and funding agencies from exploring new and innovative products. However, as CBD becomes more accepted in the U.S. and other countries, research into new methods of CBD administration is expected to increase. One such method being explored is the use of transdermal and topical delivery systems. Recognizing this potential, the National Center for Complementary and Integrative Health in the United States has expressed interest in funding CBD research. In the coming years, pharmaceutical companies or other research institutions are also likely to focus on the evaluation and development of topical or transdermal delivery systems for CBD, as this approach offers many advantages, as noted by Kamel (2015).

As antibiotic resistance becomes more prevalent, researchers are exploring new ways to treat bacterial infections. One area of interest is the use of plant compounds, such as those found in *C. sativa*, as potential antibacterial agents. Although preliminary research in this area has yielded mixed results, the potential benefits of using Cannabis extracts as antibiotics are still being studied. Factors such as the specific extracts examined and the methods used to test their activity may contribute to these varying results. Additionally, studies have shown that cannabis extracts and in particular purified cannabinoids, show promise in their ability to combat multi-drug resistant organisms, especially those Gram-positive. Furthermore, cannabinoids have been found to have antimicrobial properties against a range of bacteria, including those harmful to humans. They also have the potential to enhance the effectiveness of traditional antibiotics by acting as a natural antimicrobial agent. As a result, cannabinoids may be considered promising candidates for the development of new combination therapies to combat antibiotic-resistant bacteria (Karas et al., 2020).

The way cannabinoids impact the development and management of infections in animal models is not yet fully understood. Some studies have suggested that certain cannabinoids, such as Δ^9-THC, may suppress the immune system and make it less effective against intracellular pathogens (Schofs et al., 2021). However, other research suggests that cannabinoids may also be beneficial in protecting against bacterial infections caused by extracellular attacks and excessive immune responses. Despite the advancements made in identifying bacterial targets and developing new antimicrobial methods, more research is needed to understand the role of cannabinoids in treating various infections. Safety and toxicity concerns surrounding cannabis extract products have been alleviated using non-psychotropic cannabinoids, which have been found to possess in vitro properties that can fight against bacterial infections (Saleemi et al., 2022). Overall, the available data suggests that cannabinoids and other cannabis compounds have promising in vitro antibacterial properties that warrant further exploration as potential antimicrobial agents against clinically significant bacteria.

6. Conclusion

In summary, cannabinoids, particularly those found in *Cannabis sativa*, show promise as a potential treatment for MRSA and other antibiotic-resistant bacterial infections. Studies have shown that cannabis extracts and purified cannabinoids possess antimicrobial properties against a range of Gram-positive bacteria, including MRSA. In addition, cannabinoids have been found to have the potential to enhance the effectiveness of conventional antibiotics by acting as natural antimicrobial agents. This makes cannabinoids attractive candidates for the development of new combination therapies to combat antibiotic-resistant bacteria. While more research is needed to fully understand the mechanisms of action and potential side effects of cannabinoids in the treatment of MRSA, the available data suggest that they are a promising drug for the treatment of this and other antibiotic-resistant infections, so more efforts should be invested in this field of research.

Conflicts of Interest: The authors declare no conflict of interest.

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Scientific contribution

Chromyl Fluoride as a Strongman Representative of the Chromium (VI) Dioxodihalides Oxidizing Agent Family

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Abstract:

The chemistry of chromium (Cr) as a transition state element includes a variety of oxidation states and their specific colours. The general and most common oxidation states of chromium are (+6), (+3), and (+2). However, some stable compounds with (+5), (+4) and (+1) states are also known. The main species formed by chromium in the (+6) oxidation state are the chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) ions.

Chromium (VI) dioxodihalides represent a group of versatile oxidants that donate oxygen atoms to a variety of organic molecules. Chromium-oxotransition metal complexes in higher oxidation states have been used as models in biochemical studies, in particular to investigate how such systems can mimic biologically relevant mixed-function oxygenases and how the oxygen ligands interact in electrophilic reactions. The representative compound from this group of chromium compounds is chromyl fluoride (CrO_2F_2), which was mentioned after 1952, when its physical properties were precisely determined. It is a violet-red coloured crystalline solid that melts to an orange-red liquid at a temperature close to room temperature.

In this paper, two ways to prepare chromium fluoride are presented: (a) by reaction between its chloride analogues and gaseous fluorine and (b) by reaction between chromium(VI) oxide and anhydrous hydrogen fluoride. Raman spectroscopy was used to characterise the crude products directly in the FEP tube. Its physical properties and chemical reactivity pose a great challenge for synthesis. Special equipment is required for its production (e.g. a nickel vacuum line system).

Keywords: Chromium; Chromium(VI) dioxodihalides; Chromyl fluoride (CrO_2F_2); Fluorine; Anhydrous hydrogen fluoride; Raman spectroscopy

Citation: Jeran M, Tramšek M, Tavčar G. Chromyl fluoride as a strongman representative of the chromium(VI) dioxodihalides oxidizing agent family. Proceedings of Socratic Lectures. 2023, 8; 107-113. https://doi.org/10.55295/PSL.2023.I16

1. Introduction

1.1. Chromium compounds

Chromium (Cr) is a metal that is naturally found in the environment (soils, rocks, volcanic dusts, etc.) in two states: as trivalent Cr(III) and as hexavalent Cr(VI). The studies have shown that Cr(VI) compounds have almost 100 times higher toxicity than Cr(III) compounds (Hussain and Keçili, 2020). Chromium compounds are widely used in industry, e.g. for the production of dyes in the textile industry (potassium chromate, ammonium dichromate), for the production of printing inks, Cr-based paints, plastics (zinc chromate, sodium chromate, lead chromate and barium chromate) and as corrosion inhibitors (strontium chromate, zinc chromate and calcium chromate) (Hussain and Keçili , 2020).
Cr(VI) compounds are considered as highly toxic and industrial pollutants with dangerous effects on humans, including various diseases such as liver and kidney damage, respiratory and immune system problems (Hussain and Keçili, 2020; Cohen, et al.1993).
Chromium compounds are also important in organic synthetic chemistry. Their complexes are important as fluorinating reagents for the preparation of biologically active compounds for pharmaceuticals and agrochemicals (Haufe and Bruns , 2002). Some examples of enantioselective nucleophilic fluorination using chromosalen complexes as Lewis's acid catalysts for ring-opening *meso*-epoxidic compounds have been reported (**Figure 1**). However, these reactions lead to end products with an enantiomeric excess of only about 66% (Thornbury, et al. 2017).

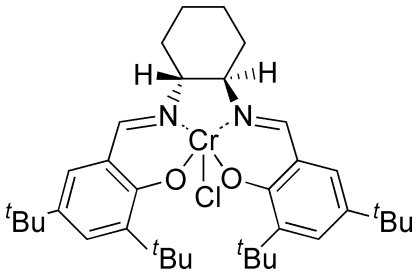

Figure 1. Jacobsen's enantiomerically pure (*R,R*)-(–)-(salen)chromium chloride complex for ring-opening epoxides in the presence of stoichiometric or slightly substoichiometric amounts (Haufe and Bruns , 2002).

1.2. Motivation for study of chromium(VI) dioxodihalides

Chromium(VI) dioxodihalides represent a group of versatile oxidants that donate oxygen atoms to a variety of organic molecules. These oxotransition metal complexes of chromium in higher oxidation states were taken as a model for the enzyme cytochrome P-450. The electronic structure of chromium(VI) dioxodichloride (**Figure 2**) was studied to determine the extent to which this remarkable system can mimic mixed-function oxygenases in biological systems and to determine the mechanism of oxygen ligand activity in electrophilic reactions (Torrent, et al. 1996).

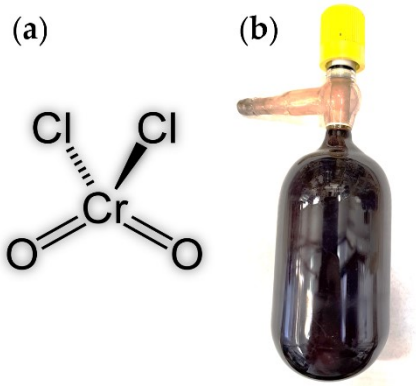

Figure 2. (**a**) Molecular structure of chromyl chloride, and (**b**) chromyl chloride as chemical reagent in a glass flask with Teflon valve.

In developing compounds with high oxidation efficiency and synthesising new materials, including materials with exceptionally high specific surface area (Tavčar and Skapin , 2019), researchers also faced various challenges in synthesis.

1.3. Chromyl fluoride (CrO_2F_2)

The literature dealing with chromyl compounds mainly reports on chromyl chloride (CrO_2Cl_2) and its reactions. The fluorinated analogue, called chromyl fluoride (CrO_2F_2), was mentioned after 1952, when its physical properties were accurately determined (Brown, et al. 1975). Infrared absorption spectra with tetrahedral structure and C_{2v} symmetry were derived from the gas phase (Brown, et al. 1976).

Brown et al. (1976) first reported the structural study of chromyl fluoride in liquid phase and presented solid phase data. The study revealed that liquid chromyl fluoride is mainly in the form of monomers with C_{2v} symmetry. The authors also confirmed the claim that the metal centres are polymerized via fluorine bridge bonds. More specifically, the solid phase forms polymers containing terminal Cr-O bonds.

Pure chromyl fluoride (**Figure 3**) reaches a pressure of 760 mm Hg as violet-red crystals at 22.6 ˚C and melts to an orange-red liquid at 31.6 ˚C. The vapor pressure at the triple point is 885 mm Hg (Engelbrecht and Grosse, 1951). Engelbrecht and Grosse (1951) noted that its solubility in HF at -78 ˚C is very low. Purification procedures took into account the fact that it forms stable and non-volatile triple addition compounds with HF and potassium or sodium fluoride (KF or NaF).

Because of its extreme reactivity with any type of organic compounds, care must be taken during handling. For example, at high temperatures, paraffinic gasses such as methane or butane ignite in its presence and burn with bright flame, producing smoke from chromium(III) oxide (Cr_2O_3) and chromium(III) fluoride (CrF_3) (Engelbrecht and Grosse, 1951).

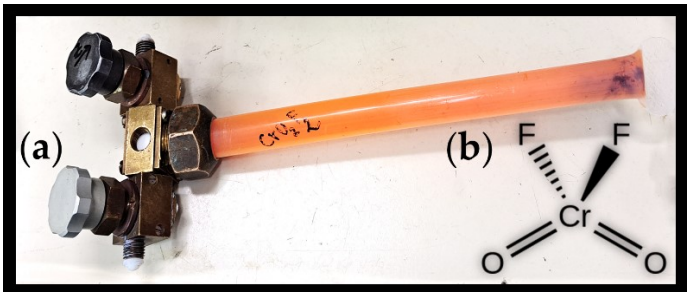

Figure 3. (**a**) Chromyl fluoride stored in fluorinated ethylene propylene (FEP) tube. Two valves on the container allow the reagent container to be connected to the nickel system. (**b**) Molecular structure of chromyl fluoride.

1.4. Method for the preparation of pure chromyl fluoride

Many of these preparation methods have been tried but proved undesirable for various reasons. The methods are often not suitable for small volumes, require difficult-to-handle reactants, or involve side reactions that lead to mixtures that are difficult to purify. Simple and robust synthesis methods that can produce a purer product were needed (Flesch and Svec, 1958; Gard and Williamson, 1986).

Chromyl fluoride can convert hydrocarbons to ketones and organic acids and is characterised by favourable conversion pathways to other chromium compounds, such as chromyl nitrate, which is easily prepared from CrO_2F_2 and sodium nitrate ($NaNO_3$). Years ago, it was necessary to work via dinitrogen pentaoxide (N_2O_5) to produce chromyl nitrate (Gard and Williamson, 1986).

Because of the extreme reactivity of chromium fluoride with both glass and quartz, it was necessary in the past to work in a metal system. The success of Engelbrecht and Grosse's (1951) method allowed laboratory equipment such as traps and tubes made of silica-free glass and inert and transparent plastic material such as polychlorotrifluoroethylene (Kel-F), which allowed visual recording of reaction monitoring and detection of products without contamination. The method was based on chromium trioxide (CrO_3) reacting readily with anhydrous hydrogen fluoride (aHF) to form the desired chromyl fluoride (**Equation 1**).

$$CrO_3 + 2HF \rightarrow CrO_2F_2 + H_2O \quad (1)$$

The synthesis reaction (**Equation 1**) is reversible, and water can readily hydrolyze the fluoride back into the starting CrO_3. The gaseous chromium fluoride hydrolyzes directly with water vapour to the usual smoky pink powder (Engelbrecht and Grosse, 1951). The studies of Engelbrecht and Grosse (1951) showed that when a high excess of anhydrous hydrogen fluoride (more than 10 moles of aHF per 1 mole of starting CrO_3) is used, a good yield of chromyl fluoride can easily be obtained (about 85% or more).
The general procedure presented by Flesch and Svec (1958) is to mix dry CrO_3 with dry CoF_3, heat the mixture in a glass or all-metal vacuum system, and collect the product in a trap cooled in a bath of solid CO_2-trichloroethylene slush.

Gard and Williamson (1986) prepared chromyl fluoride by the reaction between dry chromium(VI) oxide and carbonyl fluoride at a temperature of 185 °C (**Equation 2**). At -78 °C, the volatile components (CO_2, COF_2) were removed under dynamic vacuum. The purple-red product was transferred to another (Hoke) stainless steel tube under vacuum. They obtained the product in >99% purity.

$$CrO_3 + COF_2 \rightarrow CrO_2F_2 + CO_2 \quad (2)$$

There are a number of literature reports on the synthesis of chromyl fluoride using reagents such as fluorine, hydrogen fluoride, sulphur tetrafluoride, cobalt trifluoride, or iodine pentafluoride for reaction with chromium trioxide. Treatment of chromyl chloride with fluorine or chlorine monofluoride (ClF) has also been used to produce chromyl fluoride. These methods are suitable for the preparation of relatively small amounts of CrO_2F_2, but are not particularly practical for the preparation of the large amounts needed for chromium fluoride reaction studies (Green and Gard, 1977). The reaction of chromium trioxide with the interhalogen fluorides BrF_3, BrF_5, or ClF_3 produces chromium oxide trifluoride contaminated with the fluorinating agent, forming only a trace of chromium fluoride (Green and Gard, 1977; Clark and Sadana, 1964; Sharpe and Woolf, 1951).

Green and Grad (1977) found that an excess of ClF reacts with CrO_3 at 0 °C to form CrO_2F_2, O_2, ClO_2F, and Cl_2. When the molar ratio of ClF to CrO_3 is 1:1, CrO_2F_2, O_2, ClO_2, and Cl_2 are

formed. In the work of Green and Gard (1977), COF_2 was also found to react with CrO_3 to form chromyl fluoride and carbon dioxide. In addition, chromium trioxide was found to react with WF_6 or MoF_6 to form chromium fluoride and WOF_4 or $MoOF_4$. It was found that sulphur hexafluoride does not react with chromium trioxide. These reactions are summarized in **Table 1**.

Table 1. Green and Gard (1977) reactions of chromyl fluoride preparations.

$CrO_3 + ClF$ (*excess*) $\rightarrow CrO_2F_2 + Cl_2 + O_2 + ClO_2F$	T = 0 °C
$CrO_3 + COF_2 \rightarrow CrO_2F_2 + CO_2$	T = 185 °C
$CrO_3 + WF_6 \rightarrow CrO_2F_2 + WOF_4$	T = 125 °C
$CrO_3 + MoF_6 \rightarrow CrO_2F_2 + MoOF_4$	T = 125 °C

Chromium trioxide reacts with tungsten hexafluoride (WF_6) and molybdenum hexafluoride (MoF_6) at mild temperatures to form chromyl fluoride, while sulphur hexafluoride (SF_6) does not react even at temperatures above the decomposition temperature of chromium trioxide. The preparation of chromyl fluoride from molybdenum and tungsten hexafluoride and chromium trioxide is the simplest and most convenient route to large amounts of CrO_2F_2 reported to date. Another feature of these processes is that no high-pressure vessel is required and the reaction can be carried out in a very dry fused silica vessel. The synthesis of chromyl fluoride from molybdenum and tungsten hexafluoride and from chromium trioxide represents the simplest and most convenient route to large amounts of CrO_2F_2 known to date. Another advantage of this preparation method is that no high-pressure vessel is required and the reaction can be performed out in a very dry fused silica vessel. Since the reactants include of a non-volatile reagent and a volatile fluorinating agent, forming one volatile and one non-volatile product, CrO_2F_2 and $MoOF_4$ and WOF_4, respectively, purification is much easier. Moreover, this provides an easy route to the oxide tetrafluorides of molybdenum and tungsten, which can be obtained pure by sublimation. Chromium fluoride formation can be achieved by heating a mixture of CrO_3 and WOF_4 to 120 °C. The method leads to a (high) quantitative yield, assuming that 1 mol of CrO_2F_2 per mol of WF_6 was formed in the reaction (Green and Gard, 1977).

2. Methods

2.1 Anhydrous hydrogen fluoride (aHF)

Anhydrous hydrogen fluoride (aHF) (Linde AG, Pullach, Germany, 99.995%) was treated with potassium hexafluoronickelate(IV) (K_2NiF_6) (Advance Research Chemicals, Inc., 99.9%) for several days before use.

2.2 Synthesis of chromyl fluoride via chromyl chloride and fluorine

In 50 mL of a dried nickel reactor at -196 °C, 1.1 g (7.1 mmol) of chromyl chloride (≥99.99%, Sigma-Aldrich, USA) was condensed. Then, the aaddition of fluorine (F_2, 4 bar, approx. 7 mmol) (99.98%, Solvay Fluorand Derivate GmbH) was monitored by pressure-volume measurement

Then 4 bar (approx. 7 mmol) of gaseous fluorine (F_2) (99.98%, Solvay Fluorand Derivate GmbH) was carefully added to the reactor at room temperature. The reactor containing the reactants was heated to 200 °C overnight. The mixture was carefully cooled to room temperature, then to -196 °C, where excess fluorine was removed via natronkalk (Divelime, United Kingdom), and then to -35 °C where chlorine was removed. 748.6 mg (6.1 mmol) of chromyl fluoride was isolated (87% yield).

2.3 Synthesis of chromyl fluoride via chromium(VI) oxide and anhydrous hydrogen fluoride

In a dry and air-free FEP tube at -196 °C, 5 mL (247.38 mmol) of anhydrous hydrogen fluoride (aHF) (Linde, Germany) was added to chromium(VI) oxide (3.0 g, 30.0 mmol,

CrO_3) (p.a., Merck, Germany). Reaction vessel was slowly warmed up to the room temperature and after approx. thirty minutes, the reaction mixture turns dark green and the gas phase of the product is formed. The reaction proceeded overnight at room temperature. The reaction mixture at room temperature was transferred into a new FEP tube, cooled at -196 °C. To the crude product, additional gaseous fluorine (F_2) (99.98%, Solvay Fluorand Derivate GmbH) was carefully added (approx. 2.8 bar, 5 mmol). The tube containing the reagents was shaken on 330 rpm (SciQuip, UK) overnight at room temperature. The crude mixture was cooled at -196 ˚C and the excessive fluorine was removed at vacuum line. The crude isolate of CrO_2F_2 contains HF. The mass balance of the reaction was not possible to determine due to the presence of the HF solvent. Nevertheless, for our purposes we do not need to remove HF. This substance is suitable as a reagent for other reactions that take place in the medium HF.

2.4 Raman spectroscopy

Raman spectra were recorded on a sample placed in an FEP tube at room temperature using the Horiba Jobin Yvon LabRam-HR spectrometer (resolution of 1 cm^{-1}) equipped with an Olympus BXFM-ILHS microscope. Samples were excited with the 632.8 nm emission line of a He-Ne laser.

3. Results

The result of synthesis of chromyl fluoride was confirmed by Raman spectroscopy. The data was compared with shifts according to the Brown et al. (1976).

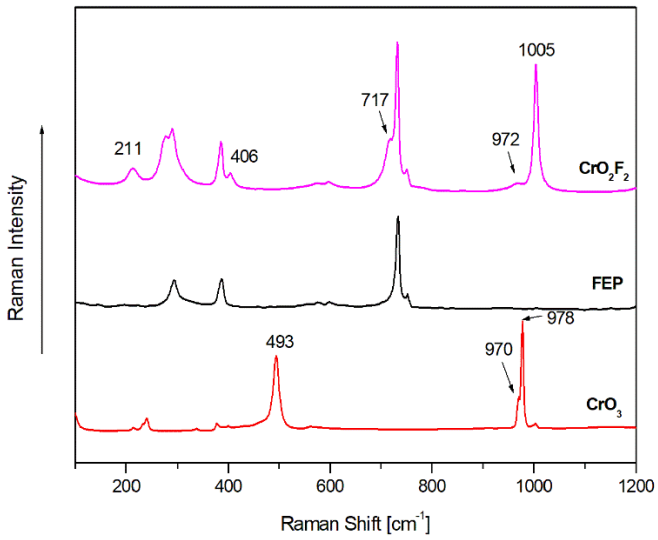

Figure 4. Raman spectra of the liquid phase of chromyl fluoride in an FEP tube, an empty FEP tube, and the starting material chromium (VI) oxide.

The results of Raman measurements show a comparison between the liquid phase of chromyl fluoride after its synthesis and chromium(VI) oxide as starting material (**Figure 4**). The strongest Raman peaks in the liquid phase at 1005 cm^{-1}, 717 cm^{-1}, and 406 cm^{-1} confirm the presence of chromyl fluoride as a product. The reported data for the vibrational frequencies (Raman peak) for pure liquid CrO_2F_2 are 995 (intensity: 100) cm^{-1}, 708 (intensity: 75) cm^{-1}, 403 (intensity: 24) cm^{-1}, 220 (intensity: 29) cm^{-1}, and 275 (intensity: 58) cm^{-1} (Brown, et al. 1975). However, the strongest peak of CrO_2F_2 dissolved in aHF was found at 1005 cm^{-1} (Besida, et al. 1989), the shift being due to interaction with HF molecules. The small peak at 972 cm^{-1} of chromyl fluoride indicates a trace of chromium(VI) oxide (John Wiley & Sons Inc, 2023). Its Raman spectra were recorded on object glass.

4. Conclusions

Chromyl fluoride is very useful as a reagent in various fields of research, especially in the preparation of new compounds. Its physical properties and chemical reactivity pose a great challenge to synthesis laboratories. Special equipment is needed for its production, in our case a nickel vacuum line system that allows safe production. Further studies on the use of this material will follow.

Characterization of the product was performed using Raman spectroscopy, which has proven to be a fast, simple and efficient method. It is a non-destructive technique that can be used to analyse samples in all states of aggregation. In addition, only a small amount of sample is required when measuring solids or liquids. In our case, the gaseous and liquid phases were successfully measured directly in the FEP tube.

Funding: This research was supported by the Slovenian Research Agency (ARRS) through core funding no. P1-0045.

Conflicts of Interest: The authors declare no conflict of interest.

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Invited lecture/Review

Diagnostic Detection of Extracellular Vesicles Using Raman Spectroscopy

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Abstract:
This article addresses concisely the possibility of using Raman spectroscopy in detecting extracellular vesicles (EVs) for disease diagnosis. The article gives an overview about EVs, including their definition, roles in normal cells, relation to disease initiation and progression and the chances and challenges of targeting EVs for disease diagnosis. Furthermore, it gives a brief background about Raman spectroscopy, its relevant techniques and to what extent it can be used for single vesicle analysis (SVA). Lastly, it presents some recent trials in using Raman spectroscopy for diagnostic detection of EVs and discusses briefly the potentiality of applying Raman spectroscopy for diagnostic detection of EVs in clinics.

Citation: Elwy M. Diagnostic detection of extracellular vesicles using Raman spectroscopy. Proceedings of Socratic Lectures. **2023**, 8; 115- 124. https://doi.org/10.55295/PSL.2023.I17

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Keywords: Extracellular vesicles; Raman spectroscopy; Diagnostic detection; Cancer diagnosis; SERS; LTRS.

1. Introduction

1.1. What is the extracellular vesicle?

Extracellular vesicles (EVs) are a heterogeneous group of particles, molecularly and physically delimited by plasma membrane, composed mainly of a phospholipid bilayer, and constitutively released by cells of diverse tissue origin through active secretion. (Lötvall, et al., 2014).

EVs have important roles in inter-cellular transferring of biomolecules such as proteins and nucleic acids, in addition to their physiological functions in many body organs and their involvement into the pathogenesis of many disorders (Yáñez-Mó, et al., 2015). In addition, it was found that the content of EVs in bio-fluids, such as saliva, blood, milk, seminal fluid and urine, is influenced by the body conditions in terms of nutrition, physical exercise, and health status. More particularly, in case of pathological conditions, the content of circulating EVs may be influenced by the diseased organ, severity and duration of the disease (Revenfeld, et al., 2014).

It was evident that various EVs types (including exosomes, microvesicles, and apoptotic bodies) contain subpopulations with unique biological functions that are highly involved in a broad range of biological processes (Willms et al., 2018; Kalluri et al., 2020).

This makes EVs a wealthy source of potential biomarkers for disease prognosis, vaccine production and drug delivery systemsms (Wiklander et al., 2019; Van Der Pol et al., 2010; Soung et al., 2017; Puente-Massagueret al., 2020). Also, EVs have an important property that it can be collected from non-accessible organs such as the brain (Shaimardanova et al., 2020). Thus, EVs-related studies have gained much attention during the past decades; nevertheless many of these studies dealt with bulk EVs and were based mainly on ensemble-averaging assays, such as proteomics for protein analysis and thin-layer chromatography for lipid analysis. These assays, despite being useful in many cases, masked the high heterogeneity between EVs in terms of their structure and composition. (Willms et al., 2018; Pick et al., 2018; Tkach et al., 2018).

For instance, ensemble measurements may be misinterpreted as a result of failing to figure out heterogeneities of molecular states of individual proteins or nucleic acids (Mathiasen et al., 2014).

In this regard, latest advances in single-vesicle analysis (SVA) have enabled deeper investigation of heterogeneity within EVs subpopulations and characterizing them on nanoscale (Pick et al., 2018).

This, undoubtedly, reveals valuable information about EVs and their potential use in clinical applications (Bordanaba-Florit et al., 2021). Wherein, SVA can reveal molecular states that govern the function and transport of the EV; also, it provides us with direct information regarding the heterogeneous composition of individual EVs, and offers statistically valid data that are often lost in ensemble-based experiments (Pick, et al., 2018; Chiang, et al., 2019).

Besides the high heterogeneity, another important motive to adopt SVA for disease diagnosis rather than bulk EVs-based methods is, in some cases, the low abundance of disease-related EVs (such as tumor-derived EVs) compared with similar-sized lipoproteins and EVs from other non-diseased organs that are usually present with much higher abundance in biofluids (Enciso-Martinez et al., 2020).

Based on the above, many researchers have developed about 20 techniques for SVA (Pick, et al., 2018; Chiang, et al., 2019). Among these techniques, Raman spectroscopy, which depends mainly on inelastic scatterings from biomolecules bearded on or contained in a single EV or a few EVs, is considered a potentially promising one.

1.2. Background about Raman Spectroscopy

Molecules can exist in different vibrational states; simply, this is the basic idea behind Raman spectroscopy. In infrared (IR) spectroscopy, when a molecule absorbs the IR radiation with the exact frequency, it will be excited from the ground state (v_0) to the first excited state (v_1), but in Raman spectroscopy, something different will happen. When we shine the material under study with a laser source, it can excite molecules into a virtual state. The

virtual state is not a real energy level of the molecule. After a very short time interval, the molecule can scatter that light back and return into its original ground state. In this case, the scattered frequency ν_r will equal the frequency of the incident laser beam ν_L (this is called Rayleigh scattering, see **Figure 1**). The Rayleigh scattering involves no energy loss, thus it is called elastic scattering (Larkin, 2017).

The other scenario can happen while the molecule is in the virtual state. The molecule can absorb a part of the laser energy, and scatter the remaining with lower frequency. In this case, the molecule will not return to its original ground state, but to the first excited state, for example. The scattered light will have lower energy and smaller frequency than the incident laser light. This is called the Stokes scattering (see Figure 1). The frequency of Stokes scattering can be expressed by the following equation (Tkach et al., 2018)

$$\nu_s=\nu_L-\nu_{01} \quad , \tag{1}$$

where ν_s is the frequency of the scattered light (Stokes), ν_L is the frequency of the incident laser source, and ν_{01} is the frequency of transition between the ground and the first excited state. It is noteworthy that ν_L is known from the laser source; also, the spectrometer itself measures ν_s; so by solving the previous equation we can find ν_{01} which is characteristic for this molecule (Larkin et al., 2017).

It is also possible for the molecule to start from an excited state, for example the first excited state. So, the laser source will excite it to a higher virtual state than that in the first case, and from that higher virtual state, the molecule can scatter all the light back and return to its ground state. It is noteworthy that the scattered photon in this case will have higher energy than the laser light, because the molecule gave up some energy. This effect is called anti-Stokes scattering (see **Figure 1**). The frequency of anti-Stokes scattering can be expressed by the following equation

$$\nu_{as} = \nu_L + \nu_{01} \quad , \tag{2}$$

where ν_{as} is the frequency of the scattered light (anti-Stokes), ν_L is the frequency of the incident laser source, and ν_{01} is the frequency of transition between the ground and the first excited state (Larkin et al., 2017).

As in the Stokes scattering, we can find ν_{01}, which corresponds to the vibrational excitation characteristic of the molecule, and this is the same information we look for in IR spectroscopy.

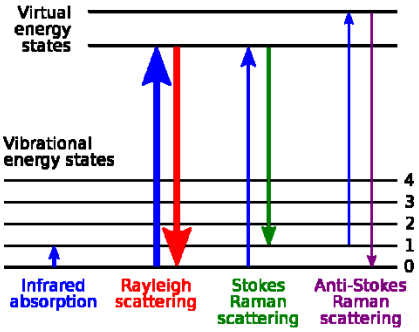

Figure 1. Schematic showing IR-absorption, Rayleigh scattering, Stokes and anti-Stokes scattering (Larkin et al., 2017)

Both Stokes and anti-Stokes lines are called Raman scattering. Raman spectroscopy yields complementary information to IR spectroscopy, as it allows the characterization of IR-inactive molecules (Larkin et al., 2017). Raman scattering is much less probable than Rayleigh scattering; and the relative probability of Stokes and anti-Stokes events is governed mainly by Boltzmann distribution. The energy of the inelastically scattered photons correspond to the chemical bonds present in the target and thus provides information regarding the presence, abundance, concentration, modifications environment of the molecules and their bi-dimensional and tri-dimensional structures (Rodriguez et al., 2006).

2. The basic principle of Raman spectroscopy-based SVA

Basically, an optical tweezer is an instrument that uses a highly focused laser beam to hold and move microscopic and submicroscopic particles such as nanoparticles (NPs), bacterial cells, blood cells and molecules such as DNA for a period sufficient to data acquisition for a single structure (Redding et al., 2015). In this context, laser tweezer Raman spectroscopy (LTRS), also known as Raman tweezer microspectroscopy (RTM), can trap an individual (or very few) vesicle in an aqueous medium, then excite them for Raman scattering which results in providing a characteristic vibrational fingerprint of surficial and internal proteins, lipids, nucleic acids and carotenoids of the single EV of interest. LTRS is considered the method of choice for globally studying the chemical and structural composition of the individually-trapped vesicles and monitoring variations in vesicle components with time (Tatischeff et al., 2012). The main advantage of this technique is the signal linearity, which allows both quantitative and qualitative characterization of a single vesicle. In addition, it requires no label and can provide a highly informative structural data (Tatischeff et al., 2012). On the other hand, the main disadvantage of LTRS is its low scattering efficiency that results in low Raman signal which highly extends the time required for data collection (Smith et al., 2015). However, the low strength of Raman signal can be compensated without losing information about single EV through using surface enhanced Raman spectroscopy (SERS).
In this context, SERS is a technique for enhancing Raman scattering from nanostructures. The scattering enhancement may be with a factor of up to 10^{11} that enables detection of a single molecules, thus characterization of a single EV, and eliminate the effect of high heterogeneity of EVs populations. (Blackie et al., 2009). In SERS, EVs are exposed to signal-enhancing substrates and/or nanoparticles to obtain an enhanced biomolecular signature (Stremersch et al., 2016). The mechanism of this enhancement is still under debate; however, there are two main theories that have interpreted this enhancement: the electromagnetic theory and the chemical theory. The electromagnetic theory proposes the excitation of localized surface plasmons, while the chemical theory proposes formation of charge-transfer complexes. (Blackie et al., 2009; Barbiellini, 2017).
Although label-free SERS can resolve the main drawback of LTRS, it brings another limitation, wherein this enhancement is highly dependent on the distance between the biomolecule and the substrate/nanoparticle, to the extent that it vanishes when this distance exceeds just a few nanometers (Cialla et al., 2014). So, this technique is mainly suitable for surfacial characterization of EVs (Bordanaba-Florit et al., 2021). Another drawback is that not all Raman modes are enhanced to the same extent, wherein those corresponding to molecular vibrations that are at right angles to the SERS surface are preferably enhanced (Cialla et al., 2014). This results in distortion of Raman spectrum and a relative difficulty in its interpretation (Bordanaba-Florit et al., 2021).

3. How Raman spectroscopy works for SVA?

3.1. Raman Microspectroscopy in Suspension

Raman microspectroscopy uses a Raman spectrometer integrated with the base of an upright optical microscope, such that a single laser beam is used for both trapping and excitation of the suspended EVs contained in a coverable glass-slide well (see **Figure 2A**). Raman scattering is collected using the objective lens that is corrected for the cover. Raman scattering is then detected using a CCD camera. Moving the objective along the *z*-axis allows focusing the laser focal spot inside the EVs suspension. The vesicular Brownian motion in the suspension is sufficient to bring them in a close proximity to the optical trap (i.e. the laser focal spot), where the net trapping force is enough to direct the EV to the focal spot (see **Figure 2A**). As long as the laser shutter is open, cumulative trapping of more vesicles occurs, until saturation of the spot volume. Once the first vesicle is trapped, it is defined through its Rayleigh spectrum, and the corresponding Raman spectrum is synchronously detected, which reveals the chemical composition of this EV. Extended monitoring time enables us to discriminate between the absence of any EVs (baseline), first individually trapped EV (1st step), and the successive trapping events. Regularly automated

clearance of laser spot, by closing the shutter, enables detection of many trapping events of single EVs (Enciso-Martinez et al., 2020a; Enciso-Martinez et al., 2020b). Using Raman spectroscopy for assessment of EVs was reviewed in (Lee et al., 2021).

An important advantage of this technique ***is*** the possibility to distinguish EVs from lipoproteins that are in the same size range, and to discriminate between various EVs from different sources (Rikkert et al., 2020). For example, EVs reveal their characteristic peaks at 1004 and 1607 cm^{-1} (Phenylalanine) in addition to their larger protein contribution at 2811 – 3023 cm^{-1} than lipoproteins (black lines in **Figures 2B & 2C**) (Enciso-Martinez et al., 2020a). Enciso-Martinez, et al. (2020a) could differentiate between individual EVs that were derived from two different prostate cancer cell lines, as shown in **Figures 2B & 2C**, i.e. PC3-cell line (blue) and LNCaP cell-line (green) and those derived from red blood cells (red).

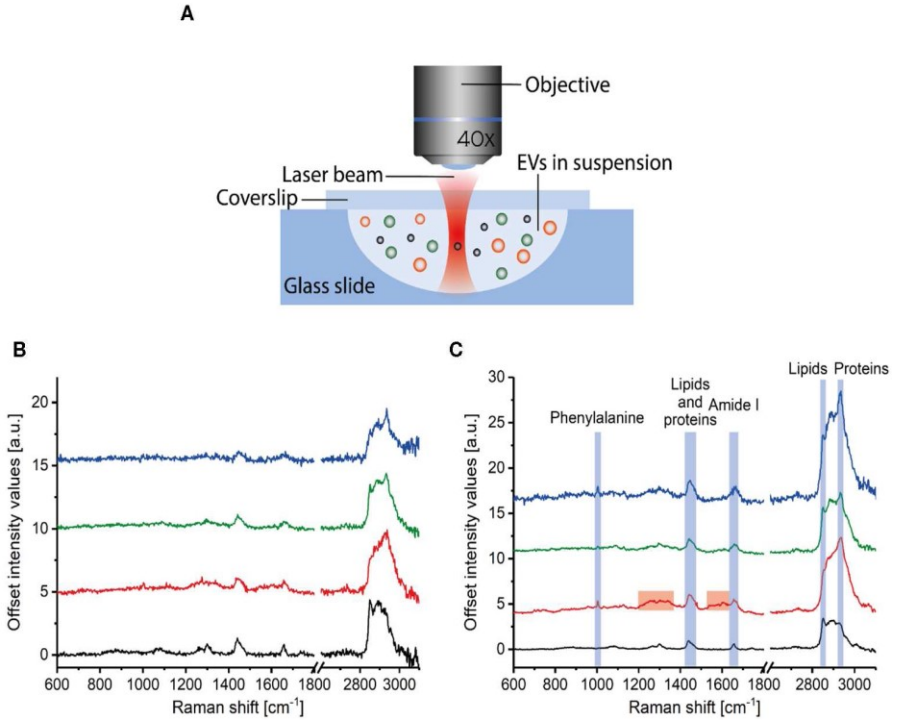

Figure 2. (A) EVs suspension loaded in the weel slide and mounted under the light microscope objective. Raman spectra correspond to single (B) and multiple (C) EVs derived from PC3-cell line (blue), LNCaP-cell line (green), red blood cells (red) and lipoproteins (black). (Enciso-Martinez et al., 2020a)

3.2. Lab-on-Chip device based Raman spectroscopy for SVA

Another interesting study adopted a lab-on-chip device for collecting Rayleigh and Raman spectra. This technique makes the advantage of the higher field gradient that results from coherently combining multiple laser beams that constructively interfere at certain spots on the chip (see **Figure 3**). Each spot serves as an optical trap for a single sub-micrometer EV; this enables trapping of smaller individual EVs for inducing Raman spectrum with the same trapping light (Rikkert et al., 2020).

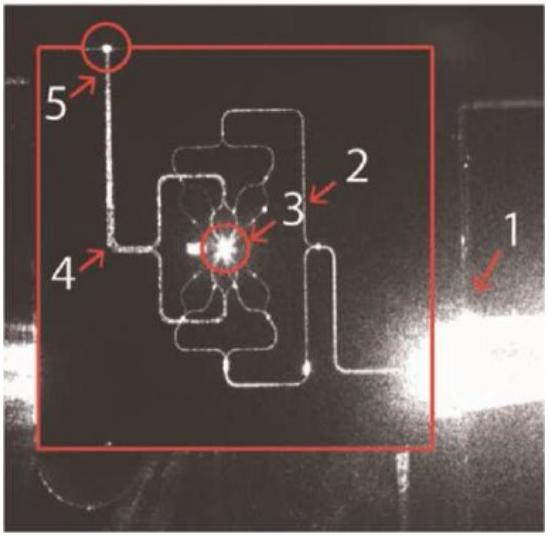

Figure 3. Lab-on-chip-based Raman spectroscopy. The device is operated with the light from an input fiber that is embedded in a fiber array unit (FAU) at the lower right-hand side. The solid red lines indicate the chip edges. 1, FAU; 2, excitation-waveguide circuitry; 3, microfluidic bath with the central trapping region; 4, detection-waveguide circuitry; 5, light from the trap that is coupled out by the detection waveguides. Here, the detection waveguides collect light as a result of direct illumination and scattering (Rikkert et al., 2020).

4. EVs-based diagnosis

The idea of using EVs in disease diagnosis is based on assessment of the disease-related EV (sub)population(s). EVs are isolated from their respective bio-fluids, getting their characteristic fingerprint using Raman spectroscopy, thus figuring out the presence, absence or progression of the disease of interest. In this context, it is noteworthy that the EVs-based diagnosis was already studied for some diseases; whereas among these, cancers diagnosis got the most attention.

4.1. Cancer

Among the first pioneering works, SERS was performed by Stremersch et al. in 2016 for discrimination between cancerous and normal cells. Gold NPs were deposited directly on the EVs and were used as a SERS probe. The researchers could optimize the main SERS parameters, including optimal EVs: NPs ratio and their incubation time for characterization of individual EVs and identifying it with specificity and sensitivity ranged from 92 to 100% (Stremersch et al., 2016).

In 2017, Park et al. deposited EVs collected from normal and cancerous cells on a precipitated gold NPs substrate for SERS experiment. The identification specificity and sensitivity ranged from 95 to 97%, but with the advantage of providing a reusable bio-sensing substrate (Park et al., 2017).

Another interesting approach integrated both immunoassay and Raman spectroscopy, wherein SERS analysis was also used successfully to identify pancreatic cancer-related EVs with amplifying the Raman signal through sandwiching the immunoassay with NPs that highly surplused the traditional ELISA (Li et al., 2018).

4.2. Skeletal Muscle Diseases

Some other pathologies, such as skeletal muscle diseases, have gained high interest in many preliminary studies that have addressed biogenesis and various roles of EVs in pathophysiology of skeletal muscle atrophy (Wang et al., 2022). Also, a recent proteomics-based study demonstrates that skeletal muscle (SKM)-interstitium EVs display unique protein and miRNA profiles that are distinct from plasma EVs. This study has defined some potential marker proteins for SkM-EVs including ATP2A1, β-enolase, calsequestrin

2, caveolin-3, and desmin. Furthermore, the same study shows that four micro RNAs (miRNAs) (including miRs-1, -206, -431, and -486), that are abundantly expressed in muscles, are significantly concentrated in the interstitium EVs. In particular, miR-1 and miR-206 in the interstitium EVs were 45- and 20-fold higher than those in plasma EVs, respectively. Thus the presence and/or relative abundance of these markers can be targeted for Raman fingerprinting-based diagnosis of skeletal muscle diseases in near future (Watanabe et al., 2022).
Positive correlation between muscle protein synthesis and degradation and muscle mass during disuse atrophy and regrowth, and the serum-EVs miR-203a-3p content was obtained by Van Pelt et al. (2020) indicating the potential of targeting serum-EVs miR-203a-3p as a biomarker for monitoring and diagnosing muscle mass and protein turnover-related diseases. In this regard, miRNAs transferable via EVs are considered key mediators for many skeletal muscle processes including their development, regeneration, functioning, and diseases. This makes miRNAs potential biomarkers for skeletal muscle disease diagnosis (Wang H and Wang B, 2016; Xu et al., 2022).
Raman spectroscopy was used in many studies for diagnosis of skeletal muscle diseases based on the whole cellular composition (Fosca et al., 2022). However, to the best of my knowledge, no studies were reported so far about using Raman spectroscopy for targeting SKM-EVs for diagnosis of SKM-disorders. The aforementioned preliminary studies, among many others, reveal some potential SKM-EV biomarkers that may be targeted for diagnostic detection using Raman fingerprinting in future.
It is worth mentioning that Raman spectroscopy was used successfully in many studies for direct in situ detection of miRNA, with detection limit reached in a recent study to 0.21 fM that is similar or even better than polymerase chain reaction (PCR), but simpler, faster, less invasive, non-destructive and less expensive (Fosca et al.,2022; Cao et al., 2017; Driskell et al., 2008). This, undoubtedly, opens doors for optimizing Raman spectroscopy in diagnostic detection of single EVs-related markers, including miRNas and proteins, in clinics.

Furthermore, it is worth mentioning that many papers have reported that EVs usually carry bioactive molecules that are significant and related to other human pathologies such as hepatopathologies (Balaphas et al., 2019), neuropathologies (Shaimardanova et al., 2020) and cardiovascular disorders (Osteikoetxea et al., 2016).

5. EVs-based diagnosis from laboratory to clinics

5.1. Single EV for disease diagnosis - Is it sufficiently mature?

Generally, using EVs for disease diagnosis faces many challenges. Basically, the identification and isolation of the disease-related vesicles from complex bio-fluids is the first and most important prerequisite. In this context, unfortunately, there are no standardized isolation and characterization protocols for different types of samples and diseases so far. This complicates the potential application of EVs in clinics (Bordanaba-Florit et al., 2021).
Another challenge arises, in my opinion, from the uncertainty that the compositional characteristics of each EV reflect the compositional properties of its parental cell. Since recent studies have shown that there are many subpopulations of EVs, with distinguished functions, originating from the same parental cell type (Bordanaba-Florit et al., 2021).
So, in my opinion, what is more critical for EVs-based diagnosis than just isolation and characterization of EVs is studying and determining the physiological or pathophysiological conditions under which the same parental cell type produces a certain (sub)population(s) of EVs. Thus, we can correlate a certain (sub)population(s) of EVs or even the relative abundance of (sub)populations to the initiation or progression of the disease.

5.2. Is Raman spectroscopy a reliable characterization technique for EVs?

In principle, Raman spectroscopy has many advantages for detecting structural characteristics of biomolecules, wherein it ensures high degree of chemical specificity, rapid analytical process because sample usually requires little or even no preparation, in addition to being harmless to biological samples, and above all it is inert to the aqueous background.

(Bordanaba-Florit et al., 2021). However, the experimental settings for applying this technique still need further optimization to be suitable for a real scenario in clinic; (Bordanaba-Florit et al., 2021) for instance, although the enhanced Raman signals provide high specificity and sensitivity, there are some limitations with regard to signal modifications under the effect of the used nanostructures. Thus, it still needs more standardization of procedures (Min et al., 2021).

So, there is a greater chance for Raman spectroscopy to be optimized for clinical applications through introduction of advanced computational approaches that can decrypt the complex information provided by Raman spectrum (Gualerzi et al., 2021).

Conflicts of Interest: The author declares no conflict of interest.

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Invited lecture/Scientific contribution

Altering the Position of Topological Defects in Nematic Shells

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Abstract:
Topological defects (TDs) in liquid crystals may have their locations experimentally altered by locally distorting the liquid crystalline (LC) order, e.g., by the melting induced by optical tweezers. In this research, we investigated the nematic ordering profiles and accompanying topological defect configurations in thin nematic liquid crystalline shells that are subject to externally forced local LC order distortions. We show that inside curved LC films these manipulations are greatly influenced by local Gaussian curvature if it displays strong spatial variability. We use a mesoscopic model in which the curvature of the surface and the nematic order parameter tensor serve to explain the shell geometry and LC orientational order. We demonstrate that TDs are rather tightly "glued" to a local Gaussian curvature on increasing the prolateness of shells.

Citation: Mesarec L, Iglič A, Kralj S. Altering the position of topological defects in nematic shells. Proceedings of Socratic Lectures. 2023, 8; 126-130. https://doi.org/10.55295/PSL.2023.I18

Keywords: Topological defects; Liquid crystals; Distortions; Gaussian curvature; Prolate shells

1. Introduction

Localized deformations in a physical field known as topological defects (TDs) are protected by topology (Mermin, 1979) and are of interest to all branches of physics due to their interdisciplinary nature (Zurek, 1985). The topological charge of TDs is their essential property, which is conserved (Mermin, 1979; Volovik, et al. 1983), thus governing transformations between different defect arrangements, such as merging and splitting (Svenšek, et al. 2004; Kralj, et al. 2017). Nematic liquid crystalline (LC) shells provide an ideal platform for studying the impact of topology and geometry on TDs (Nelson, 2002; Vitelli, et al. 2006; Skačej, et al. 2008; Lopez-Leon, et al. 2011; Rosso, et al. 2012). These shells are composed of thin nematic films that cover micrometer-sized colloidal objects and have a typical molecular length thickness.

Anisotropic LC molecules, such as rod-like molecules, form the basis of the simplest nematic LCs (Kleman, et al. 2003). These materials have both liquid-like properties and orientational order, which is described at the mesoscopic level by the nematic director field $\vec{\boldsymbol{n}}$, indicating the average molecular direction in the local region. Nematic shells are effectively two-dimensional (2D) systems, with the $\vec{\boldsymbol{n}}$ orientations confined within a curved 2D film (Nelson, 2002), which generally results in the domination of topological defects (TDs) in such structures.

Topological defects (TDs) in 2D nematic films are identified by their winding number $\boldsymbol{m}$, which can take on half-integer values due to the $\pm\vec{\boldsymbol{n}}$ invariance. This number describes the number of rotations of $\vec{\boldsymbol{n}}$ on encircling by any path the defect center counterclockwise. Defects with positive and negative values of $\boldsymbol{m}$ are referred to as defects and antidefects, respectively (Poincaré, 1886; Kamien, 2002).

Softness (strong responsivity to local stimuli) is a crucial feature of liquid crystals. Researchers have demonstrated that nematic TDs can be efficiently manipulated using laser beams (Nych, et al. 2017; Tkalec, et al. 2011; Liu, et al. 2013; Smalyukh, 2020), as the beam can locally melt the orientational order. Since orientational order is melted also within the core of defects, it is advantageous for TDs to be assembled within regions where the nematic order is reduced, as the penalty for forming the defect core is reduced. However, we demonstrate in this work that on effectively two-dimensional curved surfaces, the manipulation of nematic TD positions by laser beams is limited as TDs are relatively strongly attached to a local Gaussian curvature.

2. Methods

We utilize mesoscopic modeling to characterize the shapes of two-dimensional curved surfaces and the nematic ordering within them, employing the curvature tensor $\underline{C}$ and the nematic order tensor Q to describe the system's properties (Rosso, et al. 2012). The Weingarten curvature tensor $\underline{C}$ determines the local surface curvature:

$$\underline{C} = C_1 \ \vec{e_1} \ \otimes \vec{e_1} + C_2 \vec{e_2} \otimes \vec{e_2} \ , \tag{1}$$

where the unit vectors $\{ \vec{e_1}, \vec{e_2}\}$ are oriented along the surface principal directions exhibiting principal curvatures $\{C_1, C_2\}$. The local mean curvature H and the Gaussian curvature K can be calculated as:

$$H \coloneqq \frac{Tr[\underline{C}]}{2} = \frac{C_1+C_2}{2}, \ K \coloneqq Det[\underline{C}] = C_1 C_2. \tag{2}$$

The local nematic orientational order on the surface is characterized by the two-dimensional tensor order parameter $\underline{Q}$ (Kralj, et al. 2011). The molecules exhibiting orientational ordering are required to lie in the local tangent plane of the surface but are otherwise unrestricted. We assume rod-like molecules with head-to-tail invariance. Tensor $\underline{Q}$ can be represented in diagonal form as follows (Kralj, et al. 2011):

$$\underline{Q} = \lambda(\vec{n} \otimes \vec{n} - \vec{n}_\perp \otimes \vec{n}_\perp), \tag{3}$$

where $\vec{n}$ and $\vec{n}_\perp$ are its unit eigenvectors, while $\lambda \in [0, 1/2]$ and $-\lambda$ are the corresponding eigenvalues. When λ is equal to zero, the system is in a locally isotropic state with no orientational order. In contrast, when λ is equal to 1/2, the system is in a locally

ordered state where the molecules are rigidly aligned in the direction of the nematic director field $\vec{n}$.

The total free energy functional of the LC shell surface is given by $F = \iint f \mathrm{d}^2 r$, where the free energy density $f = f_c + f_e$ is the sum of the order condensation (f_c) and elastic (f_e) terms (Kralj, et al. 2011; Mesarec, et al. 2016). To illustrate the features of interest, we utilize a minimal model and express the nematic elasticity in terms of a single elastic constant k. The energy densities are expressed as

$$f_c = -\alpha Tr Q^2 + \beta (Tr Q^2)^2, \tag{4a}$$

$$f_e = k \mathrm{Tr}((\nabla_s Q)^2). \tag{4b}$$

Material constants α and β must take positive values to enable orientational ordering in the system. The order parameter correlation length ξ, which depends on the material properties, is defined as $\xi = \sqrt{k/|\alpha|}$. This parameter estimates the distance at which a local perturbation in the order parameter relaxes on a flat surface. We introduce R as the radius of the sphere with the same surface area as the surface area of the investigated shell. The bulk equilibrium value of the order parameter in flat geometries is $\lambda_0 = \sqrt{\alpha/\beta}/2$.
To model the laser beam, we implemented a boundary condition that locally melts the orientational order. We enforce the melting process by setting $\lambda = 0$ at certain points, while calculating the orientational ordering by minimizing the total free energy at all other points.

3. Results

We are studying how to manipulate topological defects (TDs) in nematic shells using laser-induced local distortions, which cause the nematic order to melt locally (Mesarec, et al. 2022). In our simulations, we simulate these distortions by varying the position of a melted region within prolate shells. It is well-established that local melting attracts TDs in the nematic phase (Nych, et al. 2017; Tkalec, et al. 2011; Liu, et al. 2013; Smalyukh, 2020), as both melting and TDs introduce a strong energy penalty. Since the core of a TD is essentially melted, the total energy penalty is generally reduced when the melted region and TD are in the same location. Therefore, we refer to a melted region as a distortion.
We are examining prolate shells that have a distinct spatial dependency in their Gaussian curvature (Mesarec, et al. 2022). On such shells, topological defects (TDs) tend to be closely situated near the poles where Gaussian curvature has the highest value. In Figure 1a, we show that in the absence of distortion, TDs are found close to the poles. When a distortion is introduced near the lower pole, as shown in Figure 1b, we can observe manipulation of the relative position of the TDs in the lower part of the prolate shell. One TD remains fixed within the distortion while the other shifts based on the interaction between the defects and the local Gaussian curvature. The positions of the remaining two TDs remain unchanged, indicating that their placement is mainly influenced by the local Gaussian curvature and their mutual elastic repulsion (Mesarec, et al. 2022). Similar phenomenon occurs in Figure 1c, where a distortion is again introduced near the region with high Gaussian curvature but slightly higher than in Figure 1b. If the distortion is moved outside of the area where the Gaussian curvature is high, the trapped defect is released and the defect configuration preferred by the Gaussian curvature is restored, as shown in Figure 1d. In this case, the distortion does not contain a topological defect (Mesarec, et al. 2022).

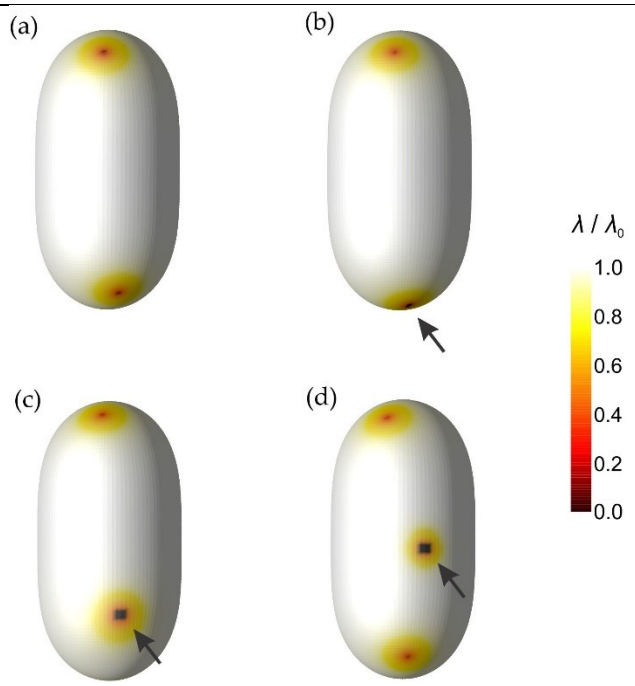

Figure 1. Equilibrium nematic ordering configurations on a prolate shell. Case without the laser beam (distortion) is presented in the panel (a), while panels (b,c,d) represent cases with different positions of the distortion (denoted by arrows). The shell shapes are presented with the superimposed nematic order parameter profiles λ. $R/\xi = 10$. Partially adapted from (Mesarec L, et al. 2022).

4. Discussion

Our study focused on manipulating TDs in nematic shells with spherical topology. We simulated the effects of introducing a localized melted region, or distortion, on the spatial distribution of TDs. Our results show that the response to distortions depends on the spatial dependence of the Gaussian curvature (Mesarec, et al. 2022). When the Gaussian curvature has a strong dependence, e.g., on prolate shape, the distortion can affect the position of TDs near poles where Gaussian curvature is high, but it cannot move TDs to the regions with low Gaussian curvature because they are strongly attracted by the regions with high Gaussian curvature. Furthermore, introducing a distortion near a certain pole on a prolate shape does not affect the TD distribution on the opposite pole (Mesarec, et al. 2022). This ability to manipulate TDs opens opportunities for various applications, such as trapping nanoparticles within their cores (Kikuchi, et al. 2002; Karatairi, et al. 2010) and forming micron-sized crystal structures (Nelson, 2002).

Funding: The authors acknowledge the support of a Slovenian Research Agency (ARRS) grants P1–0099, P2-0232, J1-2457 and J3-3066.

Institutional Review Board Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Invited lecture/Scientific

Reconfiguration of Nematic Dislocations

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Abstract:
Basic natural entities seems to be physical fields. From this perspective elementary particles should correspond to robust localized field configurations. Most probable candidates for such configurations are topological defects. They are topologically protected and they exhibit robust body-like features. Particularly adequate structures are line defects which could display also linked or knotted configurations. Such structures could be relatively easily created, manipulated and observed in nematic liquid crystals. In this contribution we focus on nematic elementary line defects characterised by winding number |m|=½. We illustrate that they behave as line-like robust elastic objects. However, they could be reconfigured into qualitatively different conformations where topological conservation rules are obeyed.

Keywords: Fields; Topological defects; Topological charge; Disclinations; Liquid crystals

Citation: Harkai S, Kralj M, Kralj S. Fields, Reconfiguration of nematic dislocations. Proceedings of Socratic Lectures. **2023**, 8; 132-139. https://doi.org/10.55295/PSL.2023.I19

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1. Introduction

Recent decades evidences that topology dominates natural behavior. It seems that key ideas introduced by Greeks and in particular by Einstein are correct: all fundamental laws of physics could be interpreted in terms of geometry. It might well be that all integers present in nature have a topological origin, i.e., they embody a topological invariant.
Topology (Kamien, 2022) deals with systems' properties which remain conserved in continuous geometrical or physical field transformations. These properties are reflected in topological invariants which are countable, discrete and conserved entities. Note that conserved quantities form the foundation of our physical understanding of the world. Topological properties reflect a global system's property, which is consequently robust and in general insensitive to local system's configurational changes. For example, a topological equivalence relates a coffee cup (Fig. 1a) and a doughnut, i.e. torus (Fig. 1b): both have in common one hole. The latter represent the topological invariant g (the so called *genus*) where g=1 fingerprints the toroidal topology.

Topological phenomena are relatively well understood in effectively two-dimensional (2D) manifolds (Singer, 1982) where mathematical treatment is relatively simple. Here manifold refers to a topological space, that is in general curved, that resembles Euclidian space near each point. Hence, n-dimensional curved manifold has a neighborhood that could be continuously morphed to the n-dimensional Euclidian space. For example, Gauss-Bonnet and Poincare-Hopf theorems (Kamien, 2022) relate the integrated Gaussian curvature of a closed 2D surface within a 3D system with the surface's Euler characteristics $\chi = 2(1-g)$ and the total winding number m of the ordering field within the manifold: $\chi = m$. Here m is the conserved topological invariant. It is also referred to as the 2D topological charge of topological defects (TDs) (Mermin, 1979) within the ordering field hosted by the 2D curved manifold. TDs refer to localized topologically protected distortions in a physical field. The key message conveyed by the theorems is illustrated in Fig. 1c, where the 2D manifold exhibits spherical geometry (represented by g=0 and $\chi = 2$) enforcing two m=1 point defects at the poles in the "axial" ordering field, where the total winding of the manifold equals to two. The two "charge one" point defects resemble point-like bodies. Note that the theorems can be generalized to other abstract (Ramirez and Skinner, 2020) 2D manifolds (e.g., the 1st Brillouin zone surface in crystals). Furthermore, topological concepts developed in 2D, where mathematics could be visualized, could be transferred to higher dimensional manifolds (Singer, 1982)

Above listed theorems are at the heart of the quantum hall effect (Ramirez and Skinner, 2020), representing one of the pioneering discoveries via which topology entered the world of physics, where it might soon become the "queen". Namely, the recent discovery of the Higgs particle confirmed the existence of the Higgs field which supports the viewpoint that physical fields represent fundamental natural entities (Hobson, 2013). This perspective suggests that TDs might embody "particles" of the standard model of physics. Note that such vortex-type theory was first proposed by lord Kelvin (Thomson, 1867) who claimed that atoms (at that time atoms were treated as fundamental particles) are topologically protected knots in the respective physical field. Such simplest knot members are illustrated in **Figures 1d,e,f**. Indeed, "tying a knot" is a metaphor for creating stability. Knots are sturdy in structure and tangled configurations persist much like a knot tied in a shoelace. Along this line of reasoning Skyrme (1962) modelled structures of hadrons and mezons as soliton excitations in the pion-field, where he stabilized these excitations by imposing rather artificial constraints. Topologically related structures (the so called skyrmions) were afterwards predicted or even observed in several other systems, including Quantum Hall magnetism (Brey et al., 1995), Spinor Bose-Einstein condensates (Ho, 1998), helical ferromagnets (Rössle et al., 2006), LC Blue Phases (Meiboom et al, 1981) to mention few of them. A typical 2D skyrmion winding configuration of magnetic skyrmion is depicted in **Figure 1g**. Recent theoretical studies suggest that such 2D structures could be in 3D twisted into complex linked and knotted objects. For example, **Figure 1h** illustrates a "nanoknot" in magnetization field (Sutcliffe, 2017). Similar structures could be realised in optical vortex configuration (Shen et al., 2023). Related knotted and linked topologically

protected structures are present in diverse tube-like structures in nature and seem to be a generic feature of complex pattern close to phase transitions and at the edge-of chaos conditions (Johnson, 2021). They appear at wide range of length scales, e.g., in the range $10^{-10} - 10^{-6}$m in superfluid vortices, $10^{-2} - 10^{2}$ m in fluid eddies and tornados, $10^{6} - 10^{10}$ m in magnetic flux tubes in universe…

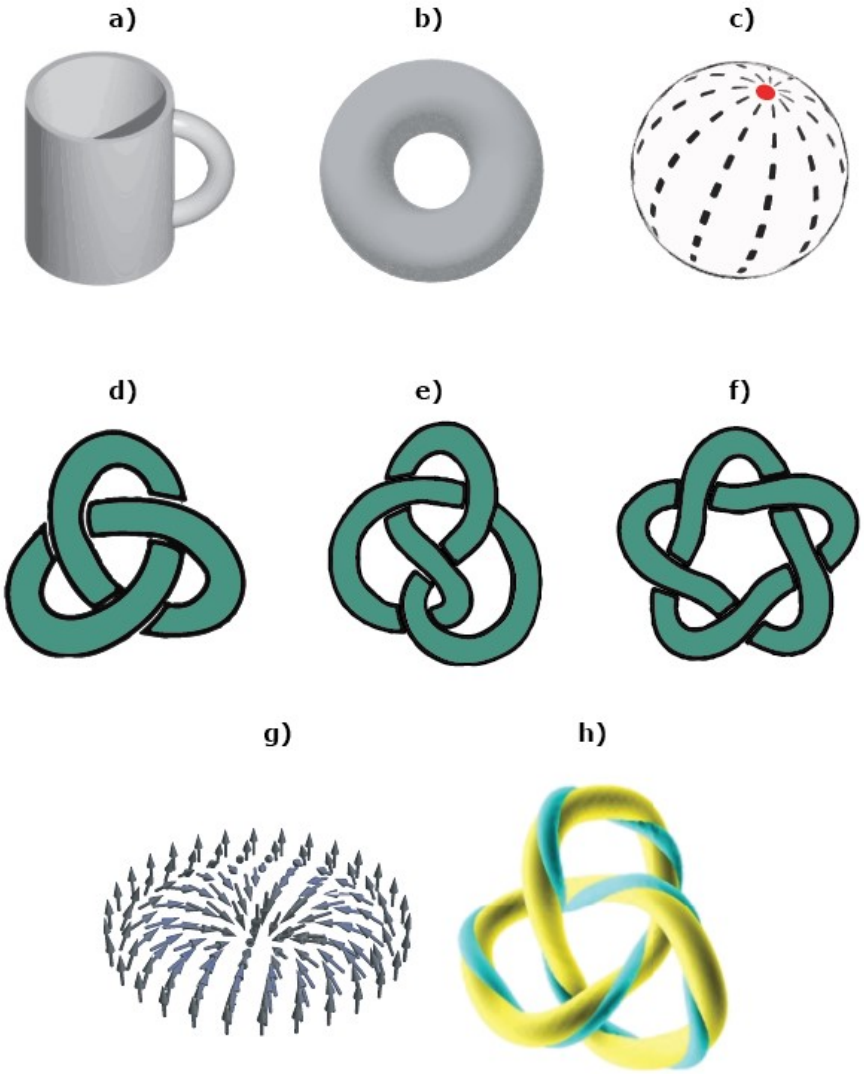

Figure 1. A cup of coffee (a) is topologically equivalent to a torus (b). (c): A sphere hosting a vector field inevitably exhibits topological defects. (d), (e), (f): topologically different knots. (g): 2D skyrmion. (h): A knot in the magnetization vector field.

Particularly adequate systems to carry out controlled and systematic studies of TDs are liquid crystals (LCs). They possess a unique and extraordinary combination of liquid character, crystalline order, softness (i.e. capability to exhibit strong responses even to weak local stimuli), complexity, and optical anisotropy. Owing to these features a rich diversity of TDs could be easily excited, stabilized, manipulated, and observed using relatively simple optic methods (e.g., using polarizing microscopy and laser tweezers). Consequently, LCs provide an excellent testbed system to reveal key features of TDs. Furthermore, TDs

in LCs could serve in various future applications, particularly in photonics and information storage and manipulation.
In this contribution we study configurational transformations of pairs of line defects in nematic LCs confined to a plane parallel cell. We illustrate that in general different reconfiguration channels exist and consequently collided pairs of line defects could in general exhibit qualitatively different post-collision configurations.

2. Methods

Nematic LC phase exhibits long range uniaxial orientational order which is in bulk equilibrium spatially homogeneously aligned along a symmetry breaking direction. At the mesoscopic level it is in general described by the tensor order parameter $\underline{\boldsymbol{Q}}$. In terms of its eigenvectors $\vec{\boldsymbol{e}}_{\boldsymbol{i}}$ and eigenvalues $\boldsymbol{\lambda_i}$ it can be expressed as (Meiboom et al, 1981; Harkai et al., 2020)

$$\underline{\boldsymbol{Q}} = \sum_{i=1}^{3} \boldsymbol{\lambda_i} (\vec{\boldsymbol{e}}_{\boldsymbol{i}} \otimes \vec{\boldsymbol{e}}_{\boldsymbol{i}}) \ . \tag{1}$$

This parametrization allows both uniaxial and biaxial states. In the former state. it is conventionally expressed with the nematic director field $\vec{\boldsymbol{n}}$ and the nemaic uniaxial order s as $\underline{\boldsymbol{Q}} = \boldsymbol{s}\left(\vec{\boldsymbol{n}} \otimes \vec{\boldsymbol{n}} - \frac{\boldsymbol{1}}{\boldsymbol{3}}\underline{\boldsymbol{I}}\right)$. The unit vector $\vec{\boldsymbol{n}}$ points along the local uniaxial direction where the states $\pm\vec{\boldsymbol{n}}$ are physically equivalent. Furthermore, the amplitude field $\boldsymbol{s} \in \left[-\frac{\boldsymbol{1}}{\boldsymbol{2}}, \boldsymbol{1}\right]$ determines the degree of anisotropic order, where s>0 (s<0) reflects prolate (oblate) uniaxial order. Biaxial states could be established at least locally if LC order is distorted. In our simulations, we express $\underline{\boldsymbol{Q}}$ in the Cartesian coordinate frame $(\vec{\boldsymbol{e}}_{\boldsymbol{x}}, \vec{\boldsymbol{e}}_{\boldsymbol{y}}, \vec{\boldsymbol{e}}_{\boldsymbol{z}})$ as $\underline{\boldsymbol{Q}} = (\boldsymbol{q_1} + \boldsymbol{q_2})(\vec{\boldsymbol{e}}_{\boldsymbol{x}} \otimes \vec{\boldsymbol{e}}_{\boldsymbol{x}}) + (\boldsymbol{q_1} - \boldsymbol{q_2})(\vec{\boldsymbol{e}}_{\boldsymbol{y}} \otimes \vec{\boldsymbol{e}}_{\boldsymbol{y}}) - \boldsymbol{2q_1}(\vec{\boldsymbol{e}}_{\boldsymbol{z}} \otimes \vec{\boldsymbol{e}}_{\boldsymbol{z}}) + \boldsymbol{q_3}\left((\vec{\boldsymbol{e}}_{\boldsymbol{x}} \otimes \vec{\boldsymbol{e}}_{\boldsymbol{y}}) + (\vec{\boldsymbol{e}}_{\boldsymbol{y}} \otimes \vec{\boldsymbol{e}}_{\boldsymbol{x}})\right) + \boldsymbol{q_4}\left((\vec{\boldsymbol{e}}_{\boldsymbol{x}} \otimes \vec{\boldsymbol{e}}_{\boldsymbol{z}}) + (\vec{\boldsymbol{e}}_{\boldsymbol{z}} \otimes \vec{\boldsymbol{e}}_{\boldsymbol{x}})\right) + \boldsymbol{q_5}\left((\vec{\boldsymbol{e}}_{\boldsymbol{y}} \otimes \vec{\boldsymbol{e}}_{\boldsymbol{z}}) + (\vec{\boldsymbol{e}}_{\boldsymbol{z}} \otimes \vec{\boldsymbol{e}}_{\boldsymbol{y}})\right)$. Quantities $\{\boldsymbol{q_1}, \boldsymbol{q_2}, \boldsymbol{q_3}, \boldsymbol{q_4}, \boldsymbol{q_5}\}$ are variational parameters. This parametrization allows both uniaxial and biaxial states.

In our study we consider nematic order within a plane-parallel cell of thickness h. The identical confining plates are placed at z=0 and z=h. We prescribe orientational order at these plates. In practice this can be realized, e.g., using AFM scribing method. At the lateral sides we impose the free boundary condition. Nematic order within the cell is calculated by minimizing the nematic free energy.

The free energy $\boldsymbol{F}$ of the system is determined by the integral of the free energy density over the LC body: $\boldsymbol{F} = \int \boldsymbol{f} \boldsymbol{d^3 r}$, where (Meiboom et al., 1981; Harkai et al., 2020)

$$\boldsymbol{f} = \frac{\boldsymbol{1}}{\boldsymbol{2}}\boldsymbol{A_0}(\boldsymbol{T} - \boldsymbol{T^*})\mathbf{Tr}\left(\underline{\boldsymbol{Q}}^{\boldsymbol{2}}\right) - \frac{\boldsymbol{1}}{\boldsymbol{3}}\boldsymbol{B}\mathbf{Tr}\left(\underline{\boldsymbol{Q}}^{\boldsymbol{3}}\right) + \frac{\boldsymbol{1}}{\boldsymbol{4}}\boldsymbol{C}\mathbf{Tr}\left(\underline{\boldsymbol{Q}}^{\boldsymbol{2}}\right)^{\boldsymbol{2}} + \frac{\boldsymbol{1}}{\boldsymbol{2}}\boldsymbol{L}\left|\boldsymbol{\nabla}\underline{\boldsymbol{Q}}\right|^{\boldsymbol{2}} \ . \tag{2}$$

Quantities $\boldsymbol{A_0}$, $\boldsymbol{B}$, and $\boldsymbol{C}$ are material constants, $\boldsymbol{T^*}$ is the supercooling temperature of the isotropic phase, and $\boldsymbol{L}$ is the representative nematic elastic constant in the single elastic constant approximation. Note that we use the minimal model to simulate phenomena of our interest.

We introduce the dimensionless temperature $\boldsymbol{r} = (\boldsymbol{T} - \boldsymbol{T^*})/(\boldsymbol{T^{**}} - \boldsymbol{T^*})$, where $\boldsymbol{T^{**}} = \boldsymbol{T^*} + \boldsymbol{B^2}/(\boldsymbol{24A_0C})$ is the superheating temperature, introduce scaled order parameter $\underline{\tilde{\boldsymbol{Q}}} = \underline{\boldsymbol{Q}}/\boldsymbol{s_0}$, where $\boldsymbol{s_0} = \boldsymbol{B}/(\boldsymbol{4C})$, and we scale distances with respect to cell thickness $\boldsymbol{h}$. The resulting dimensionless free energy density reads (Harkai et al., 2020)

$$\tilde{\boldsymbol{f}} = \frac{\boldsymbol{r}}{\boldsymbol{6}}\mathbf{Tr}\left(\underline{\tilde{\boldsymbol{Q}}}^{\boldsymbol{2}}\right) - \frac{\boldsymbol{2}}{\boldsymbol{3}}\mathbf{Tr}\left(\underline{\tilde{\boldsymbol{Q}}}^{\boldsymbol{3}}\right) + \frac{\boldsymbol{1}}{\boldsymbol{8}}\mathbf{Tr}\left(\underline{\tilde{\boldsymbol{Q}}}^{\boldsymbol{2}}\right)^{\boldsymbol{2}} + \left(\frac{\boldsymbol{\xi_b}}{\boldsymbol{h}}\right)^{\boldsymbol{2}}\left|\tilde{\boldsymbol{\nabla}}\underline{\tilde{\boldsymbol{Q}}}\right|^{\boldsymbol{2}}. \tag{3}$$

Here $\xi_b = 2\sqrt{LC}/B$ is the bare biaxial correlation length and $\tilde{\nabla} = h\nabla$. The minimization of the free energy is performed numerically deep inside the nematic phase, far below T^*.

3. Results

We analyse collisions of nematic line defects. 3D nematic LCs could display elementary line defects in orientational order (the so called disclinations) exhibiting winding number $m = \pm 1/2$. This quantity is a topological invariant and is in 2D LCs referred to as the 2D topological charge. It fingerprints the total reorientation of the principal $\underline{Q}$-eigenvector $\vec{e}_1$ on encircling by any path the defect center counterclockwise. Note that for uniaxial states it holds $\vec{n} = \vec{e}_1$. Furthermore, one can assign to disclinations also a 3D topological charge q. It reflects number of realizations of all possible $\vec{e}_1$ orientations sampled on any surface enclosing the whole line defect. Note that in bulk line defects can only form closed loops. On the contrary, in confined geometries they could emanate and terminate on a LC-limiting substrate. Elementary disclinations could exhibit either $|q|$=1 or 0. In the former case the sign of the winding number does not change along the line defect. The far-field of such enclosed defect is distorted and is topologically equivalent to a point defect (monopol) exhibiting topological charge $|q|$=1. Therefore, such defects could strongly interact with their surrounding objects which exhibit coupling with $\vec{e}_i$. On the other hand, chargeless disclinations, bearing q=0, could be surrounded by essentially spatially homogeneous nematic structure. Hence, in general they weakly interact with their surrounding.

In our simulations we impose pairs of {m=1/2,m=-1/2} line defects, which are initially essentially parallel, spanning the facing plates of the plane-parallel cell (see Fig. 2a). We enforce such structures by enforcing at each plate a pair of {m=1/2,m=-1/2} 2D defects, which otherwise impose planar orientational ordering (i.e., the nematic director field within the plates is confined to the (x,y) planes at z=0 and z=h. The separation of surface point defects at the bounding surfaces is equal to r=h/2. Experimentally, such surface boundary conditions could be realized by AFM scribing method (Harkai et al., 2020). At each plate the lines, designated by unit vectors $\vec{e}_0$ (at z=0) and $\vec{e}_h$ (at z=h) , connecting the centers of 2D neighbouring defects are initially aligned along $\vec{e}_x$ as shown in **Figure 2a**. We assume that the end-points of line defects are strongly attached to the surface-enforced defect nucleating sites. Afterward we rotate the bottom connection line $\vec{e}_0$ for the azimuthal angle θ. Figs 2b-2f illustrate representative stages on increasing θ from $\theta = 0$ to $\theta = 2\pi$. In the 1st stage the line defects become elongated, see Fig. 2b. Note that a disclination free energy penalty is for an isolated line defect linearly proportional to its length. To prevent monotonically increasing total length $l^{(tot)}$ on increasing θ the facing disclinations exchange their segments. The reconfiguration process is depicted in **Figures 2c-2d**. At the critical angle $\theta_c = 5\pi/4$ an additional chargeless loop is formed within the (x,y) plane at z=h/2 (Fig. 2d), which connects both disclinations running along the cell thickness. The latter two are also chargeless (i.e., their winding number switches its sign on crossing the mid-plane at z=h/2. Consequently, the total length of disclinations reaches the maximum at $\theta_c = 5\pi/4$ and on further increasing θ in the interval $\theta \in [\pi, 2\pi]$ the total disclination length is monotonically decreasing, reaching the minimal length $l^{(tot)} \sim 2h$ at $\theta = 2\pi$. The final configuration (**Figure 2f**) is identical to the initial configuration (**Figure 2a**). Therefore, the structural transformations exhibit periodic behaviour with the period 2π on increasing θ. Such behaviour is realized in thick enough cells.

Next, we analyse the rotation-imposed reconfiguration in a thinner cell where characteristic stages are shown in **Figures 3**. On increasing θ the total length of disclinations increases in the interval $\theta \in [0, \theta_c]$, where a characteristic pattern is shown in **Figure 3b**. However, at θ_c, the charged disclinations collide and rewire into two chargeless configurations. The latter two connect the nearby surface-imposed 2D point defects. In order to reduce their length, they become relatively strongly confined to the bounding substrates,

see Figs. 3c and Fig. 2f. Therefore, the total length of dislination at $\boldsymbol{\theta = 2\pi}$ equals roughly $\boldsymbol{l^{(tot)} \sim 2h}$. Furthermore, $\boldsymbol{l^{(tot)}}$ exhibit weak $\boldsymbol{l^{(tot)}(\theta)}$ dependence in the regime $\boldsymbol{\theta > \theta_c}$.

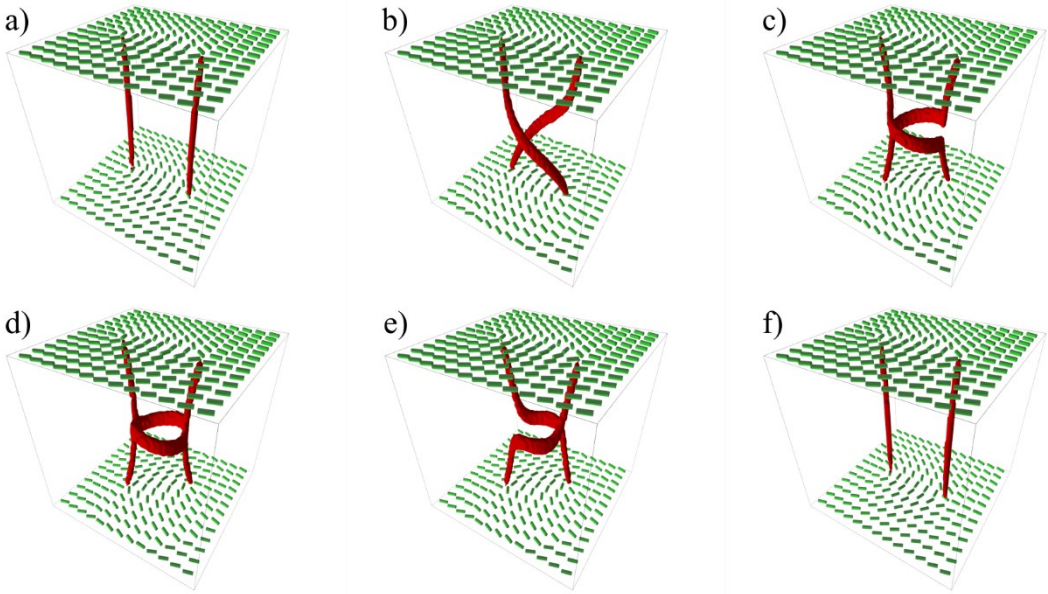

Figure 2. Structural reconfiguration of {1/2,-1/2} topological line defects on increasing θ in thin cells. (a) Initial structure at $\theta = 0$. b), (c), (d), (e): intermediate states on progressively increasing θ. (f): Final state reached following $\theta = 2\pi$ rotation.

In this case the system does not exhibit periodic behaviour on increasing $\boldsymbol{\theta}$.

4. Discussion and conclusions

We studied transformations of disclinations in nematic LCs, which correspond to line defects in the molecular field. In a bulk equilibrium, the field exhibits spatially homogeneous uniaxial orientational order along a symmetry-breaking direction. The degeneracy of competing equilibrium configurations enables the existence of topological defects. We focused on line defects. We stabilized them by appropriate surface boundary conditions. By relative rotation of confining plates, we enforced structural transformation of pairs of disclinations. We demonstrated that disclinations behave like elastic bodies that can recombine in different structures. In our study, the initial (non-rotated) structure possesses two charged disclinations exhibiting winding numbers m= -1/2 and m=1/2. The far field of such closed disclinations would resemble point defects bearing 3D topological charges q= -1 and q=1, respectively. Therefore, the total topological charge of the system equals zero. In addition, the total winding within each (x,y) plane equals zero. In our simulations, we demonstrated two qualitatively different rotation-driven reconfigurations of disclinations. In all cases the topological conservation rules were obeyed: i.e., each (x,y) plane and also the whole system were topologically neutral.

Note that physics of TDs is strongly dominated by topology which is independent from system's microscopic details. Therefore, lessons learned from detail studies in one system, which is experimentally accessible, might gain understanding on behaviour of TDs in systems, where experimental studies of TDs are difficult (e.g., study of cosmic strings in space-time fabric). Our study reveals that interacting line defects in flat geometry could not form

complex knots as first suggested by Kelvin (see **Figures 1d,e,f**). However, more complex structures could be stabilised by including into the play geometrical curvature. For example, in our setting this could be achieved by immersing a toroidal colloid into the nematic fluid, where the colloid's surface would impose isotropic tangential anchoring (i.e., all nematic director field orientations within the colloid's surface are energetically equivalent). Namely, torus possesses surface regions exhibiting positive and negative Gaussian curvature $\boldsymbol{K_g}$. Recent studies in 2D curved manifolds (Mesarec et al., 2016) reveal that surface regions exhibiting $\boldsymbol{K_g > 0}$ ($\boldsymbol{K_g < 0}$) attract TDs bearing m>0 (m<0). Therefore, one expects that chargeless loops, which possess both m>0 and m<0 segments could wind around torus geometry. We believe that by imposing strong enough excitations (for instance by switching on/off a strong enough AC external electric field, where one could vary the field amplitude and frequency) one could stabilize topologically different "torus knots"(Singer, 1982). This is the goal of our future research activity.

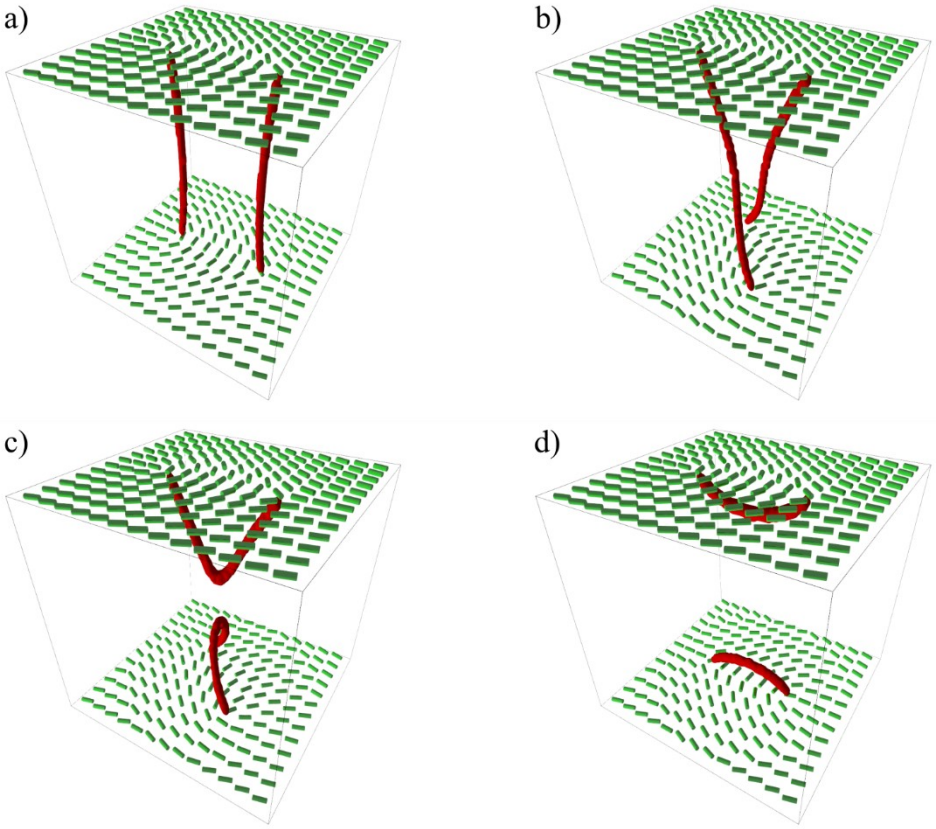

Figure 3. Structural reconfiguration of {1/2,-1/2} topological line defects on increasing θ in thick cells. (a) Initial structure at $\theta = 0$. (b), (c): intermediate states on progressively increasing θ. (d) Final state reached following $\theta = 2\pi$ rotation.

Funding: This research was supported by Slovenian Research Agency grants P1-0099 and J1-2457.

Conflicts of Interest: The authors declare no conflict of interest.

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Invited lecture/Reflection

Physics of Respiratory Pathogen Transmission Through Droplets and Aerosol

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Abstract:
Recent epidemic of the COVID-19 (CoronaVirus Disease-19), caused by SARS-CoV-2 virus exposed great gaps in the understanding of respiratory transmitted diseases in many public health institutions. Traditionally, respiratory pathogens are believed to spread through: direct physical contact (like spray of droplets onto mucous membrane), indirect contact with contaminated surfaces (known as "fomites") and inhalation of aerosols. Public health has relied on a strict split between heavy falling droplets and lighter air-lingering aerosols. In this review we take a look at this distinction, comment on potential problems of its definition and examine a few basic physical phenomena affecting airborne droplet behaviour. We conclude that droplets smaller than 10 μm tend to linger in the air for extended periods of time and that air circulation has a big impact on the presence of pathogen-carrying particles in the air, which may help craft better public health policy.

Keywords: Droplets; Aerosols; Transmission; Respiratory disease; Droplet sedimentation; Droplet evaporation; Public health

Citation: Terček J. Physics of respirator pathogen transmission through droplet and aerosol. Proceedings of Socratic Le tures. **2023**, 8; 141-147. https://doi.org/10.55295/PSL.2023.I20

1. Mechanisms of particle generation

There are several mechanisms that generate particles appropriate for pathogen transmission. These include mainly natural human respiratory activities such as talking, breathing, sneezing, and coughing. Studies suggest that breathing generates particles through condensation and high-speed atomization (Jennison, 1942). Warm gas cools down when entering the upper airways and as a result condenses and is expelled in the form of particles during exhalation. Increasingly turbulent airflow during sneezing, coughing, singing or talking results in a further atomization of particles. Recent studies also suggest that during inhalation, re-opening of small airways contributes to particle generation. (Almstrand et al., 2010; Johnson et al., 2009). Some researchers point to energetic vibration of vocal chords as a source of the majority of particle generation (Morawska et al., 2009). Recently developed time-resolved laser-light scattering method showed that far more droplets are generated than could be previously detected (Anfinrud et al., 2020).

2. Distinction between droplets and aerosols

Droplet transmission is defined as transmission of diseases by expelled particles with a propensity to, due to their size, settle quickly, generally within 1 meter of the site of generation (Wells, 1934).

To help us deal with certain diseases that transmit in these ways a distinction of droplets and aerosols is in use. This distinction leans on a few different assumptions: (i) respiratory disease transmission can be viewed in binary manner through larger droplets or smaller aerosols, (ii) this distinction depends on droplet size alone, (iii) the cut off between droplets and aerosols is set at 5 microns and (iv) there is also a strict cut off in distance at which each size of droplets matters.

This definition can be problematic because it assumes a strict exact size (5 microns) at which a droplet is too big to hang in the air and is assumed to fall to the ground or nearby surface in a few seconds. It is also assumed that droplets of size greater than 5 microns travel on average a maximum of 1 to 2 meters from the source (normally a contagious person speaking, breathing, talking, coughing or singing) and are assumed to follow a ballistic trajectory. This is not in accordance with droplet physics where droplets of various sizes, some much greater than 5 microns, can travel much further than 2 meters and can hang in the air much longer, due to various physical phenomena affecting them once surrounded in ambient air. In the case of SARS-CoV-2 the time spent in the air can even reach a few hours. This means that there is no strict discrete point at which droplets hang in the air or fall to the ground. Instead, there is a continuous distribution that depends on various external factors, most importantly relative humidity. Virus transmission is also affected by other factors such as human behaviour (staying indoors in colder seasons), ventilation, ultraviolet radiation and human immune function.

3. Influences on particle size

Many biological factors that are host-specific can influence the size of generated particles. Here we will take a quick look at the physics content, mainly relative humidity, evaporation and aggregation.

3.1 Relative humidity

A conceptually important start to understanding the relationship between evaporation, particle size and transmission are Wells curves (**Figure 1**). These curves tell us whether a particle will evaporate before reaching the ground or not (Wells, 1934). Of course this is a generalization, as a lot of factors influence particle behaviour and evaporation speed. Since the first publication of Wells curves, corrections have been made and smaller particle sizes were identified to suit the condition of fall time being equal to evaporation time. Particles with radius smaller than 50 microns completely evaporate before reaching the ground (Wan et al., 2007; Nichol et al., 2008). It was found that 95% of all particles generated by

human respiratory activity had radii of 50 microns or smaller (Duguid, 1946). Later studies showed that many droplets generated by coughing or speaking fall in the submicron radius range (Papineni et al., 2020). Further, evaporation time can be affected by relative humidity. Generally, higher relative humidity is being responsible for longer time taken to reach equilibrium size. Relative humidity can even affect the equilibrium size itself as well as the travel trajectory of particles. It is indicated that increase in vertical and lateral movement of particles is connected to decreased relative humidity (Wells, 1934; Schaffer et al.,1976).

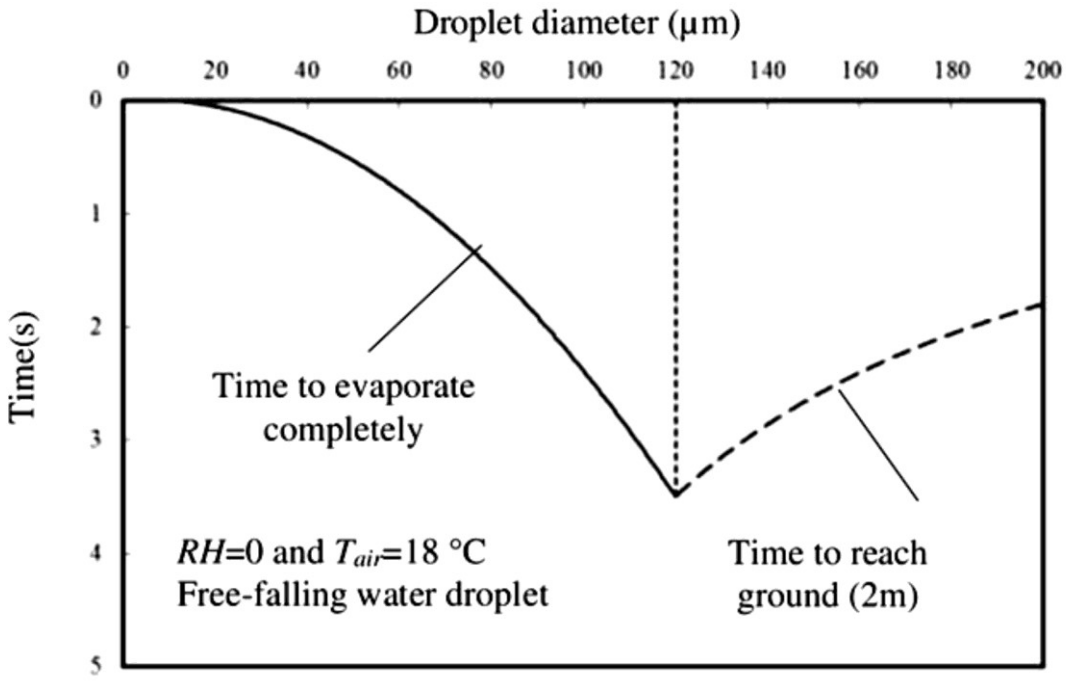

Figure 1. Wells curves showing the relationship between the size of generated particle and evaporation time. Assumed relative humidity of 0%, steady air temperature of 18 degrees Celsius and particle generation at a height of 2 meters and particles modelled as rigid spheres.

Outdoor temperature indirectly influences indoor relative humidity (RH). Especially in winter times, outdoor temperature is lower so heating the buildings dries the cold air that enters into indoor areas, resulting in a drop of RH. This causes that indoor RH in winter times would be between 10% and 40%, compared to summer indoor RH being between 40% and 60% (Božič et al., 2021).

RH influences transmission of infectious material in different ways. First, it impacts how far the droplets can travel through air. Second, stability of winter viruses in droplets is correlated with lower values of RH (between 20% and 50%), while stability of summer viruses is enhanced at higher RH values (around 80%) (Moriyama et al., 2020). Lastly, dry air dries the mucous membrane making it easier for foreign bodies such as infectious viruses to invade the respiratory tract.

1.1 Aggregation

Particles may grow in size due to aggregation with other particles. For this to happen a sufficient concentration of particles is required (Verreault et al., 2008). Aggregation speed depends on multiple factors such as particle size distribution, concentration of aerosol and thermodynamic conditions (Wichmann et al., 2000).

2. Physical phenomena affecting airborne droplets

2.1 *Droplet sedimentation without evaporation*

A basic equation used for understanding sedimentation times of droplets is

$$\tau_{sed} = C\frac{z_0}{R^2} \quad (1)$$

where the numerical prefactor turns out to be $C = 0.85 \times 10^{-8}$ m s, z_0 represents the height at which the droplet is initially placed and R represents the droplet radius (Netz, 2020). In standard practice in public health a radius of R = 5 μm is considered the threshold below which droplets are considered relevant for infections as they linger in the air for extended periods of time. The above equation is considered a good estimate for typical sedimentation times for all droplets with $R > 10$ nm. Acceleration effects can be neglected as droplets reach terminal velocity in extremely short times.

2.2 *Droplet evaporation without nonvolatile solutes*

As droplets evaporate, their radius decreases, which in turn increases sedimentation time. Evaporation of droplet at rest causes time-dependent decrease of droplet radius. This occurs in diffusion-limited evaporation scenario, valid for droplets with radii larger than 70 nm.

$$R(t) = R_0(1 - \theta t(1 - RH)/R_0)^{1/2} \quad (2)$$

$$\theta = 2D_w c_g v_w \left(1 - \frac{\varepsilon_C \varepsilon_T}{1+\varepsilon_C \varepsilon_T}\right) = 4.2 \times 10^{-10} \ \text{m}^2/\text{s} \quad (3)$$

where R_0 is the initial droplet radius, RH is relative humidity, and $\theta = 4.2\times 10^{-10}$ m^2/s at 25 °C. (Netz, 2020) and the meaning and the values of the Equation (3) parameters are given in **Table 1**,

Table 1. Parameters of Equation (3).

D_w	Water diffusion constant in air	2.5×10^{-5} m^2/s at 25 °C
v_w	Liquid water molecular volume	3.00×10^{-29} m^3 at 25 °C
c_g	Saturated vapor water concentration	7.69×10^{23} m^{-3} at 25 °C
ε_C	Linear coefficient	0.032 K^{-1}
ε_T	Temperature coefficient	55 K

Important factor that affects evaporation time is cooling of droplet surface due to evaporation. Water vapor at droplet surface has a temperature that is lower than the temperature of the surrounding air. Value of this temperature decreases in proportion to relative humidity. At RH = 0, the droplet surface temperature drops for about 20 °C. Even though this effect is significant, droplets do not freeze at ambient temperatures of 20 °C or higher. At lower temperatures however, evaporation cooling can induce freezing and even further slow down evaporation times. Evaporation cooling effect is accounted for with the factor given by

$$\left(1 - \frac{\varepsilon_C \varepsilon_T}{1+\varepsilon_C \varepsilon_T}\right) = 0.36. \quad (4)$$

If the radius of the droplet becomes smaller than 70 nm, a transition to reaction-rate-limited evaporation regime occurs. Internal mixing due to diffusion can be neglected as it is sufficiently fast for radii below about 100 μm and inhomogeneities in concentration can be neglected. Evaporation time can be approximated by

$$\tau_{ev} = \frac{{R_0}^2}{\theta(1-RH)}. \quad (5)$$

The important observation is that evaporation time increases quadratically with respect to initial radius R_0 (Equation (5)) while sedimentation time decreases quadratically with respect to R_0 (Equation (1)). For a droplet generated at height of 2 m, at RH = 0.5, critical initial radius below which droplets completely evaporate before reaching the ground is ${R_0}^{crit} = 52$ μm. As RH decreases, critical initial radius increases, with ${R_0}^{crit} = 61$ μm at RH = 0 (**Table 2**).

Table 2. Sedimentation and evaporation times. (Netz, 2020, [12])

R_0 (μm)	1	2.5	5	10	30	40	55
τ_{sed} (RH = 1)	5 h	45 min	11 min	43 s	19 s	11s	5.6 s
τ_{ev} (RH = 0.5)	0.0048 s	0.030 s	0.12 s	0.48 s	4.3 s	7.7 s	14.5 s
${\tau_{sed}}^{RH}$ (RH = 0.5)	∞	∞	∞	∞	∞	∞	7.6 s
${\tau_{sed}}^{sol}$ (RH = 0.5)	64 h	10 h	154 min	38min	231 s	99.6 s	7.6 s

R_0 represents the initial droplet radius, τ_{sed} (RH = 1) is sedimentation time without evaporation, τ_{ev} (RH = 0.5) is evaporation time at relative humidity of 50% and without any non-volatile solutes, ${\tau_{sed}}^{RH}$ (RH = 0.5) is sedimentation time in absence of non-volatile solutes at relative humidity of 50%, and ${\tau_{sed}}^{sol}$ (RH = 0.5) is sedimentation time at relative humidity of 50% and containing non-volatile solutes inside the droplet of initial volume fraction of 1%. All times are measured from height of 2 meters.

2.3 Droplet evaporation containing nonvolatile solutes

Droplets containing non-volatile solutes are unable to completely evaporate. As a result, the amount of reduction of the droplet radius through evaporation has a lower limit. Solutes in water droplets decrease the water vapor pressure and therefore limit the decrease of the droplet radius,

$$R_{ev} = R_0\left(\frac{\Phi_0}{1-RH}\right)^{1/3}, \quad (6)$$

where Φ_0 is the initial volume fraction of solutes in water droplet. Droplet evaporation all the way to its lower limit of radius is only possible at RH = 0. A good approximation for evaporation time in the presence of nonvolatile solutes in water droplets is

$${\tau_{ev}}^{sol} = \tau_{ev}\left(1 - \frac{{R_{ev}}^2}{{R_0}^2}\right). \quad (7)$$

As a result, sedimentation of droplets containing non-volatile solutes (such as pathogens) can be divided in two stages. In the first stage the radius of the droplet shrinks according to the above equations. In the second stage the droplets sediment for a prolonged time at a constant radius. Sedimentation time is given by

$${\tau_{sed}}^{sol} = \frac{Cz_0}{{R_{ev}}^2} - \frac{\tau_{ev}}{2}\left(\frac{R_0}{R_{ev}} - \frac{R_{ev}}{R_0}\right)^2. \quad (8)$$

3. Airborne time of aerosol particles

Lifetime of aerosols and air time are important in the context of viability to transmit viral loads. Pathogens capable of surviving a couple of minutes and even a couple of hours, carried in small aerosol particles are capable of circumventing a large portion of public health safety measures such as social distancing.

Since aerosols are incredibly small (typically < 10 μm) the turbulent ambient air affects them more than gravity (Somsen et al., 2020) In addition, smaller aerosols have the ability to enter deeper into the respiratory tract and pose a potential risk of a more severe infection. (Somsen et al., 2020) Whether SARS-CoV-2 can transmit by such means is still inconclusive but recent studies suggest a strong possibility for aerosol infections being possible in SARS-CoV-2. (Duval et al., 2022])

In a study on aerosol lifetime in different ventilation conditions (natural home ventilation, common mechanical ventilation and strong mechanical ventilation) (Ding et al., 2021) it was found that aerosols generated by speech decreased exponentially but remained present in the air for up to 9 hours in stagnant air with natural ventilation. For non-stagnant conditions, the number of aerosols from speaking or coughing fell exponentially across all experimental conditions and fell back to near background levels. Half-life of aerosol particles generated by coughing was slightly higher than that of speaking (by about 4% - 38%), independent of measurement methods. Half-life of aerosol particles declined with increase in air change (ventilation) (Ding et al, 2021).
We can see that social distancing is not a sufficient method for stopping the transmission of airborne aerosol-carried viruses. Proper ventilation on the other hand is a viable and effective precaution to combating virus spread.

Conflicts of Interest: The author declares no conflict of interest.

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Reflection

On Predatory Nature of Scientific Publishing

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Abstract:
A reflection on publishing activities as experienced by the author is presented. Recently, the journals of the publisher Multidisciplinary Digital Publishing Institute have been declared as "predatory". In this contribution, challenges of scientific publishing are discussed. Good practices of the MDPI publisher as experienced by the author are reflected.

Keywords: Predatory journals; MDPI; Authors; Reviewers; Editors

Citation: Kralj-Iglič V. On predatory nature of scientific publishing. Proceedings of Socratic Lectures. **2023**, 8; 149-155. https://doi.org/10.55295/PSL.2023.I21

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1. Predator

According to the dictionaries, the meanings of the word "predator" first consider the animal world. Predator is an animal that hunts, kills, and eats other animals (Cambridge, 2023), an organism that primarily obtains food by the killing and consuming of other organisms (Merriam-Webster, 2023), an animal that lives by killing and eating other animals, an animal that preys on other animals (Britannica, 2023).

Transferred to human world, predator is someone who follows people in order to harm them or commit a crime against them (Cambridge, 2023), one who injures or exploits others for personal gain or profit (Merriam-Webster, 2023), a person who looks for other people in order to use, control, or harm them in some way (Britannica, 2023), someone who tries to use another person's weakness to get advantages (Longman, 2023).

Further transferred to systems, predator is a company that buys or tries to buy another company that is in a weaker financial position (Cambridge, 2023), a person, group, or business that exploits, victimizes, or preys on others (Dictionary, 2023), a company which takes advantage of another company weaker than itself, for example by trying to buy it (Longman, 2023).

Further transferred to scientific publishing, a predatory publisher is an opportunistic publishing venue that exploits the academic need to publish but offers little reward for those using their services (Iowa State University Library, 2023).

2. Labeling bad practice in scientific publication as "predatory"

In science, publication is a key element. Publication makes possible that the ideas and results of the authors are conveyed to others interested in the subject. This creates a vivid exchange of experiences and induces new ideas that promote the knowledge. It therefore seems logical that the control over this point gives the relevant subject a great power, and eventually, also money.

With recent development, the request for publication has considerably increased and rendered scientific publication very interesting also from the financial point of view. Established publishers are now challenged by new players on the block with skills that previously did not exist or count. In order to survive, a fight for this profitable market is getting tougher. To win the confidence of the stakeholders, different classifications, rules, descriptions, etc. are being developed to justifiy the policies leading to the resources of the taxpayers' money. Bad practices are being defined and a term "predatory journal" has been assigned to those that should pertain to such (Beall's list, 2023) outlining the issues regarding editor and staff, business management and integrity. Furthermore, to lead to measurable outcomes, practical advices are suggested to the scientific society. For example, Oviedo Garcia (2021) published a thorough analysis of a particular publisher with respect to scientometric parameters and concluded:

"In summary, so as not to contribute to the continuance of malpractice: (1) researchers should neither send papers for their publication, nor cite them, nor act as reviewers for them, nor form part of their editorial committees; (2) research institutions should inform researchers of the reality of predatory journals and their iniquitous consequences at an individual and general level; and, (3) evaluation agencies and committees should ignore the registers that refer to predatory journals. Lastly, but by no means least of all, selective databases should conduct periodic controls and strengthen the criteria for the incorporation of journals, so as to prevent their good names from serving, as previously said, to prolong malpractice among journals 'that prioritize self-interest at the expense of scholarship and are characterized by false or misleading information, deviation from best editorial and publication practices, a lack of transparency, and/or the use of aggressive and indiscriminate solicitation practices (Grudniewicz et al. 2019)."

On February 22, 2023, a notice was posted on a forum Predatory Reports (2023):

"MDPI (Multidisciplinary Digital Publishing Institute) as a publisher of open-access scientific journals was spun off from the Molecular Diversity Preservation International organization. It was formally registered by Shu-Kun Lin and Dietrich Rordorf in May 2010 with its official headquarters in Basel, Switzerland. Including Switzerland, MDPI has editorial offices in 11 countries, with five offices in China, two offices in both Romania and Serbia, and offices in the United Kingdom, Canada, Spain, Poland, Japan, Thailand, and Singapore. As of February 2023, MDPI publishes 413

journals and 9 conference journals. Based on a series of information published, we decided to include the MDPI journals on the predatory publications list."

In December 2022, MDPI reached the milestone of one million articles published, from these, about 300,000 were peer reviewed and published in 2022 (MDPI, 2023). In 2022, 98 MDPI journals have earned an Impact Factor in Clarivate's Journal Citation Reports and 86 percent of journals have increased their Impact Factor from 2020 (MDPI, 2023). At the end of 2022 the workforce of MDPI counted 6750 employees (MDPI, 2023).
A question could be asked: With one million of publications authored on the average by more than one author, a number of reviewers proportional to the number of manuscripts sent, and hundreds of editors handling the manuscripts: How many of these persons (if not all) have comitted malpractices and harmed science? Or – are there some other problems or reasons leading to such labeling of this particular publisher, evidently successful in publication and in income. On the other hand, What are the roots of the success of MDPI?

3. An author's perspective

To an author, a mentor of PhD students, and an applicant for grants, the relevant questions would likely be: Does the journal have an Impact Factor and therefore the eligibility for the PhD material publication required by local academics? Will the publication be considered as a valid reference for grant applications? Will the publication be considered for personal promotion (e.g. habilitation)? How likely is it that the manuscript will be accepted? Are we able to put together the money for publication?
In the predatory chain, I see the authors that think in this way as the weakest link – the prey. On the average there is a lot of work and dedication of the authors and resources in a manuscript. Clearly, an unpublished scientific work has only some intimate value to the authors which does not justify the public money and resources that have been spent to create it. An author who does not publish will not be able to get funding for further research meaning that publication is a vital need for the authors whose jobs and careers are at stake.
As a coauthor I have published about 250 papers, of these, about 15 papers in MDPI. None of our manuscripts sent to MDPI was rejected already by the editors as "out of scope" or "not reaching the priority" which are common phrases used in rejection letters of some journals. In MDPI, all our manuscripts were peer reviewed and not all were accepted for publication. To my best knowledge, we have never considered the fees as a crucial factor in chosing a journal, and most of the journals that we have published in have charged publication in a more or less comparable amount. Some journals (but not MDPI) require money also for handling the rejected manuscripts. But, the largest losses following the rejection of the manuscript (provided that the manuscript was actually sound and later published elsewhere) are the loss of zest within the team, the loss of time and energy spent to prepare the manuscript for submission to another journal and slowing down of the research process resulting in delays for providing the references required for project reports and Ph.D. theses. These losses are eventually reflected also in money and they largely exceed possible differences in fees from different journals.

4. A reviewer's perspective

To a reviewer, the relevant questions would be: Am I able to meet the deadline for the review? Am I able to use the platform of the journal? Is the manuscript presented in such way that I can understand the message of the authors? Will the editors consider my comments?
I do not keep records on the number of reviews that I have made, but I estimate that there were about 300. I have reviewed about 10 manucsripts in MDPI journals in 2021 and 2022. When I started to review, it sometimes took me a week to study the manuscript and to write a review, but now, especially for excellent and well written manuscripts I can do it much faster, therefore, a deadline 6 days or 1 month does not mean much difference in this respect. I would expect that the majority of reviewers think likewise.

I think that it is not necessary that all the reviewers are experts on the field. Some comments are common for all fields (ethical issues, clarity, presentation, language), and sometimes, comments from other fields can bring new points of view.
An important role of the reviewer is to consider ethical issues, in particular in matters regarding living things. Besides presentation of the ethical committee permission, it is important that suggestions into clinical practice are amply argumented and that too bold statements are rephrased. It seems plausible to me to reject a manuscript if the reviewer's comments on important ethical issues were ignored in the revised version. It had happened to me once because the author would not stay away from suggestions as regards clinical practice, that were not supported by the results. However, the editor accepted the manuscript. I have written to the editor that it has no sense to require a review and then neglect what the reviewer suggested, in particular as it was not much to ask from the authors. I have obtained no answer nor any more requests for review from this journal since. It was not in the MDPI journal.

5. An editor's perspective

To an editor, the relevant questions would be: How will I get contributions? How will I get reviewers? How will I manage manuscripts and reviewers to fit my policy of publishing? I have experience as an editor in different journals, including two MDPI journals, as a guest editor. The answers to the above questions depend largely on the situation of the journal, whether it is a new one with few submissions and without an Impact Factor or an renowned one with many submissions and struggling to keep or improve the Impact Factor. It follows from the definition of the Impact Factor (IF) (in a given year, the two-year journal impact factor is the ratio between the number of citations received in that year for publications in that journal that were published in the two preceding years and the total number of "citable items" published in that journal during the two preceding years) that publishing of many papers will decrease the impact factor of the journal. As the citations follow the trend in the field, the most predictable way to keep the IF high is to keep the number of accepted manuscripts to a minimum, in other words, to reject a given number of manuscripts in any case, regardless of the possibility of their content being of interest to the readers. In an editorial board meeting of one of the journals (but not MDPI), rejecting a high percent of manuscripts was presented as a success. I wonder whether some of these manuscripts were worth publishing or could have been made so by some improvements.

6. Predatory nature of scientific publishing

Peer review issues have been exposed in the discussions regarding predatory journals as a cornerstone of sound academic publishing. In my experience, a good peer review can be of a great help in improving the manuscipt and work overall. I remember a comment of an anonymous reviewer that would merit her/him a coauthorship for we have envisaged the shortcomings of our work and considerably improved it. As a corresponding author I would gladly include this reviewer as a coauthor, but this is out of the question as the reviewers were and should have been kept anonymous to the authors. Thus I have remained thankful for ever and am looking up to this review since. But such cases were rather rare in my history of publication. Many times, after the peer review, the manuscript turned out deformed, as we pleased the reviewers to whom our course of arguments was strange. The worst experience were reviews that would not acknowledge deviations from already renowned thories. It took for years to find a journal that would publish radically new approaches, although well argumented and supported by the evidences. I found the drawback in emotional reaction of the "experts" who have moved the science forward by their contribution, but were unable to let it be moved forward by others and found new approaches as "false". The sentences like: "is now well understood" seem particularly dangerous. Also, some reviews were destructive and even rude. I remember a very short review of a manuscript sent to a renowned journal saying in a line that "the paper spent on printing would not be justified for yet another publication on the subject". Such reviews can be of no service to authors. But the editor did not see it invalid and based on it, the manuscript was rejected. The manuscript was then published in another journal (Kralj-Iglič, Iglič, 1996) and was hitherto cited 125 times.

If I sum up my experience, peer reviewers contributed a few major good ideas, that considerably improved the work. Most of the reviews presented good suggestions such as minor improvements, presentation, inclusion of additional references or grammar/ language, but did not importantly affect the scientific essence. There was also a considerable number of destructive reviews that either caused a loss of time for rejection of manuscripts that have later proved sound by publication in other journals and by citation, or in my opinion decreased the quality and marginalized the accepted papers. Here, editorial decisions not to send the paper to peer review are not counted. As I have published in many different journals, had no close connections with the editors, and as the number of submissions can be estimated as twice the number of published papers (say about 500), I dare consider my experiences as representative for many other authors who are trying to publish primarily on the account of the content of the work. Questions can therefore be asked: What is the scientific value of the peer review? and What are the ethical premises of the peer review? In my opinion, a place of the reviewer is to suggest possible improvements of the manuscript and discuss potential development of the ideas. It is however questionable on what grounds the reviewer can be considered as a judge of the quality of the scientific work. Clearly, an unpublished scientific work has no value at all for the community and only time can show whether the published work proved useful to someone. Here, a scientific approach is not apt to foresee "which grain will grow and which will not" (unless the mechanisms are in action to deliberately accelerate or decellerate its growth).

From the author's perspective I would call a predatory journal the one that rejects manuscripts in order to increase the impact factor of the journal. But as for the nature of the things, in order to keep the reputation of the journal within the existing rules, editors are forced to apply this principle. From the reviewer's point of view, predatory review is the one that accepts, rejects and deforms manuscripts for personal interests (to gain material or non-material favors or tu subdue to pressures from authors or editors). Predatory reviewing is one of the cornerstones of predatory nature of scientific publishing and is not localized within one publisher, as the same reviewers serve to different publishers. From the author's point of view, predatory practices are to adjust the manuscripts to the likings of the reviewers and editors in spite of disagreement with the requests and reporting of false interpretations, results and autorships. With such practices on all levels of publication, new players on the block need not be scientists that had first proved independent researchers and mentors but can be propelled directly to the top by the virtue of their management and communication skills. Here things become more complex because as they do not have the respective knowledge and experience to understand what is going on on all levels, albeit at its origin, they cannot be guilty of malpractice. Relaxing the ethical constraints by genuine ignorance thus widely opens the doors to achieve profit of any kind.

All these effects may be enhanced by interactions between the colleagues who perform the roles of authors, reviewers and editors. I think that it is fair to admit, that within the existing rules, scientific publishing is predatory in its essence, whereas the origin of the predatory essence is the wish of an individual to control the scientific field and its connections with monetary funds (industry, academia and governmental institutions which take care of the taxpayers' money) regardless of one's competence, references and intentions to take care of all who want to contribute. Luckily, the scientific publishing system is a human-made construction and can be adjusted by changing the rules. That is at least to some extent. The mighty law of entropy that makes things predictable if the system is large enough is acting also here. It should be kept in mind that with increasing number of involved subjects the probability of malpractice will increase on all levels.

7. Experience of goodpractice of MDPI

It is not my intention to argue on malpracticies of any particular author, reviewer, editor or publisher, although it is to be expected that they occur, by accident or intentionally, driven by a wish to find an easy way to climb the hierarhy and search for material or nonmaterial goods, and due to natural laws, the probability of such would be proportional to the number of manuscripts processed. Those who consider science and ethical issues precious should nevertheless act in best effort to minimize bad practices. Therefore, I

would like to point to good practices of the "notorious" publisher that I have experienced so far: MDPI journals were provided with Impact Factors and the publications were eligible for PhD material. Peer review was timely, and if accepted, papers were published in some days. The submission process was simple. For writing a review, a convenient platform with automatic reminder some days before the deadline was of great help. When I served as a guest editor of a special issue, soliciting of the reviewers was supported by the MDPI staff to overcome a bottleneck in processing the manuscripts. I was not pressured to decision either to accept or to reject any of the manuscripts edited. All these good practices that have to some extent relieved the burden of authorship, review and editing, do not come for granted. Enormous number of manuscripts was produced in the last years and many of these were rejected and re-submitted. I guess MDPI by its well organized processing substantially benefited from this pool, to the satisfaction of the authors and those reviewers and editors that are called to collaborate with authors to present their work in a way to be consumable by the scientific society. Although the publication fee is considered to be high, publication with MDPI proved more economical as it was efficient if one considers time and money consuming re-submissions and lobying activities that slow down the research process and contribute little or nothing to the scientific essence.
Also it is not my intention to investigate or judge whether MDPI is a more or less "predatory" publisher than any other in this profession (according to the definitions of my learned colleagues), however, I think that the scientific society would benefit if other publishers would apply the good practices of MDPI which I have experienced as an author, reviewer and editor, that is to deliver high quality papers to scientific society and thereby promote scientific work.

Conflicts of Interest: The author declares no conflict of interest.

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