UNIVERSITY OF LJUBLJANA | ZF Faculty of Health Sciences

SOCRATIC LECTURES

10TH INTERNATIONAL SYMPOSIUM,
LJUBLJANA, 9. DECEMBER 2023
PEER REVIEWED PROCEEDINGS PART I

EDITED BY:
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Socratic Lectures
10th International Symposium, Ljubljana, December 9, 2023

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Program of the Symposium Socratic Lectures, December 9, 2023, 10:30 – 14:00 (Ljubljana time)
10.30 Welcome to participants (Veronika Kralj-Iglič, University of Ljubljana) https://uni-lj-si.zoom.us/j/98919724250
10.35 -11.15 Plenary lecture: Bernd Giebel, Institute for Transfusion Medicine University Hospital Essen, Germany: Clinical Potential of MSC-EVs and Translational Challenges

Scientific sections

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11.50 - 12.10 **Malidze D:** Effect of Colchicine on atrial fibrillation onset prevention in patients with coronary artery diseases

12.10 - 12.25 **Kolar M:** Geometrical specificities of femora fractured secondary to total hip replacement

12.25 - 12.45 **Tadel Kocjančič Š:** COVID-19 and extracorporeal membrane oxygenation (ECMO)

12.45 - 13.00 **Ipavec M:** Experience with smart above knee prosthesis

13.00 - 13.15 **Amon M; Kresal F:** Therapeutic potential of hypoxia

13.15 - 13.30 **Kovačič P:** Decoding the facial expressions of cats: Insights through scientific illustration

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12.30 - 12.50 **Arko M et al.:** Extracellular particles from bovine and equine milk

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11.45 -12.00 **Nurseitov A,** Innovative Approaches to Language Learning through the Lens of Use Artificial Intelligence

12.00 - 12.15 **Protasenko O,** Digital education in the development of the company's human capital

12.15 - 12.30 **Dybach I,** Features of the formation of cross- cultural communication skills in it business

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13.00 - 13.15 **Haborets O, Kushkovyi A,** OSINT Technologies: Applications and Challenges in the Digital Age

13.15 -13.30 **Lunhol O, Torhalo P,** Artificial Intelligence in Law Enforcement: current state and development prospects

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13.10 - 13.30 **Istileulova Y,** Innovations, arts and artificial intelligence: Innovative teaching and learning through STEAM approach (SMS method)

Section 17. How to organized by **Mitja Drab**

11.30 - 12.00 **Kregar A,** How to encode the date of Younger Dryas comet impact on the stone pillars of Gobekli Tepe

12.00 - 12.15 **Drab M,** How to shape cells using membrane inclusions

12.15 - 12.30 **Turk J,** How to model small length scale deformations of injection-molded parts

12.30 - 12.45 **Jan M,** How to think about the nature of Descartes' ideas

12.45 - 13.00 **Sojecka AA,** How to think about Universal patterns of global population change: from doomsday day growth to the reversed criticality

13.00 - 13.15 **Kostanjevec J,** How to say the right thing? Remarks on expresivity and thinkability in recent debates on category theory

13.15 - 13.30 **Marin A,** How to hide: Camouflage from ultraviolet to infrared

13.35-13.55 Honorary lecture for all participants

Marjana Rupnik, European Commission, Language Department, Linguistic Professions in the European Union Institutions and Artificial Inteligence

13.55-14.00 Cultural program

Agata Angelika Sojecka - harp, Borys Urbanowicz - viola
C. Saint-Saens - The Swan
G. Faure - Apres un reve

14.00 Closing of the symposium https://uni-lj-si.zoom.us/j/98919724250

Editorial

10th Socratic Lectures consisted of three events: an Organ recital of Roberta Schmid from Naples, Italy at the Church of Assumption, Tromostovje, on Friday, 8.12. 2023. Roberta Schmid took her diploma in Organ and Organ Composition at the Musical Conservatoire "S. Pietro a Maiella" in Naples with the mentorship of prof. A.M.Robilotta and then improved her technique with maestros L. Ghielmi, E. Kooiman, M. Radulescu, D. Roth, K, Schnorr, L. F. Tagliavini, M. Torrent, P. Westerbrinck and W. Zerer. She specialized in Italian and German Organ Music Interpretation at the Musical Academy in Pistoia and in Gregorian Chant at the International Study Centre of Gregorian Chant in Cremona. She performed as a soloist and in chamber formations in XXVIII Festival Internazionale di Noale, Rassegna Antichi organi di Piacenza, Associazione Alessandro Scarlatti di Napoli, Associazione Studi Mezzogiorno, Associazione Ricercare, Accademia Organistica Campana, Rassegna organistica internazionale di Avezzano, Rassegna organistica di Pescara, Settembre Organistico Fabrianese, Rassegna organistica veneta "Musica nell'Agordino" , Rassegna "Musicalia" in Pavia, Festival Organistico Internazionale Città di Senigallia, Rassegna Organistica di Fiemme (Trento), Rassegna organistica della Svizzera Italiana, International festivals at Notre Dame de Compassion in Paris, Merano Cathedral, Amalfi Cathedral, S. Vitale's Basilica in Ravenna, S. Ambrogio' s Basilica in Milan and in Mexico City. She has recorded a CD of Bach and pre-Bach music sponsored by the Goethe-Institut, Italy. Presently she is Artistic Director of the Festival "Musica intorno all'organo" at the St. Maria della Rotonda Church in Naples and organist of Mascioni mechanical organ at St. Maria della Rotonda Church and at Santa Chiara's Basilica in Naples. www.robertaschmid.com

Program of the organ recital of Roberta Schmid

J.S. Bach: Wachet auf, ruft uns die Stimme, BWV645

N. Bruhns: Praeludium in e minor (the Great)

P. A. Yon: Toccatina for Flute

W. A. Mozart: Fantasia in f minor K594

J.S. Bach: Nun komm, der Heiden Heiland, BWV 659

C. Franck: Choral in b minor

C. M. Widor: Toccata from Symphony V

The next day, scientific symposium took place online. **The plenary lecture** on mesenchymal stem cell extracellular vesicles was donated by **prof. Bernd Giebel** from Essen, Germany, followed by 17 scientific sections. The symposium was organized by the Centre of lifelong learning of the Faculty of Health Sciences, University of Ljubljana in collaboration with Universities from Georgia, Spain, Ukraine and Kazakhstan. There were about 200 participants from 21 countries. The subjects of interest were medicine, natural sciences, green transition, digital transition, business, social sciences and education. There were two sections on extracellular particles. At the closing of the symposium **Agata Angelika Sojecka** and **Borys Urbanowicz** from Poland performed The swan (by C. Saint Saens) and Apres un reve (By G. Faure) on harp and viola, respectively.

On Monday, 11.12.2023 the accompanying social event was held at the Academy of Music, Palace Kazina. The event included classical and contemporary music performed by musicians and by scientists.

Concert on December 11 at 18.00 was in person at Pallace Kazina, Ljubljana

Program of the Concert

Classical music, Concert Hall of Julij Betteto

J.S. Bach: Wachet auf, ruft uns die Stimme BWV645, organ: Roberta Schmid

J.S. Bach: Toccata and fugue in d minor BWV 565, organ: Roberta Schmid

D. Cimarosa: Concerto for two flutes and piano, flutes: Anita Prelovšek and Sara Brumat, piano: Elena Startseva Somun

D. Cimarosa: Two sonatas for piano, piano: Matic Bogataj

M. Ravel: Blues, Moderato from Sonata No.2 for violin and piano, violin: Branko Brezavšček, piano: Elena Startseva Somun

J Pachelbel: Canon in D, piano: Elena Startseva Somun

G. Caccini: Ave Maria, soprano: Kaya Tokuhisa, organ: Jana Jamšek

Anonymus: Amazing grace, soprano: Kaya Tokuhisa, organ: Jana Jamšek

Contemporary music, Club Kazina

P. de Seneville, Marriage d'amour, classical ballet: Darja Eržen, Jelena Ficzko, Lara Elisabeth Lee, piano: Jana Jamšek

A. von Sultanova: JC Maxwell, Moja domovina, JR Oppenheimer, voice and piano: Yelena Istileulova

A. Chailleux: Andante et Allegro, saxophone: Teodor Herem

F. Kuchler: Concertino for violin and piano, violin: Živa Bratko, piano: Barbara Smrke

In the club Kazina, another memorable social event was held to create a network uniting scientists and artists.

Wellcome to the next Socratic lectures. Contact kraljiglic@gmail.com or annaromolo@gmail.com

Veronika Kralj-Iglič, Yelena Istileulova and Anna Romolo

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TEXTS

Invited lecture/Review

Extracorporeal Oxygenation (ECMO) in Patients with Acute Respiratory Failure

Tadel Kocjančič Špela[1,*]

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Abstract:
Extracorporeal membrane oxygenation (ECMO) is a method for oxygenation and removal of carbon dioxide in patients with respiratory failure in whom we cannot achieve that with standard methods of invasive mechanical ventilation. The method works as an extracorporeal bypass of blood, which we take from one central vein, then the blood goes through an oxigenator and returns oxygenated and free of carbon dioxide to another central vein. The system is run by a pump with steady non-pulsatile flow. We use two standard ECMO types. For respiratory failure alone we use veno-venous ECMO and for heart failure we use veno-arterial ECMO. We can also combine more types of ECMO according to the patient´s needs. We started using ECMO in 2009, with the number of patients is increasing every year. The majority of patients with the need of ECMO has influenza, pneumococcal pneumonia or infection with Legionella species. We also used ECMO a lot during COVID-19 pandemic. With this new method even the patients who would otherwise (even 10 years ago) die of respiratory failure, have a greater possibility to survive.

Keywords: ECMO – extracorporeal membrane oxygenation, respiratory failure, cannulas, non-pulsatile blood flow

1. Introduction

ECMO means ExtraCorporeal Membrane Oxygenation (extracorporeal life support, extracorporeal lung assist). It is a method for oxygenation and removal of carbon dioxide in patients with respiratory failure in whom we cannot achieve that with standard methods of invasive mechanical ventilation. ECMO is not a treatment and does not correct the underlying pathological insult.

We use two types of ECMO: venovenous (VV ECMO) for respiratory support and venoarterial (VA ECMO) for respiratory and hemodynamic support. We can also combine these two methods according to the patient´s needs.

ECMO was developed in 1970 for respiratory support in acute respiratory failure. It was used for adding O2 and removing CO2, but it was performed with cannulation of femoral artery and vein and limited to 5 days. In 1979, the first randomized trial showed very high complication rates and survival rates no higher than mechanical ventilation alone.

In early 1980s the switch to veno-venous was made, but after that the technology was largely abandoned due to bleeding and poor outcomes (Mosier et al., 2015).

There was a boom of ECMO during the global pandemic of influenza H1N1 in 2009-2010 and again in 2020-2022 during global pandemic od COVID-19.

Several centers reported survival benefits for ARDS secondary to influenza. Two retrospective case-control studies showed lower mortality when transferred to ECMO centers and lower mortality among younger patients who received ECMO (Schmid et al., 2015; Australia Group, 2009; Luyt et al., 2012; Zangrillo et al., 2013; Bednarczyk et al., 2014).

2. VV ECMO circuit

VV ECMO is used in isolated failure of the lungs, unresponsive to optimal ventilatory support and medical treatment. It is used in children and in adults. It consists of large conduit tubing, a blood pump, an oxygenator and additional components which may include a heat exchanger, monitors, and alarms.

Two ECMO cannulas are inserted percutaneously, ultrasound or diascopy guided. Usually they are inserted through the jugular or femoral vein, one for draining deoxygenated blood from the venous system (superior or inferior vena cava) to ECMO circuit, the other one for returning the oxygenated blood to the right atrium. Their size is dependant on the height of the patient. The cannulas are manufactured from biocompatible silicone polyurethane polymer, which may be coated with polymers that may reduce platelet activation and the inflammatory response at the blood-cannula interface (Pavlushkov et al., 2017; Lequier et al., 2013; Kohler et al., 2013).

The cannulas are constructed with a reinforced stainless steel (SS) wire. Wire reinforcement of the cannula walls is used to prevent kinking or collapse. A rigid cannula introducer is made of polyvinylchloride with an embedded SS rod (Pavlushkov et al., 2017; Lequier et al., 2013; Kohler et al., 2013; Beckmann et al., 2011; Medtronic, 2012).

Surface coatings are applied on the cannula to reduce the activation of the clotting; control of blood clotting is mandatory during the extracorporeal life support. The surfaces can be coated with heparin, bivalirudin, or tethered-liquid perfluorocarbon (Leslie et al., 2014; Wyss Institute, 2014; Yang et al., 2012).

A blood pump and an oxygenator can be joined together or separate. For adult respiratory failure the largest size ECMO machine is used which includes large conduit tubing, a blood pump capable of at least 5 L/min, and an oxygenator with rated flow over 5 L/min. A blood pump has a steady non-pulsatile flow (ELSO Guidelines, 2017).

In the absence of lung function, VV access can supply all metabolic oxygen requirements. Patient ‘s PaCO2 is controlled by the sweep gas flow (ELSO Guidelines, 2017).

3. Indications and contraindications for veno-venous ECMO

Indication for VV ECMO is potentially reversible acute respiratory insufficiency, which may be hypoxemic, hypercapnic (pH less than 7.0), and ireversible lung damage in patients who have already been accepted for lung tranplantation as a bridge to transplantation (ELSO Guidelines, 2017). Contraindications may be absolute or relative. Absolute contraindications are pre-existing conditions, incompatible with recovery, for example advanced

lung disease without possibility for transplantation, other end stage diseases. Relative contraindications are uncontrollable bleeding, very poor prognosis from primary condition, age above 65, advanced septic shock, imunosuppresive treatment (ELSO Guidelines, 2017).

4. Complications

Complications on ECMO are very common and are associated with a significant increase in morbidity and mortality. These complication could be related to the underlining pathology needed ECMO, or of the ECMO condition itself .

Patients on ECMO are very ill patients with respiratory insufficiency, usually with multiple organ disfunction. The complications are common and include complications during insertion of the cannulas, during treatment with ECMO and during decannulation.

Complications due to the insertion of cannulas are hemorrhage, venous spasm, arrhythmias, ruptured blood vessels, pneumothorax. During treatment with ECMO there can be adsorption and sequestration of drugs and blood cells on artificial materials, hemorrhage, blood clots, HIT (heparin induced thrombocytopenia), infections. There can also be neurologic complications, intracranial bleeding, infarction, cerebral edema. It is important to realize that these findings may be a consequence of the condition that prompted ECMO, rather than a complication of the ECMO process (Mateen et al., 2011; Mehta & Ibsen, 2013; Lidegran et al., 2007). These may be partially due to systemic heparinization, thrombocytopenia, coagulopathies, or systolic hypertension.

Complications due to the removal of the cannulas are hemorrhage, venous spasm and ruptured blood vessels.

5. Outcome of patients on veno-venous ECMO

According to the published data, 67% of patients with acute respiratory failure treated with ECMO were weaned off ECMO and 52% survived to hospital discharge (ELSO Guidelines, 2017; Hemmila et al., 2004; Noah et al., 2011; Pham et al., 2013). This was also confirmed by the CESAR study (Peek et al., 2009), which demonstrated that referral to an ECMO center significantly improves recovery and survival from severe ARDS.

6. Conclusion

Introduction of ECMO in the treatment of patients with acute respiratory failure has improved survival of these patients. ECMO use has risen since H1N1 influenza outbreak in 2009 and until now ECMO centres still continue to report high survival rates for patients who are supported with ECMO. It is important to emphasize that that patients, treated with ECMO return to a reasonable quality of life, although after prolonged rehabilitation.

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

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Invited lecture/Review

From Hip Arthrosis to Hip Replacement Surgery – Total Hip Endoprosthesis

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Abstract:
Hip arthrosis is a medical condition that is more commonly present in elderly people that is nowadays also becoming a healthcare burden among the younger, active population. Arthrosis is a process during which cartilage, normally present as a protective factor of the hip joint, gradually wears away, becoming frayed and rough. This results in limited protective joint space, which leads to increased friction and rubbing between hip joint components; acetabulum and femoral bone. Due to arthrosis, patients experience daily pain, limited movements and decreased quality of life. Primary treatment of osteoarthritis includes informing patients of the importance of a healthy lifestyle, including physical activity. As non-surgical alternatives to treating osteoarthritis, orthopaedic surgeons may relieve their patients' symptoms with pharmacological interventions (paracetamol and NSAIDs) and in selected cases intraarticular infiltrations. When all this is no longer effective, surgical relief of pain is recommended, including less invasive procedures, such as hip arthroscopy, hip biopsy, or simple core decompression (CD), or the most invasive, but effective hip replacement surgery - total hip arthroplasty (THA). The latter is a procedure during which diseased and necrotic tissue from the hip joint is removed and replaced with artificial materials, the hip endoprosthesis. The aim of this procedure is to reduce pain and improve patient's mobility and quality of life.

Keywords: hip osteoarthritis, hip arthrosis, hip endoprosthesis, surgery, quality of life.

1. Introduction

Hip arthrosis, also referred to as hip osteoarthritis, is a medical condition commonly present in elderly people. The condition occurs over time, as day-to-day activities gradually cause the protective cartilage of the hip joint to wear away (Swiss Medical Network, 2023). During the process of arthrosis, joint cartilage becomes frayed and rough, becoming virtually useless. Consequently, the hip joint has minimal protective joint space which would otherwise prevent rubbing between the acetabulum (socket part of hip joint) and femoral head (ball part of joint) (Foran, 2021). Friction and rubbing of bones in a joint may also cause bone spurs (irregular bone growths on edges of the bone which cause a change in the bone's shape) (Hopkins Medicine Org, 2023). Arthrosis causes patients to experience pain when engaging in daily activities, limiting them in their everyday lives. This causes patients to seek different types of orthopaedic treatment, in hopes of improving their quality of life.

2. Non-surgical treatment options for hip osteoarthritis

Osteoarthritis treatment has been vastly changing over the past decades. This is a result of earlier diagnosis, patient education, and more in-depth knowledge of the biology of tissues involved. Patient education includes teaching patients, as well as their families the importance of managing a healthy lifestyle, regular physical activity, balanced diet and weight loss. All this can help the patient stay mobile, relieve pain, and reduce risk of other medical issues or at least prolong the time they live prior to developing conditions such as arthrosis (Dunkin, 2023). Knowledge of hip joint tissues has been key in developing non-surgical treatments of hip osteoarthritis. These treatments include infiltration of joint with hyaluronic acid (viscosupplementation) or with platelet rich plasma which contains autologous growth factors (Innocenti et al., 2013). Treatment is coupled with pharmacological interventions in the form of paracetamol and non-steroid anti-inflammatory drugs (NSAID). In cases where such treatment is no longer effective, surgical relief of pain is recommended to the patient (Poulsen et al., 2011).

3. Surgical treatment options for hip osteoarthritis

Surgical treatments of hip osteoarthritis may be more or less invasive, their main goal being a delay of prosthetic hip replacement surgery. The latter is, despite its effectiveness and patient satisfaction, still considered a very final surgical solution and may be the cause of patient impediments. Less invasive surgical procedures which a surgeon may offer to their patient include hip arthroscopy. Arthroscopy is a general surgical procedure during which a surgeon creates a small incision at the joint area, through which they insert a narrow tube, attached to a fiber/optic video camera. This allows for the surgeon to view the inside of a patient's joint on a monitor (Staff, 2022). Arthroscopies may be performed on virtually any joint in the human body.

Some patients may also seek orthopaedic help due to a condition called avascular necrosis. The latter is a condition where there is necrosis of bone components due to restriction of blood supply. If untreated, avascular necrosis causes osteoarthritis and bone collapse. In such cases, a surgeon may perform hip biopsy or simple core decompression (CD). The procedure is meant to decompress the femoral head in a condition called avascular necrosis (Padmawar & Landge, 2021). It was, for many years, considered highly cost-effective, minimally invasive and had low complication rate in putting off THA. Nowadays, the procedure's results are debated and controversial, and it is considered outdated, as surgeons no longer perform it except in exceptional circumstances (Sadile et al., 2016).

4. Hip replacement surgery - total hip arthroplasty (THA)

Undoubtedly more invasive, but also one of the most common and effective orthopaedic surgical treatment methods for osteoarthritis is hip replacement surgery, or total hip arthroplasty (THA) (Varacallo et al., 2017). It is a procedure during which the orthopaedic surgeon removes diseased or necrotic tissue from the hip joint. This tissue includes bone, cartilage and surrounding muscle and fat tissue. The diseased parts of bone include femoral head and acetabulum, which are replaced with artificial materials, prosthesis (**Figure**

1). The aim of THA procedure is to reduce pain and improve patient's mobility and quality of life (Staff, 2022).

The hip endoprosthesis which a surgeon inserts into the patient's joint is made of two separate components: femoral and acetabular. Femoral component is nowadays made of two separate parts – a metallic stem and femoral head. Acetabular component is made of metal acetabular cup and acetabular interface (liner) (Baura, 2021). Endoprosthesis components may be composed of several different materials, each providing its own benefits and restrictions. Femoral stem is nowadays usually fabricated with stainless steel, titanium alloys or cobalt-chromium-molybdenum alloys (Merola & Affatato, 2019).
Cobalt-chromium (CoCr) alloy or aluminium alloy, while in the past the prosthetic component was also made from stainless steel (Baura, 2021). Prosthesis developments have, however, been largely targeted towards the articulating parts of prosthesis, which are the femoral head and acetabular component. In the past, femoral heads were mostly made from CoCr alloy, like the femoral stem, while acetabular cup was metal and its articulating interface either ultra-high molecular weight polyethylene (UHMWPE) (Gibon et al., 2013) or ceramic (Baura, 2021). Components of the hip prosthesis may be made from several different materials, which should be biocompatible and enable long-term survivability of the implant, which is the surgery's main goal (Gibon et al., 2013). Attempts to reduce friction and consequent particle debris formation have caused the metal femoral heads to be replaced with either alumina and zirconia ceramics, as well as oxidized zirconium. Ceramics as prosthetics components have consistently demonstrated reduced friction and consequent longer prosthesis lifespan, but unfortunately it was found that their toughness does not match that of metal. Still, they are the most commonly used materials for femoral heads of hip endoprosthesis today (Salehi and Hunter, 2010).

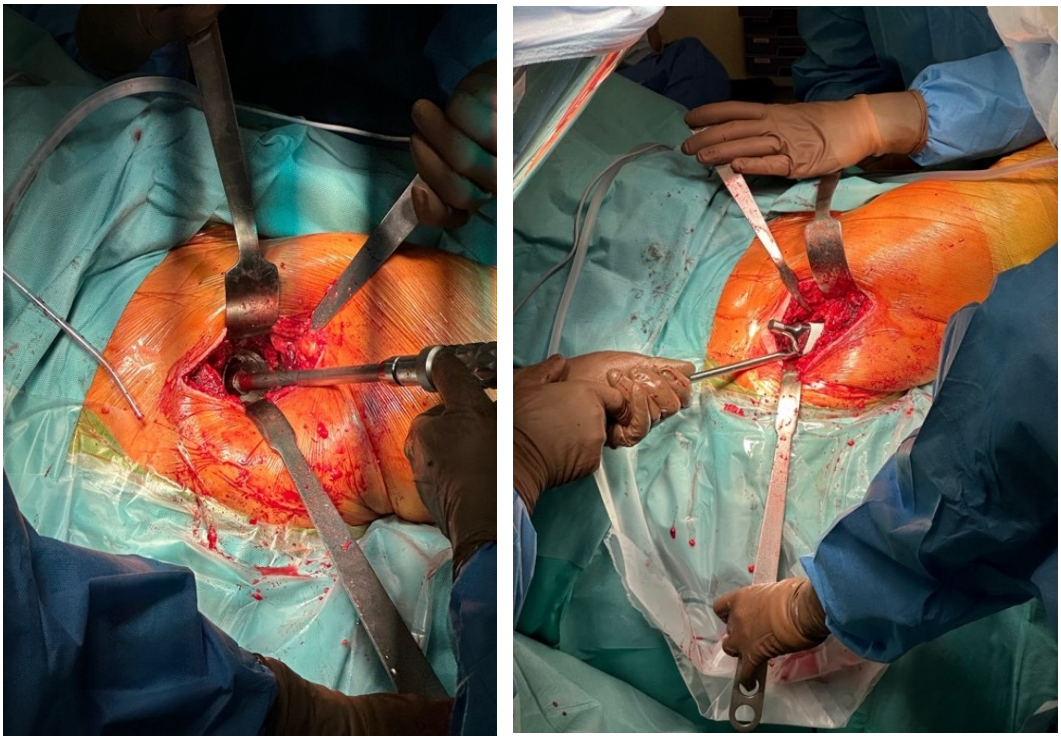

Figure 1. Surgeon reaming acetabular cup (left) and inserting femoral stem component of hip endoprosthesis (right). Source: Author's own archive.

There are three most common approaches for THA procedure (Varacallo et al., 2023). The most common approach for primary and revision cases is posterior approach. It includes blunt dissection of gluteus maximus muscle and sharp incision of fascia lata distally, avoiding hip abductors (Hyland, 2023). This approach is also favourable because of its good exposure of both acetabulum and femur, with the option for elongation of incision proximally or distally. However, some studies have cited higher dislocation rate in posterior approach compared to other surgical approaches (Varacallo et al., 2023).

The second possible THA approach is the direct anterior (DA) approach. The intermuscular interval being used with this approach is between tensor fascia lata and sartorius muscle superficially, and between gluteus medius and rectus femoris deep in the leg. The advantages of approach are the avoidance of hip abductors and reduced dislocation rates following surgery. However, its DA approach's reported disadvantages are increased wound complications, especially in obese patients, who are amongst those more at risk for arthrosis, along with limited femoral exposure and risk of paraesthesia of lateral femoral cutaneous nerve (Varacallo et al., 2023).

The less commonly used THA approach is the anterolateral or Watson-Jones approach. It utilises the intermuscular plane between tensor fascia lata and gluteus medius with a partial or complete detachment of anterior fibres of abductor muscles. In the past few years, incision or detachment of muscles or tendons has been avoided when performing the procedure (Lepri et al., 2020). The anterolateral approach theoretically offers decreased dislocation rate at the cost of postoperative limp (Varacallo et al., 2023).

Following incision and careful retraction of surrounding muscles, femoral neck osteotomy is performed. Usually, a reciprocating saw is used during this step, with the cutting beginning proximally to the lesser trochanter. Osteotomy (bone-cutting process) is continued in a proximal-lateral direction towards the base of the greater trochanter. Following this, surgeon removes surrounding soft tissue (Varacallo et al., 2023). Retractors are placed around the incision to provide acetabular visualization. Before acetabular component is placed, labrum (soft tissue surrounding hip joint) must be removed (Petis et al., 2015). This is done with a scalpel or an electrocautery. Then, acetabulum itself is prepared by reaming, starting with small-size machinery, gradually increasing sized for appropriate medialization of cup. This is demonstrated in Figure 1. Once sclerotic bone is removed and healthy bone is established, prosthetic acetabular component is inserted in a press-fit fashion. Then, corresponding liner is inserted (Varacallo et al., 2023). Then, the femoral canal is prepared for prosthesis insertion. This is done by broaches proximally and by cylindrical reamers distally. Both parts of the femoral endoprosthesis component are then inserted into the femur, which is demonstrated in **Figure 1** (right)(Fye et al., 1998). Prosthesis components may either be "press fit" into the bone, allowing it to grow around prosthesis, or they may be cemented into the bone. The quality and strength of patient's bone is a factor in choosing the right fixation method (Baura, 2021).

5. Hip arthroplasty – an increasing problem in today's aging population

In the recent years, there has been an increase in the number of both older and younger orthopaedic patients seeking surgical treatment. This might be due to the increasing number of sports injuries, as well as higher life standards and patients wanting to perform everyday activities without limitations. As the incidence of hip osteoarthritis generally increases with age, in today's aging population this is leading to a higher demand for surgical intervention. This presents an increased burden on healthcare resources and hospital budgets (Nemes et al., 2014). Indications for THA have been expanded to include younger patients who are usually more active and recover faster than older patients. Additionally, their postsurgical activity levels and their return to sports are expected to improve, consequently leading to overall higher patient satisfaction post-surgery (Fujita et al., 2022).

As patient satisfaction is an essential indicator in measuring the quality of care, several studies have attempted to measure patient satisfaction immediately and, in the years, following total hip arthroplasty (Varacallo et al., 2018; Okafor and Chen, 2019; Freudenberger et al., 2018). Said procedure is supposed to help with decreasing pain and improving motor function, patient mobility and quality of life. Freudenberger et al (2018) cited almost 60% of THA patients being highly satisfied with their hospital experience (a rating of 9 or 10 out of 10) (Freudenberger et al., 2018). Varacallo et al. (2018) reported 78% of interviewed patients 2-4 years after surgery perceived their replaced hip as "native", and 54% reported uninhibited function of replaced joint (Varacallo et al., 2018). Okafor and Chen (2019) found that older patients may experience greater post-operative, which might be due to their lower expectations of pain relief after having lived with the joint disease for years.

5. Conclusion

With today's aging population, hip arthrosis is becoming a major problem that, especially the elderly, are facing every day. Osteoarthritis is also becoming a problem amongst the younger, active population, including professional sportsmen and women. Due to the development of newer, more advanced pharmacological interventions, as well as intraarticular infiltrations, the time which passes before patients can no longer put off hip replacement surgery has been prolonged. However, with the development materials and newer, less invasive approaches to hip replacement surgery, the procedure has become one of the most cost-effective and successful orthopaedic surgeries, with patients being highly satisfied with their reduced pain levels and improved mobility and quality of life post-surgery.

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

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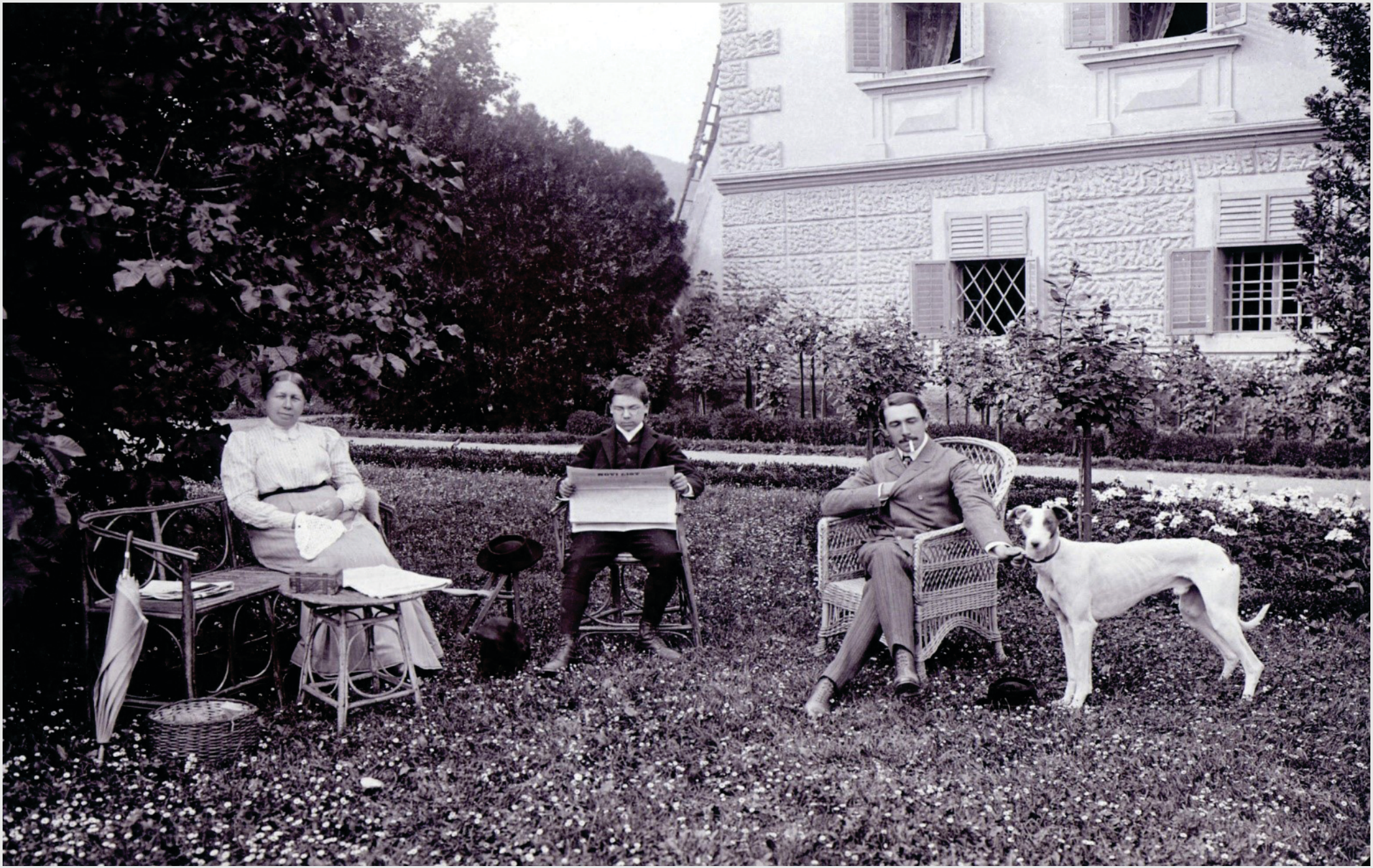

Research

Preserved Proximal Femoral Bone Stock Volume in Total Hip Arthroplasty Significantly Reduces the Risk for Periprosthetic Fractures. A Novel Modelling Technique and Preliminary Clinical Results

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Abstract:
Periprosthetic proximal femoral fractures (PPFFs) are one of the main causes for revision after total hip arthroplasty (THA), and are associated with some already known patient-/surgical-/implant-related risk factors. Despite the established increased risk of single and double-wedge femoral implants, the highest incidence in our institution has been observed with the anatomical cementless femoral component Anatomic Benoist Girard (ABG) II. The cumulative probability of PPFFs rose from 2.1% at 1 year to 6.5% at 10 years post-implantation, prompting comprehensive and multidisciplinary analysis. A novel parameter of preserved proximal femoral bone stock volume around implanted ABG II femoral stems (V_{PF}) and the modelling technique for its evaluation on the standing anteroposterior (AP) hip radiographs were introduced and estimated for each hip. Study was designed according to the standard protocol for matched case-control research. In the preliminary analysis, 5 age-/sex-/implant size-/surgeon-matched stratums, each comprising a case and 2 matched controls, were included. To calculate V_{PF}, a mathematical model was constructed by composing parts of rotational bodies and a prism, subject to geometrical parameters of the proximal femur that were assessed from radiographs. The mean value of V_{PF} in the group of cases was 141.6 ± 36.2 cm^3 and significantly lower compared to the mean volume of 254.2 ± 33.8 cm^3 in the control group ($P < 0.01$). Based on the preliminary results, the V_{PF} seems crucial for the PPFFs prevention. However, the mechanism of its effect works as a 'black box'. At this point, it can be hypothesized that insufficient bone stock from the implantation onwards interferes with adequate osseointegration by itself acutely and with increased stress shielding in the long term. The bone stock preservation should be emphasized and considered at all steps, starting from the preoperative planning. The novel parameter in THA, and the method for its evaluation were introduced and are further extensively analysed.

Keywords: Total Hip Arthroplasty, Periprosthetic Fracture, Bone Stock, Osseointegration, Stress Shielding

1. Introduction

Hip osteoarthritis (HOA) is an invalidating and prevalent disease with an estimated lifetime risk of symptomatic stage at around 25% (Katz et al., 2021; Murphy et al., 2010). Annually, more than 1 million total hip arthroplasties (THA) are performed worldwide with projections of further steady increase of primary as well as revision procedures (Ferguson et al., 2018). A number of factors, including the ageing population, the generalized demand for improved quality of life and functioning, along with the expansion of indications to the younger populations are governing the upscaling demand that has been proven insensitive even to the global economic downturns. In the next decades, a 2- to as much as 6-fold increase is projected by some studies (Shichman et al., 2023; Sloan et al., 2018).

As the incidence of primary THA continues to rise, the absolute burden of complications has increased with further growth being anticipated (Kurtz S et al., 2007; Schwartz AM et al., 2020). The periprosthetic fractures (PPFs) are one of the four most common reasons for revision after primary THA, the other three being infection, aseptic loosening and instability/dislocation (Smith PN et al., 2023). PPFs represent a complex orthopaedic pathology with significant patients' morbidity and mortality, and socio-economic implications. In more than 80% of cases, the mechanism of injury is a low-energy trauma, mainly fall from the standing height. Most of the PPFs affect the proximal femur (PPFFs), while acetabulum is involved in less than 10% of THA-related fractures (Abdel et al., 2015; Bozic et al., 2009; Patsiogiannis et al., 2021).

PPFFs are associated with some already known patient- (age, female sex, osteoporosis/osteopenia, neuromuscular diseases, cognitive disorders, Paget's disease, developmental hip dysplasia, rheumatoid arthritis), surgical- (malposition, extensive broaching), and implant-related (cementless, design/type, loosening, stress shielding) risk factors (Patsiogiannis et al., 2021; Singh et al., 2013). Despite the established increased risk of single and double-wedge femoral implants for PPFFs, the highest incidence in our institution has been observed with the anatomical cementless femoral component Anatomic Benoist Girard (ABG) II (Stryker Orthopaedics, Mahwah, NJ, USA), which is in line with some other studies in the literature (Carli et al., 2017; Catanach et al., 2015; Kropivšek et al., 2023; Mulford et al., 2022; Thien et al., 2014). The cumulative probability of PPFFs rose from 2.1% at 1 year to 6.5% at 10 years post-implantation, prompting comprehensive and multidisciplinary analysis (Kropivšek et al., 2023).

Given that, ultimately, it is the bone that fractures, and preserved bone stock is pivotal for revision procedures, the novel parameter of preserved proximal femoral bone stock volume around the implanted femoral stems (V_{PF}) has been hypothesized. The primary aim of the present study was to develop a practical and reliable method for the evaluation of this novel parameter on the widely available hip anterior-posterior (AP) radiographs, and to conduct a preliminary assessment of its validity.

2. Methods

2.1 Assessment of geometrical parameters

Geometrical parameters of the bone in contact with endoprosthesis were assessed from the standard hip AP radiographs. The images were available in DICOM format and measured by software Agfa HealthCare Enterprise Imaging (Agfa-Gevaert NV, Mortsel, Belgium). This software enabled measurements of lengths and delimited areas. The parameters x_N', x_M', x_L', $x'_{L\,LAT}$, x_N, x_M, x_L, $x_{L\,LAT}$, H_N, H_L and S that were used for calculation of respective volumes are depicted in **Figure 1**. As the magnifications of the images were not known, the parameter dimensions were scaled by considering the known diameter of the prosthesis femoral head.

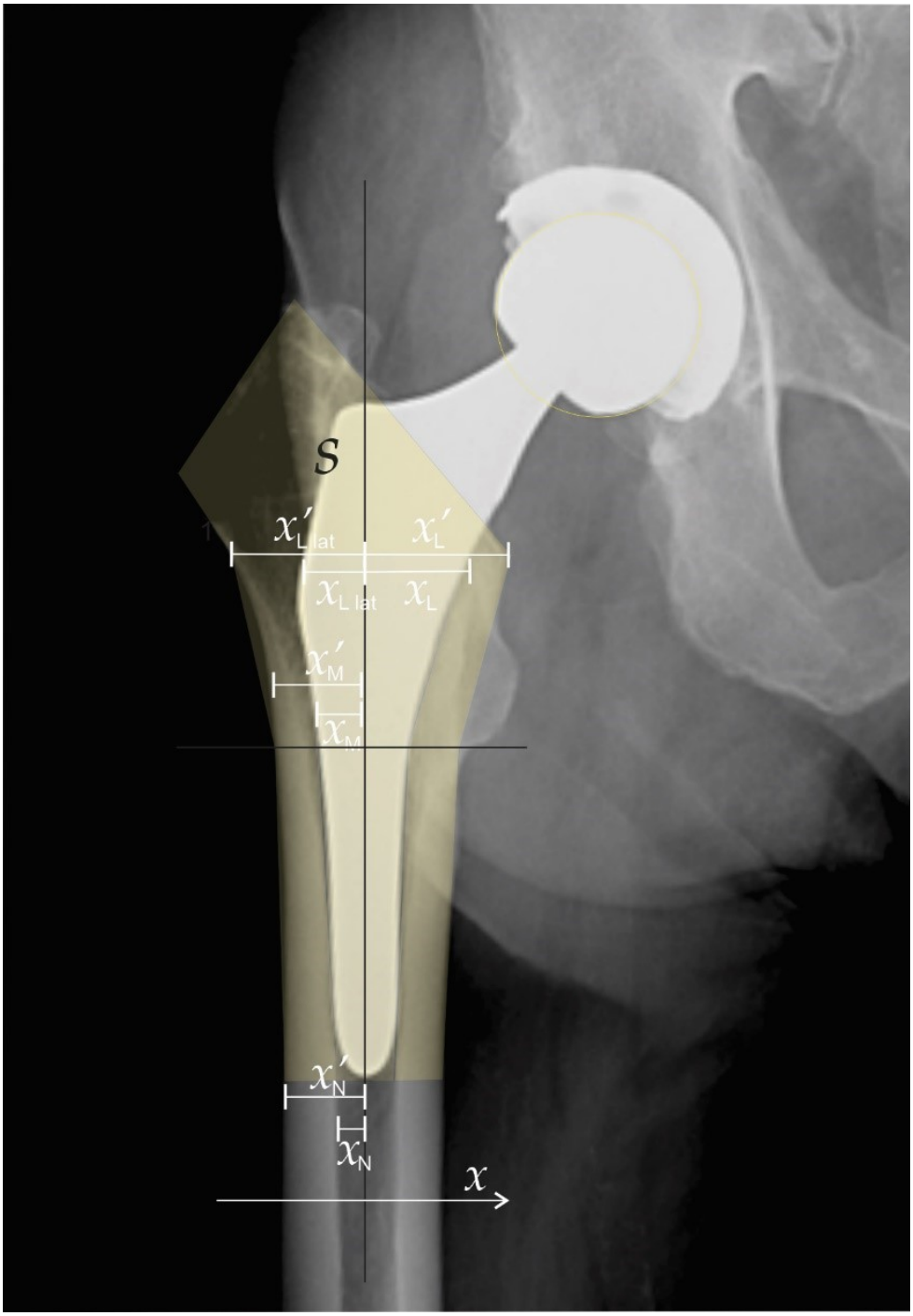

Figure 1. Geometrical parameters used as an input for the calculation of V_{PF} evaluation.

2.2 Evaluation of the V_{PF} parameter

V_{PF} is composed of hollow cut coni (modelling the bone of the femoral shaft in contact with the stem of the prosthesis) and a prism (modelling the remnants of the greater trochanter). In general, the volume of the conus with radius R at the base and height H is

$$V_{conus} = \pi R^2 H/3 \quad (1)$$

and the volume of the conus cut at height h where the radius is r is

$$V_{cut\ conus} = \pi (R^2 h + RrH + r^2 h \quad , \quad (2)$$

where

$$r = R\ (1 - h/H) \quad . \quad (3)$$

Following Equation (2), at the lower part of the shaft the volume of the hollow cut conus is

$$V_1 = \pi (x_N'^2\ H_N + x'\ x_M' H_N + x_M'^2\ H_N)/3 \quad - \quad \pi (x_N^2\ H_N + x_N\ x_M H_N + x_M^2\ H_N)/3. \quad (4)$$

In the middle part at the region of the smaller trochanter, the contour of the bone is considered asymmetric with respect to the axis along the shaft. The columns of the medial and the lateral halves are therefore calculated separately taking into account the differences between respective x_L and $x_{L\ LAT}$. The respective volumes are

$$V_2 = \pi (x_L'^2\ H_L + x_L'\ x_M' H_L + x_M'^2\ H_L)/6 \quad - \quad \pi (x_L^2\ H_L + x_L\ x_M H_L + x_L^2\ H_L)/6 \quad (5)$$

and

$$V_{2\ LAT} = \pi (x'^2_{L\ LAT}\ H_L + x'_{L\ LAT}\ x_M' H_L + x_M'^2\ H_L)/6 \quad - \quad \pi (x_L^2\ H_L + x_L\ x_M H_L + x_M^2\ H_L)/6 \quad . \quad (6)$$

It is considered that only half of each rotational body is contributing to the volume. The volume of the prism is calculated by

$$V_{prism} = 2\ S\ x_N' \quad , \quad (7)$$

where S is the area of the prism assessed by delimiting its contours. The volume of the bone in contact with the prosthesis is

$$V_{PF} = V_1 + V_2 + V_{2\ LAT} + V_{prism} \quad . \quad (8)$$

2.2 Subjects

The retrospective matched case-control study was conducted according to the standard protocol for this type of research. In the preliminary analysis, the patients with late PPFFs, minimum 1 year postoperatively, were enrolled from the observational cohort of all implanted primary total hip arthroplasties with uncemented ABG II femoral stem between January 1, 2012, and January 31, 2013, at a single tertiary hospital (University Medical Centre Ljubljana, Department of Orthopaedic Surgery, Ljubljana, Slovenia). Clinical investigational plan was approved by the National Medical Ethics Committee (permit No. 0120-605/2021/3). In the group of cases, 5 patients with late PPFFs were included. An example of PPFF around the ABG II femoral stem is represented in **Figure 2**. For each case, 2 con-

trols matched for age, sex, implant size, and surgeon were found from the whole observational cohort: 1531 uncemented ABG II femoral stems implanted between January 1, 2012, and December 31, 2018. As a result, the control group of 10 patients was formed, and 5 matched stratums, each comprising a case and its 2 controls, were analysed. Patients' demographics, medical history, stress shielding (Engh Grading Scale**)** (Engh, et al. 1987), Canal Flare Index (CFI) (Noble PC, et al. 1988), and length of radiographic follow-up evaluation were documented (**Table 1**).

3.3 *Surgical intervention*

Patients were operated under spinal or general anesthesia, in the supine position with the direct lateral approach, or in the lateral decubitus position with the posterior approach to the hip joint. The cementless ABG II femoral stems were combined with either acetabular cup ABG II or acetabulum from another manufacturer. All surgical procedures were performed in the two operating rooms of the same operating suite of a single tertiary university hospital. Perioperative antibiotic prophylaxis, thromboembolic prophylaxis and postoperative rehabilitation protocol were uniform for all patients at a given time-point, but they have been changing between 2012 and 2018 in accordance with the national guidelines. The patients were followed from the initial primary total hip arthroplasty until the eventual outcome assessment on October 31, 2023.

Table 1. Demographics, medical history, and follow-up time of the radiographs' evaluation in both groups. Categorical variables are presented as frequencies (percentages), while continuous variables as mean (standard deviation). For comparison of both groups Student t test (continuous variables) or Chi square test (categorical variables) were applied.

Characteristic	All (n = 15)	Cases (n = 5)	Controls (n = 10)	Comparison (P values)
Age (years)	70.2 ± 5.0	68.2 ± 4.2	71.1 ± 5.3	0.31
Sex (n)				
Female	9 (60%)	3 (60%)	6 (60%)	1.0
Male	6 (40%)	2 (40%)	4 (40%)	
Height (m)	168.6 ± 8.9	168.6 ± 9.4	168.6 ± 9.1	1.0
Weight (kg)	78.3 ± 7.4	76.8 ± 7.0	79.1 ± 7.8	0.59
BMI (kg/m2)	27.7 ± 3.0	27.0 ± 1.7	28.0 ± 3.6	0.60
Follow-up (months)	59.7 ± 32.7	59.6± 35.2	59.7 ± 33.8	0.99
Osteoporosis (n)	2 (13%)	1 (20%)	1 (10%)	0.60
Stress shielding (Engh Grading Scale)	1.6 ± 1.0	2.6 ± 0.5	0.8 ± 0.4	0.01*
CFI	2.9 ± 0.4	2.8 ± 0.3	3.0 ± 0.4	0.58

CFI– Canal Flare Index, BMI – Body Mass Index, m – meter, kg – kilogram, kg/m2 – kilogram per square meter

Figure 2. Case of PPFF around the ABG II femoral stem. Type B2 (unstable implant, sufficient bone stock) according to the Vancouver classification (Duncan CP et al., 1995) .

2.4 Statistical analysis

Descriptive statistical analysis was used to describe patients' demographics, medical history, stress shielding (Engh Grading Scale) (Engh et al., 1987), Canal Flare Index (CFI) (Noble et al., 1988), and length of radiographic follow-up evaluation. Continuous variables were presented as means with standard deviations (SD), and categorical variables as frequencies with corresponding percentages. For comparison of both groups either Student t test (continuous variables) or Chi square test (categorical variables) were applied. Statistical analysis was performed with SPSS (Version 25.0; IBM, Chicago, IL, USA). The level of statistical significance was set at $P < 0.05$.

3. Results

The mean value of V_{PF} in the group of cases was 141.6 ± 36.2 cm^3 and significantly lower compared to the mean value of 254.2 ± 33.8 cm^3 in the control group ($P < 0.01$). Moreover, in all 5 age-/sex-/implant size-/surgeon-matched stratums, the mean V_{PF} of both controls was lower than V_{PF} of the case (**Table 2**).

Table 2. V_{PF} in the 5 age-/sex-/implant size-/surgeon-matched stratums. In each stratum, the V_{PF} of the case (PPFF) and the mean V_{PF} with standard deviation of both controls are presented.

Stratum No.		V_{PF} (cm^3)
1	PPFF	90.0
	Controls mean	241.5 ± 1.6
2	PPFF	180.2
	Controls mean	280.1 ± 3.8
3	PPFF	118.5
	Controls mean	217.4 ± 27.3
4	PPFF	154.4
	Controls mean	239.3 ± 18.9
5	PPFF	165.0
	Controls mean	292.5 ± 12.1

V_{PF} – Volume of Preserved proximal Femoral bone stock around femoral stems, PPFF - Periprosthetic Proximal Femoral Fracture, cm^3 – cubic centim

4. Discussion

The present study introduces a novel method for the evaluation of preserved proximal femoral bone stock volume around the implanted femoral stems on the widely available hip AP radiographs. The preliminary results are promising.

Despite the longstanding awareness of the increasing incidence and consequences of PPFFs, and the rationale protective role of the preserved bone stock, no method for its evaluation, with the potential for routine clinical application, has been available. Interestingly, the research and development have been for decades intensively focused mainly on the artificial implants, their materials, composition, design, and other characteristics, while the local host environmental factors have remained mostly unaddressed (Burchard et al., 2023; Carli et al., 2017; Glassman et al., 2006; Huiskes et al., 1992; Rivière et al., 2018; Sumner et al., 1992).

The introduced modelling technique aims to tackle the increasing PPFFs problem, and proposes a novel V_{PF} parameter, which seems to significantly influence the risk of PPFFs and could be controlled to some degree. The method utilizes readily available standing hip AP radiographs that are part of every routine diagnostic assessment of patients with the indication for the primary THA. Moreover, its simplicity, quick learning curve, and time efficiency, taking only a few minutes after some examples measured, enable the surgeon to plan and control the bone stock preservation for every patient. Based on the preliminary results, the V_{PF} seems crucial for the PPFFs prevention. However, the mechanism of its effect works as a 'black box' (Mavčič et al., 2012). The interplay between the two well established risk factors, osseointegration and stress shielding, with this novel third parameter of the preserved bone stock could be proposed (Savio et al., 2022). Bone preservation supports initial stability and enhance osseointegration acutely, while reducing stress shielding and preventing loosening (ensuring stable osseointegration) in the long term. Therefore, the preserved bone stock volume may be considered as the common biological denominator of osseointegration and stress shielding. Moreover, the observed significant difference in the mean grades of stress shielding between the groups supports the proposed correlations. However, the constitutional law between these factors at interplay has not been fully established yet. Despite only the preliminary analysis being performed, the

results clearly indicate the importance of considering bone stock preservation at all steps, starting from the preoperative planning.

5. Conclusion

The introduced method for V_{PF} evaluation demonstrated intuitive and promising results. Its wide availability, simplicity and significance promise the implementation into routine clinical practice. The novel parameter in THA, and the method for its evaluation were introduced and are further extensively analysed.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki. Clinical investigational plan was approved by the National Medical Ethics Committee (permit No. 0120- 605/2021/3).

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

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Invited lecture /Rewiev

Urinary Tract Infections in Dogs and Cats

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Abstract:
Urinary tract infection (UTI) is a very common health problem in dogs and cats, treated with antimicrobial drugs. As the antimicrobial resistance problem increases, clinicians should be more aware of how to treat patients with antibiotics. This article aims to highlight the importance of antimicrobial susceptibility testing before antimicrobial treatment and remind clinicians to follow updated guidelines of the International Society for Companion Animal Infection Diseases (ISCAID) recommendations (2019) and to monitor their local antimicrobial resistance situation. The short review includes sporadic bacterial cystitis, recurrent bacterial cystitis, pyelonephritis, and subclinical bacteriuria (Weese et al., 2019).

Keywords: Cystitis; Subclinical bacteriuria, Pyelonephritis; Antimicrobials; Canine; Feline

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1. Introduction

Urinary tract infections (UTIs) are a prevalent health concern in dogs and cats. Various microorganisms, mostly pathogenic bacteria and rare infecting fungi, viruses or parasites, can instigate these infections.

UTIs are a common health issue in dogs and cats and one of the main reasons for antibiotic prescription. It is estimated, that 14% of dogs and 3-19% of cats will develop UTI at least once in their lifetime (Ling, 1984; White et al., 2013; Dorsch et al., 2016).

Bacterial cystitis occurs more commonly in female dogs and cats (Byron, 2019). UTI is more common in senior female cats, older than 10 years of age, mostly with comorbidities (Dorsch et al., 2014).

The development of UTI depends on the host immune system and defenses on one side and bacterial virulence on the other. Genital, rectal and perineal bacteria are the main reservoars for UTI (Osborne et al., 1991; Johnson et al., 2003). The most common cause of UTI is uropathogenic Escherichia coli (UPEC) (Moyaert et al., 2017).

Veterinarians often prescribe antibiotics without urinary microbial culture and antibiotic susceptibility testing. Antibiotics commonly used in UTI are fluoroquinolones and 3rd generation cephalosporines (De Bruyne et al., 2014). These two antibiotics are on the list of highest priority critically important (Lhermie et al., 2020)

Antimicrobial resistance is a growing concern in veterinary and human medicine. We must also be aware of our responsibility for public health, as AMRD genes can transfer between animals and human pathogens (Ewers et al., 2012).

For this reason and for better therapeutic results, new recommendations regarding antimicrobial drugs, doses, duration and frequency of treatment should be followed and the health status of each patient should be carefully monitored.

2. Sporadic bacterial cystitis

Management of sporadic bacterial cystitis is based on ISCAID guidelines (2019) where bacterial cystitis results in inflammation and clinical signs such as stranguria, hematuria and pollakiuria occur. Sporadic bacterial cystitis is diagnosed when fewer than 3 episodes of cystitis in the preceding year occurred with or without comorbidities. The diagnosis includes clinical signs, urine analysis, and urine bacterial cultures. Urinalysis is the major diagnostic test and includes macroscopic (dipstick, specific gravity) examination and microscopic examination (urinary sediment). Microbiological analysis is preferred, and urine for bacterial culture should be obtained by ultrasound-guided cystocentesis in cases where other pathologies of the urinary bladder can be evaluated (such as masses, uroliths, etc.) If cystocentesis is risky for the animal, another option for obtaining urine is catheterization or a voided sample. Proper urine storage and quick laboratory analysis are important for accurate results (Weese et al., 2019).

Young and middle-aged cats rarely have bacterial UTIs. Feline idiopathic cystitis (FIC) and urolithiasis are the main reasons for feline lower urinary tract disease (Dorsch et al., 2014).

Bacterial urine culture and antimicrobial susceptibility tests in cats are necessary to make a diagnosis of UTI and treat patients properly. Empirical antimicrobial treatment for dogs with no or limited history of antibiotic treatment without urine culture is acceptable (Weese et al, 2019).

ISCAID guidelines (2019) recommend amoxicillin (with clavulanic acid in case amoxicillin alone is not available) or trimethoprim-sulphonamides for antibiotic treatment of sporadic bacterial cystitis. The duration of therapy is 3-5 days. When urine culture and sensitivity have been performed, with resistance to empirical antibiotic detected but clinical signs have resolved, there is no need to repeat the urinalysis after treatment. Even if the antibiotic was in line with the results of urine culture and sensitivity but clinical signs persist, clinicians should reevaluate the diagnosis and find the reason (Weese et al., 2019).

The guidelines emphasizes that clinicians should consider local known antimicrobial resistance and presume alternate antimicrobials for better outcomes in empirical treatment. Treatment can be started with non-steroid anti-inflammatory drugs (NSAIDs) because of

inflammation (side effects of NSAIDs should be considered) and continued with antibiotics in case where clinical signs do not improve (Weese et al., 2019).

3. Recurrent bacterial cystitis

ISCAID guidelines (2019) defines recurrent bacterial cystitis as: three or more episodes of clinical bacterial cystitis in the preceding 12 months or two or more episodes in the preceding 6 months.

Recurrent UTIs include various scenarios: relapse of infection (with the same microorganism after successful treatment of the UTI, often seen in diseases like urolithiasis or pyelonephritis); reinfection (infection with a different microorganism after the initial microorganism responded to therapy, common in disease like diabetes mellitus); persistent infection (positive urine cultures with the same organism during treatment with appropriate antimicrobial agents); and superinfection (infection with new organisms that develop during antimicrobial treatment for the initial infecting organism, observed in diseases such as neoplasia or urinary catheterization) (Barsanti, 2009; Weese et al., 2019).

Concomitant factors and underlying causes are mostly the main reasons for recurrent UTI. Advanced diagnostics should be performed to identify the underlying disease responsible for the recurrent UTI (endocrinopathy, kidney disease, obesity, abnormal vulvar conformation, congenital abnormalities of the urogenital tract (e.g. ectopic ureter), prostatic disease, bladder tumor, polypoid cystitis, urolithiasis, immunosuppressive therapy, rectal fistula, urinary incontinence/retention). Diagnostic imaging with ultrasound, radiography, contrast imaging and cystoscopy is warranted. In some cases, biopsy of urinary bladder mucosa for pathophysiologic and microbiological examination is necessary (Olin & Bartges, 2015; Weese et al., 2019).

Proper diagnosis, treatment, and controlling diseases like Cushin's disease, diabetes mellitus, correction of ectopic ureter, vulvar skin fold, and others can end the cycle of recurrence of UTI.

The clinician must ensure adequate antibiotic concentration in the bladder, check dosage, regiment client compliance, etc.

Treatment should be based on the results of urinary culture and sensitivity. While awaiting urine bacterial culture results, NSAIDs can be administrated, and if an empirical antibiotic is necessary, first-line amoxicillin or trimethoprim-sulphonamide should be initiated. In case of microbial invasion of urinary bladder wall, antimicrobials that are effective against *Escherichia coli* in tissue are prescribed. After obtaining urine culture and sensitivity results, clinical response to therapy should be considered. In case of clinical failure, bacterial culture-based antimicrobial changes should be indicated (Weese et al. 2019).

Treatment duration in case of reinfection is short, typically 3-5 days, and monitoring is based on clinical response. In cases of persistent and relapsing infections, longer treatment durations of 7-14 days are indicated, with repeating urine culture after 5-7 days of treatment being prudent. A positive urine culture mandates checking compliance with therapy and further evaluation of the patient. Negative results of urine culture do not guarantee microbiological cure and these are also authors' experiences. After 5-7 days post - treatment, urine culture should be repeated if clinical signs resolve. In cases of positive urine culture (relapse, reinfection, persistent) with the absence of clinical sign, subclinical bacteriuria is diagnosed, and referral to a specialist should be considered (Weese et al., 2019).

4. Upper urinary tract infections (pyelonephritis)

Pyelonephritis is an inflammation of the renal parenchyma and pelvis, mostly caused by an ascending urinary infection rather than hematogenous spread (Parry, 2005). Leptospira should be considered in case of pyelonephritis (Sykes et al., 2011). Pyelonephritis can have acute or chronic course. Acute pyelonephritis is associated with fever, uremic signs (anorexia, lethargy, vomiting), painful kidneys, polyuria/polydipsia, anuria, lumbal pain, etc. Chronic pyelonephritis can present with mild (or even absent) clinical signs and slowly progressive azotemia, whicht can lead to kidney failure. Diagnosis of pyelonephritis is based on clinical signs, urinalysis, positive urine culture, compatible ultrasonographic

changes of the kidney, leukocytosis (neutrophilia) and azotemia. It is important to remember that not all patient with pyelonephritis have leukocytosis or azotemia (Olin & Bartges, 2015; Bouillon, 2018). In cases of negative urine culture, immunosuppressed animals, or those with fever, a blood culture is needed (Kim et al., 2017, Westropp & Sykes, 2017).

In a study of pyelonephritis in dogs, Bouillon et al. (2018) found, that 74.5 % of dogs with pyelonephritis presented with comorbidities.

Treatment should be started with antimicrobial agents effective against Enterobacteriaceae, taking into account regional antimicrobial susceptibility. Veterinary fluoroquinolone or cefpodoxime are the first choice. Cefotaxime and ceftazidime are important for intravenous application. If results of antibiogram show resistance to the initial antimicrobial and clinical response is not sufficient, substitution with a susceptible antimicrobial is necessary (not necessary if the clinical response is good). If therapy is initiated with two antimicrobials and both are susceptible, one of them might be discontinued if there is a good clinical response. If results of urinary culture and susceptibility identify multidrug - resistant microbes, consultation with a specialist is recommended (Weese et al., 2019).

The duration of the treatment is 10-14 days. One to two weeks after discontinuation of antimicrobials, a recheck is recommended (physical exam, urinalysis, bacterial culture, blood analysis). In cases of full clinical recovery and positive results of clinical culture, subclinical bacteriuria should be suspected (Weese et al., 2019).

5. Subclinical bacteriuria

Subclinical bacteriuria is defined as the presence of bacteria in urine as determined by positive bacterial culture from a properly collected urine specimen, in the absence of clinical evidence of infectious urinary tract disease. It is not uncommon in healthy animals, patients with comorbidities, and those treated with immunosuppressive drugs. In a Norwegian study of the prevalence of subclinical bacteriuria, in a cohort of 108 healthy cats with a mean age of 4 years, 0.9% were positive (Eggertsdottir et al., 2011). With age, prevalence increased. Another study reported 67 cats older than 7 years of age with a prevalence of subclinical bacteriuria in 10-13% (White et al., 2016).

Lamoureux et al. (2022) found that 32% of 62 dogs with chronic kidney disease (CKD), including 8 dogs in International Renal Interest Society (IRIS) stage 1, had positive urine culture, and only 8% showed clinical signs of urinary tract disease. Another study in cats with CKD reported a prevalence of 22-29% (Mayer-Roenne et al., 2007).

Current guidelines recommend that antimicrobials should not be used for treatment in subclinical bacteriuria. There are recommendations for treatment of specific conditions, such as animals with suspected pyelonephritis, those undergoing surgical or endoscopic procedures of the urinary tract likely to cause bleeding, etc. Clinicians should evaluate each case individually and decide whether to treat or not (Weese et al., 2019).

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Invited lecture/Review

Physiotherapy Approach for Treating Lateral Epicondilalgia

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Abstract:
Lateral epicondylitis, a tendinopathy affecting forearm extensor muscles, results from overuse and commonly manifests as pain over the lateral humeral epicondyle. The extensor carpi radialis brevis (ECRB) is implicated, particularly during forceful gripping. The condition is prevalent in the 35-45 age group and it affects 1-3% of the population, with symptoms lasting two years and reoccurring after asymptomatic periods. Considering the economic impact of lateral epicondylalgia-related sick leave, therapeutic focus must emphasize long-term as well as short-term efficacy. Treatment approaches include drugs, rest, physiotherapy and surgery. Platelet-rich plasma proves highly effective, reducing pain and work absenteeism. Severe cases may require surgical approach, with studies questioning the benefits of surgery over a placebo. Pain management involves shockwave therapy, ultrasound, friction massage, eccentric exercises, stretching, and orthoses. Shockwave therapy stands out for its rapid pain relief and long-term efficacy. The combination of exercises and kinesiotaping is effective, while high-intensity laser therapy has limited evidence. Functionality improves with manual therapy, stretching, and strengthening exercises. Orthoses may negatively impact hand function. Grip strength correlates closely with functionality, with therapeutic exercise and manual therapy showing significant long-term results.

Keywords: Lateral epicondylalgia, tendinopathy, rehabilitation, physiotherapy approach

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1. Introduction

Lateral epicondylitis is a tendinopathy of the forearm extensor muscles, often caused by overuse or repetitive use (Tarpada et al., 2018). Individuals suffering from tennis elbow often experience pain localized around the lateral humeral epicondyle. Pain tends to intensify during elbow flexion, particularly when the wrist is in a pronated position and extended against resistance. The consensus among many researchers is that the origin of the extensor carpi radialis brevis (ECRB) is the key contributor to the symptoms associated with lateral epicondylitis. From a biomechanical standpoint, forceful gripping places the ECRB under maximum strain, especially when the forearm is pronated, and the wrist is flexed and ulnar deviated. Lateral epicondylitis is most prevalent in the individuals aged 35-45, affecting approximately 1-3% of the general population. Symptoms usually last for 2 years, but it usually relapses after asymptomatic periods (Sandhu et al., 2020; Bisset & Vicenzino, 2015).

Due to the increasing economic impact of lateral epicondylalgia-related sick leave, attention must also be given to the short-term effects of therapies that will reduce the duration of work absenteeism.

Different treatment approaches have been proposed, such as drugs, rest, physiotherapy and surgery (Zhong et al., 2020). The first therapeutic step usually involves rest and administration of drugs, providing short-term pain relief but also yielding poor results for problem resolution and relapse prevention (Boden et al., 2019).

Platelet-rich plasma treatment proves to be a highly effective therapy; research indicates that, in addition to reducing pain and improving function, the time of absence from work is shortened even more compared to non-invasive therapies (Alessio-Mazzola et al., 2018). But there is 2-11% of cases severe enough that need surgical removal of diseased tissue. With the surgical approach, immediate pain relief is achieved in 80-97% of cases, with more than 75% of patients with no or minimal pain after one year. Although 1.5% of patients undergo a second surgical procedure in the following 18-24 months (Degen et al., 2018; Holmedal et al., 2019). On the contrary, study by Kroslak and Murrell (2018) showed that surgical excision of the degenerative portion of the ECRB offers no additional benefit over and above placebo surgery for the management of chronic tennis elbow.

Physiotherapeutic treatment is shown to be the most effective, but it should include manual therapy performed under the pain threshold to relieve pain and improve joint range of motion (Zhong et al., 2020).

When assessing the effectiveness of rehabilitation techniques, focus is on pain, limb function, maximum grip strength, and wrist range of motion.

2. Pain management

Research indicates that pain intensity decreases most rapidly (between 3 and 9 sesions) with therapies involving shockwave therapy, ultrasound, friction massage, eccentric exercises, stretching exercises, and orthoses (Landesa-Piñeiro & Leirós-Rodríguez, 2022). Shockwave therapy demonstrates a notable decrease in pain threshold after only three sessions (Yalvaç et al., 2018), whereas ultrasound therapy yields comparable effects after ten sessions, as evidenced by Yan et al. in 2019. The analgesic efficacy of these therapies arises from the stimulation of pain receptors and the activation of unmyelinated C fibers and A delta fibers, initiating the gate control theory of pain (Dedes et al., 2018). Notably, shockwave therapy employs higher energy waves, leading to a more pronounced stimulation of pain receptors (Yan et al., 2019). Moreover, shockwave therapy induces a localized metabolic response characterized by heightened vascularity and reduced adhesion formation. These contribute to the facilitation of the inherent healing processes (Orhan et al., 2004). The effect of shockwave therapy lasts longer than the effect of conventional therapy (thermotherapy, ultrasound, TENS). Shockwave therapy is more effective in the short term for chronic patients, but in the long term, it has a better impact on acute patients (Köksal et al., 2015).

Another randomized contolled study showed the combination of exercises and kinesiotaping can be more effective than exercises alone or with sham taping for up to one month after treatment. There is some high quality evidence that shows positive effect on pain after performing all kind of strenghtening exercises combined (Eraslan et al., 2018).

There is only one high quality study that show positive effect of high-intensity laser therapy in comparison with placebo laser therpy, but the tickness of common extensor tendons did not change at all (Dundar et al., 2015).
For long term pain relief there is evidence that shockwave therapy, manual therapy, eccentric strengthening exercises and corticosteroid infiltration have positive effects up to 1 year after therapy (Köksal, 2015; Olaussen, 2015).

3. Functionality and grip strenght

The relationship between functionality and grip strength demonstrates a notable correlation, wherein grip strength serves as a quantifiable and objective metric. Interventions based on therapeutic exercise and manual therapy obtained good results in the long term and even significantly higher results compared to those based on shockwave therapy, orthoses, and corticosteroids (Landesa-Piñeiro & Leirós-Rodríguez, 2022).
The effect of conventional therapy (stretching and strengthening exercises with education) showed good result but recovery time depends, to a great extent, on the frequency of their execution (Stasinopoulos & Johnson, 2004). Studies show that no kind of strenghtening exercises is superior than other, but there is lack of studies that isolate the effect of particular kind of exercises (Lepley et al., 2017; Stasinopoulos & Stasinopoulos, 2017). It is important to point out that studies show improvement in range of wrist motion after only eccentric exercises (Nowotny et al., 2018). This modality of strenght training also instigates collagen production, diminishes the incidence of inflammation and neovascularization, and mitigates pain through the augmentation of tendinous resistance and desensitization of central nervous pathways involved in pain transmission (Peterson et al., 2014).
Shockwave showed good results but is less efective compared to kinesiotaping which should be used in combination with exercises for longer effect (Giray et al., 2019). Eraslan et al. (2018) assert that the combination of conservative therapy and kinesiotape application is more effective than the combination of conservative therapy and shockwave therapy. According to the three parameters of the Patient-Rated Tennis Elbow Evaluation Scale, the difference before and after therapy is statistically significant in both groups. However, the progress in the group with kinesiotape application is statistically significantly superior. Kinesiotape reduses pain and edema and facilitate motor activity by relieveing motor abnormal muscle tension. They also stimulate mechanoreceptors by applying pressure on the skin, which are effects that have a direct impact on the generation of strenght (Pieters et al., 2020).
Studies show that the use of orthoses can have a negative effect on hand function, reducing mobility and preventing normal elbow movement (Stasinopoulos & Stasinopoulos, 2017). The use of orthoses for 6 weeks in combination with mobility exercises and stretching exercises daily has no positive effect (Yi et al., 2018).

4. Conclusion

Taken together, researchers still haven't agreed on the most effective long-term therapy due to lack of studies showing lasting effects. Additionally, considering the economic consequences related to work absenteeism there is need to find therapies with best short term effect.
The combination of manual therapy, involving deep friction massage, along with streching and eccentric strenghtening exercises yields the most favorable results for functional improvement and increased grip strength in lateral epicondylalgia, furthermore, their cost-benefit ratio is very favourable. Other therapy techniques such as shockwave and ultrasound therapy have the greatest effect on reducing pain intensity. Moreover, research indicates that shockwave therapy achieves the fastest results in pain management.
According to available literature, the rehabilitation of lateral epicondylalgia should be divided into three phases. In the first and second phases, the focus is on reducing pain and improving hand dysfunction. The literature suggests therapy using shockwave therapy in combination with manual therapy (deep friction massage) under pain threshold and stretching exercises. In the second phase, isometric exercises are added to strengthen the wrist extensors and finger extensors. These exercises are then progressed in the third phase, when we focus on grip strenght, where concentric and eccentric

exercises are introduced for the wrist extensors, supinators, and pronators. In all three phases, the application of kinesiotaping can be added. However, it is important that it is applied by a trained person, as incorrect application may not bring positive results.

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

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Invited lecture/Review

The Impact of Sleep Deprivation on Physical Activity

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Abstract:
Proper amount of sleep has a critical role in biological processes and overall health and is linked to negative health outcomes, such as diabetes, heart disease, brain stroke, weight changes, pain, mood swings, Alzheimer's disease, cognitive health, chromosome changes etc. Despite dedicating one-third of our lives to sleep, a substantial portion of the world's population faces sleep deprivation. Insufficient sleep goes beyond simple tiredness, and it is affecting humans metabolic, cardiovascular, cognitive, and emotional dimensions. When it comes to physical activity and athletic performance, sleep deprivation manifests in compromised reaction time, accuracy, vigour, submaximal strength, and endurance. These challenges also affect athlete's cognitive functions such as judgment and decision-making. In the context of athletic performance, even partial sleep deprivation can lead to significant impairments. Relationship between sleep and physical activity was found as emphasizing the importance of adequate sleep for optimal performance and shows that good sleep hygiene and recommended amount of physical activity really go hand in hand contributing to overall well-being including physiological and mental health.

Keywords: Sleep deprivation; Physical activity; Athletic performance, Metabolic health; Cognitive functions, Global prevalence.

1. Sleep hygiene and sleep deprivation

1.1. What is sleep and why we need it

Sleep is defined as natural and reversible state marked by reduced responsiveness to external stimuli and relative inactivity, accompanying a loss of consciousness (Rasch & Born, 2012). Sleeping represents the supreme support for human health, constituting a fundamental biological imperative. An average human dedicates approximately one-third of their lifespan to this crucial physiological function (Hirshkowitz et al., 2015). Furthermore, sleep serves as a key opportunity for the body's reparative processes (Bandyopadhyay & Sigua, 2019). It does not represent just a state where our responses are reduced, but it has 4 stages and they all have their own important role (Rasch & Born, 2012). Stages of sleep are presented in **Figure 1**.

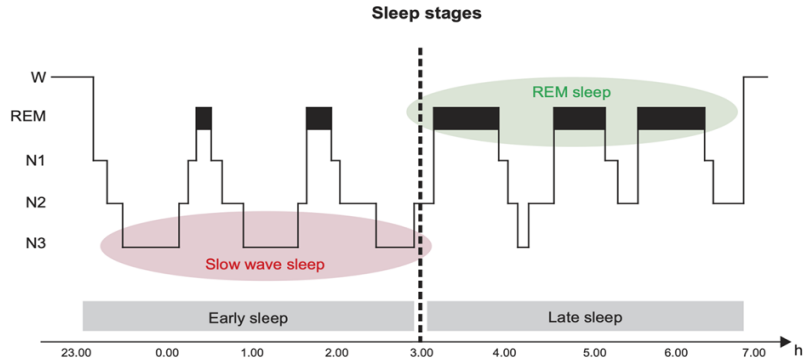

Figure 1. Stages of sleep; N1 = a stage of falling asleep/light sleep, N2 = stage of slower heart rate and decrease of body temperature, N3 = slow-wave sleep; stage of deep sleep and body repairation prosesses, REM sleep (stands for rapid-eye movement sleep) = phase of dreaming and skeletal muscle atonia (Racsch & Born, 2012).

It is visible in Figure 1 that slow wave sleep (N3) predominates in the early hours of sleep and REM sleep becomes more dominant in the later hours of sleep (Rasch, Born, 2012). But sleep is not just a collection of physiologic processes that occur together at the intersection of rest and activity. It is a non-negotiable biological state that is necessary for the maintenance of human life. Ensuring a plentiful duration of quality sleep, has numerous advantages on our health and it is the key to a good quality of life, such as elevated immunity, mental health and emotional resilience, cognitive advancements, physical fitness and energy balance, cardiovascular benefits, and metabolic regulation (Hirshkowitz et al., 2015). As such, it is important to consider developmental changes in sleep, which reflect directly on brain development. In preschool-aged children, decreases in napping have been associated with higher vocabulary and enhanced memory. Recent studies also report associations between sleep micro-structure and brain maturation in youths (Mason et al., 2021).

1.2. Sleep deprivation and recommended duration of sleep

Obviously sleep deprivation occurs when a person is not able to get enough sleep. The amount of sleep needed to feel refreshed, and function well depends on the individual and varies across the ages. Manny people refer to 5 or 6 hours as good enough amount of sleep, but that is not enough time for our body to regenerate properly. As said earlier sleep is a very important part of our development, so it is expected that babies and very young kids need more time to dedicate to this important part of our 24h cycle (Mason et al., 2021). Recommended amount of sleep for every age group is visible in **Figure 2**.

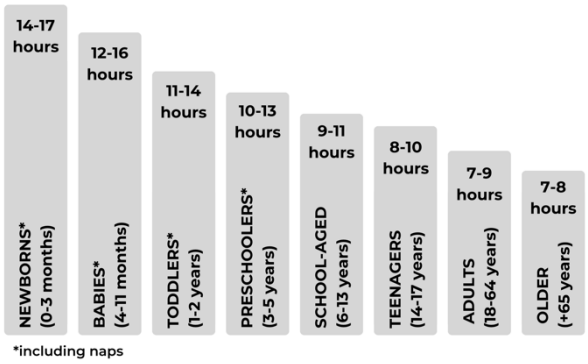

Figure 2. Recommended duration of sleep (Hirshkowitz et al., 2015).

2. Prevalence of sleep deprivation and its effects on human body

Sleep deprivation became a great problem, affecting a substantial portion of the world population. According to research conducted by the American Thoracic Society (Bandyopadhyay & Sigua, 2019), one-third of the US adult population fails to achieve the recommended seven hours of sleep. This prevalent problem is also noticeable across other age groups, with an approximately 50-70 million adults in the United States suffering from sleep disorders (Baranwal, 2023). The impact of insufficient sleep also represents a great problem with students, especially those in high schools and universities. A study conducted by Albqoor and Shaheen (2021) on university students in Jordan has shown poor sleep quality among the student population. Interestingly, the study found that students with lower income, those who were smokers, physically inactive and used media devices before bed, experienced the highest increase in sleep deprivation. The prevalence of sleep deprivation is alarming among high school students, with 73% receiving less than 8 hours of sleep and 58% of middle school students getting less than 9 hours of sleep per night.

Obviously not getting enough sleep is the most common cause of sleep deprivation, but other causes also include poor sleeping habits, circadian rhythm disturbance (e.g. delayed sleep phase), sleep disorders like insomnia, restless leg syndrome and sleep apnoea, use of medications or drugs, jet lag when traveling across multiple time zones, late shift work etc. (Hirshkowitz et al., 2015). Reducing your sleep time by even 1 hour can impact your thought process and reaction time the following day, which was found to be in correlation with car crashes and other accidents. Lack of sleep can also lead to chronic fatigue, potentially affecting work or school performance (Lo et al., 2016). The main impact of sleep deprivation extends well beyond the fact that we can be tired after not getting enough sleep, but it lives consequences on various dimensions of health. Inadequate sleep disrupts fundamental physiological processes, such as metabolic or cardiovascular health. Insufficient nocturnal sleep leads to impaired glucose processing, which is elevating the risk for developing type 2 diabetes. Cardiovascular health can be negatively effected by consequences such as elevated blood pressure, increased inflammation, and physiological stress responses, contributing to a 48% increased likelihood of heart disease and a 15% increased risk of stroke in adults that sleep regularly less than 6 hours per night (Liew & Aung, 2021).

The role of sleep in vascular restoration becomes evident, with deficiencies potentially leading to vessel damage and severe conditions such as aneurysms, atherosclerosis, and ischemia (Liew & Aung, 2021). Cognitive functions can also be effected by sleep deprivation, since sleep-deprived brain need significantly more effort to complete any tasks, which can lead to bad decisions or work mistakes. Beyond the physical outcomes, sleep deprivation can also impact mood, energy levels and irritability, which is reflecting the intricate our mental health and well-being (Killgore, 2010). Another result of sleep depri-

vation can be disrupted hormonal regulation, which is influencing child's growth and affecting appetite and glucose breakdown in adults (Davies et al., 2014). On a molecular level, sleep deprivation affects chromosome telomeres, contributing to genetic instability and potentially reducing cell lifespan. Disruptions extend to brain circuits, molecules, and genes governing the sleep-wake cycle, affecting both cognition and emotional responses (Murillo-Rodriguez et al., 2012). Poor sleep has an influence on executive function and declarative memory (Lo et al., 2016) and is prospectively linked with the development and progression of Alzheimer's disease (Grandner & Fernandez, 2021). Chronic sleep loss provides oxidative stress, which can potentially increase DNA damage and slow down reparation processes that can also negatively produce various disorders, including reproductive and nephrological issues (Liew & Aung, 2021).
Since sleep deprivation has also been associated with several medical conditions, we can easily say prioritizing quality sleep should be seen as a fundamental strategy for optimal physical and mental health.

3. Impact of sleep deprivation on physical activity and athletic performance

In exploring the relationship between sleep and physical activity, researchers have found compelling evidence of sleep deprivation influencing various of performance components. Sleep deprivation not only impairs reaction time, accuracy, vigour, submaximal strength, and endurance but also compromises cognitive functions such as judgment and decision-making (Vitale et al., 2019). The consequences extend to resistance training outcomes and metabolic health, with just three consecutive nights of sleep restriction proving sufficient negative impacts (Knowles et al., 2018). The significance of the postural control system in sports and physical activity cannot be overlooked. Sleep deprivation has higher impact on men, which we can explain with higher Centre of Pressure (COP) values in postural control testing, underscores the gender-specific sensitivity to sleep disruption. This heightened sensitivity translates into reduced postural stability, posing an increased risk of injury during physical activity in both genders (Olpinska Lischka et al., 2021). Research by Wilms et al. (2020) indicates that the impact of sleep loss on physical activity is more pronounced when sleep is restricted during the second half of the night compared to the first half. Taking a closer look at athletes, even partial sleep deprivation, amounting to just 4 hours of sleep, can result in subtle yet potentially significant impairments in athletic performance (Cullen et al., 2019). The heightened risk of injury in athletes due to decreased attention and impaired judgment is further highlighted, with evidence suggesting that obtaining less than 8 hours of sleep per night is associated with an increased risk of injury (Tsukahara et al., 2023).

One such novel concept is "banking sleep," a practice involving sleep extension before a planned night of intentional sleep deprivation before a sporting event. This approach, as proposed by Vitale et al. (2019), suggests that intentionally extending sleep in anticipation of sleep deprivation may serve as a proactive strategy to enhance performance. The concept of "banking sleep" challenges conventional perspectives on the immediate consequences of sleep loss and underscores the potential benefits of prioritizing sleep in the days leading up to a crucial athletic event. Considering these findings, it becomes evident that the relationship between sleep and athletic performance is substile, not only for the consequences of sleep deprivation but also for potential advantages of strategic sleep extension.

4. Conclusion

We can conclude that sleep and physical activity go hand in hand when it comes to influence of sleep deprivation on physical activity and the impact of physical activity on sleep patterns. Despite their positive interaction, maintaining even a relatively low volume of physical activity can decrease the risks for insufficient sleep duration. Engaging in physical activity in the morning has been especially beneficial to optimize sleep hygiene, as suggested by Valente et al. (2023). The results in research of Kizilkilic et al.

(2023) are showing the importance of physical activity as a powerful intervention for an overall well-being. Moreover, the importance of sleep as a crucial daily regulator of both physical and mental health throughout all life stages is highlighted. Sleep disorders are linked to significant morbidity and are associated with various medical and psychiatric conditions. It is established that sleep deprivation, especially when sleep duration is lower than 7 hours per night, can result in adverse health outcomes. Recognizing sleep as a vital physiological function, it is crucial to acknowledge its paramount role in facilitating effective exercise recovery. In conclusion, both adequate sleep and regular physical activity are integral components of a holistic approach to maintain optimal health and well-being.

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

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Invited lecture/Review

Physiotherapy Approach for De Quervain Tenosynovitis During and After Pregnancy

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Abstract:
De Quervain's tenosynovitis is an attritional and degenerative process. Pain results from resisted gliding of the abductor pollicis longus and the extensor pollicis brevis tendons in the fibro osseus canal. This review aims to evaluate the current literature surrounding evidence on physiotherapy approach for treating De Quervain's tenosynovitis during and after pregnancy. One third of pregnant females are suffering from the pain in their wrist. Pain in the wrist is the third most common musculoskeletal pain during pregnancy after low back pain. Fluid retention, oedema, and repetitive forceful hand movements may cause stenosing tenosynovitis of the hand and wrist tendons. An overweight mother with her first pregnancy is at highest risk for developing De Quervain's tenosynovitis. Treatment is aimed at reducing inflammation, preserving movement in the thumb and preventing reoccurrence. The symptoms should improve within four to six weeks. If non-invasive treatment is not successful, corticosteroid injections can be applied.
De Quervain's tenosynovitis has a good prognosis and non-surgical treatments are usually effective for pregnancy-related hand and wrist problems.

Keywords: de Quervain's tenosynovitis, pregnancy, post-partum, wrist pain, Finkelstein test

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1. De Quervain's tenosynovitis

1.1. Stenosing tenosynovitis

De Quervain's tenosynovitis (DQT) is a stenosing tenosynovitis of the first extensor compartment of the wrist (Ilyas et al., 2007). It is described to be an attritional and degenerative process, triggered by stenosing inflammation of the tendon sheath in the first dorsal compartment of the wrist (Ramchandani et al., 2022). Pain results from resisted gliding of the abductor pollicis longus and the extensor pollicis brevis tendons in the fibro osseus canal (Ilyas et al., 2007). This review aims to evaluate the current literature surrounding evidence on physiotherapy approach for treating De Quervain's tenosynovitis during and after pregnancy.

1.2. Epidemiology

According to previous reports, DQT occurs up to six times more frequently in women than men and is associated with the dominant hand use during middle age (Ilyas et al., 2007). Often it is noted in pregnancy and during the postpartum period (Ramchandani et al., 2022).

1.3. Etiology

In a descriptive study conducted by Kesikburun et al. (2018) 61 out of the 184 pregnant females complained of musculoskeletal pain in the hand and wrist in pregnancy. These problems were the third most common musculoskeletal complaints, after low back pains (Afshar & Tabrizi, 2021). Balik et al. (2014) studied the hand and wrist problems in 383 pregnant females and reported that 125 (32.6%) of pregnant females were asymptomatic patients and 67.4% of the pregnant females were suffering from hand and wrist problems (Balik et al., 2014). This condition is also the second most common hand and wrist problem during pregnancy and during the postpartum period after carpal tunnel syndrome (Thabah & Ravindran, 2015).

Fluid retention, oedema, and repetitive forceful hand movements may cause stenosing tenosynovitis of the hand and wrist tendons (Kesikburun et al., 2018). Read et al. (2000) studied the histopathological appearances. Histopathological examination of the tendon sheaths revealed that myxoid degeneration was responsible for the remarkable thickening observed in the sheath. In addition, characteristic intramural deposits of mucopolysaccharides were present, predominantly in the sub synovial region. However, acute, or chronic inflammatory changes were not seen (Read et al., 2000). Etiology of this condition is combination between mechanical factors, anatomical dimorphism, and hormonal-driven pathological changes (Thabah & Ravindran, 2015).

1.4. Risk factors

People between the ages of 30 and 50 have higher risk of developing De Quervain's tenosynovitis than people in other age groups (Kesikburun et al., 2018). The condition may be associated with baby care, lifting a child and moves that involve repetitive movements using the thumbs as leverage. Studies showed that women who had caesarean section and multiple gestation were at higher risk for De Quervain's tenosynovitis, while baby weight was not associated with the condition (Bae et al., 2023). Daglan et al. (2023) concluded that an overweight mother with prolonged pregnancy and her first pregnancy is at highest risk for De Quervain's tenosynovitis.

2. Treatment

2.1. Symptoms

Symptoms of De Quervain's tenosynovitis include pain near the base of the thumb, swelling, difficulty moving the thumb as well as the wrist and also some sensation can be felt in the thumb while moving it (Ilyas et al., 2007). The pain gets worse while grasping, lifting and during any motion where ulnar deviation of the wrist is present. Tenderness over the radial styloid can be felt as well as thickened first extensor retinacullum (Read et al., 2000).

2.2. Physiotherapy examination

We ask the patient to perform Finkelstein test in which patient bends their thumb across the palm and bend their fingers over the thumb. Then they bend their wrist towards little finger. If that manevour causes pain on the radial side of the wrist, then the patient most likely has De Quervain's tenosyovitis.

2.3. Physiotherapy treatment

Possible treatment is application of the brace, especially during the night, in worse cases steroid injection is possible. On rare occasions that the non-surgical treatments fail to resolve the symptoms within 4-6 months, surgical release of the first extensor compartment can be offered (Ramchandani et al., 2022). Pidgeon (2022) reported that 50 % - 80 % of patients are successfully treated nonsurgically. Treatment is aimed at reducing inflammation, preserving movement in the thumb and preventing recurrence. The symptoms should improve within 4-6 weeks if the treatment starst early. But it is more likely that the condition will get better around the end of either pregnancy or at the end of the breastfeeding. (Balik et al., 2014).

If any of ther intervations are not successfull, the corticosteroid injection can be applied, (Peters-Veluthamaningal et al., 2009). With safe dose of Methylprednisolone 10 mg (Avci et al., 2002) no contraindications during pregnancy or while lactating were seen (Larsen et al., 2021).

3. Conclusion

De Quervain's disease of pregnancy and lactation is self-limited condition and non-surgical treatments are usually effective for pregnancy-related hand and wrist problems. In general, pregnancy-related De Quervain's tenosynovitis has a good prognosis and usually resolves after reducing inflammation and movement preservation.

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Invited lecture/Research

Effects of Therapeutic Approaches in Treating Varus Malalignment of the Knee Joint

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Abstract:
Knee malalignment is shown to be an independent risk factor for osteoarthritis progression. The knee adduction moment is directly correlated with varus malformation and can be decreased with changes in gait pattern, external foot rotation and external support. The aim of this literature review was to determine the effects of different therapeutic approaches in treating varus malalignment of the knee joint. The literature search was conducted in the PubMed and EBSCO databases. We used a combination of English keywords. Studies were screened regarding the inclusion and exclusion criteria. We included five studies investigating the effects of therapeutic approaches in participants with or without osteoarthritis onset. Statistically significant decrease in knee adduction moment was reported in one study, which was implementing modified gait pattern with real time feedback. Other outcome measures were also indicative of potential efficacy in different therapeutic approaches. There is a bigger potential for treating varus malalignment before osteoarthritis (OA) onset. The results indicate that weight-bearing exercise and gait modification in combination with a corrective training protocol provide a potential useful approach to reduce varus malalignment.

Keywords:
Varus malalignment, knee joint, knee adduction moment, therapeutic approaches

1. Introduction

1.1. Mechanical forces of the lower extremity

The lower extremity is in the frontal plane aligned through a mechanical axis which is in a neutral positioned extremity running from the hip joint, medially or through the middle of the knee joint to the ankle joint (Tetsworth and Paley, 1994). A malalignment is present when the axis is shifted medial or lateral of the knee joint, creating a moment arm and causing a disturbance in the load bearing of the joints (Sharma et al., 2010). Those changes can be visualized in the frontal plane as a dynamic worsening of the varus alignment as the lower extremity accepts weight in the stance phase (Chang et al., 2010). In young individuals the changes in the frontal plane are shown to be directly associated with changes in the transverse plane including increased internal foot placement and increased tibia rotation (Stief et al., 2014).

1.2. Varus malalignment and OA

Knee malalignment is shown to be an independent risk factor in OA progression (Tanamas et al., 2009). Where greater knee varus at baseline is associated with greater odds of medial compartment OA progression (Sharma et al., 2010). OA is among others one of the most prevalent diseases in older adults and is limiting independence and functional activities, such as stair climbing, home chores, carrying bundles (Guccione et al., 1994). The knee adduction moment (KAM) is directly corelated with varus malalignment (Foroughi et al., 2009) and is consequently together with other mechanical forces, such as varus thrust associated with higher chances of OA progression in the medial compartment of the knee joint (D'souza et al., 2022).

1.3. Treatment of varus malalignment

The KAM and the load on the medial knee compartment can be reduced by changing the gait pattern, external foot rotation or external support (Tetsworth and Paley, 1994). Conservative approaches can include knee bracing. Considering the results of the systematic review Yan et al. (2022) valgus braces could reduce symptoms of OA through decreasing the KAM and the varus angle and with it the improper distribution of load forces. In patients with unicompartmental OA in early degenerative stages high tibial osteotomy can be indicated to correct the varus deformity. The surgical procedure can decrease the symptoms and delay the need for knee replacement (Dowd et al., 2005).

1.4. Purpose

The aim of this literature review was to determine the effects of different therapeutic approaches in treating varus malalignment of the knee joint.

2. Methods

The literature search was conducted in the PubMed and EBSCO databases. The last review was carried out on the December 5, 2023. Following combinations of keywords were used: varus AND knee AND treatment; knee adduction moment AND varus AND treatment; knee adduction moment AND varus AND gait. Inclusion criteria were articles in English language, populations of subjects with varus malalignment with or without OA, interventions addressing the varus malalignment directly or through changes in KAM. Studies that investigated effects of orthotic interventions and studies that didn't measure the change in varus malalignment or KAM were excluded.

3. Results

After screening and eligibility assessment, five studies published between 2010 and 2021 were included in the review (Barrios et al., 2010; Bennell et al., 2010; Bennell et al., 2014; Choi & Shin, 2021; Jafarnezhadgero et al., 2018).

The characteristics of the participants in the included studies are presented in **Table 1.** In three of the included studies (Barrios et al.,2010; Choi & Shin, 2021; Jafarnezhadgero et al., 2018) the mean age of participants was between 11.21 and 23.0 years. In two studies (Bennell et al., 2010; Bennell et al., 2014) the mean age of participants was between 62.2 and 64.6 years.

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

Study	Pathologies	Samples (n)	
		F	M
Barrios et al. (2010)	Varus, no OA	1	7
Bennell et al. (2010)	Varus, with OA	43	46
Bennell et al. (2014)	Varus, with OA	52	48
Choi & Shin (2021)	Varus, no OA	4	4
Jafarnezhadgero et al. (2018)	Varus, no OA	0	28

OA: osteoarthritis; F: female; M: male

In three of the studies (Bennell et al., 2010; Bennell et al., 2014; Jafarnezhadgero et al., 2018) the participants were divided in to an experimental and a control group which enabled a between group comprehension. In two studies (Barrios et al.,2010; Choi & Shin, 2021) only an experimental group was present and comparison of the outcome measures before and after the implementation of the intervention was performed. The interventions and outcome measures used in the included studies are presented in **Table 2.**

Table 2: Interventions and outcome measures and time of assessment used in the included studies.

Study	Intervention	Outcome measure	Time of assessment
Barrios et al. (2010)	Gait retraining with real-time feedback. 8 weeks	Gait analysis Effort	Baseline After intervention After 1 month
Bennell et al. (2010)	Hip strengthening exercise 12 weeks	Gait analysis Knee pai, physical function Strength measures Other	Baseline After 13 weeks
Bennell et al. (2014)	Neuromuscular vs quadriceps strengthening exercise 12 weeks	Gait analysis Pain, physical function Strength measures Physical performance Health related quality of life Other	Baseline After 13 weeks
Choi & Shin (2021)	Medial foot loading in gait 8 weeks	Gait analysis Adverse effects	Baseline After initial training After 8 weeks
Jafarnezhadgero et al. (2018)	Corrective training protocol 16 weeks	Gait analysis Joint kinematics Quadriceps angle	Baseline 6 days after intervention

In the gait retraining study (Barrios et al., 2010) there was a statistically significant reduction in peak KAM in comprehension of the baseline and after intervention modified gait ($p = 0.027$). In comprehension of the baseline and after 1 month follow up modified gait a reduction in KAM was again present ($p = 0.019$). When comparing the baseline and both post intervention natural gaits no differences were observed. In the hip strengthening exercise study (Bennell et al., 2010) there were no between group differences for the KAM found ($p = 0.193$). In the neuromuscular vs quadriceps strengthening exercise study (Bennell et al., 2014) there was no withing group differences for changes in peak KAM from baseline for both groups. There were also no between group differences in changes of peak KAM ($p = 0.23$). The primary outcome measure used in the medial foot loading study

(Choi & Shin, 2021) was the knee adduction angle which served as an indicator of KAM. A significant change in the knee adduction angle was found at initial contact, mean angle during gait cycle and maximal abduction and adduction angle ($p < 0.01$). Similarly, the KAM wasn't directly measured in the corrective training protocol study (Jafarnezhadgero et al., 2018). Within and between group differences were found for some of the hip, knee, and ankle joint angles for the dominant and nondominant lower extremity compared to baseline ($p < 0.05$). Statistically significant differences in other outcome measures used in the included studies are presented in **Table 3** and **Table 4**. Results for within group differences were also presented for the outcome measures in the studies Bennell et al. (2010) and Bennell et al. (2014) although the statistical significance was not determined.

Table 3: Within group differences for other outcome measures - before and after intervention.

Study			**Other outcome measures**
Barrios et al. (2010)	Modified gait	Post intervention	SS ↑ peak hip IR
		Follow up	SS ↑ peak hip IR
	Natural gait	Post intervention	No difference
		Follow up	No difference
Choi & Shin (2021)			SS ↑ walking speed, step length SS ↓ knee ADD angle (initial contact, mean, maximum) SS ↓ knee ABD angle (stance phase) SS ↑ hip ADD angle (initial contact, mean, maximum) SS ↑ ankle eversion (initial contact) SS ↑ foot ER (initial contact, mean, maximum)
Jafarnezhadgero et al. (2018)	EG	Dominant extremity	SS ↓ peak DF, foot IR, knee IR, hip ABD and ER SS ↑ peak knee ER
		Nondominant extremity	SS ↓ peak ankle inversion and eversion, foot IR, knee IR, hip ABD and ER

SS: statistically significant; ↑: increased; IR; internal rotation; ↓: decreased; ADD: adduction; ABD: adduction; ER: external rotation; EG: experimental group; DF: dorsal flexion

Table 4: Between group differences for other outcome measures.

Study	**Other outcome measures**	
Bennell et al. (2010)		SS ↑ ipsilateral pelvic drop – EG SS ↓ pain – EG SS ↑ physical function – EG SS ↑ strength (hip ABD and ADD, knee EXT) – EG
Bennell et al. (2014)		SS ↑ single leg stance – NS
Jafarnezhadgero et al. (2018)	Dominant extremity	SS ↑ knee ER – CG SS ↓ peak foot IR, knee IR, hip ER and ABD – EG
	Nondominant extremity	SS ↑ peak ankle inversion – CG SS ↓ peak ankle eversion, foot IR, hip ER – EG

SS: statistically significant; ↑ higher; EG: in favor of the experimental group; ↓: lower; ABD: abduction; ADD: adduction; EXT: extension; NS: in favor of the neuromuscular group; ER: external rotation; CG – in favor of the control group; IR: internal rotation

4. Discussion

The results are indicating that effectiveness of the therapeutic approaches is greater when they are implemented to younger participants with no OA present (Barrios et al.,2010; Choi & Shin, 2021; Jafarnezhadgero et al., 2018). This coincides with the fact that the loss of bone and cartilage as a result of OA can also contribute to malalignment progression (Tanamas et al., 2009). Another possible explanation for more encouraging results in these studies could be usage of load bearing therapeutic approaches. Those are directly addressing the varus trust present as worsening of the varus malalignment when the lower extremity bears weight (Chang et al., 2010). However, it needs to be considered that the number of included participants was low (Barrios et al. (2010); Choi & Shin (2021) n = 8; Jafarnezhadgero et al. (2018); n = 28).

As seen in the study by Barrios et al. (2010) the gait retraining program caused changes in the modified gait through time with gait becoming a less difficult task to perform. Since changes didn't transfer into the natural gait pattern, this indicates that a 8-week training program was too short for a permanent change to occur. Nevertheless, the potential of this results is supported by the systematic review Richards et al. (2016) where gait retraining with real-time biofeedback was shown to be useful to reduce KAM in healthy controls. To improve the motor learning of gait modification, it's also important to consider the practice structure in a blocked or random manner, the amount and timing of feedback provided and social-cognitive-affective aspects of learning such as motivation and attention (Charlton et al., 2020).

The mayor limitation of this review is a small number of studies eligible for inclusion. Heterogeneity between the studies was limiting a direct comparison of their results. There were also limitations of the included studies such as low number of participants (Barrios et al., 2010; Choi & Shin, 2021; Jafarnezhadgero et al., 2018), no control group (Barrios et al.,2010; Choi & Shin, 2021; Jafarnezhadgero et al., 2018; Bennell et al., 2014) and no follow-up assessment except in one study (Barrios et al., 2010).

An important question to consider is how the change in the gait pattern would affect not only the knee but also the other joints of the lower extremity. An impact reduction walking used in the study Tajima et al. (2018) was shown to decrease the ground reaction force, the external joint moments and loading rate. This approach of gait changing could potentially be useful for individuals with varus malalignment.

Different therapeutic approaches have shown to have different impact on treating varus knee alignment. There is a bigger potential for treating varus malalignment before OA onset. Where weight-bearing exercise and gait modification in combination with a corrective training protocol provide a potential useful approach to reduce varus malalignment. Future research on bigger samples with lengthy protocols and direct varus alignment measures is needed to determine the effectiveness of this approaches.

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

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Invited lecture/Research

Validation of Interferometric Light Microscopy for Assessment of Extracellular Particles in 250 samples of Diluted Plasma: Preparing the Path for Future Clinical Practices

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Abstract:
In recent years, the analysis of extracellular particles (EPs) has become instrumental in deciphering intercellular communication and disease biomarkers. Interferometric light microscopy (ILM) has emerged as a powerful label-free technique for real-time characterization of nanoscale particles. This contribution presents for the first time measurements of EPs directly in diluted plasma by ILM on populations of samples. The study involves the measured number density of EPs, the corrected number density of EPs (taking into account dilution of the sample) and the size of EPs. Analysis involves plasma of multiple species—canine, equine, and human. Most of the D_h in all three species were confined to the interval between 130 nm and 200 nm. We found no statistically significant correlation between the corrected n and D_h indicating good performance of the method. The correlation between the measured n and D_h was statistically significant indicating hindered movement of EPs due to their higher number density. These results showed that high throughput measurement of number density and size of EPs in plasma is feasible.

Keywords: Extracellular vesicles; Interferometric Light Microscopy; Plasma analysis; Nanoparticle characterization; Videodrop technology; Nanoparticles

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1. Introduction

In recent years, the study of EPs has emerged as a crucial avenue for understanding intercellular communication and disease biomarkers (Welsh et al., 2024). Among various analytical techniques, ILM has proven to be a powerful tool for real-time, label-free characterization of nanoscale particles of different types (Romolo et al., 2022). This technology already proved its utility by quantifying viruses in a river (Roose-Amsaleg et al., 2017) or by analysing aquatic biotic nanoparticles (Boccara M et al., 2016).
In this contribution, we present the application of ILM to the measurement of extracellular particles in diluted plasma. ILM enables assessment of number density and size of particles in the size range of 80 to 500 nm. A few microliter sample is needed. The aim of this contribution is to analyse populations of samples of plasma from different species by ILM.

2. Material and methods

2.1. Preparation of the samples

Animal blood samples were taken from expired transfusion bags. Three bags of canine blood and 1 bag of equine blood were used. Human blood samples were donated by the authors. Collection was established in the morning after fasting for a minimum of 12 h overnight. A G21 needle (Microlance, Becton Dickinson, Franklin Lakes, NJ, USA) and 2.7 mL evacuated tube with trisodium citrate (BD Vacutainers, 367714A, Becton Dickinson, Franklin Lakes, NJ, USA) were used. Blood samples were centrifuged (centrifuge Centric 400/R, Domel, Železniki, Slovenia) at 18°C and different centripetal accelerations (between 50 × g and 1000 × g) for different times (from 5 min and 30 min) to obtain plasma. We analysed 250 samples (81 canine, 113 equine and 56 human). For assessment with ILM, plasma was diluted to such extent to enable saturation of the interferred light. As the medium we used saline for injections 9 mg/mL (B Braun, Melsungen AG, Melsungen, Germany). The probe dilution was 50 ×.

2.2. Interferometric light microscopy

The number density of EPs in the sample (n) and the average hydrodynamic diameter of EPs in the sample (D_h) were determined by ILM using Videodrop (Myriade, Paris, France). Signals from the medium (saline for injections) was under the detection limit. The threshold value of 4.2 was used. Seven microliters of sample were placed between cover glasses and illuminated by 2 W of blue LED light. The light scattered on the particle was imaged by a bright-field microscope objective and allowed to interfere with the incoming light. The image was recorded by a complementary metal-oxide-semiconductor high-resolution high-speed camera. The number density of the particles is the number of detected particles within the detected volume, which depends on the microscope characteristics and the particles' size. The typical detection volume was 15 pL. D_h was estimated by tracking the position of the imaged particle within the recorded movie. It was assumed that particles undergo Brownian motion due to collisions with surrounding particles. The diffusion coefficient D of the motion of the particle is taken to be proportional to the mean square displacement d of the particle between two consecutive frames taken in the time interval Δt, $\langle d^2(\Delta t)\rangle = \langle 4D\,\Delta t\rangle$, while D_h was estimated by assuming that the particles were spherical and using the Stokes–Einstein relation $D_h = kT/3\pi\eta D$. Each particle that was included in the analysis was tracked and processed individually, and the respective incident light signal was subtracted from each image. Processing of the images and the movies was performed by using the associated software, QVIR 2.6.0 (Myriade, Paris, France). More details are given in Romolo et al., (2022).

2.3. Design of the study

We assume that centrifugation of blood within the above range of centripetal accelerations and times of centrifugation would not sediment EPs. However, larger centripetal accelerations and longer times more effectively sediment erythrocytes than smaller centripetal accelerations and shorter times of centrifugation. Motion of mutually interacting erythrocytes pushes plasma carrying EPs upwards into the compartment that is gathered for analysis (Božič et al., 2022). We do not expect changes in the average size of the EPs but we could expect that there would be different number densities of EPs in plasma obtained

by different centrifuge settings. Recommendations of the producer of the ILM (Myriadelab, Paris, France) indicate that measurements of number density between 5×10^8 /mL and 5×10^9 /mL are reliable. We therefore hypothesized that the dependence of n on D_h in the range where the measurements are reliable would be constant. We considered measured n of the diluted plasma sample and n corrected for dilution. The corrected values give information on the EP content of plasma while the measured values are of interest to analyse the performance of the instrument.

3. Results

Figure 1 shows dependence of EP D_h on n in canine, equine and human plasma. Different colors represent different species (orange – dog, blue – horse, black – human). In upper panels (A and B) n were corrected for dilution and lower panels (B and D) show measured n. Most of the D_h in all three species were confined to the interval between 130 nm and 200 nm **(Figure 1)**. There were four points considerably out of this range in Panels A and C, therefore we performed analysis also by excluding these 4 outliers (Panels B, D). While corrected n of EPs in human samples extended more or less evenly over a wide interval, clusters of blue and orange points pertaining to a particular animal can be distinguished in Panels A and B.

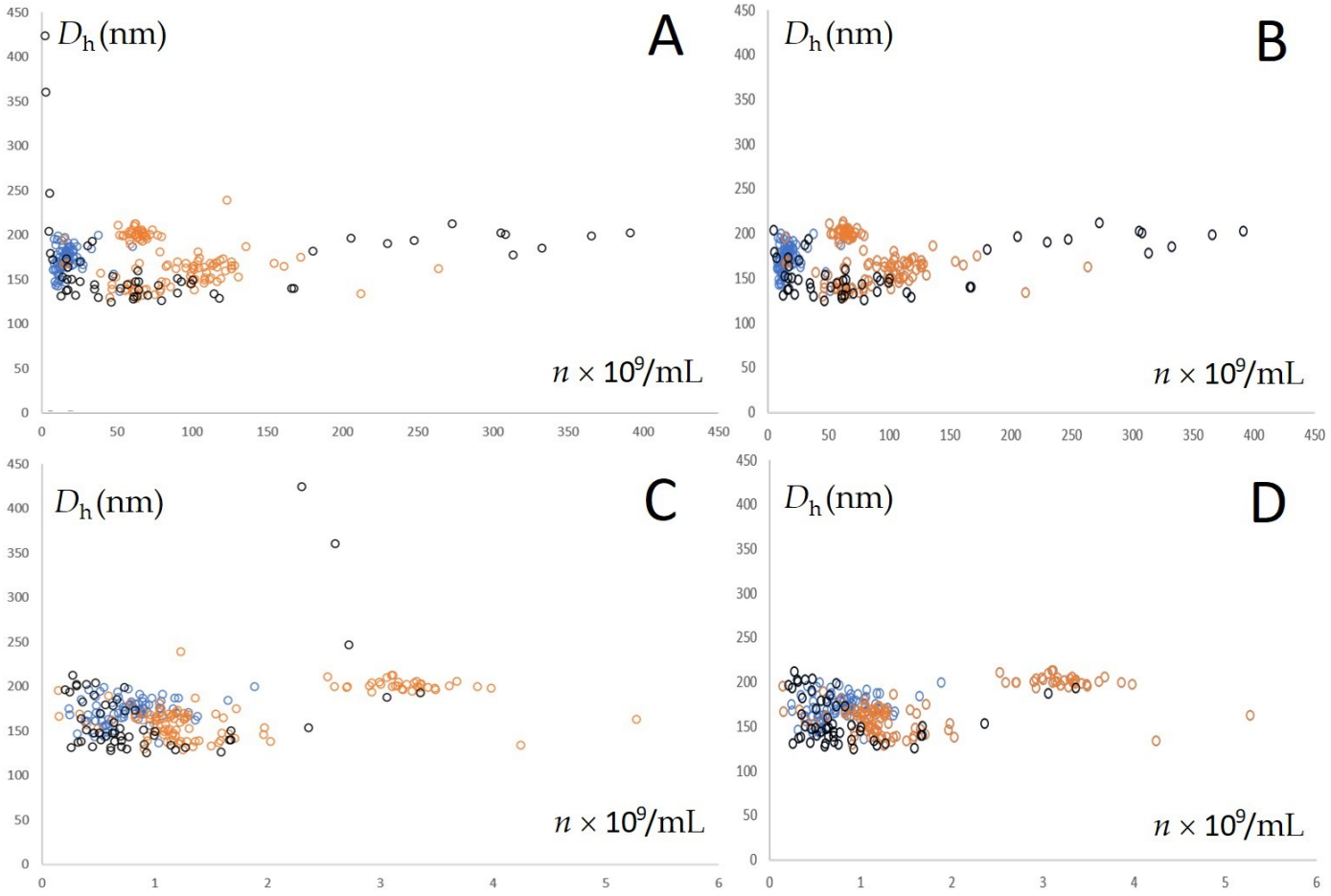

Figure 1. Hydrodynamic diameter of EPs in plasma (D_h) in dependence on the number density of EPs (n). Panels A and B: corrected n. Panels C and D: measured n. In Panels B and D, 4 outliers with D_h over 230 nm have been removed from respective Panels A and C. Orange circles: canine samples, blue circles: equine samples, black circles: human samples.

Table 1 shows Pearson correlation coefficient between n and D_h. We found no statistically significant correlation between the corrected n and D_h while the correlation between the measured n and D_h was statistically significant. Removing 4 outlier points from the graph did not considerably affect the statistical analysis results.

Table 1. Pearson coefficients of the correlation between the corrected n and D_h and between the measured n and D_h.

	All data		4 outliers removed	
	Corrected D_h/n	Measured D_h/n	Corrected D_h/n	Measured D_h/n
Pearson coefficient	<0.001	0.38*	0.08	0.41*
p	>0.05	$<10^{-4}$	>0.05	$<10^{-4}$

Asterisk denotes statistically significant result.

4. Discussion

We have for the first time performed measurement of EPs directly in diluted plasma in a large number of samples. We observed that the hydrodynamic diameter of EPs was in all species between cca 130 and 200 nm (**Figure 1**). The number densities of EPs in the samples varied and seemed to be characteristic to a donor (**Figure 1**). We observed no correlation of the corrected n of EPs in plasma and D_h (**Table 1**) which we assumed to be in agreement with the dynamics of the sedimentation of particles during centrifugation. In contrast, we observed a statistically significant positive correlation between measured n and D_h (**Table 1**). As D_h is determined by using the Einstein equation and the diffusion coefficient of the medium is assessed from the record of the movement of the particles, it is possible that the structure of plasma may hinder the movement of the particles depending on their concentration. If there are more particles in the plasma, they are restricted in their motion which is reflected in an overestimated size. However, in practice we are interested in concentration of EPs in blood or in plasma, both of which are relevant for therapeutic purposes. ILM performed well (**Figure 1**) as D_h and corrected n showed no correlation.

It was reported that the number density of extracellular vesicles in ex-vivo samples of human blood plasma were estimated at 10^{10} particles/mL but may be increased as a result of various pathological conditions (Božič et al., 2019). We have obtained n values up to the order of 10^{11} EPs per mL. Estimating viscosity of plasma by the viscosity of water, hydrodynamic diameter D_h remained between 130 and 200 nm, which agrees with the results on extracellular vesicles isolated from plasma obtained by dynamic light scattering (150 nm) (Božič et al., 2019).

5. Conclusions

We report on the first measurements of EPs in diluted plasma by ILM. The estimation of size and number density are in agreement with the data from the literature. It is indicated that high throughput measurement of number density and size of EPs in plasma is feasible. The hydrodynamic diameter of EPs in plasma did not depend on the concentration of EPs in the sample while number density of EPs measured in diluted plasma positively correlated with the hydrodynamic radius indicating hindered movement of EPs due to higher number density. Further research is needed to explore the potentials and limitations of ILM, yet our results indicate that it is a method that should be reckoned with in the study of EPs and extracellular vesicles.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, blood was donated voluntarily by the authors of the study.

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

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Invited lecture/Review

Glycosylation Research in Bovines-the Significance and Recent Updates

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Abstract:
Glycosylation is an enzymatic process of attaching carbohydrate chains, glycans, to biomolecules, thereby influencing their biological features. Understanding the glycosylation patterns and mechanisms in bovines (*Bos taurus*) has the potential to bring improvements in various fields, aspects such as reproduction, herd health management, and the quality and safety of milk and meat products. The article, starting with a glimpse into glycobiology, will continue with overviewing the previous 5-year achievements of glycosylation in bovines, collated during a recent PubMed search. Hereafter, more details about the four studies will follow as the selected examples and go along with the concluding remarks and general future research directions.

Keywords: data mining, glycosylation, bovines

Glycosylation represents the enzymatic formation of a glycoconjugate, which consists of carbohydrate chains, or glycans, covalently linked to different biomolecules such as proteins, lipids, or RNA (Reily et al., 2019; Trbojević Akmačić et al., 2022). Glycobiology is a comprehensive science focusing on glycans, with the research spanning their biological chemistry, biosynthesis, evolution, (patho)physiological roles, analysis, and biotechnology aspects. It initially appeared among natural sciences and rapidly attracted interest from many other fundamental, biomedical, and biotechnology disciplines (Varki et al., 2022).

The article will start with a brief explanation of glycobiology key postulates. It will continue with an insight into the reasons for researching glycosylation in bovines. After, four examples illustrating the relevance will follow. Concluding the article will provide a summary of the current findings' relevance and future research directions.

1. A glimpse into glycobiology

Mammals are estimated to possess trillion different branched glycan structures, formed through the combination of 17 monosaccharides with multiple glycosylation sites, numerous enzymes involved, and the potential for α or β stereochemical conjugations (Reily et al., 2019). Various biomolecules serve as substrates for glycosylation, resulting in the formation of glycoproteins, proteoglycans, glycosphingolipids, glycosaminoglycans, or glycoRNA. In addition, glycobiology also grants interest for free oligosaccharides, adding further complexity to the field (Trbojević Akmačić et al., 2022).

Macro- and microheterogeneity are crucial concepts in understanding glycobiology. For example, in glycoproteins, multiple glycosylation sites on the polypeptide "backbone" constitute macroheterogeneity, while microheterogeneity indicates the presence of different glycans on the same glycosylation site. Finally, if only the N-glycan supplement distinct glycoproteins, they represent glycoforms (Varki et al., 2022).

In eukaryotes, N- and O-glycans are two major types of glycans. The N-glycans attach via the side chain of an asparagine (Asn) residue within the peptide sequence Asn-X- serine (Ser)/threonine (Thr), where X stands for any amino acid except proline. Their structural core consists of two acetylglucosamines (GlcNAc) conjoined with a mannose structure (Man), which holds two additional mannoses lined at positions 3 and 6 (Varki, Cummings et al., 2022). According to biosynthetic maturation, indicated by the structures extending the core, four glycan types exist: pauci-mannosidic, oligo-mannosidic, complex, and hybrid. In pauci-mannosidic extensions are lacking, while in the oligo-mannosidic, they form only of Man residues. The characteristic of the complex glycans is the presence of up to five antennae, whose structures start with the GlcNAc and continue with the repeating N-acetyllactosamine residues. Hybrid glycans have oligomannose extensions at core position 6, while up to two antennae appear at position 3. More precise biosynthesis phase annotation comes with additional derived traits like antennarity, sialylation, galactosylation, fucosylation (core/antennary), or bisection (de Haan et al., 2022). The attachment sites for O-glycans are Ser or Thr residues. The O-glycans (patho)biological contexts typically diverge into features related to O- N-Acetylgalactosamine (GalNAc) and O-GlcNAc glycans. For O-GalNAc glycans, four core structures are available, each with GalNAc bound on Ser or Thr residue, differing by the presence of Gal and GlcNAc. The structure of O-GlcNAc glycans is less complex because they contain only GlcNAc attached (Varki et al., 2022).

The endoplasmatic reticulum and Golgi complex are intracellular locations of the three-step N-glycosylation process, involving the generation of the lipid-linked oligosaccharide donors, co-translational glycan shifts onto glycosylation sites on polypeptide chain, and further processing of the glycans. Regulation of this comprehensive process relies on modifying the activity of enzymes involved, glycosyltransferases and glycosidases, at the transcriptional or posttranscriptional level (phosphorylation, glycosylation, availability of chaperones). Besides, the availability of their substrates also contributes to the regulation (Esmail et al., 2021).

At the molecular level, N-glycans play a substantial role in proper protein folding, assembling of multimeric proteins, and stabilizing their structures. In the physiological context,

N-glycosylation is involved in a network of comprehensive mechanisms such as cell adhesion, recognition of foreign pro- and eucaryotic cells, or receptor activation. N-glycosylation also attracted the attention of pathophysiology and clinical disciplines. Congenital disorder of glycosylation syndrome Type I, an autosomal recessive multi-system disorder, has a depleted N-glycosylation as the cause. Changes in cell membrane N-glycome are characteristic of malignancy, thus raising the potential for advanced oncological biomarkers discovery. Terminal sialylation and fucosylation usually change, while increased branching relates to growth, infiltration, and metastasis. N-glycome alterations are frequent in chronic diseases. The best example from that field is rheumatoid arthritis (RA), an autoimmune arthritis associated with changes to the galactosylation of Ig G, where the disease severity relates to these changes in antigen-specific IgGs. Research advancements prompt additional exploration into efforts on N-glycosylation as a potential therapeutic target (Esmail et al., 2021).

2. What is the importance of researching glycosylation in bovines?

Understanding the glycosylation patterns and mechanisms in bovines (*Bos taurus*) has the potential to bring improvements in various fields, aspects such as reproduction, herd health management, and the quality and safety of milk and meat products (Beletić et al., 2023a). For example, the results of a recent proteomic study in cows with the retained placenta (Beletić et al., 2023b) suggested as potential biomarkers lipopolysaccharide-binding protein and haptoglobin, both of which are glycoproteins (https://www.uniprot.org). A proteomic study of milk from dairy cows with subclinical mastitis (Beletić et al., 2022) indicated that a higher abundance of thrombospondin-1, a glycoprotein (https://www.uniprot.org), could differentiate between samples with *Staphylococcus* spp. and *Streptococcus* spp. as the causative agents.

Recently, we have performed a PubMed search for "glycosylation" and "*B. taurus*" using the following filters: full text available, the publication date of five years, and the preprints excluded. From the 244 initially retrieved results, the content analysis identified 88 as eligible, among which only one was a review article. These studies primarily focused on functional aspects and glycan profiles, with milk and tissues being the most common sample types. Among them, ten studies provided data on the total glycome of milk or tissue samples, while many others analyzed glycosylation of individual proteins, with fetuin being the most frequently studied (Beletić et al., 2023a). Apart from the associated (patho)biological relevance, which is still challenging, bovine fetuin is also worth mentioning in the analytical context as a frequent testing analyte during the development of glycoproteomics analytical approaches (Achim et al., 2023). Milk-related studies brought the data about total glycome, or individual proteins, like casein or IgG (Beletić et al., 2023a), which are of particular relevance in the assessment of milk quality and nutritive value (O'Riordan et al., 2014).

3. The recent updates-selected examples

In a recent paper, Dilimulati et al. (2023) presented intriguing findings about the involvement of N-glycosylation in the interaction between sperm and oocyte proteins in bovines. In mammalian species, a layer called zona pelucida (ZP) surrounds oocytes and has essential functions in oogenesis, fertilization, and preimplantation. The ZP structure has long interconnected fibrils harboring ZP glycoproteins (ZPGs), and ZP thickness, protein content, and N-/O-glycosylation represent a species specificity (Wassarman, 2008; Yonezawa, 2014). ZP in bovines contains three ZPG3 annotated bZP2, bZP3, and bZP4. In vitro results identified the N-terminal domain of bZP4 (ZP-N1) and the middle region of bZP4 as substantial for sperm binding but without evidence for N-glycosylation of ZP-N1 as necessary for this purpose (Dilimulati et al., 2022). Further research of the bZP4 middle region (including the hinge domain crucial for the bZP3-bZP4 complex formation, which is essential for sperm binding) showed species-specific sperm binding. Additional analyses associated the bZP4 middle region function with N-glycosylation at Asn-314 and marked N-glycosylation sites at Asn-314 (near the hinge region) and Asn-146 (within the hinge of the bZP3-bZP4 complex) as required for the unhampered sperm binding (Dilimulati et al., 2023). These findings significantly contribute to a better understanding of between-

species differences in ZP functions. Identification of sperm-associated factors responsible for sperm-ZP interactions and validation via in vivo bovine fertilization research represents the primary research efforts to achieve reliability in bovine reproduction practice (Dilimulati et al., 2022).

The review by Zlatina and Galuska (2021) comparatively assessed N-glycosylation traits of the bovine and human lactoferrin (Lf), primarily guided by the fact that the bovine variant often serves as a model for developing innovative therapeutic strategies. Lf is a whey protein present in most mammals. Human Lf (hLf) also occurs in other organs and fluids such as kidneys, lungs, liver, prostate, saliva, plasma, and immune cells, having numerous effects: antimicrobial defense, immunomodulation, antioxidant protection, or microbiome homeostasis (Kowalczyk et al., 2022). The primary structures of hLf and bovine Lf (bLf) have a 69% analogy. Five N-glycosylation sites exist on bLf (two more than hLf). Four sites have 100% occupancy, and the fifth varies between 15% in mature milk and 30% in colostrum. Consequently, two bLf variants appear, differing in glycosylation pattern, molecular mass, and colostrum/mature milk abundance. Structural analyses of bLf and hLf suggested the N-glycans' significance with potential effects on their functionality. Their removal prominently reduces the iron-binding properties, the primary Lf function, and the "weapon" against bacteria. The site-specific presence of N-glycans on bLf is a potential inhibitor of proteases. The large amount of high-mannose N-glycans, rare among extracellular proteins of mammalian species, also differentiates bLf from hLf and might induce a quick clearance of the gastrointestinal tract and bloodstream mediated by mannose-recognizing receptors on macrophages. Another feature of bLf glycome is the presence of N-Glycolylneuraminic acid, a non-human form of sialic acid, which, besides enhancing the clearance of dietary bLf, also links to antigenicity and potential adverse effects on the efficiency of the immune response. Notwithstanding the relevance of the described advances for deciphering the bLf effects in humans, prudence is necessary in their interpretation, originating from the comprehensive network of molecular (like amino acid sequence), physiological (breed, lactation time, or diet), and methodological features (Zlatina et al., 2021).

Fat, casein, and whey represent the three milk components (Haug et al., 2007). According to their amount, the whey proteins belong to highly (α-lactalbumin, β-lactoglobulin, serum albumin, IgG, glycosylation-dependent cellular adhesion molecule 1 (GlyCAM-1), and Lf), moderate (IgA, IgM, lactoperoxidase, and osteopontin) and minor (lysozyme and folate binding protein) abundant group (O'Riordan et al., 2014; Tacoma et al., 2016). Whey proteins are ingredients in numerous food products, including infant formulas. Considering N-glycans' relevance for the glycoprotein functional properties, an assessment of the bovine whey total glycome profile would be significant for predicting the quality and safety of whey-containing food products. Valk-Weeber et al. (2020) analyzed the role of individual glycoproteins in determining the comprehensive N-linked glycoprofile of bovine whey. In accordance with the protein abundance, approximately 95% of *N*-glycans in the total glycome originated from Lf, IgG, and GlyCAM-1. As expected, Lf dominantly participated with oligomannose-type glycans. IgG was the source of fucosylated di-antennary glycans with N-Acetyllactosamine domains. GlyCAM-1 was the only whey glycoprotein carrying highly fucosylated and sialylated tri- and tetra-antennary glycans. Analyzing differences between early and late colostrum and mature milk revealed the complex interactions between the glycoprofile alterations (dominanly higher sialylation and fucosylation degree in early colostrum) and dynamics of glycoproteins concentrations.

Food fraud is a growing general issue in food safety and quality and is particularly common among animal products. Species identification is one of the key tasks in preventing adulteration problems with foods of animal origin (Smaoui et al., 2023). Tai et al. (2023) developed a glycopeptide-based analytical pipeline to identify six meat species: pork, beef, mutton, chicken, duck, and turkey. The first phase of their experiment was the sarcoplasmic proteome analyses using one-dimensional gel electrophoresis, which showed that enolase could differentiate livestock and poultry origin. Applying ultra-high-performance liquid chromatography with quadrupole time-of-flight mass spectrometry confirmed the species specificity of enolase glycoprofiles. The validation procedure, aiming to

identify livestock, poultry, and mixed meat, yielded promising results, which might be even superior to the currently available analytical platforms.

4. Conclusions

The pioneer insight provided in this article allowed for the conclusion on the availability of reliable data about glycosylation in the bovines and the incarnated challenges. As such, they are eligible as the starting point for further scientific efforts on their continuous appending, systematization, and multidisciplinary analyses.

Conflicts of Interest: The authors declare the following competing interests: G.L. is the founder and owner of Genos Ltd., a private research organization that specializes in high-throughput glycomic analysis and has several patents in this field. A.B., I.D.O., T.P., and J.K. are employees of Genos Ltd.

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Invited lecture/Research

Omics Mass Spectrometry Analysis of Canine Plasma

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Abstract:
Blood derived products, such as autologous plasma, have high clinical importance and are applied in numerous therapeutic fields. The preparation of autologous plasma from the patient's own blood is easy to perform by centrifugation, however, the preparation procedure can significantly affect the blood cells, platelets and vesicles in the sample. Therefore, it is of utmost importance to understand the impact of sample processing on the chemical composition of plasma preparations as well as on their biological activity. Here, we present a mass spectrometry-based plasma profiling method in which three compound groups: lipids, proteins and glycoproteins are analysed in a single workflow. Analysis of the chemical composition of plasma samples prepared by different centrifugation protocols revealed differences in the lipid and glycoprotein profiles, demonstrating the importance of standardized protocols for the preparation of plasma products.

Keywords: Plasma; Lipids; Proteins; Glycoproteins; Mass Spectrometry, Ion Mobility

1. Introduction

Analytical characterisation of complex biological samples is still a challenging and time-consuming task, especially if a comprehensive analysis is planned. Mass spectrometry (MS) is a leading analytical method for the chemical analysis of biological samples, as MS-based measurements can be easily combined with other analytical and separation techniques. For this reason, mass spectrometry has a key role in "omics" research, where the characterisation of different sets of biomolecules is performed.

Plasma can be produced from blood collected in tubes treated with an anticoagulant. The blood is then processed with centrifugation to remove cells and the autologous plasma is often used for therapeutic purposes (Troha et al., 2023). For example, the eradication of a wound in the middle ear was demonstrated after the application of autologous plasma (Božič et al., 2020). Nevertheless, the exact composition of human plasma varies from individual to individual and is largely affected by the production and storage process. It is therefore important to develop methods to characterise the molecular composition of plasma-derived products. The first step, the centrifugation procedure by which blood plasma is prepared has not yet been standardised.

In this work, the lipid, glycoprotein and protein content of blood plasma was investigated using a newly developed multi-step sample preparation combined with mass spectrometry. Dog (Canis lupus familiaris) plasma samples, subjected to two different centrifugation steps, were compared to detect possible chemical differences due to differences in the preparation.

2. Methods

2.1 Blood sampling

Blood was from expired transfusion bag (**Figure 1**). The donor was a 2 years old female dog without record of the disease. Before pouring the blood the content of the bag was homogenized by gently squeezing the bag. Blood was aliquoted into 3 ml tubes by gently pushing it through attached plastic tubes.

Figure 1. A bag of expired canine transfusion blood.

2.1 Preparation of plasma samples

Two different canine plasma sample sets were prepared by centrifugation at 2000 g for 5 min (at 30 °C), and by centrifugation at 4000 g for 30 mins (at 30 °C). 4-4 technical replicates were prepared for both samples.

2.2 Sample preparation for mass spectrometry

In the first step, lipids were extracted with the following protocol: 150 µL methanol was added to 20-20 µL plasma samples. After mixing, 500 µL methyl-*t*-butyl ether (MTBE) was added and the samples were incubated at room temperature for 1 hour under shaking at 350 rpm. 125 µL MilliQ water was added, and the incubation was repeated for 10 mins. Samples were centrifuged for 10 mins at 14800 rpm. The upper organic phases were collected, and the extraction of lipids was repeated by 500 µL MTBE. The combined organic

phases were vacuum dried. The lower aqueous phases, containing the proteins were further processed in the next steps. 6-6 µL of 0.2%-os RapiGest SF detergent solution were added to 100-100 µL aqueous phases, as well as 2 µL 10 mM dithiotreiol (DTT) solution. Samples were reduced at 60 °C for 30 mins. After cooling to room temperature, 15 µL 200 mM ammonium hydrogen carbonate solution and 2 µL 20 mM iodoacetamide solution were added and the proteins were alkylated for 30 mins in the darkness. Then, 1-1 µL of 0.1 mg/ml trypsin was added and the samples were incubated overnight 37 °C under gentle shaking. 3 µL of formic acid was added to quench the enzymatic reaction and the samples were vacuum dried. In the last step, glycopeptide enrichment was performed. Briefly, the samples were reconstituted in 50 µL of 1% formic acid and 500 µL ice-cold acetone was added to the samples and the samples were then kept overnight at -20 °C. Samples were centrifuged for 10 mins at 12000 rpm to isolate to pellet enriched in glycopeptides. The pellet and the supernatant were then separated and vacuum dried. Peptide solutions were further purified using Pierce™ C-18 Spin columns (the glycopeptide fractions were analysed without additional clean-up)

2.3 Mass spectrometry

Chromatographic separations were performed using a Waters Acquity I-Class UPLC System, while MS experiments were performed on a high resolution and high mass accuracy hybrid Q-TOF equipped with cyclic ion mobility separation cell (Waters Select Series Cyclic IMS, Waters Corporation) and an electrospray ionization Z-spray source. Single Lock Mass (leucin-enkephalin) was used for mass correction.

Lipid fractions were dissolved in 100 µL 50% A eluent / 50% B eluent mixture (*V*/*V*). Lipidomic LC-MS measurements were performed on an ACQUITY UPLC CSH C18 column (1.7 µm, 2.1x100mm) at 55 °C column temperature and 0.35 mL/min flow rate. Eluent A was 600/390/10 acetonitrile/water/1 M ammonium formate solvent mixture containing 0.1% formic acid (*V*/*V*). B eluent was 900/90/10 isopropanol/ acetonitrile /1 M ammonium formate mixture containing 0.1% formic acid (*V*/*V*). Gradient elution profile was the following: 0 min: 50% B, 0.5 min: 53% B, 4 min: 55% B, 7 min: 65% B, 7.5 min: 80% B, 10 min: 99% B. MS analysis was performed in negative ionization mode in the *m*/*z* 50-1200 range. Collision induced dissociation (CID) was performed in the 25-45 V collision voltage range. Single pass cyclic ion mobility separation was included in the measurements. Data were evaluated and lipids were identified by the Progenesis QI software.

Peptide fractions were dissolved in 25 µL 2% acetonitrile, 0.1% formic acid (*V*/*V*) solvent mixture. Proteomic LC-MS measurements were performed on an ACQUITY UPLC Peptide CSH C18 column (1.7 µm, 1x150mm) at 45 °C column temperature and 20 µL/min flow rate. Eluent A was 0.1% formic acid in water, B eluent 0.1% formic acid in acetonitrile (*V*/*V*). Gradient elution profile was the following: 0 min: 5% B, 1 min: 5% B, 45 min: 35% B, 46 min: 85% B. MS analysis was performed in positive ionization mode in the *m*/*z* 50-2000 range. Collision induced dissociation (CID) was performed in the 19-45 V collision voltage range. Data were evaluated and the proteins were identified by the ProteinLynx Global Server software.

3. Results

The objective of this work was the characterisation and comparison of blood plasma samples in a comprehensive way by a multi-step sample preparation workflow. The sample preparation was divided into three main steps: first, the lipid content of the plasma was isolated using MTBE as organic solvent. Then the lipid-depleted samples containing the plasma proteins were subjected to enzymatic cleavage by trypsin. The resulting peptide mixture was further fractionated. A fraction enriched in glycopeptides was isolated by ice-cold acetone precipitation and the peptide fractions depleted in glycopeptides were also isolated. All three fractions were analysed by UPLC-MS(/MS) and the compounds were identified by database search. Lipids were analysed using cyclic ion mobility separation[3] (cIM) to increase the number of compounds (e.g. isomers) that could be differentiated. **Figure 2**. shows the ion mobility heat map (drift time vs. retention time) of the lipids by LC-

MS under the optimized gradient elution. Using a single pass cyclic ion mobility separation, we were able to resolve several isomeric lipids, which otherwise elute at the same or highly similar retention times.

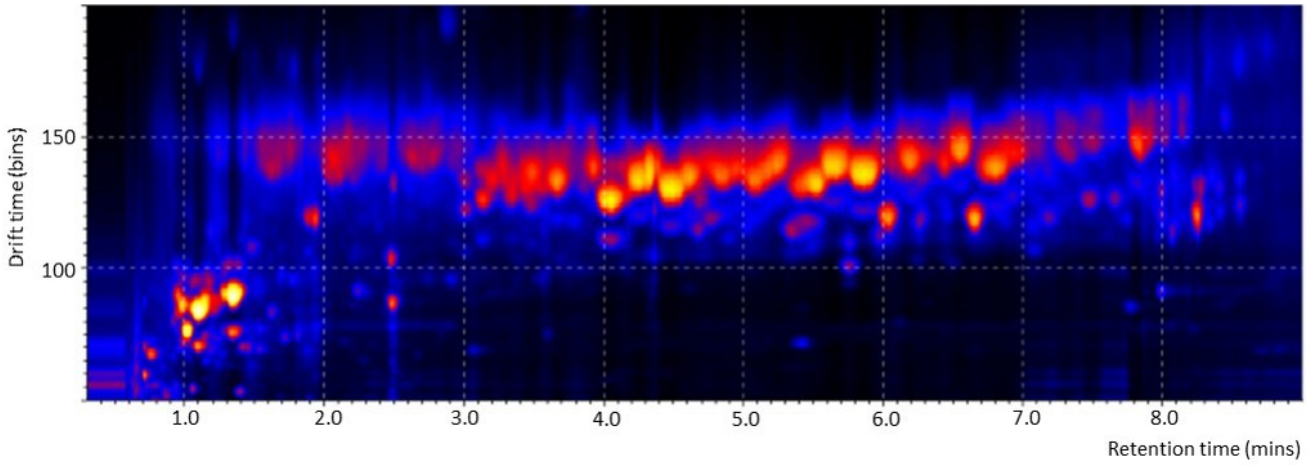

Figure 2. 2D LC-cIM-MS heat map (mobilogram) of the extracted lipids from canine plasma centrifuged at 2000 g for 5 min.

Main lipids were identified by database search. Analytical data and possible structures of the most intensive lipid species are summarized in Table 1. Note that in many cases, the exact structure of the lipids is ambiguous, due to identical masses and similar fragmenta tion patterns. Here, the collision cross sections (the drift times) are different. The most intensive species correspond to phosphatidylcholines (PC), sphingomyelins (SM) and ether-linked phosphatidyl-ethanolamines (PE). Under these conditions, formate adducts ($[M+FA-H]^-$) were dominant in the mass spectra.

Table 1. The 15 most intensive plasma lipids identified by LC-cIM-MS from canine plasma centrifuged at 2000 g for 5 min.

m/z	Retention time (min)	CCS (Å)	Formula	Possible lipids	Adduct type
747.5681	4.03	286.1	C39H79N2O6P	SM(34:1)	$[M+FA-H]^-$
750.5459	6.64	278.3	C43H78NO7P	PE(O-38:5)	$[M-H]^-$
802.5631	4.48	291.2	C42H80NO8P	PC(34:2)	$[M+FA-H]^-$
804.5766	5.48	291.2	C42H82NO8P	PC(34:1)	$[M+FA-H]^-$
826.5615	3.66	294.3	C44H80NO8P	PC(36:4)	$[M+FA-H]^-$
826.5624	4.23	295.4	C44H80NO8P	PC(36:4)	$[M+FA-H]^-$
828.5776	4.58	296.4	C44H82NO8P	PC(36:3)	$[M+FA-H]^-$
830.5932	5.86	297.5	C44H84NO8P	PC(36:2)	$[M+FA-H]^-$
832.6086	6.79	299.6	C44H86NO89	PC(36:1)	$[M+FA-H]^-$
854.5922	5.24	299.5	C46H84NO8P	PC(38:4)	$[M+FA-H]^-$
854.5932	5.6	300.5	C46H84NO8P	PC(38:4)	$[M+FA-H]^-$
856.6079	6.16	301.6	C46H86NO8P	PC(38:3)	$[M+FA-H]^-$
857.6759	7.81	307.8	C47H93N2O6P	SM(42:2)	$[M+FA-H]^-$
882.6243	6.53	308.7	C48H88NO8P	PC(40:4)	$[M+FA-H]^-$
885.5517	4.32	300.3	C47H83O13P	PI(38:4)	$[M-H]^-$

CCS: Collision Cross Section

The type of the major lipids was identical in the samples, however, comparison of the samples prepared by different centrifugation revealed differences in the intensity of specific lipid species. **Figure 3** shows the intensities of two lipid ions in the LC-MS chromatograms. While the intensity of PC(O-36:4) did not change, there is a remarkable decrease in the intensity of PE(O-38:5) due to centrifugation with elevated spin and longer time.

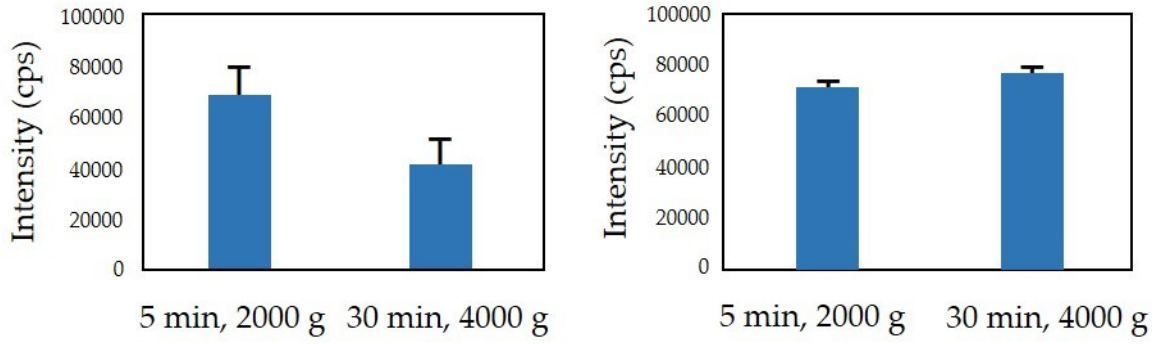

Figure 3. Effect of the centrifugation on plasma lipids. A.) Intensity of *m*/*z* 750.5459, PE(O-38:5); B.) Intensity of *m*/*z* 812.5822, PC(O-36:4).

Similarly, the protein and glycoprotein content of the samples were also compared. Protein contents of the samples were analysed after tryptic digestion. Fractions enriched and depleted in glycopeptides were prepared and proteins were identified by MS/MS peptide sequencing and database search. Database search revealed the presence of the most typical and abundant plasma proteins, including for example Apolipoproteins, Hemoglobin, Serum albumin, Haptoglobin and Fibronectin. While the peptide fraction did not show significant differences between the two sample groups, the intensity of the glycopeptides was increased in the plasma sample set which was centrifuged at higher spin and longer time (**Figure 4**). Based on glycopeptide marker ion intensities, larger spin and longer centrifugation time increased the intensity of glycopeptides, demonstrating that the relative ratio of glycoproteins increases in the prepared plasma. **Figure 4** shows the total intensity of the glycopeptide MS/MS fragment ions at *m*/*z* 366.14 for the samples prepared by different centrifugation protocols.

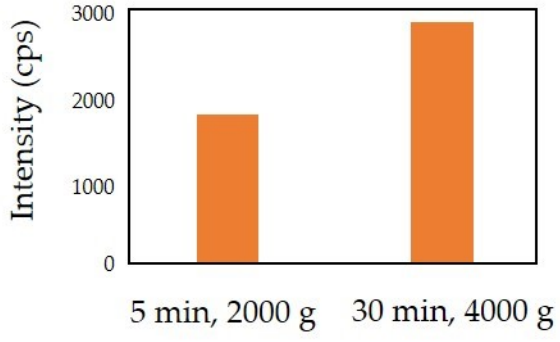

Figure 4. Effect of the centrifugation on plasma glycoproteins. Intensity of *m*/*z* 366.14 glycopeptide marker fragment ion in the MS/MS spectra of the LC-MS/MS chromatograms.

4. Discussion

LC-MS-based comparison of plasma samples produced by different centrifugation methods revealed differences in their chemical compositions. In the case of lipids, certain species showed significant decrease while other species showed increase based on their LC-MS intensities. Differences were also observed in the glycopeptide fraction obtained by tryptic digestion of differently centrifuged samples: longer centrifugation times resulted in a more intense glycopeptide pattern. Our results demonstrate that the centrifugation steps, used for the preparation of plasma products, changes the chemical composition. Therefore, it is essential to obtain more information on the effect of preparation protocols and the composition of plasma products in the future.

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

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Invited lecture/Research

Extracellular Particles from Equine Milk

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Abstract:
Equine and bovine milk extracellular particles were investigated by scanning electron microscopy, interferometric light microscopy and flow cytometry. Scanning electron microscopy revealed micro-sized globular particles and numerous nano-sized particles. Higher concentration of micro and nano-sized particles was found in skimmed milk when compared to the whole milk and the differences were statistically significant.

Keywords: Extracellular vesicles; Milk; Interferometric light microscopy; Flow cytometry; Scanning electron microscopy; Concentration of particles

1. Introduction

1.1. Milk as a complete food

Milk is a complete food with all nutritionally important components required for a newborn mammal – it comes in liquid form and contains proteins (corpuscular casein and dissolved whey proteins), fat (fat globules) and carbohydrates (milk sugar, i.e., lactose) (Musaev et al., 2021, Malacarne et al., 2002).

1.2. Milk as a source of a variety of particles

Milk is also a source of a variety of small particles including molecules, their complexes, and extracellular particles (Ong SL et al., 2021). It contains bioactive components derived from various cellular sources (Musaev, A et al., 2021) and immunologically active cells (Palmeira P et al., 2016). Here, we investigated the extracellular particles from equine milk. The concentration was measured by interferometric light microscopy and flow cytometry. To observe the morphology of particles, scanning electron microscopy micrographs were obtained from equine milk.

2. Methods

2.1. Milk Sampling

For flow cytometry and interferometric light microscopy animal was milked by hand from a Posavje (breed) mare on 91st day of lactation. Milk was collected into tubes VACUETTE® TUBE 3 ml Z No Additive 13x75 white cap-black ring, non-ridged (Greiner AG, Kremsmünster, Austria). To obtain skimmed milk, whole milk was centrifuged at 300 g for 15 min and the cream was removed from the top using a pipette with the tip shortened for 2 mm by scissors. The procedure was repeated twice. For scanning electron microscopy, the milk samples were obtained at different days of lactation from Posavje (breed) mare.

2.2. Scanning electron microscopy (SEM)

The milk samples were incubated with added 1% OsO_4 for 1 hour, washed 3 times for 10 minutes with dH_2O, dehydrated in graded ethanol (EtOH): 30%, 50%, 70%, 80% and 90% for 10 minutes at each concentration and in absolute EtOH 2-times for 10 minutes, incubated with added absolute EtOH and HMDS (ratio 3:7) for 10 minutes, incubated in added absolute EtOH and HMDS (ratio 1:1) for 10 minutes, incubated in added 100% HMDS for 10 minutes and depleted of HMDS by evaporation in exicator with silica gel for 12 hours. After fixation, samples were gold-sputtered and observed by the scanning electron microscope (SEM, JEOL JSM-6500F).

2.3 Flow Cytometry (FCM)

The particle numbers in samples from equine and bovine milk were estimated by flow cytometry, using a MACSQuant Analyzer flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) and the related software. Before measurement, the milk was diluted by saline to obtain a measurable dilution (200x bovine and equine whole and skimmed milk). The following instrument settings were employed: FSC: 458 V; SSC: 467 V with a trigger set to 1.48; B3: 300 V; and R1: 360 V. Particles were detected from the forward (FSC) and side scatter parameters (SSC).

2.4 Interferometric Light Microscopy (ILM)

The average hydrodynamic diameter (D_h) and the number density of small particles in milk were determined by interferometric light microscopy using Videodrop (Myriade, Paris, France). Before measurement the milk was diluted by saline for injections (Braun, Melsungen, Germany) to obtain a measurable dilution. The range of dilution was 8x - 500x for bovine whole milk, 32x - 500x bovine skimmed milk and 16 x - 50x for equine whole and skimmed milk. Signals of the saline were under the detection limit. The threshold value 4.2 was used. 7 µL of sample was placed between cover glasses and illuminated by 2W blue LED light. The light scattered on the particle was imaged by a bright-field microscope objective and allowed to interfere with the incoming light. The image was recorded by a complementary metal–oxide–semiconductor high resolution high speed camera. The

obtained pattern that includes contrasting black and white spots was recognized as a particle and its position in the sample was assessed. Number density of the particles is the number of the detected particles within the detected volume (e.g. 15 pL). D_h was determined by tracking the position of the imaged particle within the recorded movie. It was assumed that particles undergo Brownian motion due to collisions with surrounding particles. The diffusion coefficient D of the motion of the particle is taken to be proportional to the mean square displacement d of the particle between two consecutive frames taken in the time interval Δt, $\langle d^2(\Delta t)\rangle = \langle 4D\ \Delta t\rangle$ while D_h was estimated by assuming that the particles were spherical and using the Stokes-Einstein relation $D_h = kT/3\pi\eta D$. Each particle that was included in the analysis was tracked and processed individually and the respective incident light signal was subtracted from each image. Processing of the images and of the movies was performed by using the associated software QVIR 2.6.0 (Myriade, Paris, France).

2.5. Statistical analysis

All measurements were performed in triplicates and presented by the average values and standard deviations. The differences were evaluated by the t-test using the Excel software. The value p = 0.05 was taken as a threshold for statistical significance.

3. Results

Concentration and size of the particles in the equine milk was obtained by FCM and ILM **(Table 1).**

SEM micrographs revealed presence of globular particles' morphology (**Figure 1**).

Table 1. Concentration and size of particles in the equine whole and skimmed milk determined by FCM and ILM. The measurement was done on the 91st day of lactation. Average ± Standard Deviation assessed from 3 measurements are given.

Sample	ILM (size range 80 nm - 400 nm)	ILM	FCM
	Number density ($\times 10^9$/mL)	D_h (nm)	Number density ($\times 10^9$ /mL)
91st day whole milk	28.89 ± 2.14	308 ± 12	0.23 ± 0.06
91st day skimmed milk	36.80 ± 2.00	292 ± 7	2.14 ± 0.41
p (t-test)	0.009	0.12	<0.00001

4. Discussion

According to Walstra (1969), in the bovine milk there is a subpopulation of milk fat globules with a diameter smaller than 1 μm. They seem to represent 80% of all milk fat globules but only approximately 5 % of the volume of the milk fat (Walstra, 1969).We found significantly higher concentration of EPs sized 80 – 500 nm in the skimmed milk than in the whole milk indicating that fat globules occupy larger space but are not very numerous. Equine milk fat globules were reported to be relatively small, their average volume-surface diameter being about 2.75 μm (Welsch et al., 1988). In the cow's milk, a population of medium globules (ranging from 1 to 8 μm) was measured; it was estimated that these globules constitute approximately 94% of the fat volume (Walstra, 1969). However, when the average size of EPs in the equine milk was assessed by ILM, there was no significant difference between the whole and skimmed milk, which might be explained by the low fat content in the equine milk.

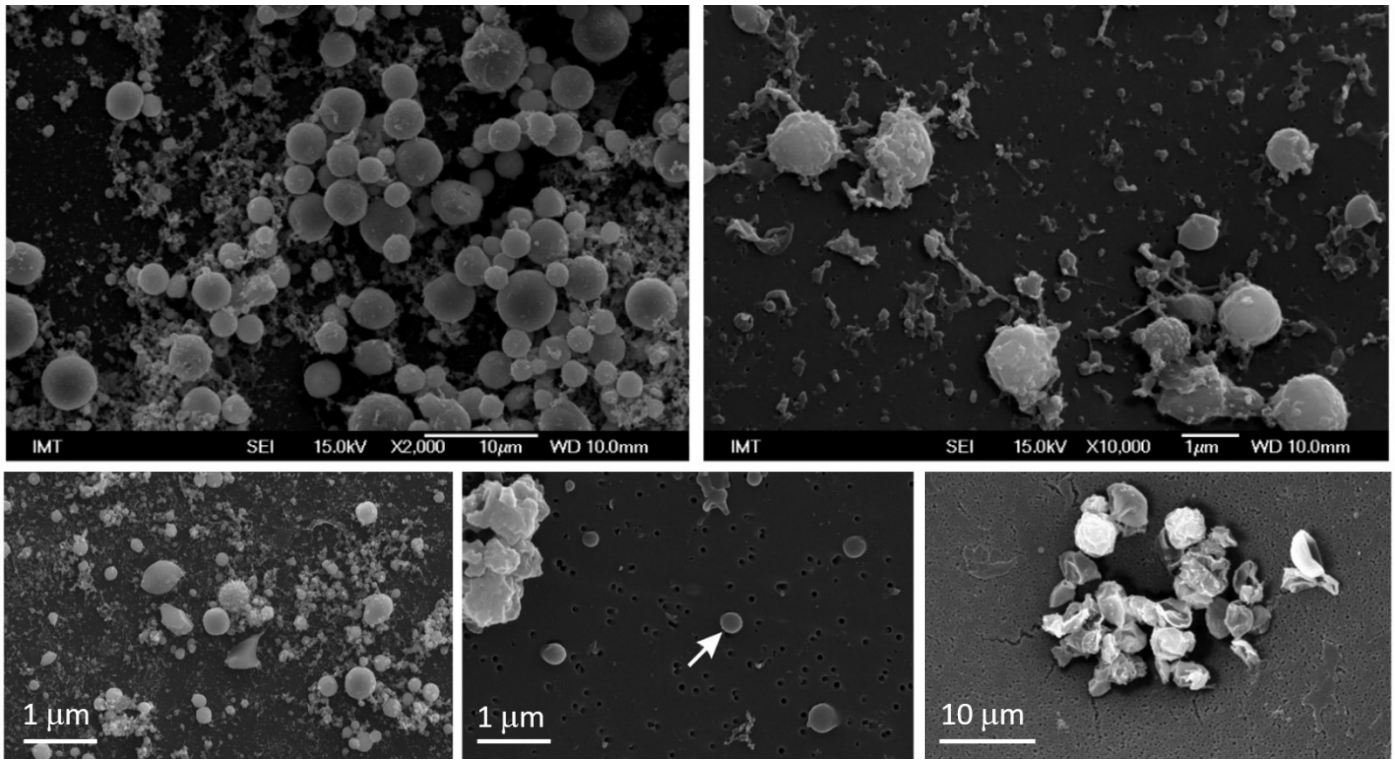

Figure 1. SEM micrographs of equine milk: A – Equine milk on the 1st day after parturition, B – Equine milk on the 1st day after parturition, C – Equine milk on the 2nd day after parturition, D – Equine milk on the 3rd day after parturition, E – Equine cream.

The content of fat in the equine milk was found lower (1,21%) than in the bovine milk (3,61%) and in the human milk (3,64%) (Malacarne M, et al., 2002). Similarly, the concentration of particles measured by FCM was significantly higher in the skimmed milk and lower in the whole milk. The average size of the EPs measured by ILM was significantly higher in the whole milk which might be due to milk fat globules (present in the whole milk) and casein micelles (present in the whole and skimmed milk).

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Review

An Insight into the Use of Cannabis in Medical and Veterinary Dermatological Applications and its Legal Regulation

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Abstract:
Cannabis contains more than 140 cannabinoid compounds, of which cannabidiol and tetrahydrocannabinol are the most exposed ones, as they have the potential for numerous applications in various fields of health and medicine. Many scientific findings and numerous clinical studies over the last decade report results related to pain relief, treatment of chronic diseases and certain neurological disorders in human medicine. In line with the growing trend of research and application in human medicine, the research and application potential in veterinary medicine is also increasing. Of the biologically active molecules contained in the plant, cannabidiol has attracted the most interest. The cultivation of cannabis for medical and pharmaceutical purposes requires, among other things, compliance with legal regulations. An analysis of the legal acts regulating the use for medical and pharmaceutical purposes shows that the current regulation at state level is complex and should be amended.

Keywords: Cannabis, Cannabinoids, Human medicine, Veterinary medicine, Inflammation, Legal Regulation

1. Introduction

Cannabis sativa stands as a botanical enigma deeply ingrained in human history, weaving a rich tapestry across ancient cultures. From being a crucial agricultural crop and industrial raw material to its multifaceted roles in contemporary society – medical, nutritional, and industrial cannabis has evolved as a subject of intense scientific inquiry. Its origins date back to more than a millennium BC and cannabis played a central role in agriculture until the late 19th century (Zuardi, 2006). The year 2019 marked an important turning point with the European Parliament's resolution supporting the medicinal use of cannabis (Pečan et al., 2021). This has driven efforts towards decriminalisation in the European Union, the promotion of research and the integration of medicinal cannabis in EU countries. At the same time, the Commission on Narcotic drugs recognised cannabis, particularly Δ-9-tetrahydrocannabinol (Δ^9-THC, THC) (**Figure 1** (**b**)), as a substance that primarily causes no harm, which shaped the international monitoring framework (Pečan et al., 2021; Štukelj et al., 2019).

Cannabis, which botanically belongs to the Cannabaceae family, is a dioecious, flowering plant characterised by remarkable adaptability. The leaves are palmately compound or toothed with toothed leaflets. The phenotypic expression of the plant is strongly influenced by environmental factors, which makes it an interesting subject for detailed botanical studies (Amaducci et al., 2008).

Modern scientific research has identified over 420 compounds in cannabis (Atakan, 2012). Trichomes, specialised secretory glands concentrated in the female inflorescences, contain cannabinoids, terpenes and flavonoids, fundamental components of the secondary metabolism of cannabis (Tanney et al., 2021). Cannabinoids, more than 90 different substances, have a binding affinity to cannabinoid receptors and thus trigger a spectrum of physiological effects (Andre et al., 2016).

As the primary psychoactive component, THC plays a central role in the pharmacological profile of cannabis (Bridgeman and Abazia, 2017). Classified as a narcotic, THC acts as a partial agonist at the cannabinoid receptors CB1 and CB2, particularly in the central nervous system and the immune system, and thus unfolds its psychotropic effect (Pertwee 2008). THC is approved for medical applications and plays an important role in drugs such as Marinol®, Cesamet™ and Sativex® (Tavčar Benković et al., 2019).

Cannabidiol (CBD) (**Figure 1** (**a**)) was reported in 1940 (Burstein, 2015) and has developed into an important phytocannabinoid with a wide range of applications. CBD has analgesic, anti-inflammatory, antineoplastic and chemopreventive effects and differs from THC in that it is not psychoactive (Martínez et al., 2020). Its complex mechanisms include stimulation of endoplasmic reticulum stress, inhibition of AKT/*m*TOR signalling and activation of autophagy, which offers diverse therapeutic potential (Pertwee, 2006).

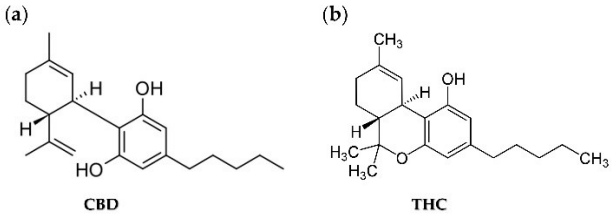

Figure 1. Structures of (**a**) cannabidiol (CBD) and (**b**) Δ-9-tetrahydrocannabinol (THC).

Cannabis and its derivatives have made a name for themselves in the pharmaceutical industry, particularly for dermatological problems (Martins et al., 2022). Cannabinoids such as CBD have anti-inflammatory and analgesic properties, making them promising candidates for the treatment of conditions such as psoriasis and eczema (Baswan et al., 2020). Pharmaceutical formulations containing cannabis extracts are being investigated for their potential for topical treatments that provide targeted relief (Baswan et al., 2020).

In veterinary medicine, cannabis and its derivatives are currently being carefully investigated for their potential therapeutic benefits. Studies suggest that cannabinoids may

be useful in the treatment of certain conditions such as osteoarthritis and epilepsy in animals (Di Salvo et al., 2023). This emerging field offers the possibility of tailored veterinary treatment, taking into account species-specific responses and dosing considerations (Gupta et al., 2019).

Cannabis, deeply rooted in antiquity, is evolving into a complex botanical entity with profound implications for historical understanding and contemporary applications. Research into the complex botanical and pharmacological dimensions of cannabinoids such as THC and CBD forms the basis for careful scientific investigation. The dynamic legal landscape and increasing applications underscore the need for rigorous research that places cannabis at the forefront of scientific exploration, offering potential advances in human and veterinary medicine and opening new dimensions for specialized pharmaceutical formulations (Pečan et al., 2023).

2. Cannabis extracts and cannabinoids for the treatment of skin diseases in human medicine

The long history of the use of cannabis for medicinal and recreational purposes has also led to its use for various skin applications. Cannabis extracts have been investigated as a potential remedy for skin problems and disorders (Shao et al., 2023). The therapeutic potential of the cannabinoid CBD has been particularly researched and studied as it is abundant in the cannabis plant and has a relatively favourable legal status compared to THC. Recently, extensive review articles have been published on the therapeutic potential of phytocannabinoids, particularly cannabidiol, focusing on its effect on skin health, disease (Baswan et al., 2020; Martins et al., 2022) and delivery mechanisms (Filipiuc et al., 2023). Natural cannabinoids, including CBD, have been shown to have a positive effect in the treatment of many skin diseases (Baswan et al., 2020). Their effect is mainly due to the fact that the endocannabinoid system (ECS) has an important regulatory function in the skin (Baswan et al., 2020) and therefore effective preparations can be used when applied topically. Cannabinoids are an interesting field of research in dermatology due to their anti-inflammatory, antipruritic and antinociceptive properties (Shalaby et al., 2018).

2.1. Atopic dermatitis and eczema

Atopic dermatitis (AD) and eczema are chronic conditions that lead to inflammation, redness and irritated skin. Many factors can lead to atopic dermatitis, such as a damaged skin barrier, an unwanted immune response and an imbalance in the microbiome (Dimitriu et al., 2019). The anti-inflammatory effect of CBD was investigated in an experimental model (Petrosino et al., 2018). This study has shown that CBD inhibits the production of the chemokine MCP-2 and several other proinflammatory cytokines in poly-(I:C)-stimulated keratinocytes more effectively than other non-psychotropic phytocannabinoids tested. Clinical studies have yet to be conducted. Several studies have suggested using the skin ECS as a potential treatment for AD (Mounessa et al., 2017). Studies in mice showed that activation of cannabinoid 1 receptor (CB1R) in skin cells has a positive effect on epidermal barrier function and reduces a Th2-type inflammatory response (Gaffal et al., 2014; Kim et al., 2015).

In an earlier study, the consumption of cannabis seed oil was compared with olive oil (Callaway et al., 2005); in a 20-week randomized, single-blind crossover study with AD patients, the treatment was carried out with a daily intake of 30 mL cannabis seed oil or olive oil (Callaway et al., 2005). The intake of cannabis seed oil led to significant changes in plasma fatty acid profiles and a reduction in skin dryness, skin irritation and itching compared to olive oil. The authors assume that this effect is due to the high proportion of polyunsaturated fatty acids (PUFAs) in cannabis seed oil.

An unbalanced skin microbiome could also have an important influence on the severity of dermatitis; the studies suggest that the main antimicrobial effects of cannabis come from its essential oil (Mediavilla et al., 1997), *i. e.* the volatile component of cannabis. Its main components are terpenes such as myrcene, α-pinene, β-caryophyllene and others. The antimicrobial effect has been proven against *S. aureus* (Zengin et al., 2018).

2.2. Acne and seborrhea

Acne and seborrhea are well-known skin diseases characterized by irregularly increased sebum production by the sebaceous glands (SC). Acne as a skin disease occurs when the hair follicles become clogged with oil and dead skin cells. Sebum overproduction, inflammation and altered sebocyte proliferation are the most important factors in the appearance of acne on the skin. The endogenous endocannabinoids (ECBs) are involved in the maintenance of homeostatic sebum production in the human sebaceous glands (Dobrosi et al., 2019). Several studies confirm that CBD could play an important role in the treatment of acne due to its effect on acne-related factors (Oláh et al., 2014). An important study was conducted to test the anti-inflammatory effect of CBD on human sebocytes (Oláh A et al., 2014). The study suggests that CBD is not limited to a direct ECS interaction, but has a more generalized effect. More importantly, CBD not only acts as a lipid-lowering agent but also stabilizes lipid imbalance by normalizing lipogenesis (Jin and Lee, 2018). In addition, CBD has been shown to reduce overall cell proliferation at some relevant doses (Oláh et al., 2014). The same research group has tested the anti-inflammatory potential of CBD and demonstrated its effect (Oláh et al., 2014). The anti-inflammatory effect was attributed to the A2a adenosine receptor-dependent upregulation of Tribbles homolog 3 (TRIB3) and inhibition of the NF-κB signaling pathway. The study concludes that CBD could be a promising therapeutic agent for the treatment of acne due to its combined lipostatic, antiproliferative and anti-inflammatory effects (Oláh et al., 2014).
An unbalanced skin microbiome can contribute to the occurrence of acne, especially the overgrowth of *C. acne* (Platsidaki and Dessinioti, 2018). The study (Jin and Lee, 2018) using cannabis seed hexane extract showed that it has an antimicrobial effect on *C. acnes*. It also induced inflammation and lipogenesis in sebocytes. Cannabis seeds have a minimal CBD content (Callaway, 2004), so we cannot draw parallels regarding their effect on the acne skin microbiome. More promising results could be expected from the use of cannabis essential oil, which mainly contains terpenes (Vuerich et al., 2019). These have been shown to have an antimicrobial effect against *C. acnes* (Raman et al., 1995).

2.3. Psoriasis

Psoriasis is a chronic, inflammatory autoimmune disease of the skin in which the immune system becomes overactive and causes hyperproliferation of skin cells, followed by skin changes known as psoriatic plaques and inflammation (Michalek et al., 2019). Recent research (Sangiovanni et al., 2019) has shown that CBD and cannabis extract (*C. sativa*) standardized to 5% CBD (CSE) inhibit TNF-α-induced NF-κB inhibitory effects in HaCaT cells. NF-κb is a family of transcription factor protein complexes that regulate DNA transcription, cytokine production and cell survival (Wu et al., 2003). It plays an important role in inflammatory skin diseases, usually associated with psoriasis (Sangiovanni et al., 2019). CSE treatment reduced all 26 genes associated with inflammation, while CBD alone reduced only 15 genes (Sangiovanni et al., 2019). This suggests that a broad range of cannabinoids could achieve better results than CBD alone. The use of phytocannabinoids for the treatment of psoriasis should be used with caution. A study on the treatment of psoriasis with CBD (Casares et al., 2020) has also shown that it has proliferative effects on keratins 16 and 17. This could lead to additional complications for psoriasis patients.

3. Cannabis in veterinary dermatology

Dermatologic diseases are the main reason for consultations in general veterinary small animal clinics after preventive health care (Hill et al., 2006). Skin diseases encompass a variety of different conditions, but in most cases an inflammatory response triggered by an infectious, parasitic or immunologic stimulus is the main mechanism behind the pathologic process (Scott & Paradis, 1990). Therefore, pruritus is the most commonly observed sign, especially in dogs (Khoshnegah et al., 2013). In cats, swelling of the skin appears to be the predominant clinical presentation (Hill et al., 2006). Bacterial skin infections are also common in dogs, with *S. pseudintermedius* being the main cause in most cases (Bourguignon et al., 2013). Consequently, the most commonly prescribed drugs for the treatment of skin diseases in small animals are systemic antibiotics and glucocorticoids

(Hill et al., 2006). Topical treatments may also be prescribed for localized skin lesions (Mueller et al., 2012). The commercially available formulations used in veterinary medicine are very similar to those used in humans. They are mainly topical creams and ointments with antibacterial or anti-inflammatory agents to heal skin lesions.

The inclusion of novel molecules with anti-inflammatory, immunomodulatory and antimicrobial effects such as cannabinoids (Izzo et al., 2009) could expand the therapeutic arsenal for dermatological diseases.

The endocannabinoid receptors (CB1 and CB2) are expressed in different cell types of the epidermis and dermis of healthy dogs (Campora et al., 2012), cats (Miragliotta et al., 2018) and horses (Kupczyk et al., 2022). CB1 receptors have also been found in the inner root sheath cells of the primary and secondary hair follicles of dogs (Mercati et al., 2012). The ECS appears to play a protective role in inflammatory dermatological conditions, as ECS expression has been reported to be increased in canine atopic dermatitis (Campora et al., 2012) and in cats with hypersensitivity dermatitis (Miragliotta et al., 2018).

Cannabidiol (CBD) and Δ-9-tetrahydrocannabinol (THC) are the best-studied phytocannabinoids in veterinary medicine (De Mendonça Lima et al., 2022). The use of CBD and THC is considered safe for dogs (Vaughn et al., 2020) and cats (Kulpa et al., 2021). CBD is better tolerated than THC, as escalating doses of CBD resulted in mild adverse effects in both dogs and cats (Vaughn et al., 2020; Kulpa et al., 2021). High doses of THC can cause moderate to severe adverse effects in pets, such as lethargy, hypothermia and ataxia (Vaughn et al., 2020; Kulpa et al., 2021). However, THC has a high therapeutic index, as maximum doses of this compound do not cause death in dogs (Beaulieu, 2005).

Pharmacokinetic studies in dogs have shown that the oral bioavailability of CBD is low, ranging from 13 to 19% (Samara et al., 1988). Although other administration methods have also been tested, oral administration is still the preferred route of administration in most pharmacological studies on cannabinoids in veterinary medicine (Corsato Alvarenga et al., 2023). For focal dermatological conditions, cannabinoids can be administered via the transdermal route to maximize skin concentration while allowing absorption into the bloodstream (Bartner et al., 2018; Hannon et al., 2020). To this end, cannabinoids could be formulated as ointments, preferably as native acid derivatives, as acidic forms of CBD and THC have been shown to be better absorbed than their decarboxylated counterparts (Hannon et al., 2020).

In veterinary medicine, the clinical efficacy of CBD has been tested mainly in osteoarthritic pain, epilepsy and behavioural disorders (De Mendonça Lima et al., 2022; Di Salvo et al., 2023). For skin diseases, two studies focused on testing the potential benefits of cannabinoids in atopic dermatitis in dogs (Loewinger et al., 2022; Mogi et al., 2022). In both studies, cannabinoids were able to reduce itching, although no clinical differences in skin lesions were observed compared to the control groups (placebo) (Loewinger et al., 2022).

Infectious diseases of the skin could be another potential clinical target for cannabinoids. Infections on the skin surface of animals can not only prolong the patient's recovery time, but also spread to other parts of the body, invade the bloodstream and migrate to the internal organs, which can prove fatal if left untreated for too long. Amoxicillin and cefalexin are the most commonly prescribed systemic drugs for skin infections in veterinary dermatology (Hill et al., 2006). Cannabis derivatives exert antimicrobial properties on a broad spectrum of pathogenic bacteria (Schofs et al., 2021). Cannabinoids could act against *S. pseudintermedius*, the main cause of skin infections in dogs (Bourguignon et al., 2013; Aiemsaard et al., 2022), but also against skin infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), a pathogen important for both public health and animal health (Haag et al., 2019; Pečan et al., 2023). These antimicrobial effects of cannabinoids could be further explored in clinical trials in veterinary medicine to test *in vivo* efficacy against skin infections.

Laboratory animal models are not always of translational use, as in many cases they have not been able to predict clinical efficacy and toxicity in humans (Ritter et al., 2020; Marshall et al., 2023). As part of a "One Health" approach and utilising naturally occurring diseases in dogs and cats, cannabinoids can therefore be used as a potentially useful tool in both veterinary and human medicine.

4. Legal regulation of medical cannabis in Slovenia

The history of cannabis regulation is marked by an evolving understanding and shifting legal frameworks. Initially used for medicinal and recreational purposes in various cultures, cannabis' perception underwent a significant transformation in the 20th century. This period saw the international community, through agreements like the 1961 Single Convention on Narcotic Drugs, categorize cannabis as a controlled substance, significantly restricting its use globally (United Nations, 1961). However, recent decades have witnessed a re-evaluation, especially in the context of medical cannabis. This shift has been propelled by a growing body of scientific research that underscores the therapeutic potential of cannabis, particularly in managing chronic pain and other medical conditions (Whiting et al., 2015). Consequently, many countries have begun to adapt their legal frameworks, creating distinct regulations for medicinal and recreational cannabis use. Today's regulatory landscape of cannabis is a complex amalgamation of medical research, legal policies, and evolving societal attitudes (Hall & Lynskey, 2016). This article focuses specifically on cannabis as a plant and does not address cannabinoids that are synthesized independently of the cultivation of the plant.

4.1. Cannabis as an illegal drug

Zakon o proizvodnji in prometu s prepovedanimi drogami (Production of and trade in illicit drugs act: ***Law 1***) specifies the conditions under which the production and trafficking of illegal drugs, as well as the possession of illegal drugs, are permitted. The law requires the Government of the Republic of Slovenia, upon the proposal of the minister responsible for health, to adopt the classification of illegal drugs into the following three groups:

- *Group I:* Plants and substances that are extremely dangerous for human health due to the severe consequences that can result from their abuse and are not used in medicine;
- *Group II:* Plants and substances that are extremely dangerous due to the severe consequences that can result from their abuse but can be used in medicine;
- *Group III:* Plants and substances that are moderately dangerous due to the consequences that can result from their abuse and can be used in medicine.

According to the currently valid *Uredba o razvrstitvi prepovedanih drog* (Decree on the classification of illicit drugs: ***Decree 1***), cannabis, or scientifically *Cannabis sativa* L., as a plant, or its resin and extracts are classified in Group II.
Cannabis is thus an illegal drug according to Slovenian law.

4.2. Under the gaze of Article 186: The regulation of cannabis in Slovenia

In the context of illegal drugs, the Slovenian *Kazenski zakonik* (Criminal code: ***Law 2***), specifically Article 186, addresses the unauthorized production, processing, sale, offer for sale, or acquisition for the purpose of sale or distribution of illegal drugs. This legal framework is crucial for understanding the criminal implications of cannabis-related activities in Slovenia.
Article 186 states that anyone who unjustifiably produces, processes, sells, offers for sale, purchases, stores, or transports drugs, or facilitates their sale or purchase, is subject to legal penalties. This article is applied in situations involving cannabis when these activities are conducted without proper authorization or in violation of existing regulations.
The application of Article 186 to cannabis-related activities underscores the seriousness with which the Slovenian legal system treats the control of illegal drugs. This includes cannabis, which, despite its varying legal status in different jurisdictions (including Slovenian), remains under strict regulation in Slovenia. The law aims to prevent unregulated and potentially dangerous drug trafficking, ensuring public health and safety by controlling the production and distribution of such substances.

4.3. Exclusion of illegality

Although in the case of the specific criminal act under Article 186, we could even talk about the non-fulfillment of the statutory characteristics, as it emphasizes unauthorized production, sale, *etc*., due to systematic correctness and the possibility of future

amendments to the text, the author adopts a position that will always exist regardless of the text of the criminal code, as it originates from the theory of criminal law.

To understand how exactly legal acts then permit its use for medical purposes, it is necessary to briefly explain the theory of a criminal offense. For an act to be considered criminal, an individual's conduct must meet specific conditions, which are described in the Kazenski zakonik (***Law 2***).

When an individual does this, it is necessary to examine whether they acted in circumstances that, despite meeting all the characteristics, make their conduct completely in accordance with the law. This is called the exclusion of illegality. One way to exclude illegality is through exclusion by another law, meaning another law, not the criminal code, permits a certain behaviour that would otherwise be criminal according to the criminal code.

4.4. Regulation of registered cannabinoid medicines in Slovenia

Under the Slovenian Law on the *Zakon o proizvodnji in prometu s prepovedanimi drogami* (Production of and trade in illicit drugs act: ***Law 1***), registered medicines that contain cannabis and its naturally derived active substances are not considered illegal drugs, provided they are obtained and handled legally. Article 6 exempts such substances from being categorized as illegal drugs when they are used in accordance with specific regulations (*Zakon o lekarniški dejavnosti* (Pharmacy practice act: ***Law 3***) in *Zakon o zdravilih* (Medicinal products act: ***Law 4***)). Consequently, these substances are not implicated in the criminal offense described in Article 186 of the Criminal Code, which deals with the unauthorized production and trafficking of illegal drugs. As of the time of research, there are 24 such medicines registered in the central database of drugs, including those that are only authorized for import and not for distribution within Slovenia.

4.5. Supply of cannabis for pharmaceutical production

In the context of importing active substances, the exclusion of illegality operates in a somewhat different manner which will be described in the following paragraphs. This approach is essential in jurisdictions where the use of certain substances is permitted for specific purposes, but production is not and therefore all active substances in Slovenia are imported.

The import of active substances for the manufacture of (magistral) drugs with cannabinoids in Slovenia is governed by a complex legal framework, ensuring that only high-quality and safe ingredients are used. The primary legal documents regulating this area are the *Zakon o zdravilih* (***Law 3***) specifically Articles 119 to 122, and the *Government rules (*Rules on the manufacture, wholesale distribution and import of active substances and on registers of manufacturers, wholesalers and importers of active substances*:* ***Rules 1***). These laws stipulate that only registered importers, who are either holders of a medicinal product manufacturing license, active substance manufacturers, or wholesalers of active substances, are authorized to import such substances.

In terms of magistral drugs with cannabinoids, Slovenian regulations permit the prescription of isolated or synthetically obtained cannabinoids, standardized cannabis flower extract, and phytocannabinoids in the form of floral and fruit tops of medical cannabis. However, the import of standardized cannabis flower extract and its components requires a demonstrated medical need (JAZMP, 2023).

Furthermore, the import of drugs without marketing authorization in Slovenia is permissible only with a specific import permit. The Agency for Medicinal Products and Medical Devices of the Republic of Slovenia (JAZMP) issues these permits within 30 days upon receipt of a complete application for drugs necessary for individual emergency treatments, essential or urgently needed medicines, and in extraordinary cases or for reasons of public health protection (JAZMP, 2023). The applicant for these permits must be a holder of a wholesale drug trading license or a manufacturer authorized to import drugs.

In summary, the Slovenian legal framework ensures that the import of active substances for (magistral) drugs with cannabinoids is strictly regulated. Importers must be registered and comply with EU or equivalent production standards. Prescribing and importing

cannabinoid-based drugs are subject to stringent controls, emphasizing the necessity of medical justification and adherence to public health interests.

4.6. Industrial hemp cultivation regulations in Slovenia

In Slovenia, the cultivation of industrial hemp is strictly regulated under the *Pravilnik o pogojih za pridobitev dovoljenja za gojenje konoplje in maka* (Rules on conditions for obtaining a permit for hemp and poppy cultivation: ***Rules* 2**). This legislation mandates that cultivation is permissible solely for certain varieties that are included in the national "Sortna lista" for 2023. These varieties must be listed within the EU and contain a THC content of less than 0.3%.

To engage in hemp cultivation, farmers are required to satisfy various criteria. These include possessing a minimum land area of 0.1 hectares, which is reduced to 0.01 hectares for organic farmers. Additionally, they must register their cultivation activities, providing comprehensive details about the area of cultivation and its intended use.

These stringent measures ensure that Slovenian hemp cultivation adheres to both national and European Union standards. It's crucial to note that the cultivation of medical cannabis, or cannabis suitable for the production of medicines, is currently not permitted in Slovenia. This distinction is important as the term "medical cannabis" specifically refers to varieties used for medicinal purposes, different from those allowed for industrial cultivation.

5. International Resonance and Comparative Perspective

The significance of medical cannabis in the context of human rights has also already been acknowledged. The European Court of Human Rights (ECHR) in the case of Thörn v. Sweden (***Case 1***) examined whether domestic authorities violated the appellant's right to respect for private life by not exempting him from general criminal liability, which applies to such actions. The case emphasizes the cultivation of cannabis for personal medical use and it underlines the necessity for domestic authorities to consider individual circumstances in criminal prosecutions involving medical cannabis cultivation, balancing public drug regulation interests against individual health needs, considering THC content, distribution risk, medical necessity, and legal available alternatives.

In contrast to Slovenia, some EU countries like Germany and the Czech Republic have more liberal approaches towards medical cannabis. Germany has a regulated medical cannabis program with strict quality control measures and a tightly regulated supply chain to ensure patient safety and product consistency. The regulation also covers the import and manufacturing of medicinal cannabis, with strict licensing requirements (Düwel et al., 2023). The Czech Republic permits medical cannabis use with a prescription and allows individuals to cultivate up to five cannabis plants for personal use. As of 2022, decrees regulate the cultivation and processing of cannabis plants for medical use, setting the rules for proper cultivation practice and licensing for Czech growers. There have been efforts to increase the number of suppliers, enable export, and reduce patient costs. The Czech legal framework involves reimbursement of medical cannabis costs and ongoing developments in the regulation of cultivation and supply (KOPAC, 2023) which can sometimes prove ineffective (Medical cannabis network, 2019).

The benefits of liberal medical cannabis regulation in the EU have been recognized. More EU Member States are viewing the clinical benefits of cannabis and its derivatives favorably. There is a movement towards ensuring fair and equal access to medical cannabis products and establishing a harmonized policy framework at the EU level (Lipnik-Štangelj and Razinger, 2020).

6. Conclusion

Cannabis has been very popular throughout human history due to its versatility, as it can be used in various fields such as the textile, paper, food, furniture and energy industries. It contains more than 140 cannabinoid compounds, of which cannabidiol and tetrahydrocannabinol are the most exposed as they have the potential to treat diseases (Čulić et al., 2021). Numerous scientific findings and clinical studies over the last decade agree that the use of cannabis in medicine has undoubted clinical benefits for many

conditions. The best-known effects of cannabis use are pain relief and treatment of chronic diseases and certain neurological disorders in human medicine (Čulić et al., 2021).

The use of cannabis products by humans is increasing worldwide. In veterinary medicine trend is related to the growing interest of clients and veterinarians in the treatment of animal diseases with these molecules. In general, CBD is of primary interest in veterinary medicine (Temmerman, 2023).

In summarizing Slovenia's approach to cannabis regulation, it is essential to recognize the nuanced interplay between legal rigidity and emerging recognition of cannabis's medicinal value. The journey through Slovenia's legal frameworks, particularly the *Zakon o proizvodnji in prometu s prepovedanimi drogami* (***Law 1***) and Article 186 of the *Kazenski zakonik* (***Law 2***), highlights a cautious yet evolving stance towards cannabis.

A critical aspect yet to be fully explored is the potential for domestic production of medical cannabis. The current legislative framework, while stringent on illicit drug activities, has not fully embraced the cultivation of cannabis for medicinal purposes (Štukelj et al., 2018). Developing a regulatory system that permits controlled cultivation for medical production under strict quality standards could significantly enhance Slovenia's healthcare landscape and contribute to global medicinal cannabis research.

This review of Slovenia's cannabis regulation reveals a nation balancing control with emerging medicinal perspectives. The potential for regulatory reform, accommodating both public safety and medical advancement, looms as a promising horizon. Such a future, where regulation aligns with the scientific and compassionate use of medicinal cannabis, could position Slovenia as a leader in this field, reflecting a commitment to both legal integrity and medical innovation.

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List of regulation documents

1. ***Law 1***: Zakon o proizvodnji in prometu s prepovedanimi drogami (Uradni list RS, št. 108/99, 44/00, 2/04 – ZZdrI-A in 47/04 – ZdZPZ)
2. ***Law 2***: Kazenski zakonik (Uradni list RS, št. 50/12 – uradno prečiščeno besedilo, 6/16 – popr., 54/15, 38/16, 27/17, 23/20, 91/20, 95/21, 186/21, 105/22 – ZZNŠPP in 16/23)
3. ***Law 3***: Zakon o lekarniški dejavnosti (Uradni list RS, št. 85/16, 77/17, 73/19 in 186/21)
4. ***Law 4***: Zakon o zdravilih (Uradni list RS, št. 17/14 in 66/19)
5. ***Decree 1***: Uredba o razvrstitvi prepovedanih drog (Uradni list RS, št. 69/19, 157/20, 162/21, 8/23 in 113/23)
6. ***Rules 1***: Pravilnik o proizvodnji, prometu na debelo in uvozu učinkovin ter registrih proizvajalcev učinkovin, veletrgovcev z učinkovinami in uvoznikov učinkovin (Uradni list RS, št. 79/16)
7. ***Rules 2***: Pravilnik o pogojih za pridobitev dovoljenja za gojenje konoplje in maka (Uradni list RS, št. 40/11, 36/15, 33/18 in 61/23)
8. ***Case 1***: European Court of Human Rights. (2022). Case of Thörn v. Sweden (Application no. 37075/09).

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Invited lecture/Research

A Multiplex GC-MS/MS Analysis for the Quantitative Monitoring of Bilobalide, Ginkgolides and Ginkgotoxin in *Ginkgo biloba*-Derived Products and Biomaterials

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Abstract:

The bioactivity *Ginkgo biloba*-derived extracts and other preparations is attributed to the presence of secondary metabolites, especially terpene trilactones (ginkgolides and bilobalide), flavonoids but also toxic constituents, like ginkgotoxin. In this study, we set up a multiplex method using a gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) for the simultaneous quantitative analysis of six of these characteristic metabolites, namely ginkgolide A, B, C, J, bilobalide and ginkgotoxin. Parameters were set up and optimized for multiple reaction monitoring (MRM) to allow the sensitive and selective monitoring of specific collision induced dissociation transitions for each analyte. The method was applied to quantify and compare the above ingredients after derivatization in the methanol extracts of a commercial supplement, ginkgo nuts, ginkgo cells from suspension and callus cultures, and microvesicles (MVs) and nanovesicles (NVs) isolated from ginkgo seeds. As a result of the MRM analysis, the commercial supplement contained 7,77% terpene lactones per tablet. The seed samples contained 50 parts per million (ppm) ginkgolide A and 14 ppm ginkgolide B, but ginkgolide C and J were below the limit of quantitation. Ginkgo biloba cells from in vitro cultures, MVs and NVs contained considerably less bilobalide and ginkgolides than the seed and the commercial supplement.

Keywords: *Ginkgo biloba***;** extracellular vesicles; GC-MS/MS; derivatization; quantitative analysis, multiple reaction monitoring, ginkgolides, bilobalides, ginkgotoxin

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1. Introduction

Ginkgo biloba L., the only survivor of genius ginkgo is a living fossils revered for its longevity and elegance of its leaves (Chi et al., 2020). Different parts of ginkgo tree such as leaves, seeds and roots have been used in traditional Chinese medicine for thousands of years. A standardized extract of ginkgo biloba (GB) dried leaf, Egb 761 containing 24% Ginkgo Flavone Glycosides and 6% Terpene lactones is among the most studied and most popular herbal supplements today (Nor-E-Tabassum, 2022). Egb 761 represents the only herbal alternative to synthetic antidementia drugs in the therapy of Alzheimer's disease and cognitive decline (Nor-E-Tabassum, 2022). Sora *et al* developed and validated a liquid chromatography tandem mass spectrometry (LC–MS/MS) assay of terpene trilactones in (GB) extracts and pharmaceutical formulations through standard addition method and found bilobalide content 2.8-3.2 mg and ginkgolides (A, B, C) content 2.9-3.2 mg (Sora et al., 2009). Gas chromatography - mass spectrometry (GC-MS) has also been successfully applied to quantify ginkgolides in ginkgo dietary supplements (Deng and Zito, 2003).

The edible seeds of GB are also used in traditional Chinese medicine for different purposes, including the treatment of senility, asthma, bronchitis, and kidney and bladder disorders. GB seeds known to contain toxic compounds like ginkgotoxin (N-methyl pyridoxine), an analogue of Vitamin B6, that raised concern about its use (Boateng and Yang, 2022). LC-MS and LC-MS/MS methods have been developed for the identification and quantitation of ginkgotoxin (Scott et al., 2000) and determined 174 ppm ginkotoxin in seed extracts.
In this study, our goal was to develop a multiplex analytical method based on gas chromatography-tandem mass spectrometry (GC-MS/MS) for the simultaneous quantitation of five terpene lactones (ginkgolide A, B, C, J and Bilobalide) and ginkgotoxin (**Figure 1**).

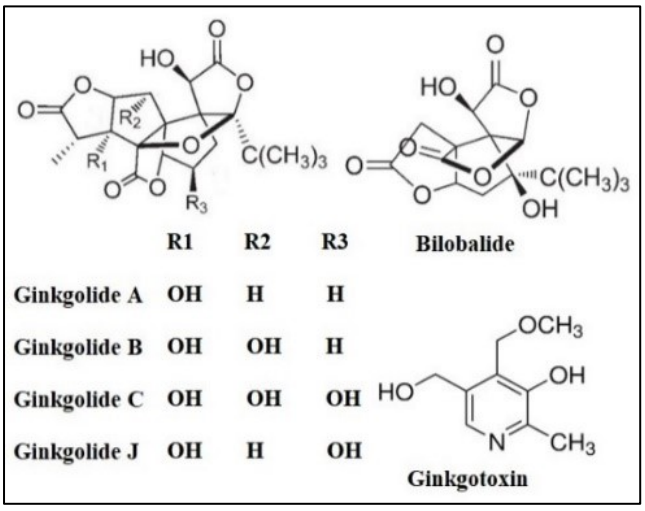

Figure 1. Chemical structures of ginkgolides A, B, C, J, bilobalide and ginkgotoxin.

The developed method was used to investigate the quantities of these primary ginkgo specific ingredients in the methanol extract of ginkgo nuts, ginkgo cells from suspension and callus cultures, micro- and nanovesicles isolated from seeds as well as a commercial supplement (**Figure 2**). While there have been LC-MS/MS based studies on terpene lactones that investigates commercial pharmaceutical products containing standardized extracts (Sora et al., 2009; Scott et al., 2000), to the best of our knowledge there is no GC-MS/MS method available today for the simultaneous analysis of terpene lactones and ginkgotoxin.

Figure 2. Samples analysed; A) Gloryfeel ginkgo biloba supplement prepared from ginkgo leaves, B) ginkgo seeds C) embryo, callus and cell suspension cultures.

2. Materials and methods

2.1. Sample preparation and standards

GB dietary supplement was from Gloryfeel BmbH (Hamburg, Germany). Tablet (274.05 mg) was pulverized in a mortar and sample (235.6 mg) was extracted in 5 mL methanol (Romil MS grade) for 30 minutes under orbital shaking at room temperature. Samples were centrifuged at 14000 x g at 26 °C for 20 minutes two times.

GB seeds were collected in Naples Piscinola in November, 2023. Seed shell and inner layer were removed and 5 samples containing 5 seeds were dried for 72 h at 40 °C. Samples were pulverized in a mortar and 50 mg were extracted in 600 µL methanol.

Callus and cell suspension cultures were established using GB embryos as starting material. The seeds were surface sterilized with sodium hypochlorite and subsequently, viable GB embryos were isolated and put for callus induction on Gamborg B5 growth medium (Duchefa Biochemie), supplemented with 2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4D) and 2 mg/L of naphthyl acetic acid (NAA). White and friable callus cultures were obtained after 8 weeks of cultivation in dark conditions and were maintained by subculturing every 4 weeks. To initiate cell suspension cultures, 5 g of callus were resuspended in 50 mL of the same growth medium and incubated at 26 °C in dark conditions under constant orbital stirring (110 rpm). The cultures were maintained by subculturing every 7 days. To prepare the samples, 40-45 days old callus cultures and 7 days old suspension cultures were used. Samples were homogenized in methanol using a Tissuelyser (Qiagen) at 30 Hz, 5 x 1 minutes.

Microvesicles (MVs) and nanovesicles (NVs) were isolated from the homogenate of GB seeds using the differential ultracentrifugation method (Bokka et al., 2020). Protein concentration was measured by Qubit assay (Invitrogen). 300 µg MVs and NVs expressed in protein quantity were extracted in 300 µL methanol. The samples were homogenized in methanol using a Tissuelyser (Qiagen) at 30 Hz, 5 x 1 minutes.

Five replicates were prepared of the suspension and callus cultures, seeds and tablet, and triplicates for MVs and NVs samples. All methanol extracts were centrifuged at 15000 x g for 20 min. 600 ng D-Mannitol-^{13}C6 (Cambridge Isotope Laboratories, Canada) was added as internal standard (IS). Samples were vacuum dried and then further dried under nitrogen flow. Derivatization was performed using 50 µL silylation derivatization reagent, (N,O-bis[trimethylsilyltrifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Sigma Aldrich) and 35 µL pyridine (Merck) at 70 °C for 1 hour at 500 rpm agitation in a thermomixer.

The calibration solutions were prepared in the following ranges: ginkgolide A, B, C and J from 0.05 µg to 50 µg; bilobalide from 0.05 to 30 µg and ginkgotoxin from 0.01 to 20 µg in 85 µL. The quantity of the IS was maintained at 600 ng in 85 µL in each standard solution.

2.2. GC-MS/MS measurement parameters

GC-MS/MS analyses were performed using a Thermo Scientific Trace 1300 GC coupled to a TSQ 8000 Duo mass spectrometer equipped with an electron ionization (EI) ion source and a triple quadrupole (QqQ) ion analyzer. Separation was performed using a TG-SQC 30 m, 0.25 mm x 0.25 µm capillary column. GC parameters were as follows: measurement time: 35 minutes, column flow: 1.2 mL/min (helium), injection volume: 1.0 µL, injection type: splitless, inlet temperature 230 °C. MS parameters; transfer line temperature: 280 °C, ion source temperature: 250 °C. GC temperature ramp; initial temperature: 70 °C hold for 1 min, ramp rate 1: 10 °C/min to 280 °C, ramp rate 2: 6 °C/min to 313 °C, ramp rate 3: 0,6 °C/min to 314 °C, ramp rate 4: 6 °C/min to 316 °C, ramp rate 5: 2,4 °C/min to 318 °C, ramp rate 6: 6 °C/min to 320 °C hold for 2 min.

Dionex Chromeleon 7 Chromatographic Data System version 7.2.10 (Thermo Scientific Fisher, USA) was used for data acquisition and processing. The tentative identification of the compounds was based on computer matching with the National Institute of Standards and Technology (NIST) library. Collision induced dissociation (CID) was performed using Argon as collision gas. Two ion transmissions of the single analytes were selected by using the Thermo Scientific AutoSRM application run under Chromeleon 7 software package. Concentrations of the analytes in the samples were calculated using calibration curves values corrected against the internal standard.

3. Results and Discussion

Full scan MS-analysis using chemical derivatization to make the compounds volatile provides an unbiased identification of metabolites in plant extracts (Fiehn, 2016). In this study, after silylation derivatization we have performed full scan GC-MS analyses of the methanol extracts of the following ginkgo biloba-derived samples: i) seed, ii) callus iii) cells from suspension culture, iv) microvesicles isolated from GB seeds, and v) nanovesicles isolated form GB seeds. Compounds that were identified in at least 3 samples were considered. Appendix A shows the compounds tentatively identified in each sample. Mostly primer metabolites, i.e. aminoacids, lipids, organic acids, aldehydes, ketones and alcohols and sugars could be identified in this analysis. 100 compounds were identified in the GB seed extract, 60 in callus culture, 68 in cell suspension derived cells, 35 in microvesicles and 39 in nanovesicles derived from GB seeds. Venn diagrams compare the presence of different metabolites in seed and seed-derived micro- and nanovesicles samples (Figure 3A) and in seed and seed explant-derived callus and suspension cultures (Figure 3B). Only 10 compounds were commonly present in MVs, NVs and seed extracts, 7 compounds in NVs and seed, 8 compounds in MVs and seed, and 13 compounds in MVs and NVs (Figure 3A). 30 compounds were commonly identified in callus, suspension cultures and seed, 3 compounds in callus and seed, 6 compounds in suspension cultures and seed, and 19 compounds were commonly identified in callus and suspension cultures (Figure 3B). Interestingly, only two secondary metabolites, the flavonoid monomeric unit containing catechin (2R-trans) and epigallocatechin, were detected in callus and suspension cultures. Characteristic GB metabolites, like bilobalide, ginkgolides or ginkgotoxin could not be identified through the full scan GC-MS analysis in seed and seed derived cells cultured in vitro or seed-derived micro and nanovesicles.

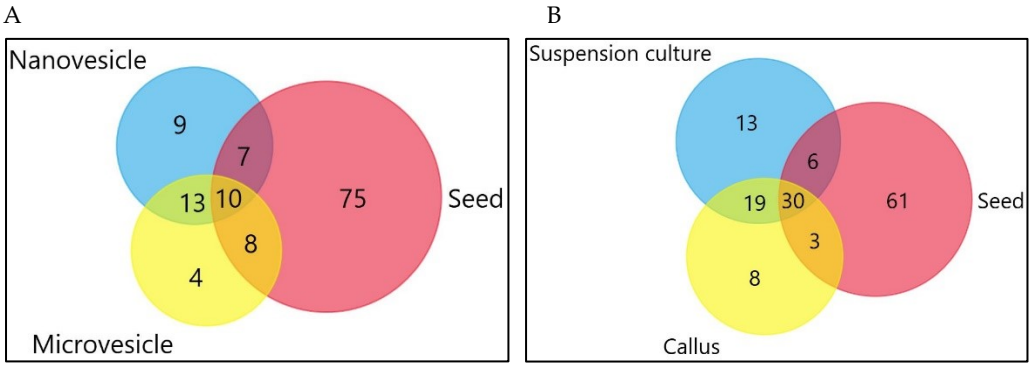

Figure 3. Venn diagram of A: the metabolites identified in MVs, NVs and ginkgo biloba seeds and B: callus, suspension cultures and ginkgo biloba seeds (lists of compounds are reported in **Appendix A**).

To setup the GC-MS/MS targeted analysis method, we have determined and optimized two characteristic precursor-to-product ion transitions for the internal standard mannitol $^{13}C6$ and six ginkgo biloba secondary metabolites, i.e. ginkgotoxin, bilobalide and ginkgolides A, B, C and J. These are reported in **Table 1**. Collision energy (CE) for each transition was optimized using the Chromeleon AutoSRM software and the optimal CE values are reported in **Table 1**.

Table 1. Precursor / product ion's *m/z* as well as collision energy values.

Compound	m/z Precursor/Product Ion	Collision Energy (eV)
ginkgotoxin	280.1 / 206.1	10
	295.1 / 280.1	10
D-mannitol $^{13}C6$	426.1 / 264.1	10
	426,1 / 336.14	10
bilobalide	299.1 / 271.1	10
	398.3 / 223.1	10
ginkgolide A	537.2 / 187.0	25
	537.2 / 391.2	10
ginkgolide B	625.3 / 479.2	10
	625.3 / 597.2	5
ginkgolide C	713.3 / 567.1	10
	713.3 / 595.1	10
ginkgolide J	478.3 / 264.2	10
	478.3 / 422.2	10

Based on these results we setup a quantitation method suitable for the monitoring of these analytes in a single data acquisition. **Figure 4** shows a representative MRM spectrum of ginkgotoxin, bilobalide, ginkgolide A, B, C, J and internal standard D-mannitol-$^{13}C6$ in MS quantitation mode. GC conditions were set to achieve baseline separation for all the four ginkgolides studied.

The data of calibration curves, i.e. retention times, ranges, R2 values, slopes are reported for each standard in Table 2. The results of the quantitative analysis are reported in **Table 3**. Based on our analysis we found that the commercial supplement contained 7,77% terpene lactones per tablet. The seed samples contained 50 ppm ginkgolide A and 14 ppm ginkgolide B, but ginkgolide C and J were below limit of quantitation (LOQ), methanol extracts of Ginkgo biloba cells from callus and cell suspension, MVs and NVs contained considerably less bilobalides and ginkgolides than the seed and supplement extracts. We

found that MVs sample contained 269 ppm, NVs 61 ppm, suspension culture (dry) 3 ppm, seed 266 ppm and tablet 37 ppm of ginkgotoxin.

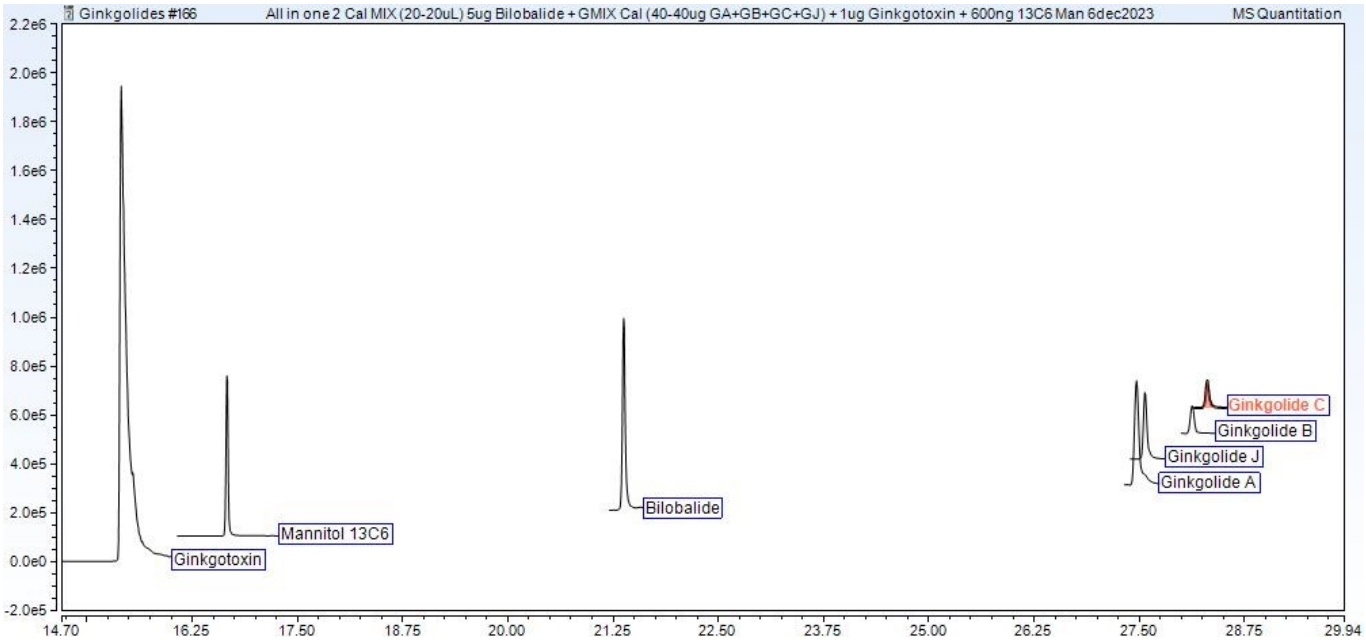

Figure 4. Representative MRM spectra of ginkgotoxin, bilobalide, ginkgolide A, B, C, J and internal standard the D-mannitol-^{13}C6 (MS quantitation).

Table 2. Calibration data (Retention Times (RT), Ranges, R^2, Slopes).

Compound	RT (min)	Calibration points	Range (µg)	R^2	Slope
ginkgotoxin	15.40	7	0.01-20	0.9944	13502.73
bilobalide	21.41	6	0.05-30	0.9917	343.02
ginkgolide A	27.53	5	0.50-50	0.9939	16.15
ginkgolide B	28.20	5	0.50-50	0.9965	14.44
ginkgolide C	28.35	5	0.50-50	0.9982	14.55
ginkgolide J	27.60	5	0.50-50	0.9804	14.76

Table 3. Results of the quantitative GC-MS/MS analysis of nanovesicles, microvesicles, cells from suspension culture wet and dry, callus wet and dry, seed and ginkgo supplement tablet – weight: mass used for the methanol extraction in milligrams (mg, average values); ginkgotoxin, bilobalide, ginkgolide A, B, C and J analytes expressed in part per millions (ppm) (average values). LOQ is for limit of quantitation.

Sample	Weight (mg)	Ginkgotoxin (ppm)	Bilobalide (ppm)	Ginkgolide A (ppm)	Ginkgolide B (ppm)	Ginkgolide C (ppm)	Ginkgolide J (ppm)
Nanovesicles (wet)	0.3	61	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Microvesicles (wet)	0.3	269	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Suspension culture (wet)	123.3-190.9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Suspension culture (dry)	11.97	3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Callus (wet)	127.8-182.9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Callus (dry)	12.41	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Seed (dry)	49.20-52.04	266	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Tablet (dry)	274.05	37	11303	55525	4309	5008	1514

4. Conclusion

The ginkgolides are known to inhibit platelet aggregation, while the bilobalide protects against neuronal death caused by brain ischemia. On the other hand, ginkgotoxin is a neurotoxin structurally related to vitamin B6, naturally occurring in GB nuts and leaves. The untargeted full scan analysis of the different GB biomaterials could not identify any of the above, main GB-related secondary metabolites in the samples studied (**Appendix A**). These natural ingredients are present at very different quantities in the different GB derivatives and their simultaneous quantitation is neither trivial nor easy. The full scan analysis turned out to be useful in identifying primary metabolites (aminoacids, lipids, organic acids, aldehydes, ketones, alcohols, and sugars). Moreover, it identified two secondary metabolites, namely the flavonoid monomeric unit containing catechin (2R-trans) and epigallocatechin in both callus and suspension cell cultures. Cathenkins are known to be potent antioxidants that have already been identified in GB products. A recent study shows strong inhibition of β-amyloid peptide aggregation and destabilization of preformed fibrils by GB-derived cathekins and suggests that they represent viable approaches for the prevention and treatment of Alzheimer disease (Xie et al, 2014).

To achieve our goal towards the identification of the six GB secondary metabolites (**Figure 1**), we have setup a GC-MS/MS MRM method that can quantify these metabolites in a single run. To do that, we have improved the separation of 4 ginkgolides in the GC timescale by the careful setting of temperature ramping. Moreover, we have selected a pair of single reaction monitoring (SRM) precursor and product ions transitions for each analyte as well as optimized the collision energy for each transition (**Table 1**) and setup calibration curves for each analyte (**Table 2**). We found that the quantity of terpene lactones in grown ginkgo cells in vitro, MVs and NVs are lower than the quantitation limit (**Table 3**). In MVs we detected more ginkgotoxin than in NVs (**Table 3**). This could be explained by the lower purity grade of the MVs. In fact, MVs sample was obtained after a series of low velocity centrifugation steps from the GB seed homogenate, while the NVs were isolated after the ultracentrifugation step. Therefore, the higher amount of ginkgotoxin in MVs could be associated with its nonspecific association to the vesicles.

As a result of MRM analysis, the commercial supplement contained 7,77 % terpene lactones per tablet. The method could be useful for the quality control of similar products. The seed sample contained 50 ppm ginkgolide A and 14 ppm ginkgolide B, but ginkgolide C and J were below LOQ. Ginkgo biloba cells from callus and cell suspension, MVs and NVs contained considerably fewer bilobalide and ginkgolides than the seed and supplement extracts (**Table 3**). We measured that the quantity of ginkgotoxin were 269 ppm in the microvesicles samples, in NVs 61 ppm, suspension culture in dry condition 3 ppm, seed 266 ppm and tablet 37 ppm. This shows that the targeted MRM method can be useful for screening ginkgotoxin in different GB products.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki; blood was donated voluntarily by the authors of the study.

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

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Appendix A

1. Compounds identified in the Microvesicles sample in the full scan GC-MS analysis.

Compound	compound class
2,3-Butanediol	aldehyde, ketone and alcohol
Isoborneol	
D-Pinitol	
5-tridecaol	
2,6-Di-Tert-butylphenol	
cyclopropene-1-(2-Hydroxypropyl)-2-ol	
6-tridecanol	
Alanine	amino acid
Glycine	
Leucine	
13-Octadecenoic acid (E)	fatty acids
5,8,11-Eicosatrienoic acid, (Z)-	
9,12-Octadecadienoic acid (Z,Z)-	
Palmitic acid	
2-Butenedioic acid, (E)-	other organic acids
Butanedioic acid	
Citric acid	
Hydracrylic acid	
Lactic acid	
Oxalic acid	
1,2,2,3,4-Butanepentacarbonitrile	other compounds
4-Hydroxy-4-methylhex-5-enoic acid, tert.-butyl ester	
4-Pyridinol	
9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	
Octadecane, 6-methyl-	
Pyridinium, 1-(2-hydrazino-2-oxoethyl)-, chloride	
t-Butyldimethyl(2-styryl[13]dithian-2-yl	
carbamate	
Myo-Inositol	sugar
ß-D-Tagatopyranose	
α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-OH-, cyclic methylboronate	
D-Mannitol	

2. Compounds identified in the nanovesicles sample in the full scan GC-MS analysis.

Compound	**Compound class**
2,6-DI-Tert-butylphenol	aldehyde, ketone and alcohol
Alanine	amino acid
Glycine	
Leucine	
L-Isoleucine	
L-Methionine	
L-Proline	
L-Threonine	
L-Valine	
Phenylalanine	
5,8,11-Eicosatrienoic acid, (Z)-	lipid
9,12-Octadecadienoic acid (Z,Z)-	
Palmitic acid	
Petroselinic acid	
Aconitic acid (E)	organic acid
Citric acid	
Hydracrylic acid	
Lactic acid	
1,2,2,3,4-Butanepentacarbonitrile	other
4-Hydroxy-4-methylhex-5-enoic acid, tert.-butyl ester	
4-Pyridinol	
5-Dimethy tridecane	
6-Dimethyl tetradecane	
Cephaloridine	
Decane	
D-Gala-l-ido-octonic amide	
Dodecane, 2,6,11-trimethyl-	
Isoborneol, pentamethyldisilanyl ether	
N-4,5-dihydrothiazol-2-amine	
Octadecane, 6-methyl-	
Pyridinium, 1-(2-hydrazino-2-oxoethyl)-, chloride	
Silanoltrimethyl-phosphate(3:1)	
t-Butyldimethyl(2-styryl[13]dithian-2-yl	
carbamate	
α-D-Glucopyranose	sugar
α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-OH-, cyclic methylboronate	

3. Compounds identified in the ginkgobiloba seed sample in the full scan GC-MS analysis.

Compound	Compound class
(2R,3R,4R,5S)-Hexane-1,2,3,4,5,6-hexol	aldehyde, ketone and alcohol
2,3-Butanediol	
(S)-5-Methylhydantoin	
1,3,5-Pentanetriol	
2,6-DI-Tert-butylphenol	
Erythritol	
Ethanolamine	
Glycerol	
L-Arabinitol	
L-Threitol	
Propanetriol, 2-methyl-, tris-O-(trimethylsilyl)-	
trans-Coniferrylalcohol	
4-Aminobutanoic acid	amino acid
Alanine	
Asparagine	
DL-Pyroglutamic acid	
Glycine	
L-Aspartic acid	
Leucine	
L-Glutamic acid	
L-Isoleucine	
L-Methionine	
L-Proline	
L-Threonine	
L-Valine	
Phenylalanine	
Serine	
ß-Alanine	
11-Octadecenoic acid, (E)-	lipid
9(Z),11(E)-Conjugated linoleic acid trimethylester	
9,12-Octadecadienoic acid (Z,Z)-	
9-Octadecenoicacid (E)-	
Palmitic acid	
Stearic acid	
2-Butenedioic acid, (E)-	organic acid
Shikimic acid	
(E)-Ferulic acid	
2,3,4-Trihydroxybenzoic acid	
2-Amino-2-cyclopropylacetic acid	
2-Aminomalonic acid	
4-Hydroxybutanoic acid	
4-Trimethylsiloxy(trimethylsilyl)valerate	
Butanedioic acid	
Butanoic acid, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	
D-Gluconic acid	
Glyceric acid	

Glycolic acid	
Lactic acid	
Malic acid	
Pentanedioic acid, 2-[(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	
Pyrrolidine-3-carboxylic acid	
Quininic acid	
Ribonic acid	
(3R,4S,5R)-3,4-Dihydroxy-5-((2S,3S)-1,2,3,4-tetrahydroxybutyl)dihydrofuran-2(3H)-one	other
16-Heptadecyn-4-one, 1,2-dihydroxy-	
2,3-Dihydroxybutanedihydrazide	
2-Pyrrolidinone	
3-Vanil-1,2-bis(trimethylsilyloxy)propane	
5-Dimethyl(trimethylsilyl)silyloxytridecane	
6-Dimethyl(trimethylsilyl)silyloxytetradecane	
Erythrono-1,4-lactone	
Methyl-3-(4-hydroxyphenyl)-2-[(4-hydroxyphenyl)methyl]-4-methoxy-5-oxofuran-2-carboxylate	
Pentasiloxane, dodecamethyl-	
Silanoltrimethyl-phosphate(3:1)	
Uridine	
1,5-Anhydroglucitol	sugar
3-α-Mannobiose (isomer 1)	
D-(-)-Fructofuranose (isomer 1)	
D-(-)-Fructofuranose (isomer 2)	
D-(+)-Cellobiose (isomer 2)	
D-(+)-Talofuranose (isomer 2)	
D-(+)-Turanose	
D-Allofuranose	
D-Arabinopyranose (isomer 2)	
D-Glucitol	
D-Mannitol	
D-Pinitol	
Galactinol	
Galactitol	
Lactulose	
Maltose	
Myo-Inositol	
ß-Arabinopyranose	
ß-D-(+)-Mannopyranose	
ß-D-(+)-Talopyranose	
ß-D-Galactopyranoside, methyl 2,3-bis-O-(trimethylsilyl)-, cyclic butylboronate	
ß-D-Galactopyranoside, methyl 2,3-bis-O-(trimethylsilyl)-, cyclic methylboronate	
ß-D-Glucopyranoside, 2-hydroxy-2-methyl-3-buten-1-yl	
ß-D-Glucopyranosiduronic acid, 3-(5-ethylhexahydro-2,4,6-trioxo-5-pyrimidinyl)-1,1-dime-	
ß-D-Tagatopyranose	
ß-Lyxopyranose	
Sucrose	
Sweroside	
Talose	
α-Chloralose (isomer 2)	

α-D-(+)-Talopyranose	
α-D-Glucopyranose	
α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-OH cyclic methylboronate	
β-D-Lactose (isomer 2)	

4. Compounds identified in the cell suspension culture sample in the full scan GC-MS analysis.

Compound	**Compound class**
(S)-5-Methylhydantoin	aldehyde, ketone and alcohol
Ethanolamine	
Glycine	
4-Aminobutanamide	amino acid
Alanine	
Asparagine	
DL-Pyroglutamic acid	
Homoserine-4-imino-N,O-bis(trimethylsilyl)-trimethylsilylester	
L-5-Oxoproline	
L-Asparagine	
Leucine	
L-Glutamic acid	
L-Glutamine	
L-Isoleucine	
L-Proline	
L-Threonine	
L-Valine	
L-β-Homoglutamine	
Serine	
ß-Alanine	
9-Octadecenoicacid (E)-	lipid
Palmitic acid	
[(4,6-Diamino-2-pyrimidinyl)sulfanyl]acetic acid	organic acid
1-Aminocyclopropanecarboxylic acid	
2,3,4-Trihydroxybutyric acid	
2-Keto-L-gluconic acid	
4-Cyclohexene-1,2-dicarboxylicacid	
Butanedioic acid	
Glycericacid	
Lactic acid	
Malic acid	
Quininic acid	
Shikimic acid methyl ester	
3-Piperidineamin-N-(6-bromopyridin-2-yl)-1-trimethylsilyl-	other
3-Piperidinecarboxamide	
5-amino-3-isoxazolol	
D-(-)-Erythrose, tris(trimethylsilyl) ether, methyloxime (syn)	
Methyl-3-(4-hydroxyphenyl)-2-[(4-hydroxyphenyl)methyl]-4-methoxy-5-oxofuran-2-carboxylate	
Phosphoric acid, bis(trimethylsilyl)monomethyl ester	
Putrescine	

Silanoltrimethyl-phosphate(3:1)	
Stigmast-5-ene, 3β-(trimethylsiloxy)-, (24S)-	
Uracil	
Catechine(2R-trans)	secondary metabolite
Epigallocatechin	
3-α-Mannobiose (isomer 1)	sugar
a-D-Galactopyranose	
D-(-)-Fructofuranose (isomer 1)	
D-(-)-Fructofuranose (isomer 2)	
D-(+)-Turanose	
D-Altrose	
D-Fructose	
D-Glucopyranose	
D-Mannitol	
D-Psicopyranose (isomer 2)	
Galactinol	
Lactulose (isomer 1)	
Myo-Inositol	
ß-Arabinopyranose	
ß-D-Galactopyranoside, methyl 2,3-bis-OH-, cyclic methylboronate	
ß-D-Tagatopyranose	
ß-D-Xylopyranose	
ß-Lyxopyranose	
Sucrose	
Talose	
α-D-Galactopyranoside, methyl 2,6-bis-OH-, cyclic methylboronate	
α-D-Glucopyranose, 2,3,4,6-tetrakis-OH-phosphate	
α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-OH-, cyclic methylboronate	

5. Compounds identified in the callus sample in the full scan GC-MS analysis.

Compound	**Compound class**
(S)-5-Methylhydantoin	aldehyde, ketone and alcohol
Ethanolamine	
1-Aminocyclopropanecarboxylic acid	amino acid
4-Aminobutanoic acid	
Alanine	
Asparagine	
DL-Pyroglutamic acid	
Glycine	
Homoserine	
L-Asparagine	
Leucine	
L-Glutamic acid	
L-Norvaline	
L-Proline	
L-Serine	
L-ß-Homoglutamine	
L-Threonine	
L-Valine	
Serine	
ß-Alanine	
ß-Glutamic acid	
Palmitic acid	lipid
2,3,4-Trihydroxybutyric acid	organic acid
D-Gluconic acid	
Glyceric acid	
Lactic acid	
Malic acid	
Quininic acid	
Shikimic acid	
(R)-1,1,1-Trimethyl-N-(1-oxybutan-2-yl)amine	other
3-Piperidineamin-N-(6-bromopyridin-2-yl)	
3-Piperidinecarboxamide	
5-amino-3-isoxazolol	
Methyl-3-(4-hydroxyphenyl)-2-[(4-hydroxyphenyl)methyl]-4-methoxy-5-oxofuran-2-carboxylate	
Putrescine	
Silanoltrimethyl-phosphate(3:1)	
Stigmast-5-ene, 3β-(trimethylsiloxy)-, (24S)-	
Catechine(2R-trans)	secondary metabolite
Epigallocatechin	
2-α-Mannobiose (isomer 1)	sugar
D-(-)-Fructofuranose (isomer 1)	
D-(-)-Fructofuranose (isomer 2)	
D-(+)-Turanose	
D-Altrose	
D-Fructose	
D-Mannitol	

D-Mannopyranose, phosphate	
D-Psicopyranose (isomer 2)	
Galactinol	
Myo-Inositol	
ß-Arabinopyranose	
ß-D-Galactopyranoside, methyl 2,3-bis-OH-, cyclic methylboronate	
ß-D-Tagatopyranose	
ß-D-Xylopyranose	
ß-Lyxopyranose	
Sucrose	
Talose	
α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-OH-, cyclic methylboronate	
α-D-Mannopyranoside, methyl 2,3-bis-OH-, cyclic butylboronate	

Invited lecture/ Review

Use of Gaseous Plasma for Dental Applications

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Abstract:
Plasma technology is a rapidly growing field of science that permeates various branches of medicine and dental medicine. In dental medicine, cold or nonthermal gaseous plasma can be used directly in the oral cavity for the surface treatment of hard dental tissues, periodontal tissues, or oral mucosa or indirectly for treating dental materials before intraoral use or placement. Simplified atmospheric-pressure plasma devices in plasma pencils or jets have broadened the spectrum of plasma technology applications for safe plasma treatment of living tissues. Cold gaseous plasma also allows surface treatment of various heat-sensitive materials. Dental alloys, polymers, waxes, and ceramics can be decontaminated or disinfected with plasma treatment. Antimicrobial properties of cold atmospheric plasma have been demonstrated to facilitate the treatment of oral mucosa infections, dental caries, and endodontic space infections. Various dental materials can also be functionalized through plasma surface treatment to improve their biocompatibility, adhesive properties, wettability, or permeability. Promising results of cold plasma treatment have also been shown in the bleaching of teeth with external or internal staining and the enhancement of the adhesion of dental composites to dentin. Plasma may also serve as a method of dental armamentarium cleaning and sterilization. Although the use of cold gas plasma is not yet part of the standard procedures in the daily clinical practice of dentists, promising results from preclinical and clinical research are encouraging further development and exploration of this technology.

Keywords: cold gaseous plasma; atmospheric pressure plasma; plasma technology; dental medicine; surface treatment

1. Introduction

1.1. Plasma technology in dental medicine

Plasma medicine, a rapidly developing scientific discipline, is revolutionizing the medical landscape by introducing transformative treatments across diverse specialties. Although cold gas plasma is not yet a part of the standard procedures in routine dental clinical practice, promising results from preclinical and clinical research are encouraging for further development and exploration of this technology. Therefore, it is expected that the clinical use of this technology in dentistry will become increasingly widespread in the future.

1.2. Definition of gaseous plasma and cold (nonthermal) gaseous plasma

Gaseous plasma (i.e., *gas* plasma) is defined as the fourth state of matter that occurs when matter in the gaseous state is supplied with energy, leading to partial ionization of the gas (Cheruthazhekatt et al., 2010; Kim et al., 2014). This phenomenon was discovered by the British physicist Sir William Crookes in 1879, and the term "plasma" was first used to designate it by the American chemist Irving Langmuir in 1929 because of its physical properties resembling electrolytes in medicine and biology (Arora, 2013; Cheruthazhekatt et al., 2010). The term gas plasma is used more commonly to avoid confusion with blood plasma, the non-cellular component of blood.

Ionized gases constitute more than 99% of visible matter in the universe, making plasma the most common state of matter (Arora, 2013; Cheruthazhekatt et al., 2010). In everyday life, plasma can be seen in the form of several natural phenomena (flame, lightning, the solar corona, stars, aurora borealis) and is also encountered as a crucial technological foundation for many devices that have changed the way we live (e.g., mobile phones, computers, plasma screens) (Kim et al., 2014).

Ionization of the gas may be induced by supplying heat or, more commonly, by exposing the gas to an electric or electromagnetic field, leading first to the disruption of molecular bonds and subsequently to the ionization of atoms. Thus, plasma contains a roughly equal amount of electrons, positively and negatively charged ions, neutral and excited atoms and molecules, providing it electrical conductivity, in contrast to gases, which are usually electrical insulators (Cheruthazhekatt et al., 2010; Kim et al., 2014).

Plasmas can be devided to thermal and nonthermal plasmas. The thermal plasma is fully ionized or simply hot plasma, which reaches temperatures in the ranges of a tenth of thousands and up to millions of Kelvins. Such plasmas are less applicable for surface treatment processes. On the contrary, nonthermal plasmas are generated by exposing gases to non-equilibrium conditions which partially ionize them. For nonthermal plasma, electrical energy is usually used, either by directly applying a voltage to the electrode(s) or indirectly by using coils and electric current to generate strong magnetic fields to enable ionization of the gas (Laroussi et al., 2007). These types of nonequilibrium or cold gas plasma have unique applications in various scientific fields and medicine. Unlike thermal plasma, the temperature of heavy particles in cold plasma is significantly lower than the temperature of electrons, reaching values of less than 40°C (Arora, 2013). Thus, it is suitable for the surface treatment of various heat-sensitive materials and even for direct use on tissues (Wu et al., 2016).

2. Atmospheric-pressure plasma appliances

Cold plasmas can be further divided into low-pressure and atmospheric-pressure plasmas. The former are generated in near-vacuum conditions via sophisticated low-pressure plasma systems that enable gas evacuation. On the contrary, atmospheric-pressure plasma is generated in less controlled conditions at atmospheric pressure and is more applicable for use in medicine (Cheruthazhekatt et al., 2010). Atmospheric pressure plasma medical devices use various noble gases or gas mixtures, usually argon, helium, or neon, as sources of gaseous plasma, with or without an added reactive gas (O_2, N_2, or air).

For gas ionization to be achieved at a pressure of 1 atm or more, high strength of the electric field is required, usually more than 30 kV/cm; hence, the distance between electrodes is often small, limiting the applicability of conventional dielectric barrier discharge

(DBD) devices for treatment of small objects that can be placed directly in the discharge field. This obstacle has been successfully surpassed by the development of devices with special nozzles (plasma pencils, plasma jets), allowing the creation of a plasma jet where radicals or charged particles reach several centimeters beyond their source, enabling safe treatment of heat-sensitive substrates (Wu et al., 2016). The gas travels through a tube, and plasma is formed in the discharge region between the inner rod-shaped electrode and the surrounding ring electrode (Wu et al., 2016).

3. Applications of plasma technology in dental medicine

Cold gaseous plasma in dental medicine can be used in two ways: directly in the oral cavity for the surface treatment of hard dental tissues, periodontal tissues, or oral mucosa, and indirectly for the treatment of dental materials before their intraoral use or placement (Li et al., 2017). Its use has been successfully tested in several areas of dental medicine.

3.1. Application as a sterilization method

Cold gaseous plasma can be utilized to sterilize surgical instruments and dental armamentariums. Effective cleaning of hard-to-reach areas of instruments (grooves and threads of endodontic instruments) and removal of protein contamination are achieved combining peroxide as a highly reactive sterilization agent and plasma, which enables full removal of degradation products. As an alternative sterilization method, it is beneficial for heat-sensitive materials that cannot be autoclaved, as it allows sterilization at room temperature (Sarkar et al., 2018). Recently, novel technological devices for simultaneous sterilization and activation of surfaces by gaseous plasma have also been developed.

3.2. Application in the field of pediatric dentistry and endodontics

Numerous *in vitro* studies have demonstrated that cold atmospheric pressure plasma has a bactericidal effect on bacteria in planktonic form and biofilms, with its effect comparable to 2% chlorhexidine. It successfully kills *E. faecalis*, a gram-positive facultatively anaerobic coccus often isolated from root canals or periapical lesions of teeth with persistent chronic apical periodontitis after unsuccessful endodontic treatment. An 8-minute treatment with He and O_2 plasma has a lytic effect on *C. albicans*, an opportunistic pathogen in the oral cavity, while a 30-second treatment also eliminates *Streptococcus mutans*, a key pathogen in carious lesions (Wu et al., 2016). Plasma can decontaminate carious cavities, an alternative to conventional mechanical removal of infected dentin with drilling or lasers. Plasma also successfully destroys bacteria on irregularly shaped carious cavities, penetrating into deeper parts of carious lesions. Simple plasma devices have been developed for safe plasma treatment of root canals by disinfecting the endodontic space (Singh et al., 2014). Atmospheric plasma successfully penetrates *in vitro* biofilms, making it suitable for disinfecting root canals, although, in some studies, it has shown less effectiveness than NaOCl (Wu et al., 2016).

3.3. Application in esthetic and restorative dentistry

In everyday clinical practice, patients increasingly express a desire for teeth whitening. Cold gaseous plasma has also been tested for this purpose. In a study by Lee et al., the tooth bleaching effect was more significant in the group with direct atmospheric pressure plasma surface treatment of stained extracted teeth than in the group with a standard 35% hydrogen peroxide gel treatment. A more pronounced change in tooth color was achieved with simultaneous plasma treatment and application of a 35% H_2O_2 bleaching gel compared to using the bleaching gel alone (Lee et al., 2009). Plasma may successfully remove both external staining via the plasma cleaning effect (removal of surface proteins) and internal staining through the plasma-induced production of hydroxyl ions. In a study by Wang et al., enamel changes after plasma treatment were comparable to changes caused by the H_2O_2 gel.

Plasma treatments have also been attempted to improve the adhesion of dental composites to dentin: a 30-second treatment with atmospheric argon plasma after a 15-second etching with phosphoric acid increased the bond strength (TBS) of composite resin to dentin by 64% compared to the standard adhesive protocol, with the improvement limited only to the peripheral layers of dentin and only for treatments lasting up to 100 seconds, whereas after more extended treatment (i.e., 5 min), the bond strength was lower (Ritts et

al., 2010). Stasic et al. (2019) successfully increased dentin's wettability and surface free energy with plasma treatment, which did not significantly differ from the values in the control group with the conventional phosphoric acid etching. Plasma treatment may also improve dentin wetting with self-etch adhesives (Stasic et al., 2019). Treatment with cold plasma significantly improves the penetration of hydrophilic monomers of adhesive systems (HEMA) into demineralized dentin after etching (Zhang et al., 2014). Plasma-induced deposition of a thin layer of organic polymer on the enamel surface improves the bond strength between the composite and enamel, but the durability of the bond is inferior to that achieved with the standard etching protocol (Han et al., 2014).

Several studies have also attempted plasma treatment as an alternative method for polymerizing dental composites and adhesive systems to achieve greater crosslinking and more time-efficient monomer conversion (Sarkar et al., 2018).

In prosthodontics, the indirect uses of cold plasma have been researched more extensively, especially for surface modification of various dental materials: alloys, polymers, waxes, and ceramics, thereby improving their properties without affecting the chemical and physical properties of the bulk material (Cheruthazhekatt et al., 2010). Material surfaces can be activated and functionalized, thus improving their biocompatibility, adhesive properties, wettability, and permeability. Bactericidal substances (metal ions), therapeutic agents, fluorides, and extracellular matrix proteins for cell adhesion and proliferation may be attached to the material surface via plasma technology (Li et al., 2017).

In dental implantology, researchers have aimed to improve the osseointegration of dental implants or prevent bacterial colonization to prevent biological complications of implant-retained prosthodontic rehabilitation. Plasma etching and plasma spraying of titanium implants are extensively reported in the literature, providing a rough surface for improved osseointegration.

Numerous studies focus on the surface treatment of dental ceramics, especially zirconium oxide ceramic, where treatment with cold plasma increases its wettability and reduces contamination without increasing its surface roughness or changing the material's crystalline structure. Thus, improved bonding of zirconium oxide ceramic to composite cement is achieved, with the bond strength comparable to that achieved through the standard sandblasting protocol, which changes the crystal structure of ZrO_2, causes micropores in the surface, and thereby affects the material's mechanical properties. Plasma also successfully decontaminates zirconia frameworks before veneering. Surface contamination with saliva is reduced more efficiently compared to an isopropanol bath, but plasma fails to clean Fitchecker residues efficiently. Through plasma treatment, TBS of veneered porcelain to zirconia frameworks comparable to that in the group without saliva contamination was achieved (Hallmann et al., 2016; Ito et al., 2016a, 2016b; Piest et al., 2018; Valverde et al., 2013). Experimental research is also being conducted on the plasma treatment of glass ceramics before their adhesive cementation to replace the standard hydrofluoric acid etching protocol, which is technique-sensitive and hazardous (Rener-Sitar et al., 2022). Cold plasma treatment can improve the wettability and adhesive properties of the glass-ceramics (Birk et al., 2023).

3.4. Application in the fields of periodontology, oral diseases, and oral surgery

In vitro studies on the use of cold gaseous plasma on cultures of human periodontal mesenchymal stem cells have shown that plasma treatment may potentially contribute to periodontal regeneration by stimulating cell proliferation and osteogenic differentiation and inhibiting cell migration (Miletić et al., 2013). A favorable effect of plasma has been demonstrated in treating oral mucosal infections caused by *C. albicans*, such as angular cheilitis, denture stomatitis, and linear gingival erythema (Sarkar et al., 2018). Gaseous plasma has also been proven to benefit healing after surgical interventions in the oral cavity by stimulating fibroblast proliferation, extracellular matrix formation, and angiogenesis (Jha et al., 2017).

For oral tumors, plasma treatment at increased power or duration can potentially disrupt the "S" phase of tumor cell mitosis, thereby inducing their necrosis or apoptosis. The studies on the use of plasma technology in oncology have encouraging results in both *in vitro* and *in vivo* conditions, but our knowledge is still insufficient to implement this method in everyday clinical practice (Sarkar et al., 2018).

4. Conclusion

Plasma treatment shows high potential in dental applications, not only for treatment of materials but also for direct treatment of tissues and cells. Despite favorable short-term results in *in vitro* and animal studies, the long-term studies are still needed to evaluate the safety of gaseous plasma treatment for intraoral dental applications (Jablonowski et al., 2019). Concerns regarding potential carcinogenicity, particularly in individuals with pre-existing risk factors, necessitate comprehensive assessments to determine its long-term suitability as an alternative treatment option.

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

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Review

Endangered Butterflies and their Conservation: the Decline of *Parnassius apollo* and *Phengaris spp.* in Europe and Slovenia

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Abstract:

This article addresses the alarming global decline in insect biomass and biodiversity and the decline of European butterfly populations, more notably in Slovenia. Between 1989 and 2016, a 76% decrease in insect biomass raises concerns for ecosystems reliant on pollinators and intricate food webs. Butterfly populations, echoing this decline, witnessed a 50% reduction between 1976 and 2021. Key contributors, including habitat loss, chemical pollution, and climate change, necessitate urgent conservation efforts. Focusing on the Apollo (*Parnassius apollo*) and genus *Phengaris*, the study emphasises the threats posed by global warming and habitat loss. Swift and comprehensive conservation measures are crucial to ensure the survival of these iconic species, moreover recognizing butterflies as "umbrella species" that safeguard broader ecosystems.

Keywords: Butterflies, Decline, *Phengaris*, *Parnassius apollo*, Biodiversity, Conservation

1. Introduction

Insects have a crucial role in ecosystems around the globe. For the past few decades there has been an alarming decline in insect biomass. Between the years 1989 and 2016 the insect biomass declined for more than 76% (Hallmann et al., 2017). Loss of insect biomass and diversity is certain to affect the ecosystems negatively, as insects are crucial in many food webs and in the role of pollination (Müller et al., 2023; Hallmann et al., 2017). One of the most recognizable, well-known, and remarkable groups of insects are butterflies (Lepidoptera). It is composed of day butterflies (Rhopalocera) and moths (Heterocera). While butterflies are comprised of about 17.500 species (Smithsonian, n. d. - a), most of the species in the order Lepidoptera are moths with more than 160.000 species (Smithsonian, n. d. - b). Butterflies serve as an important environmental indicator as they react hastily to changes in the environment, as their presence does not follow vegetation-based indicators (Dennis et al., 2003). As the population of insects has dwindled over time, a parallel decline has been observed in the population of butterflies. It is estimated that between the years 1976 and 2021 overall numbers of butterflies decreased by around 50% (Warren et al., 2021). Further studies have concluded that butterfly numbers started decreasing long ago, with a 80% decline between the years 1890 and 1940 (Warren et al., 2021). Main factors that contribute to the rapid decline are habitat loss, chemical pollution, and climate change (Warren et al., 2021).
The aim of this article is to shed light on the factors of rapid decline and how they affect butterfly populations in Slovenia and conservation programs that have and are helping butterfly populations.

2. Biology of the butterfly

2.1. Adaptations to specific habitat

When assessing the endangerment level of a particular species, significant consideration is given to its degree of specialism (generalism). Two terms are widely used: specialists and generalists. Species described as specialists are often confined to specific ecological parameters and are more susceptible to change, whereas generalists whose ecological niche is broader are less vulnerable and thus better cope with ongoing environmental shifts. In a stable environment, specialists typically outperform generalists. This is due to the additional expenses of generalists linked with utilising multiple resources and developing expensive adaptations to cope with fluctuating environmental conditions (Dapporto & Dennis, 2013; Richmond et al., 2005). Current conditions, influenced by human interference, are far from stable and therefore specialised species are impacted to a greater extent. Several characteristics like number of suitable host plants and nectaring flowers, mobility index, voltinism (number of broods per year) and many others are considered when assessing how specialised a certain species is (Dapporto & Dennis, 2013). It is worth noting that employing either the term specialist or generalist for classifying a species might not yield the most precise characterization, as there clearly exists a continuum between the two extremes. Some species can be specialists for some resources and generalists for others (Dapporto & Dennis, 2013).

2.2. Grassland indicator

The European Grassland Butterfly Indicator program represents the collective population trend of 17 selected grassland species. Initiated in 1990 and ongoing, it has identified substantial butterfly declines across Europe, with published data extending until 2020. When interpreting these findings, it is important to realise that at the start of the monitoring lower population coverage was available and that butterfly populations fluctuate significantly from year-to-year. The number of transects is also limited. With new countries joining and new data becoming available, trends can change and differ from previous versions of the indicator. The indicator shows a linear decline of 36% in the last ten years in Europe, with the 2020 value being significantly lower than the start value (van Sway, et al. 2022).

3. Apollo (*Parnassius apollo*)

3.1. Description

One of Europe's most iconic butterflies, belonging to the family Papilionidae, is the Apollo (*Parnassius apollo*) (Linneaues, 1758) (**Figure 1**). The Apollo is a medium sized butterfly, with 50–80 mm wingspan and great flying capabilities (Brommer & Fred, 1999). It is a butterfly with rounded, chalky white wings, with grey markings, black spots, and red spots with a lighter smaller spot in the centre on the hindwings. The Apollo expresses sexual dimorphism, with male and female having different patterns on fore and hindwings. It has more than 200 (Todisco et al., 2010) subspecies through its territory with the variation in size and wing shape, pattern, the density and intensity of grey markings and black spots, while hindwings always contain the striking red spots. The Apollo was widely distributed from Europe to Asia, although its range has been declining because of loss of habitat. Their populations are often isolated (Collins & Morris, 1985).

Figure 1. **a)** *Parnassius apollo*. **b**) *Sedum album,* host plant of *P. apollo.* Photo: Luka Šturm (with permission)

3.2. Habitat and ecology

The Apollo is a relic of the glacial epoch (Collins & Morris, 1985). The butterfly is found in ranges from 500–2400 m, generally above 1000 m in S Europe (Tolman & Lewington, 2008). It inhabits diverse, rocky, subalpine regions that are not only rocky but also in proximity to deeper soils, that are able to support nectar-rich plants for imagos to feed on (Tolman & Lewington, 2008). The Apollo is univoltine, overwintering in the egg stage. Caterpillars of the Apollo feed exclusively on stonecrops (*Sedum* spp.) (Collins & Morris, 1985), principally *Sedum album* and less often on *Sedum telephium* (Tolman & Lewington, 2008). The larval host plant thrives in dry, rocky outcrops which aren't suitable for nectar-rich plants, therefore the resources for the imago and its larval phase are segregated spatially (Brommer & Fred, 1999).

3.3. Decline of the Apollo

The Apollo was and still is prized by many insect collectors, especially the more lucrative subspecies. Over-collecting isolated populations can easily bring them to extinction. However, over-collecting is not the main factor driving the rapid decline (Collins & Morris, 1985).

Studies have shown that the Apollo is temperature sensitive and 35% of the populations moved northward by 35-50km in the span of a few decades. Populations in the lowland habitats have been in serious decline because of the aforementioned factors, moreover it has been discovered that the Apollo is unable to exist in ranges below 850m in southern

France and appearing earlier as a consequence of global warming. Unpredictable weather has shown to have devastating effects on the populations of the Apollo, especially when its populations are smaller. Such small populations often experience inbreeding and as a result of that higher mortality because of occurrence of deformations. Natural forest expansion is directly limiting and fragmenting the habitat of the Apollo as it prefers open, sunny habitats as are abandoned grasslands in early successional stages. One of the ways that suitable habitats are formed is through forest fires, which can reshape a forest into a grassland. One of the factors of the habitat reduction is abandonment of livestock pasture, as it allows for shrub and forest succession to take place. The impact of predators and parasites is negligible on the decline of the Apollo (Nakonieczny et al., 2007).

Loss of habitat due to human interference is one of the most pressing challenges for the Apollo. Forest management and intensive farming have led to severe shrinkage of its habitat and consequently its numbers. To negate deforestation as a consequence of the industrial revolution many countries started afforestation with the intent to replenish and help the local ecosystems. With the introduction of foreign tree species (spruce and pine) that increased soil acidity, before-present plant species were unable to thrive and create an environment suitable for the Apollo. The afforestation played a major role in fragmentation and isolation of Apollo habitats. Open mines, settlements, roads and quarries effect and any other profound land transformations have a negative impact on the species. Moreover, pollution plays a crucial role in the rapid decline of the Apollo (Nakonieczny et al., 2007).

3.4. Protection of the Apollo

To conserve the Apollo and its habitat many actions have taken place. It was the first invertebrate to be included in Appendix II of Convention on International Trade in Endangered Species (CITES) (Collins & Morris, 1985). It is also included in the Bern Convention and European Union (EU) Habitats Directive and EU regulation of trade of fauna and flora (European Environment Agency, n. d.). In many countries active efforts have been taken to conserve their habitat with shrub and tree removal in areas where larvae or imagos were present. Many countries monitor populations, and some have reintroduced the species in parts where it was extinct. Inside the EU the butterfly and its habitat is protected under the Natura 2000 project. This is of big importance in Slovenia, as there are yearly transect monitorings. Preserving the Apollo's habitat is of great importance as it also protects the complex plant and animal communities living in such habitats and serves as an "umbrella species" for the whole ecosystem (Nakonieczny et al., 2007).

3.5. Apollo in Slovenia

Sadly, the numbers of imagos have been on a sharp decline as in line with the rest of Europe. In Slovenia we face significantly bigger problems as our base population is much smaller than in other countries due to lack of habitat. The numbers of adult Apollos counted in transects has dwindled by more than 90% in the last 8 years (Zakšek et al., 2023). Currently there are no active programs in Slovenia dedicated to protecting and conserving the Apollos habitat as it isn't prioritised as much as other wetland butterflies.

4. Genus *Phengaris*

4.1. Description

Within the Lycaneidae family, the genus *Phengaris* stands as one of the most extensively researched and studied due to its unique and fascinating life cycle (Thomas et al., 1989; Nowicki et al., 2007). Four European species are currently recognised: *Phengaris alcon* (Denis and Schiffermüller, 1775); *Phengaris arion* (Linnaeus, 1758); *Phengaris teleius* (Bergsträsser, 1779) and *Phengaris nausithous* (Bergsträsser, 1779) (**Figure 2**) (Wiemers et al., 2018). All four species are also present in Slovenia (Verovnik et al., 2012). The genus is thought to have evolved in the steppes of central Asia (Als et al., 2002;

Sibatani et al., 1994), so that the European *Phengaris* species were pre-adapted to survive and spread in traditional European agricultural landscapes. Their existence in Western Europe is currently threatened with extinction, likely attributable to recent alterations in land use practices (Als et al., 2002; Kljun et al., 2016). In Europe, *Phengaris* butterflies occur in 37 countries with northernmost populations in Finland while southernmost populations reside nearby in the Asian part of Turkey (Oliveira et al., 2013). Their status is deemed to be stable in only seven countries (Oliveira et al., 2013; Wynhoff, 1998).

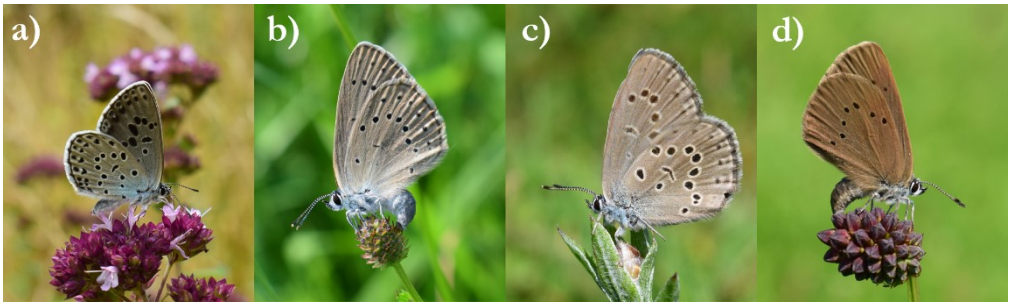

Figure 2. **a**) *Phengaris arion* on its host plant *Origanum vulgare*. **b**) *Phengaris teleius* on its host plant *Sanguisorba officinalis*. **c**) *Phengaris alcon.* **d**) *Phengaris nausithous* on its host plant *Sanguisorba officinalis.* Photo: Luka Šturm (with permission)

4.2. Habitat and ecology

Phengaris species live across various vegetation types, typically found in relatively poor soil. As a result, *P. teleius* and *P. nausithous* occur in wet grasslands, *P. arion* occupies dry grasslands, and *P. alcon* inhabits moist heaths and bogs. Within these habitats, mentioned species predominantly reside in isolated populations with minimal migration or dispersal tendencies (Oliveira et al., 2013).

The larvae of all species exhibit a high level of specialisation by briefly feeding on a specific plant. The host plants differ between species: *P. teleius* and *P. nausithous* use exclusively *Sanguisorba officinalis* (Kőrösi et al., 2012; Oliveira et al., 2013), *P. arion* uses *Thymus* spp. and *Origanum vulgare* and *P. alcon*, uses *Gentiana pneumonanthe* as its main host plant (Oliveira et al., 2013).

After the phytophagous stage is over they spend 11–23 months underground, acting as social parasites within colonies of *Myrmica* ants (Thomas & Schönrogge, 2019). Various species of *Phengaris* engage in parasitic relationships with usually one or two different ant species, depending on the region and habitat they occur in (Oliveira et al., 2013). With such complex and specialised life cycle big challenges must be overcome for success in complete development. The first is the selection of the right host plant, preferably in the vicinity of host ants (Dyck et al., 2000; Oliveira et al., 2013). The second challenge arises after the larva reaches 4th instar, when it drops to the ground and awaits the adoption by specific host ants. Foraging worker ants of a particular species mistake the *Phengaris* larvae for their own brood, due to chemical mimicry, and carry them to their nests (Oliveira et al., 2013). It is key that the ants do not recognise them as impostors. When these butterflies lay their eggs in ant nests that are not of the suitable host species, the ants quickly identify the hatched caterpillars as impostors, as they are unable to mimic the necessary cues or signals to deceive the ants. In such instances they do not manage to leave the nest alive (Thomas et al., 1989).

4.3. Decline of the genus Phengaris

Because of the complex and specialised foremost mentioned lifecycle the butterflies of the genus are highly susceptible to environmental changes and have suffered severe declines in Europe. For example in the Netherlands four of the *Phengaris* species occurred at the beginning of the century, but in the seventies all but one became extinct. *P. arion* went extinct from the United Kingdom (UK) in 1979 (Wynhoff, 1998; Oliveira et al., 2013).

One of the main factors contributing to the rapid decline of the Large blue butterflies is the direct habitat loss caused by human activities. In a recent study spanning roughly two decades a nearly 15% decrease in total habitat area has been detected (28.8 ha of the original area exceeding 200 ha was lost). Two thirds were lost through conversion to built-up areas complemented by abandonment that resulted in vegetation overgrowth and the disappearance of host plants (Kajzer-Bonk & Nowicki, 2023). The study was conducted near Krakow in Poland monitoring populations of *P. teleius* and *P. nausithous* and their habitat. The estimated loss of butterflies in vanished patches was considerable in both investigated species, oscillating around several hundred to thousands of adult individuals per year (Kajzer-Bonk & Nowicki, 2023).

The problem of abandonment of patches by farmers has also become apparent. It results in increased vegetation height providing cover and a subsequent loss of microclimates suitable for ants, whose nests support the Large Blue butterflies (Oliveira et al., 2013). Overgrowing may also cause the reduction in available host plants, also resulting in diminishing butterfly populations. An often problem when talking about overgrowth are invasive plant species which frequently colonise new environments as a result of human activities. One of the most notable ones are goldenrods (*Solidago* spp.), which In Central Europe, have become dominant in up to 90% of former grasslands, leading to the creation of homogenous habitat (Kalarus, 2023). The sizes of local populations of the *P. teleius* and *P. nausithous* butterflies were both lower in patches with higher goldenrod cover (Kalarus, 2023).

Another issue affecting large blue butterflies is the improper meadow management, particularly mowing at inappropriate times. If the latter occurs during the flowering of host plants and flight period of the adults (July-September) it prevents imagos from accessing nectar sources. More importantly it prevents females from accessing suitable locations for egg-laying if host plants are not there (Oliveira et al., 2013).

4.4. Protection of the genus Phengaris

Many actions have taken place to conserve the genus of the Large blue butterflies. P. *arion*, *P. teleius* and *P. nausithous* have all been included in European Union Habitats Directive App. II and IV and IUCN Red List of European Butterflies (van Swaay et al., 2010). All *Phengaris* butterflies are also included in the red lists of most European countries (Czekes et al., 2014; Kljun et al., 2016). Various reintroduction programs have taken place to assist with the declining *Phengaris* populations. These notably include the reintroduction of the *P. arion* in the UK in 1986 and reintroduction of *P. teleius* and *P. nausithous*, in the Netherlands in 1990. Both of these were classified as successful, as the populations have developed well and increased in numbers (Oliveira et al., 2013). A key consideration limiting the success of reintroductions is low habitat quality. Ensuring the long-term survival of these species critically hinges upon maintaining habitats that offer essential resources as mentioned earlier: flowering host plants available from July to September and the presence of suitable host ants (Kalarus, 2023; Nowicki et al., 2007).

4.5. Genus Phengaris in Slovenia

As previously mentioned, all four European species are found in Slovenia. Among them, *P. arion*, *P. nausithous*, and *P. teleius* have been under monitoring schemes as a part of Natura 2000 for over a decade. The outcomes of these monitoring programs consistently indicate an unfavourable population status for these species (Zakšek et al., 2023). While monitoring of *P. alcon* is not conducted annually, it holds an 'endangered' (EN) status and stands as one of the most rapidly declining butterfly species in Slovenia, as indicated by Verovnik et al. in 2012. It has vanished from significant portions of its range, potentially facing extinction in the Koroška region. Only a few scattered populations remain in the Štajerska, Gorenjska, and Bela krajina regions (Verovnik et al., 2012; Kljun et al., 2016).

Main factors that contribute to the endangerment of the genus in Slovenia are overgrowth of dry grasslands caused by their abandonment, excessive grazing, and too frequent mowing. Inappropriate timing of mowing, alongside with habitat fragmentation and

degradation due to intensification also pose serious threats to Slovenian populations (Verovnik et al., 2012; Zakšek et al., 2023).

5. Conservation

Butterfly conservation is carried out at several levels, from the programs founded by the EU to an individual's garden. The more that we realise the importance of biodiversity, the more we have to do to conserve it. Sadly, much of the damage we have done is practically irreversible, such as global warming, which the United Nations are trying to keep to a max + 1.5 degrees Kelvin (United Nations, n. d.). One of the core issues that many grassland butterflies face is too many grass cuttings through the summers. Studies have shown that delayed mowing and delayed first grazing has outstandingly positive effects for the invertebrate, plant and bird species studied (Dicks et al., 2020). For that reason, inside the European Union there have been stimuluses to defer the farmers from mowing too often and restrict overgrazing to protect Natura 2000 species (European Commissions, 2018). Removal and restriction of invasive species is also crucial as they can completely displace authentic species and prevent the growth of host plants (Kalarus, 2023). Of course, loss of habitat due to human interactions is unavoidable, but with careful road-planning and protection of endangered habitats it is possible to leave a smaller impact. With the reintroduction of controlled cattle grazing, it is possible to restore mountain pastures and provide much needed habitat for butterflies to thrive in (Nakonieczny et al., 2007).

Most of the aforementioned points require government-sided programs, but there are still many things an individual can do. One of the more vital steps is spreading awareness, as many people are not informed about the loss of biodiversity and insect biomass that is currently happening. Programs like the Big Butterfly Count, which is a UK-wide environment assessment survey, are bringing awareness of wildlife and butterfly protection to the public (Big Butterfly Count, n. d.). Moreover, many gardens can be transformed into adequate habitats for butterflies with the addition of nectar-rich and host plants for butterflies to develop (Teasdale, n. d.).

6. Conclusion

Among all the species on the decline it is important to remember that many others are as important as these two species. Species like Cranberry blue (*Plebejus optilete*) face similar issues as the Apollo, as it is also a glacial epoch and is facing serious decline in Slovenia. We mustn't forget about other wetland butterfly species, which are currently disappearing the most. One of the most indicative of them is False ringlet (*Coenonympha oedippus*), for whom we have special programs for restoration in Slovenia (Čelik, 2021). There are many generalists whose populations are also on a decline, but many of them are so prevalent that there isn't too much concern about their downfall yet, however we believe it is crucial to act accordingly and conserve their diverse habitats before it is too late. It is important to note that with the protection of many butterfly species that are so-called "umbrella-species" we protect many other species of animals and plants that are heavily specialised to that region.

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Invited lecture/Review

Uncovering algae biomass potentials: from wastewater to biostimulants

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Abstract:
The need to minimize human impact on the environment encourages alternative methods and solutions which could provide new steps towards green transition. Population is growing and climate is changing which is causing distress in food production and to recycle resources is getting ever more important. It is already known that municipal wastewater is a nutrient-rich medium, furthermore there is great potential to implement new technologies for nutrient recovery. We must provide sustainable wastewater treatment and at the same time we must try to maximize utilization of all available resources found in wastewater. Wastewater is suitable for growing algal biomass which can later be used as biostimulants in crop production which is in line with the goals of circular economy. This article provides a short overview of several studies on algal wastewater treatment and types of biostimulants produced from algae biomass. Further research on algal biostimulants production using municipal wastewater need to be focused to pilot experiments and real wastewater since the majority of reviewed studies are done on the lab scale with synthetic wastewater.

Keywords: Nature-based solutions; High-rate algae pond; Agriculture; Reuse; Phytohormones.

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1. Introduction

Growing population is causing global warming, extreme weather events and environmental changes, therefore striving to the green transition is essential, including environmentally friendly technologies, nature-based solutions and reuse of resources (Kapoore et al., 2021). Because resources are scarce, sustainable solutions to improve agricultural technologies and effective strategies for plant production are needed for providing the outcome without the negative consequences within production. Pressure on global food systems will be increasing also due to pollution caused by agriculture which is critical and causing ecosystem problems. Therefore, focus should be on finding new technologies and solutions for problems in agriculture that we are and will be facing (Calabi-Floody et al., 2018; La Bella et al., 2021; Shahzad et al., 2021).

In recent decades, algal biomass is gaining attention as it is versatile and can be used in a variety of industries, including wastewater treatment (Razaviarani et al., 2023). The use of algae in wastewater treatment was pioneered less than a century ago and have since been established worldwide (Oswald et al., 1957; Ho & Goethals, 2020). Process is mainly based on the biodegradation of organic matter and pollutants, driven by bacterial consortia and the implementation of microalgae as part of wastewater treatment depends on their ability to consume the organic or inorganic carbon, inorganic nitrogen and phosphorus in the wastewater for their growth, reducing concentration of certain substances in the water (Mohsenpour et al., 2021).

Microalgae (**Figure 1**) have shown great potential to grow in various types of wastewaters (Mohsenpour et al., 2021), moreover they have shown a high potential to remove contaminants from industrial waste or municipal sewage (Abdelfattah et al., 2023). High-rate algae ponds (HRAPs) contribute to the resource recovery, represent an alternative to conventional wastewater treatments and have great potential for the municipal wastewater treatment in locations with enough exposure to the solar radiation (Mehrabadi et al., 2017; Škufca et al., 2021). According to recent study by Kohlheb et al. (2020), HRAP is beneficial both environmentally and economically, contributing especially to CO_2 sequestration and reduction of eutrophication. There is also the potential of wastewater treatment using HRAP for production of low-cost biofuel (Mehrabadi et al., 2017). However, the performance of algal wastewater treatment is a remaining challenge due to variations in parameters like algal culture, wastewater composition, photobioreactor types, temperature and light intensity. Additionally, lack of an efficient method of harvesting microalgae from the culture medium has been a well-known problem (Zenouzi et al., 2013; Chawla et al., 2020).

Figure 1. *Scenedesmus* sp. under a microscope (400x).

It is known that phytohormones, obtained from the diverse algae and cyanobacteria, may be applied commercially in agricultural land to enhance the crop productivity (Singh et al., 2017). Phytohormones are one of the biostimulants that can be produced by algae (Kapoore et al., 2021). Biostimulants can enhance the growth and quality of crops, improve mineral nutrient uptake and increase plant tolerance to abiotic stresses (Navarro-Lopez et al., 2020; Gonzalez-Perez et al., 2022). It is also important, that they could be applied in small quantities (Sharma et al., 2014). It is known that both, micro- and macroalgae, have biostimulant potential to support plant development and growth along with improved tolerance to abiotic stresses, but microalgae cultivation and harvesting is more difficult (Michalak et al., 2017) and have been explored less in the case of agricultural applications (Craigie, 2011). Because biostimulants have similar regulatory roles in microalgae as in higher plants (Lu & Xu, 2015), the application of microalgal specific components as biostimulants are gaining wide attention (Kapoore et al. 2021). Biostimulants represent sustainable alternative to synthetic plant protection products, are environmentally friendly, safe, and respect the human health, not generating chemical residues (Povero et al., 2016; Du Jardin et al., 2015). Therefore, they play a key role in sustainable intensification through efficiency of nutrients uptake from plants, enhancing productivity, yield and health of crops (Gonzalez-Perez et al., 2022). However, a major challenge remains in unravelling the mode of biostimulant action of specific bioactives due to variability of algal and crop species and their interactions, which depend on abiotic factors (Bulgari et al., 2019; Kapoore et al., 2021).

In this paper, we analysed the diversity of biostimulants from microalgal biomass that are relevant to agriculture and production technologies.

2. Methods

The literature review was carried out in the November 2023 using the Google Scholar (GS), Scopus and Web of Science (WOS) databases. Research through GS using search terms ''microalgae, biostimulants, agriculture, wastewater'' provided with more than 600 review articles, therefore more combinations of terms were used to narrow down the results. We searched through Scopus and WOS using keywords microalga* AND wastewater* AND biostimulant*, resulting 44 in Scopus and 32 searches in WOS. Without ''*'', there were only 26 results in both databases. We added keyword ''agriculture'', resulting in 15 hits in WOS (and 14 in Scopus). Search results were divided in two groups – review articles and primary research articles and thoroughly reviewed to determine if their content is relevant to the topic of algal biomass from wastewater and biostimulants.

3. Results and discussion

3.1. Biostimulatory compounds from algae biomass

Various bioactive components, including phytohormones, betaines, oligosaccharides elicitors, and microalgal protein hydrolysates, can be recovered from microalgae for production of plants. They could also contain amino acids, humic acids, fulvic acids, polysaccharides, antioxidants, vitamins, enzymes and others (Oancea et al., 2013; Kapoore et al., 2021). Some major groups of bioactive components are presented in **Table 1**. For example, in *Chlamydomonas* sp. and *Chlorella* sp. phytohormones like Gibberellic acid (GA) and ethylene (ET) play roles in growth, senescence, and other biological activities, therefore suggesting their potential commercial application in agriculture (Yordanova et al., 2010; Park et al., 2013; Tate et al., 2013).

Table 1. Bioactive molecules with stimulatory effects, found in algae.

Biostimulants and their effects		**Examples**	**Growth media**	**References**
Auxins*	Initiate cell elongation and root formation, provide stress tolerance (drough, salinity, heat) (Kapoore et al., 2021).	1. *Chlorococcum* sp., *Micractinium pusillum*, *Scenedesmus* sp., *Chlorella* sp. 2. *Chlorella vulgaris*	1. Tris-Acetate- Phosphate (TAP) medium. 2. Bristol Liquid Media.	1.Rupawalla et al., 2022. 2. Stirk et al., 2013.
Cytokinins*	Control cell division, fruit and flower development, stress tolerance (drought and heat…) (Kapoore et al., 2021).	1. *Chlorococcum* sp., *Micractinium pusillum*, *Scenedesmus* sp., *Chlorella* sp. 2. *Chlorella vulgaris*	1. Tris-Acetate- Phosphate (TAP) medium. 2. Bristol Liquid Media.	1.Rupawalla et al., 2022. 2. Stirk et al., 2013.
Gibberellins*	Initiate stem elongation, seed germination, flowering (Kapoore et al., 2021).	1. *Chlorococcum* sp., *Micractinium pusillum*, *Scenedesmus* sp., *Chlorella* sp.	1. Tris-Acetate- Phosphate (TAP) medium.	1.Rupawalla et al., 2022.
Abscisic acid*	Associated with stomatal closure, shoots growth inhibition, seed dormancy (Kapoore et al. 2021).	1. *Chlorococcum* sp., *Micractinium pusillum*, *Scenedesmus* sp., *Chlorella* sp.	1. Tris-Acetate- Phosphate (TAP) medium.	1. Rupawalla et al., 2022.
Proteins and amino acids	Major category for example: regulating nutrient uptake and enzymes, improve plant growth, metabolic signaling (Kapoore et al., 2021).	3. *Chlorella vulgaris* 4. *Spirulina* sp.	3. Sea or fresh water. 4. Fresh water.	3 and 4. Christaki et al., 2011.
Polysaccharides	Plant metabolic pathways, overall crop improvements, biotic and abiotic stresses protection (Rachidi et al., 2020; Kapoore et al., 2021).	5. 16 different species of mariculture microalgae	5. Medium t'2, medium fE containing EDTA or medium G2.	5. Brown, 1991.
Antioxidants, pigments, micronutrients	Various compounds, including some vitamins, chlorophylls and phenolics, often secondary metabolites (Kapoore et al., 2021).	6. 57 different strains (phylum *Chlorophyta* and *Cyanophyta*). 7. Two microalgal consortia made up of different strains (phylum *Chlorophyta* and *Cyanophyta*).	6. Coastal waters of western India. 7. Modified Bold's and Basal Medium, sewage wastewater.	6. Paliwal C et al., 2016. 7. Renuka N et al., 2017.
Auxin- and cytokinin like activity	Promote plant growth, improvements in germination and root formation (Navarro-Lopez et al., 2023; Renuka et al., 2018).	8. *Chlorella* sp., *Scenedesmus quadricauda*, *Coenochloris* sp., *Chlorosarcina* sp., *Tetracystis* sp., *Chlamydomonas* spp. 9. *Scenedesmus* sp. 10. *Scenedesmus obliquus*	8. Tamiya. 9. Synthetic treated wastewater. 10. Brewery wastewater.	8. Stirk WA et al., 2002. 9. Navarro-Lopez E et al., 2023. 10. Navarro-Lopez E, et al., 2020.

*phytohormones

3.2. Wastewater as medium for biostimulants production

High production costs of bioactives from algal biomass is a major obstacle for wider application, and wastewater as a nutrient-rich environment represents low-cost possibility for reuse of resources and fits circular economy definition (Kapoore et al., 2021). Currently, despite a high interest in developing natural biostimulants from microalgae, there is a lack of commercially available products, therefore it is crucial to identifiy strains with capacity to grow in wastewater and are highly productive (Morillas-Espana et al., 2022). Most of the studies are laboratory-based and should be taken on a pilot scale. Microalgae, such as *Scenedesmus obliquus*, grown in brewery wastewater, have the potential to act as plant biostimulants, contributing to sustainable and healthy food production in addition to wastewater treatment (Navarro-Lopez et al., 2020). *Scenedesmus* sp. was recently grown in simulated treated wastewater to obtain biostimulants, where cytokinin and auxin-like activity were found, improvements in germination and root formation appeared (Navarro-Lopez et al., 2023). Furthermore, Alvarez-Gonzalez et al. (2023), proposed wastewater as a suitable medium for growing microalgae strains *Synechocystis*, *Phormidium* and *Scenedesmus*, which can produce biostimulants in form of phytohormones. Cultivating microalgae in urban wastewater is offering new possibilities to identify and quantify the production of biostimulants, along with decreasing production costs of these high-value compounds (Alvarez-Gonzalez et al., 2023). Overall applications of algae biostimulants represent a promising approach in agriculture to reduce or substitute use of harmful chemicals when maintaining plant productivity (Ferreira et al., 2021; Dagnaisser LS et al., 2022).

Although studies have shown that algae are efficient in wastewater treatment, the compassion of effect of algal culture is still difficult due to several practical and economic challenges (Chawla et al., 2020; Mohsenpour SF et al., 2021). According to Kapoore et al., 2021, the major problem is risk of contamination with heavy metals, chemicals, self-care products and pharmaceuticals. There is a major hole of knowledge about pathogens and processes associated with transferring genes (Li et al., 2022). There are also difficulties with maintaining monoculture due to species or bacterial contamination and variety of seasonal and nutrient levels that are changing growth profiles. All these factors can highly affect composition of the end product of algae biomass (Kapoore et al., 2021).

4. Conclusions

Microalgae contribute to sustainability in crop production and wastewater treatment, stating the importance of integrating new technologies into environmental and agricultural practices. Production of biostimulants with algae-based technologies have big potential to achieve sustainable agriculture. We believe that it is possible to define methods to produce abundance of microbial biostimulants by growing and harvesting algae in municipal wastewater under controlled environmental conditions. The right balance between temperature, nutrient levels, water quality and other variables impacting successful microalgae growth and production of bioactive will be therefore the focus of our future research.

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

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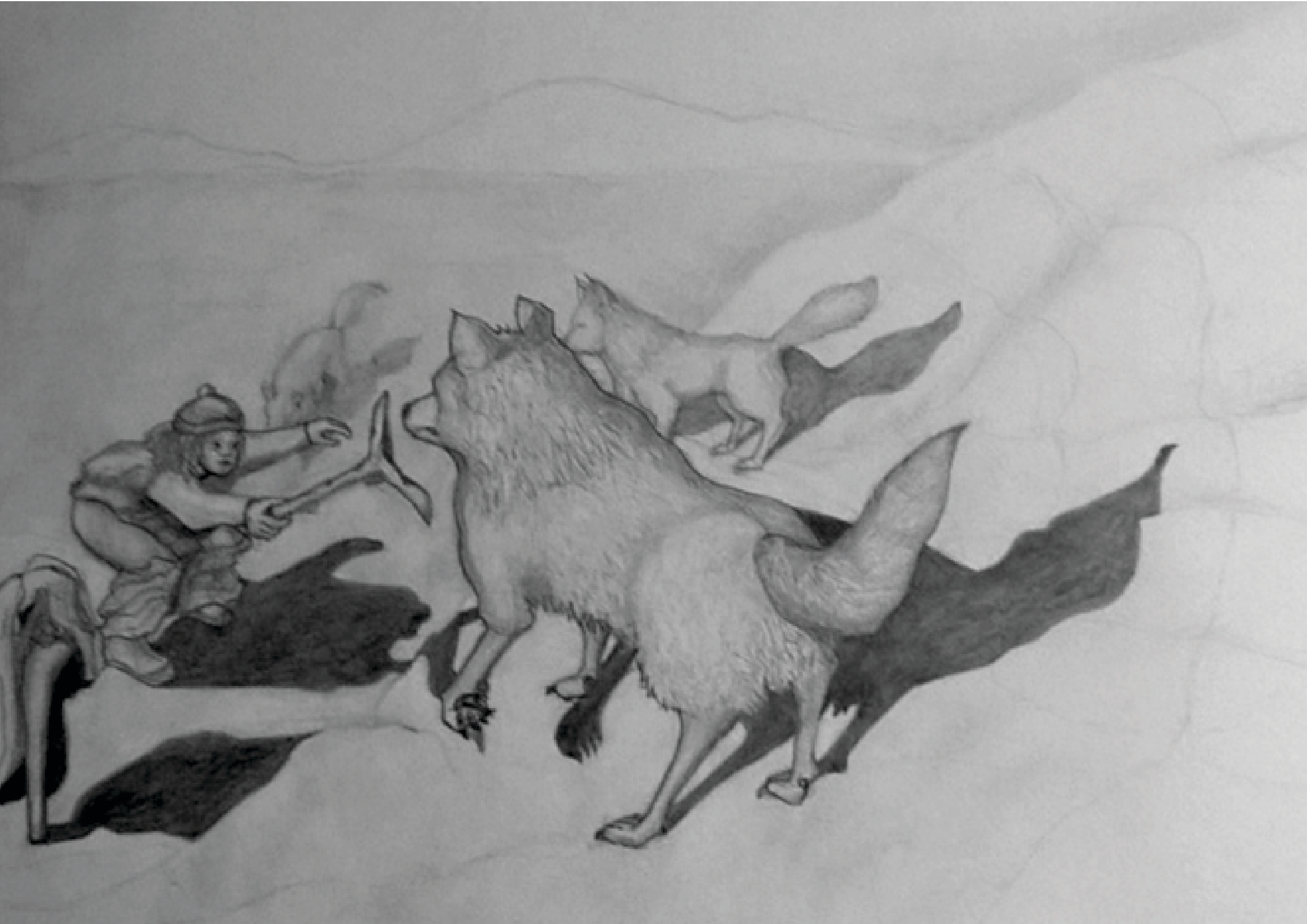

Invited lecture/Review

Green Transition in Slovenian Building and Civil Engineering Industry: 10 Years of Research on Alkali-Activated Materials and Alkali-Activated Foams

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Abstract:
The building and civil engineering industry yearly causes more than 40% of man-made CO_2 and consumes raw materials for two-thirds of Mont Everest. To decrease the carbon footprint and consumption of raw materials, alkali-activated materials (AAMs) are researched as an alternative to conventional building and civil engineering products like cements, mortar, and ceramics. Ideally, locally available waste materials are used as ingredients: (i) as precursors that react with alkali and form an aluminosilicate network, and (ii) as fillers that get permanently encapsulated and safely stored in AAMs. The addition of gas bubbles and lightweight fillers transforms AAMs into alkali-activated foams and alkali-activated lightweight materials that have the potential to be used as thermal and acoustic insulation materials. Although AAMs are researched worldwide, this review focuses on the state-of-the-art localised solely to Slovenia, particularly on the materials and curing procedures used, as well as on the scientific contribution of the basic research. Besides, the year 2024 marks 10 years of research on alkali activation of raw and waste inorganic materials in Slovenia.

Keywords: Secondary raw materials; Alkali-activated materials; Alkali-activated foams; Low-temperature curing; Microwave curing; Circular economy

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1. Introduction

The amount of waste is increasing worldwide with a steep slope (by 2050 increase of solid waste is predicted to be 70%, i.e. $3.4 \cdot 10^9$ t) because of the increased consumption and small recycling rate (less than 20%) (Alves, 2023). At the same time, the non-renewable natural resources are being depleted due to constant (large-scale) excavation and outtake from nature: with the current consumption rate, coal will be depleted in 175 years, and oil and natural gas in 45 years ("Natural Resources Depletion," 2022).

Globally, mankind excavates more than $500 \cdot 10^9$ t of raw materials every year. This amount can be estimated as two-thirds of the mass of Mount Everest. Half of all excavated natural resources are consumed by the building and civil engineering industry, which causes 40% of global CO_2 emissions (Miller, 2021). However, the need for building and civil engineering will be always present, therefore, the challenges have to be recognised and solved.

Among building and civil engineering products available on the market, conventional cement represents a challenge that has to be addressed among the first. Namely, besides that for the production of cement available on the market raw materials are needed, these raw materials are treated at temperatures up to 1450 °C (Shepherd and Rankin, 1911). Such high energy has an additional negative environmental impact connected to cement. Its use in civil and building engineering is wide because it is used also as an ingredient in other products, like mortar and concrete, making the "cement" issue even more prominent.

Although raw materials are already partially replaced in cement by (fly) ash, municipal wastes, and (ground granulated blast furnace) slag (Nwankwo et al., 2020; Rashad, 2018), the replacement is just partial. Therefore, material, that can be made solely from waste material, and at temperatures below 100 °C, and still have at least similar properties as cement, will sooner or later replace nowadays cement. One of the qualifying options is alkali-activated materials (AAMs) (Roy, 1999) which are being researched worldwide regarding the availability of local secondary materials (waste materials) and are showing promising results regarding mechanical properties (Ding et al., 2016) and durability (Wang et al., 2020).

AAMs can be made either from natural resources or waste materials that contain a sufficient amount of silicon (Si) and aluminium (Al) in amorphous content. Si and Al that are present in precursor dissolve in liquid alkali (usually hydroxides and alkali silicates) and rearrange into an aluminosilicate network (ASN). The rate of dissolution depends on many factors, however, the most important is the size of the particles in the precursor, as is presented in **Figure 1**.

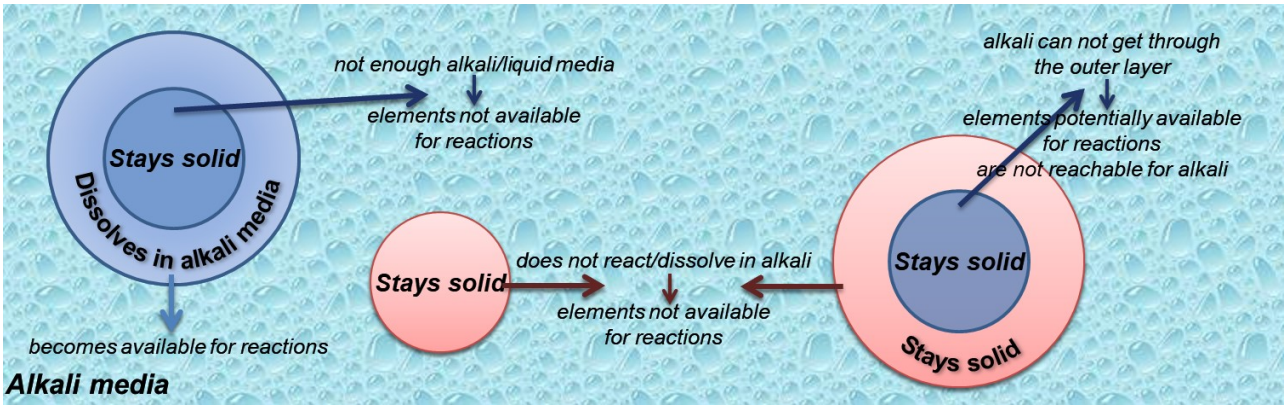

Figure 1. The dissolution potential of powder material containing amorphous Si and Al in alkali liquid. Red does not dissolve in alkali, lighter blue can get dissolved in alkali, darker blue cannot (blue circle on the left: not enough alkali to reach the core blue circle in red on the right: alkali cannot reach blue through the red).

If the alkali cannot reach the part that is prone to dissolve or if there is not enough alkali available, not everything will have the possibility to dissolve. If the addition of alkali is

limited by avoiding the efflorescence, the molar ratio between alkali metal and amorphous Al can be equal to or lower than 1 (Horvat & Ducman, 2019). The reason for aiming the molar ratio Al and alkali metals to be equal to 1 is that alkali metals compensate the extra bond of Al with O in the tetrahedron that is connected through oxygen bridges with other tetrahedra that have in the centre either Si or Al (Škvára, 2007). These tetrahedra are building blocks in a chain, so they represent an inorganic polymer system. If the precursor is metakaolin (calcined clay), this inorganic polymer can be called geopolymer (Davidovits, 1991, 1989), otherwise it should be addressed as AAM.

AAMs can encapsulate many different materials: hazardous and radioactive waste (Shi and Fernández-Jiménez, 2006), organic and inorganic, fibrous and "spherical" (Elijah, 2015), materials that enhance the desired properties regarding the intended purpose of the product and materials that just have to be safely stored. However, due to European legislation, AAMs are not utilised, especially as large-scale building and civil engineering products. On the other hand, in Australia, which has different legislation than Europe, there are already several big-scale projects finished and in operation: (i) aircraft pavements at Brisbane West Wellcamp Airport, Toowoomba (in operation since November 2014; main ingredient was slag) (Glasby et al., 2015), (ii) a 4-story public building, University of Queensland's Global Change Institute, Queensland ("World's first public building with structural Geopolymer Concrete – Geopolymer Institute," 2013), where fly ash and slag were used.

Slovenian researcher, dr. V. Ducman, brought AAMs to Slovenia and designed a mixture from fly ash that was used as a sculpture material by J. Kočica. This sculpture has been permanently placed at the Slovenian National Chemical Institute since 2012/2013 and marks the beginning of art from AAMs in Slovenia. However, the official beginning of scientific research on AAMs was the year 2014, with the Slovenian national project "Mechanisms for the strengthening of different types of ash by means of a geopolymerization process" led by Dr V. Ducman. With this review, we are celebrating 10 years of research on AAMs in Slovenia. The selection of the bibliography was performed using the database SICRIS, however, only studies (or their parts) fully performed in Slovenia, relevant original research and online-available conference papers were taken into account. The works are presented time-wise from older to the newest published to show the progress in the field.

2. Review of 10 years of basic research on alkali-activated materials in Slovenia

After testing the viability of AAMs in larger sculptures, the first research was performed using local low-calcium fly ash as a precursor, and sodium hydroxide (NaOH) and liquid sodium silicate (Na-silicate) as alkalis, cured at room conditions (Kramar & Ducman, 2015). The amounts of alkali and water were varied in the study. Compressive strength showed dependence on water content, i.e., the lower the amount of water, the higher the compressive strength and the fuller the ASN. The highest compressive strength reached in this study was ~50 MPa (Kramar & Ducman, 2015).

With the addition of foaming agents, i.e., solid pulverized Al and (liquid) hydrogen peroxide (H_2O_2), fly ash that was activated with NaOH and Na-silicate, and cured for 24 h at 70 °C before being kept at room conditions, foamed (Ducman & Korat, 2016). The highest addition of Al was 0.2 mass percentage (m%), and H_2O_2 was 2 m%. Total porosity was up to ~60%, which resulted in low mechanical strengths (less than 5 MPa for samples with a density lower than 1 kg/l). However, using H_2O_2 resulted in smaller pores (Ducman & Korat, 2016).

Foaming of fly ash with H_2O_2 (up to 1.5 m%) during alkali activation (using NaOH and Na-silicate) was controlled by stabilizing agent sodium dodecyl sulphate (SDS; up to 4 m%). The mixture was cured for 24 at 70 °C, after that they were kept at room conditions. The addition of a stabilising agent decreased the compressive strength and density because it was successful at stabilizing-separating bubbles in the ASN. At lower additions of H_2O_2, the limit value of density and compressive strength was reached at 4 times higher addition of SDS (Korat & Ducman, 2017).

Upcycling with alkali activation technology was studied on waste mineral wools, i.e., rock and glass wool, both as construction and demolition wastes, delivered to the landfill. Both

types of wool should have been completely amorphous and differ only in chemical compositions, but as they were waste materials, they both had 5 to 10 m% of minerals. Alkali used in the study was 10 M NaOH mixed with Na-silicate solution in a mass ratio of 1 to 1. Dry precursor, milled and sieved below 90 μm, was added to the alkali mixture until viscosity was not measurable anymore. The mass ratio precursor to 10 M NaOH to Na-silicate solution was for glass wool 1.5:1:1 and rock wool 2:1:1, respectively. However, the amount of used alkali exceeded the amount of needed alkali, i.e. efflorescence took place (thermonatrite formed). Alkali-activated glass wool had ~3 times lower shrinkage, ~4 times higher bending strength and ~2 times higher compressive strength (Horvat et al., 2018).

Although waste foundry sand is not a promising material for alkali activation, its usefulness for the reaction has been evaluated by the addition of KOH/NaOH and K/Na-silicate solution in different ratios, as well as the addition of extra water and molarities of KOH/NaOH. The baseline ratio of amorphous Si to Na normalised to amorphous Al was set to be 1.9 to 1 to gain the highest compressive strength and avoid efflorescence according to the literature (Duxson et al., 2005). Curing of alkali-activated local waste foundry sand was performed at room conditions and 70 °C for 24 h, then left to further dry at room conditions, in a drier at 110 °C for 24 h or with microwave irradiation (in a microwave oven with magnetron source working in cycles, 2.45 GHz, 700 W, 2.33 min). If curing was performed solely at room conditions, samples did not solidify. A smaller amount of liquid phase resulted in higher mechanical strengths, both for NaOH and KOH. Maximal strength was reached without the addition of hydroxides, i.e., compressive strength reached ~30 MPa, which is an impressive result for material with less than 40 m% of amorphous content, than 10 m% of amorphous Si, and 4 m% of amorphous Al. Activating precursor with Na-silicate solution instead of K-silicate solution had 2 times higher compressive strength. Volumetric drying (microwave irradiation) was, regarding the shrinkage, faster than drying at 110 °C, which was faster than drying at room conditions. However, faster drying causes bigger cracks and a decrease in bending strength but increases compressive strength (Horvat et al., 2019a).

The potential of different local foundry wastes was evaluated regarding their usefulness in alkali activation at room conditions. Different waste foundry sands, foundry flue gas, and waste casting cores were activated with a mixture of 10 M NaOH and Na-silicate solution (mixed in the mass ratio of 1 to 1). The addition of precursor to alkali mixture was determined experimentally, i.e. the chosen ratio was when viscosity was not measurable anymore. One of the foundry sands was mixed with “green waste ceramics”, and with “green waste ceramics and (bottom) ash”, to shorten the time of solidification from 1.5 years to less than a week (all solid ingredients were mixed in an equal mass ratio), i.e., time of solidification can be controlled by selection of a mixture of precursors when curing ovens must be avoided. Waste casting cores were once ground and sieved below 90 μm, and once gently ground and sieved below 600 μm. The first did not solidify in more than 2 years (remained rubber-like), and the latter fell apart when demoulded. Compressive strengths for all other samples were from 5 to ~10 MPa. Therefore, these materials can be used as reactive fillers (Horvat et al., 2019b).

Another potential precursor, which has not been widely researched up to date (Soriano et al., 2023), was local green ceramics waste that was activated using NaOH and Na-silicate, but in calculated ratios following molar ratios of Al, Si and the sum of the chemical elements of the first group of the periodic system, as also the mass of whole water (from all sources) content present in the mixture. Besides, mathematical formulas running behind calculations were published and software for the calculations and mixture design with the ability to choose the alkalis was developed. AAMs were also chemically foamed using powder Al, powder Na-perborate (monohydrate), liquid H_2O_2, and a stabilizing agent SDS. All were varied from 0 to 10 m%. Curing conditions were room temperature, 70 °C and 90 °C, for different amount of time. From the time development of mechanical strengths, it was concluded that the chemistry of the mixture is the most important parameter, i.e., molar ratios of amorphous Si to Na (normalised to amorphous Al) should be 1.9 to 1, respectively. Mixtures with this ratio gained a limit value of mechanical strengths fastest no matter the curing temperature. If the ratio was not optimal, the mixture might have reached high early compressive strength, which decreased by more than 50% of the

early value. In the study, the influence of the viscosity on the mechanical properties was reported: lower viscosity and higher temperature led to lower mechanical strength (which was reached faster), and higher viscosity and lower temperature led to higher mechanical strengths because curing was gentler, but to reach the limit values took more time. The density of foamed samples, where compressive strength was between 1 and 5 MPa, was lowest at 0.7 kg/l (Horvat & Ducman, 2019).

Different fractions of local electric arc furnace slag were alkali activated with K-silicate solution with the addition of water in mass ratios equal for all samples which were cured for 3 days at 70 °C. The highest compressive strength reached samples made from the smallest fraction (~60 MPa samples from slag with particle size below 63 μm, compared to ~20 MPa for samples from slag with fraction between 90 and 125 μm), which showed the highest reactivity and had the biggest surface area. Using this fraction resulted also in the smallest total porosity of alkali-activated slags (Traven et al., 2019).

The influence of curing/drying methods was further researched on alkali-activated waste casting cores that did not solidify in more than 2 years at room conditions no matter their particle size (Horvat et al., 2019b). Both granulations (below 600 μm and 90 μm) were activated with 10 M NaOH and Na-silicate solution, all mixed in a mass ratio (2.5/3.3/3.75):1:1, respectively, and cured for 24 h at 70 °C. This curing regime was not enough for any mixture or tested granulation. However, for larger granulation of precursor, additional curing/drying at 110 °C for 24 h or microwave treatment for less than 1 minute (with a microwave oven working at 700 W, with 2.45 GHz, and a magnetron source working in cycles) resulted in the compressive strength ~20 MPa. In the smaller fraction used, the highest compressive strength was achieved when the largest proportion of precursor was additionally treated at 110 °C for 24 h (~25 MPa), while microwave treatment for less than 1 min resulted in a compressive strength of 5 MPa. If the addition of precursor was lowered, so was compressive strength, and if microwave treatment was prolonged, the mixture foamed (density of 1.6 kg/l lowered to 0.6 kg/l, compressive strength to 2 MPa). Self-foaming was observed as a consequence of degradation of the organic compound while being irradiated with microwaves (Horvat and Ducman, 2020a).

Besides testing waste materials regarding their potential to be used as precursors and fillers, waste bottle glass and waste cathode-ray tube glass were studied focusing on their usefulness for the synthesis of alkali activators. Activators were produced using hydrothermal synthesis changing the synthesis time and temperature, as well as the particle size of the waste glass. The highest concentration of dissolved Si (19 g/l) and Al (0.9 g/l) was prepared at 120 °C treated for 24 h. However, using the best hydrothermally synthesised alkali for alkali activation of local fly ash resulted in a compressive strength of ~30 MPa, while when commercial Na-silicate solution was used, the compressive strength was ~70 MPa (König et al., 2020).

Self-foaming was further researched with the reaction between waste refractory materials and Na-silicate solution in the mass ratio of 5 to 3 for precursor, at 70 °C for 24 h. The precursor was only sieved below different fractions, where the smallest fraction was 1 mm. All fractions had more Al than Si in the amorphous content, which led to foamed material, but only in the smallest fraction. All bigger fractions had big enough voids among particles for the released gasses to accumulate, while in the smallest fraction, released gasses had accumulated in the ASN. The highest compressive strength had a fraction between 1 and 2 mm, 20 MPa. This fraction was tested further to evaluate the dependence of the temperature and time of curing: at room conditions, it did not cure, i.e., after 1 month the compressive strength was 2 MPa, which was reached in 1 week if the sample was cured at 40 °C for 1 week, but if the sample was cured at 70 °C for 1 week, compressive strength was 80% of the compressive strength when the sample was cured at the same temperature for only 1 day. However, after 1 month the compressive strength of the sample treated at 70 °C for a longer time reached the same value as it was treated for a shorter time (Horvat & Ducman, 2020b).

Local fly ashes, alkali activated with Na-silicate solution and NaOH, were foamed with Na-perborate and stabilised using SDS, cured at 70 °C for 24 h and exposed to 1000 °C. The lowest density obtained was ~0.3 kg/l, highest compressive strength was ~6 MPa. The best performance had fly ash that contained the highest amount of Si and Al, as well as the highest amount of amorphous content. Also when exposed to elevated temperature,

i.e., its density did not change, while compressive strength increased by ~50%. Fly ashes with a lower amount of Si and Al, and a smaller amount of amorphous content, showed higher shrinkage after exposure to 1000 °C, therefore the density increased, as the compressive strength (for ~800%) (Korat & Ducman, 2020).

A "cradle-to-cradle" life-cycle assessment was performed for the prefabricated alkali-activated façade cladding panels made from large fractions of recycled construction and demolition waste (wood, and as alternative waste inorganic materials like fired clay, mortar, and concrete) used as fillers. Precursors used were metakaolin, ground granulated blast furnace slag, and class F fly ash, while alkali activators were Na-silicate and K-silicate solutions; the final mixture was obtained by project partners (Panizza et al., 2018). The biggest environmental burden comes from the manufacturing process, which can be lowered by optimising the need for electricity requirements. However, during the use of the product, the energy needed for heating decreases by ~20 to ~40%. Comparison to façade cladding panels made from different virgin materials was calculated on base "cradle-to-gate". Results suggest that products made via alkali activation and incorporating waste fillers are environmentally friendlier solutions, particularly when the heating during the use stage is considered. Therefore, not only that alkali-activated materials are environmentally friendlier when compared to conventional materials like cement, mortar, concrete and ceramics, but so are the final construction products assessed in the study (Kvočka et al., 2020).

Alkali-activated thermally insulating façade panels with inorganic construction and demolition waste used as filler, which were evaluated regarding their life cycle performance, were analysed regarding their mechanical strength and durability tests: capillary water uptake, water vapour permeability, impact resistance, bond strength, freeze-thaw behaviour, freezing in the presence of de-icing salt, resistance to carbonation, alkali-silica reactivity, and sulphate resistance. Samples performed comparably to cement-based panels, except at the test evaluating resistance to freezing in the presence of de-icing salt, where alkali-activated panels ended up severely damaged (Frankovič et al., 2020).

Further development of alternative alkali activators from pulverised waste materials (rock and glass wool, bottle glass and cathode-ray tube glass) was performed at 120 °C at normal pressure (no hydrothermal synthesis) for 4 and 24 h. In 200 ml of 10 M NaOH, 5 and 20 g of pulverized glassy waste was constantly stirred. Synthesised alkali was tested with local thermopower plant fly ash, which was used as received, and electric arc furnace slag from the metallurgical industry, which was milled and sieved below 90 μm; the mass ratio precursor to alkali was kept constant. As a reference 10 M NaOH was used. In the case of fly ash, mechanical strengths did not show noticeable changes, while for slag ~70% increase of already low compressive strength happened when activators based on waste bottle glass and waste glass wool were used. Activators might be better than 10 M NaOH, but are not yet comparable to the commercial Na-silicate solutions (König et al., 2021).

Waste glass and rock wool, and their mixtures, were studied as sole precursors in alkali activation with 5 M NaOH, and with different ratios of NaOH dissolved in Na-silicate solution, cured at room conditions and for 3 days at 40 °C. Higher temperature allowed faster reaching higher mechanical strength values, rock wool solidified faster, but did not reach as high final compressive strength as glass wool. However, a mixture of 25 m% of rock wool and 75 m% of glass wool resulted as an optimal choice, i.e., samples gained faster the early mechanical strengths but also reached higher final values (Pavlin et al., 2021).

The development of deformation during the early stages of curing was studied on ladle slag and electric arc furnace slag, both activated with Na-silicate solution, in dependence on the temperature (room temperature, 40 and 60 °C) and humidity (30 and 90%). The focus was placed on autogenous and drying shrinkage in the first 72 h. The autogenous shrinkage had the highest rate at the highest tested temperature. The highest tested temperature and lowest humidity also gave the highest rate of drying shrinkage, i.e., the conditions were most favourable for water evaporation. The smallest deformations happened when the curing temperature was 40 °C for the first 24 h (Češnovar et al., 2021).

Alkali-activated foams, foamed with H_2O_2 and stabilized with SDS, based on fly ash were thermally improved by the replacement of fly ash with metakaolin and the replacement of Na-based with K-based alkalis (hydroxides and silicate solutions). The replacement of

fly ash with metakaolin delayed the start of the shrinkage from ~600 to ~700 °C, the replacement of Na-based with K-based activator from ~600 to ~800 °C, and the replacement of fly ash with metakaolin along with the replacement of Na-based with K-based activator from ~600 to ~900 °C (Traven et al., 2021).

Alkali-activated local fly ash and slag mixture (ladle slag and electric arc furnace slag, in mass ratios of 1 to 1), activated with Na-silicate solution and NaOH, foamed with Na-perborate and H_2O_2, and stabilised with triton, with/without added polypropylene fibres, embedded lightweight aggregates (expanded clay, perlite, polystyrene, expanded glass). While bending strength was below 1 MPa, and compressive strength was below 5 MPa, thermal conductivity was between ~100 and ~200 mW/(m·K). If lightweight aggregates had large open pores, alkali-activated foam expanded into them and mechanically fixated aggregates into the ASN. Otherwise, under the scanning electron microscope, a chemical reaction between alkali-activated slurry and aggregates was not observed (Traven et al., 2022b).

Alkalis based on glass wool, rock wool, bottle glass and cathode-ray tube glass were synthesized in KOH (5 and 20 g of glassy waste was treated in 200 ml of 10 M KOH for 4 and 24 h at 120 °C). Their usefulness in alkali activation was tested on electric arc furnace slag and compared to activation with commercial K-silicate solution and KOH, and compared to the study where alkalis were prepared in NaOH. Compressive strength results of alkali-activated slag were higher when alternative activators were made in KOH than in NaOH, and higher or comparable to reference activated with KOH and NaOH. However, compressive strength results were lower compared to slag activated with commercial Na-silicate and K-silicate solution 3 to 4-fold (Pavlin et al., 2022c).

The influence of (three-roll mill) homogenization of the freshly mixed alkali-activated slurry on mechanical strength was studied on different precursors: fly ash, fly ash with metakaolin (mixed in mass ratio of 3 to 1 for fly ash), glass wool, waste green ceramics, and slag mixture (ladle slag and electric arc furnace slag in mass ratios of 1 to 1). Precursors were activated by NaOH, Na-silicate solution, and laboratory-produced alkali based on waste cathode-ray tube glass, in recipes that were not comparable, so that the only parameter left would be homogenization and no homogenization. Therefore, curing regimes (60 and 70 °C) and drying methods (room temperature and 105 °C) also differed. Moreover, the slag mixture was foamed using Na-perborate and stabilized by triton, and the mixture of fly ash and metakaolin with Na-perborate and stabilized by SDS. Homogenization lowered the viscosity, therefore workability and moulding were easier, the distribution of particulates and pores was more uniform, and mechanical strengths were higher. In the case of fly ash, the non-combusted cellulose got combusted during homogenization (smoke was visible during the procedure and under the scanning electron microscope no cellulose was found after homogenization). If the onset of the foaming was delayed, homogenization could be performed for foamed materials without loss of gasses and increase of density, ending with smaller pores (Horvat et al., 2022).

To improve mechanical properties, different fibres (polypropylene, polyvinyl-alcohol, basalt, and waste glass wool) in several quantities (0 .5, 1.0, 1.25, 1.5, and 2.0 volumetric percentage) were added into alkali-activated foam based on a mixture of ladle slag and electric arc furnace slag (their mass ratio is 1 to 1). As an activator Na-silicate solution was used, as a foaming agent H_2O_2, and as a stabilising agent the surfactant triton. Compressive strength showed improvement in all samples while bending strength showed improvement only when polypropylene and polyvinyl-alcohol were added (Traven et al., 2022a).

Besides the addition of fibres into alkali-activated slag foams, 1 m% of different fibres (basalt, cellulose of 2 types, glass, polypropylene, polyvinyl alcohol and steel fibres) were added to alkali-activated milled waste rock wool (activator used was Na-silicate solution), that is fibrous on its own. Bending strength after 1 month was highest when glass fibres were used, while compressive strength was high for all fibres except basalt and cellulose packed in cubes (Pavlin et al., 2022b).

Waste rock wool was further investigated regarding its potential to be used as a precursor in alkali-activated foams. The activator used in the study was a Na-silicate solution, the foaming agent was H_2O_2 (added from 1 to 3 m%), and the surfactant used was triton (1.5 m%). The samples were cured at 70 °C for 3 days. The sample with the lowest density (~0.5

kg/l) had a compressive strength ~1.5 MPa and total porosity below 80%. The lowest thermal conductivity was ~90 mW/(m·K) (Pavlin et al., 2022a).

Alkali-activated fly ash and ground-granulated blast furnace slag were analysed as adhesives to different substrates (concrete, ceramic tiles, wood-based geopolymer and high-density geopolymer). They were mixed in different ratios and activated with K-silicate solution and KOH. Good bonding (high pull-off strength) was achieved in connection with concrete and ceramic tiles, while not with either of the geopolymeric samples. On the interfaces it was confirmed, that also chemical adhesion took place, but the mechanism is not yet understood (Wisniewski & Ducman, 2022).

Alkali-activated materials made from waste precursors have a lower carbon footprint (Kvočka et al., 2020) compared to conventional building and civil engineering alternatives. However, the environmental imprint can be further lowered by decreasing the need for energy and time for production. The potential candidate is a replacement of the surface with volumetric heating, therefore microwave irradiation of alkali-activated materials in their early stage was studied. Alkali-activated metakaolin slurry was irradiated with microwaves at 100 W and 1000 W for 1 min (microwave of inverter type working at 2.45 GHz) and compared to the sample cured solely at room conditions. Microwave irradiation with low powers resulted in a higher early compressive strength, while irradiation with high powers foamed and solidified alkali-activated slurry. If the dehydration during irradiation was hindered, compressive strength was higher. However, alkali activation particularly enhanced the leaching of As, while irradiation with 1000 W increased the leaching of Cr and As, all compared to the precursor. With the decreased potential of immobilisation of Cr and As, microwave irradiation has the potential to be used for the removal of heavy elements and as a cheap and efficient method for recovery or remediation (Horvat et al., 2023).

Rock wool mixed with lime, metakaolin, and electric arc furnace slag, was alkali activated with Na-silicate solution with added NaOH, and treated at room conditions, 40 and 60 °C, and at 30, 60 and 90% humidity. At upscaling, curvature and highly varying mechanical properties arose, which was because of unevenly milled batches of rock wool. This was the reason for the constant need to change the recipe, however, when the milling was of constant quality, so was the recipe. The final choice for curing to avoid curvature of the façade panels was 3 days at room temperature in the PVC bag (to hinder dehydration), and an additional 3 days at 60 °C and 60% humidity demoulded on a level metal mesh to allow uniform drying (Pavlin et al., 2023).

Calcined river sediments, rich in clay, were evaluated regarding their potential to be used as precursors in alkali activation with Na- and K-silicate solution, and with NaOH and KOH. Clay was calcined at 900 °C, and alkali-activated samples were cured at 60 °C for 3 days. Replacement of the calcined sediment was done up to 30 m% with fly ash and ladle slag. Calcined clay can be used as a precursor, however, the addition of more reactive ingredients is beneficial for mechanical strengths (Žibret et al., 2023).

The influence of microwave irradiation in the early stage of alkali-activated local ladle slag, local thermopower plant fly ash and metakaolin was evaluated regarding the time and power of irradiation (2.45 GHz, magnetron source). Mixtures with Na-silicate and NaOH synthesised had the needed addition of alkali calculated by fixing the Si at an Al to Si molar ratio of 1 to 1.9. Then the first group of the periodic system was set to avoid efflorescence by not exceeding the molar amount of amorphous Al, if possible. Besides, the amount of liquid to solid was varied to study the dependence of water content in the alkali-activated slurry during microwave irradiation. Even if the material absorbed the same amount of dose, it is clear that it is important if the dose was received gently and slowly, or fast under harsh conditions. This was different for every mixture, not just every precursor. Additional water decreased the compressive strength, which was slightly higher after irradiation. However, there were no mineralogical changes observed because of the irradiation.

3. Conclusions

10 years of research on alkali-activated materials in Slovenia brought the research to the edge, i.e., with just chemical and mineralogical evaluation of any material containing silicon and aluminium, the optimal algorithm for calculating the recipe was proposed. This recipe is the starting point in experimental analysis on the TRL4 level, while 10 years ago everything started on the TRL1 level. With a theoretical approach, several pilot products and testing fields in cooperation with the local industry and from local waste were designed.

Besides levelling up the start of the research from the TRL1 to TRL4 with a theoretical approach, foaming through chemical reactions and heat was researched. Chemical foaming was performed through the introduction of different foaming and stabilizing agents into the raw ingredients (not into the fresh mixture) as induced foaming, while a correct choice of precursors in an alkali environment led to a delayed self-foaming. As self-foaming did not need an introduction of additional chemicals, so did not physical foaming with volumetric heating. However, the last curing-foaming technique is still ongoing research in the first national project on the influence of microwave irradiation on alkali-activated materials.

Nonetheless, if the waste is collected and stored as raw materials are, in big enough quantities, the future of the building and civil engineering products made with alkali activation of the secondary materials is viable but has to be tested first on a bigger scale. However, bigger countries with different industries that produce different wastes, have a much wider pallet of materials to choose from and select the most suitable regarding the desired properties of the product and environmental impact.

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Invited lecture/Review

Unveiling PFAS-free Solutions for Hydrophobic and Oleophobic Textile Coatings

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Abstract:
Per- and polyfluoroalkyl substances (PFAS) are a large group of synthetic compounds containing carbon-fluorine (C-F) bonds. They are used in almost all industries, including the textile industry, to impart hydrophobic, oleophobic, stain-repellent or non-stick properties to various materials. They are also valued for their excellent thermal, chemical and mechanical stability. However, increasing environmental and health concerns about PFAS have led to an urgent need to find sustainable alternatives. Due to their chemical composition, which contains strong carbon-fluorine bonds, they are difficult to degrade and tend to bioaccumulate in the environment and in human tissue. Human exposure to PFAS has been linked to a number of adverse health effects, including immunosuppression and cancer. As a result, academia and industry are increasingly focused on developing alternatives to PFAS in a variety of applications, to reduce the environmental and human health impacts associated with these persistent substances. This review article explains the basic properties of PFAS, the principles of textile wettability and discusses possible PFAS-free solutions in the field of environmentally and human friendly hydro-/oleophobic textile coatings.

Keywords: PFAS, hydrophobicity, oleophobicity, bio-based, coatings, textile

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1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a large group of synthetic chemical compounds containing carbon-fluorine bonds. They are highly valued in various industrial applications due to their exceptional thermal, chemical and mechanical stability. As reported by the Environment Directorate of the Organisation for Economic Co-operation and Development (OECD), there are more than 4,700 different chemical compounds that belong to the PFAS family (OECD, 2018). PFAS are mainly known for their hydrophobic and oleophobic properties, which means that they impart water and oil repellent properties to treated surfaces. However, as they contain carbon-fluorine (C-F) bonds, which are among the strongest bonds in organic chemistry, they are difficult to degrade. As a result, their persistence in the environment, bioaccumulation and potential adverse effects on the environment and human health have raised significant concerns. As a result, the science and industry are increasingly focussing on developing alternatives to PFAS in various applications, including textiles, to reduce the environmental impact associated with these persistent substances.

2. PFAS in the textile industry

PFAS have played a pivotal role in the textile industry as coatings to improve repellent properties of textiles. Moreover, textile industry is considered the largest PFAS consumer, accounting for approximatelly half of the worldwide PFAS use (Lassen et al., 2015). Due to the uniquefunctional, mechanical, chemical and thermal properties, they are especially used for products that need to be exceptionally durable under various conditions. Their ability to repel water and oil allows coated surfaces to be highly resistant to stains and liquids, making them essential for safety, health and comfort, in the protective, medical or apparel industry. The most common water and oil-repellent products found in textiles are sportswear, leisure wear, uniforms, workwear, upholstery and automotive fabrics, awnings, sunblinds, curtain fabrics, tents, umbrellas, table and bed linen and carpets (Schindler & Hauser, 2004). They are often found under trade names such as NanoTex and GoreTex (Zheng & Salamova, 2020).

2.1 PFAS properties

Generally, PFAS are divided into two main groups: polymers and non-polymers (**Figure 1**). While the polymer group includes two commonly used materials, polytethrafluoroethylene (PTFE, Teflon) and ethylenetetrafluoroethylene (ETFE, Tefzel), the non-polymers are more often commonly in the environment (Meegoda et al., 2020). Non-polymeric PFAS are categorised into subgroups depending on whether hydrogen atoms are completely (perfluorinated) or largely (polyfluorinated) replaced by fluorine atoms (Brunn et al., 2023).

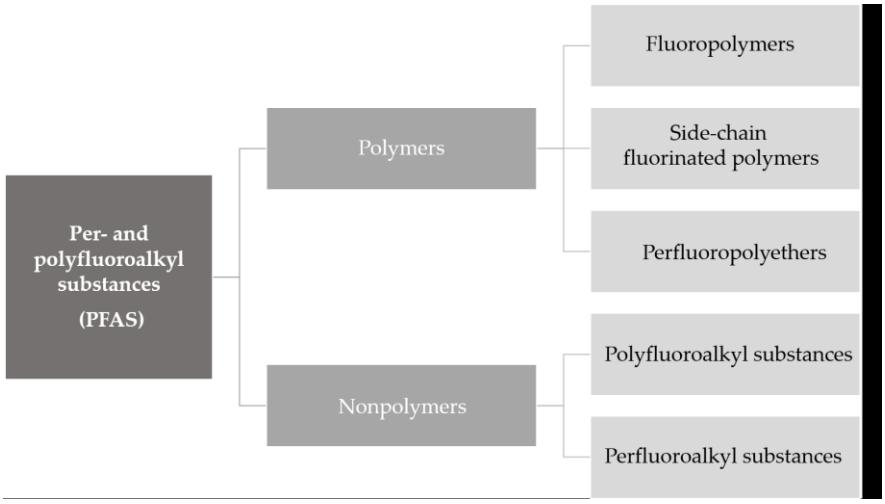

Figure 1. Classification of PFAS compounds, adapted from Meegoda et al., 2020.

This is especially important because the exceptional durable and functional properties of PFAS are closely linked to their chemical composition. The unique properties result from the presence of fluorine atoms bonded to a carbon backbone, which leads to a strong and stable molecular structure. The high electronegativity and small size of fluorine make the C-F bond one of the strongest covalent bonds, which means that a large amount of energy is required to break it (Meegoda et al., 2020). This robust chemical structure gives them remarkable thermal, chemical and mechanical resistance.
To achieve water, oil and stain-repellency of textiles, perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are mainly used, which belong to the nonpolymeric perfluroalkyl substances group. Due to the existing restrictions on the use of PFOS and PFOA (Pontius, 2019), PFAS with shorter carbon chains, that are less persistent are also used (Zheng and Salamova, 2020). However, the chemical structure (especially the length of the carbon chain) strongly influences the properties of the compound. Long-chain PFAS compounds are associated with increased hydro-/oleophobicity (Meegoda et al., 2020).

2.2 Basic principles of textile surface wettability

Three different types of repellencies can be achieved by modifying the surface of textiles. The first is hydrophobicity, where the water droplets do not spread or wet the surface of the textile fabric. The second one is known as superhydrophobic or ultrahydrophobic, in which the contact angle between the liquid droplet and solid surface (θ) (**Figure 2**) is greater than 150° and the droplet has a small slip-off angle (Shirtcliffe et al., 2010; Ueda & Levkin, 2013), meaning that superhydrophobic fabrics do not only repel water, but also allow the water droplet to roll off the surface. This phenomenon can also be found in nature and is known as the lotus leaf effect, which has a self-cleaning effect due to the high surface roughness. The third type of repellency is oleophobicity, where the surface repels oily liquids.
The boundary between wetting and repellency is defined by the contact angle of the liquid droplet on the textile substrate (**Figure 2**). If the contact angle is less than 90°, the textile is wettable, but if the contact angle is greater than 90°, the textile substrate is repellent. In other words, an increase in the contact angle means an increase in repellency.

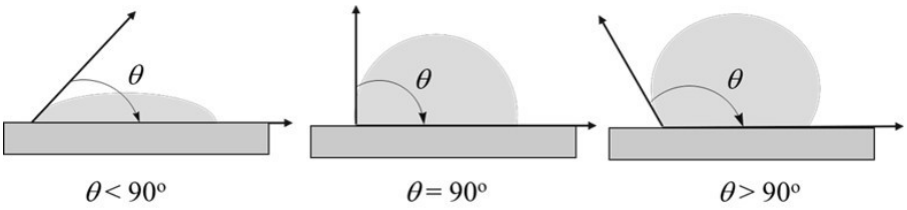

Figure 2. Wetting or repellency depending on the contact angle of the liquid droplet (θ).

We outline two theoretical models that describe the wettability of rough and heterogeneous (non-uniform, consisting of different chemistries) surfaces, the Cassie-Baxter and the Wenzel model (Huang & Gates, 2020; Roach et al., 2008; Crick & Parkin, 2010; Wang et al., 2020). According to Wenzel, the water droplets have full contact with a rough surface, and there are no air pockets under the liquid droplets (Shahid et al., 2022; Crick & Parkin, 2010). According to the Cassie-Baxter model, a water droplet sits on top of a rough surface, due to the trapped air between the grooves of the rough surface. The area where the air is trapped is not wetted by the liquid, creating a separation between the liquid and the grooves of the rough surface (Shahid et al., 2022; Darband et al., 2020).
Achieving good repellency is to a large extent dependent on two properties, surface energy and surface roughness. The surface energy depends on the chemical structure of the surface and can be altered by chemical modification of the fibres (Simončič, 2012). In order to achieve a water and oil repellency it is necessary to reduce the surface energy of the fibres. Roughness, or more specifically nanoroughness, can be achieved by changing the

morphology of the fibres through the application of coatings containing nanoparticles or nanocomposite films.

Applying a repellent coating to the surface of the textile changes its properties by reducing the surface free energy of the fibres. The extent to which the surface free energy of the finished textile is reduced depends on whether it is hydrophobic or oleophobic. The lower the surface free energy of the fibre, the less wettable the fabric is. The critical surface energy or surface tension (γ_C) of fibres must be much lower than the surface tension of the liquid (γ_L) that is being repelled (Schindler & Hauser, 2004). Commercially available repellents contain either hydrocarbon or perfluorinated hydrocarbon groups in their chemical structure. If the repellent contains a hydrocarbon chain (**Figure 3a**) in its structure, it lowers the surface energy of the substrate to 22–30 mN/m (Simončič, 2012). Such a surface is only water repellent because the surface free energy is lower than the surface tension of water (73 mN/m) (Schindler & Hauser, 2004), but not lower than the surface tension of oils (30–40 mN/m) (Simončič, 2012). If the repellent has perfluorinated hydrocarbon groups (**Figure 3b**) in its structure, it reduces the surface energy of the substrate to a value 6–30 mN/m, providing oil repellency in addition to water repellency (Simončič, 2012). In this case, the surface free energy is much lower than the surface tension of the oils.

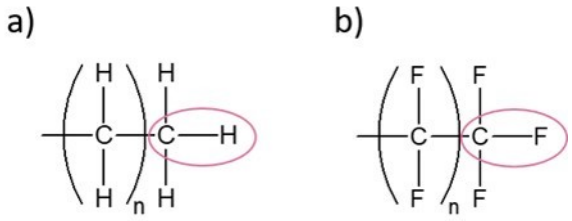

Figure 3. A hydrocarbon chain (a) which is included in the structure of commercial water repellents and perfluorinated hydrocarbon groups (b) which are included in the structure of commercial oil repellents.

Another principle for achieving repellency of a textile material is by increasing surface roughness. This can be achieved by changing the morphology of the fibres. On a rough hydrophobic surface, the contact area between the water droplet and the surface is reduced. The water droplet sits on the grooves of such rough surface and forms an almost spherical shape with a high contact angle (**Figure 4**). The presence of air pockets increases the interfacial area between water and air and decreases the interface between the solid and water. The water droplet sits partly on a solid surface and partly on air pockets, which greatly reduces the adhesive forces between the water and the solid.

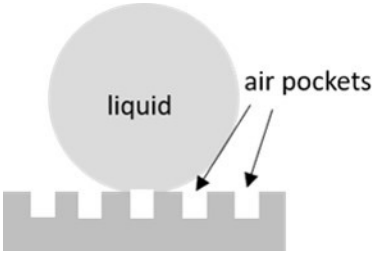

Figure 4. A spherical shape of water droplet with high contact angle on rough surface.

3. Environmental and human health concerns

Due to their chemical structure, PFAS are very resistant to decomposition and prone to accumulation, which as a sighnificant impact on the human health and the environment. Their persistence is causing air, water (including drinking water), soil and wildlife

contamination. A major source of exposure is also household dust (Karaskova et al., 2016), which largly consists of short textile fibres. In addition, the long-range transport further contributes to their global distribution, as they have already been detected in the remote areas, i.e., in Arctic (Lin et al., 2020). People are therefore being exposed to PFAS through the environment, drinking water and food. PFAS have already been detected in human urine, breast milk and blood samples. Studies have also shown that the highest concentrations of PFAS in human blood have been detected are in the industrialised areas (Worley et al., 2017). Exposure to PFAS throught the skin, inhalation and ingestion has been associated with a wide range of adverse health effects, such as infertility, fetal development, thyroid hormone and kidney dysfunction, weakend immune system, reduced effectivness of vaccines, developmental problems in children and even cancer (Kleinman & Stevenson, 2021; Bil et al., 2023; Blake at al., 2018; Looker et al., 2014; EPA – United States Environmental Protection Agency, 2023). As a result, the health risks associated with PFAS have led to extensive regulatory controls and restrictions. Regulatory authorities around the world are taking steps to regulate the use, manufacture, and disposal of PFAS. In particular, the acceptable concentrations of certain PFAS compounds in water, soil and air have already been limited in the recent decades, in order to to restrict their release into the environment and reduce the associated risks. Restrictions have also been placed on the production and use of certain PFAS, such as perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). As a result, the industry, including the textile industry, is forced to look for PFAS-free alternatives to fulfil these regulations.

3. PFAS-free alternatives for the textile industry

3.1 Characheristics of ideal PFAS-free alternatives

Ideal PFAS-free alternatives for hydro-/oleophobic textile coatings should exhibit specific properties to ensure effective and long-lasting performance while addressing environmental and health concerns. These alternatives should have durable hydro-/oleophobic properties, that ensure the treated textiles effectively repel water and oil for multiple uses and wash cycles and withstand the exposure to sunlight, moisture and mechanical stress without compromising performance. Futhermore, in addition to ensuring the functional properties of the textiles, the durability of the coating contributes to the overall sustainability of the product by minimising the need for freequent reapplications. At the same time, the end-of-life aspect should also be considered when developing new coatings. These alternatives should also be versatile enough to be applied to a variety of fabrics without compromising their basic textile properties, i.e., breathability or flexibility. Furthermore, the PFAS-alternative coating should be cost-effective as this is particularly important to achieve a widespread industrial use. The alternatives should be economically viable for manufacturers, to make the transition financially feasible. The ideal alternative should not only offer competitive pricing, but also minimise production costs without compromising on performance, durability or safety. A balance between functionality, durability and sustainability is essential for the successful development and adoption of PFAS-free hydro-/oleophobic textile coatings.

3.2 Promising PFAS-free alternatives

Several promising PFAS-free alternatives are emerging as the textile industry searches for sustainable and environmentally friendly solutions. One promising research path involves the use of bio-based materials, such as biodegradable polymers from renewable sources. Natural polysaccharides, as well as oils and waxes from plants can be used to reduce the surface tension of the material and thus increase hydrophobicity. These alternatives not only provide an effective water and oil repellency but also, address concerns about persistence in the environment due to their bio-degradability. Another way to achieve hydro-/oleophobicity is to utilize the principle of increasing the surface roughness of the material, which can be achieved by using nanoscale materials. The use of nanoparticles allows precise control of coating thickness and uniformity and in addition to increasing the roughness of the textile fibres, can create a protective barrier between the

fibre and water or oil. Moreover, additional functionality of the material can be achieved through the specific properties of the added nanoparticles, offering the potential to improve functionality and performance.

Several methods have been developed to produce superhydrophobic textiles using bio-derived compounds as low surface energy chemicals and/or nano/micro scale surface roughening components (Shahid et al., 2022). Two main approaches can be found in the literature. The first is a two-step application where the first step is surface roughening followed by superhydrophobisation (coating with low surface energy materials) and the second is a one-step coating or simultaneous surface roughening and superhydrophobisation.
In the first approach, the surface can be roughened by enzyme etching or coating with different nanostructures based on natural compounds such as chitosan, cellulose, lignin, bovine serum albumin and natural polyphenols. They all contribute to nanoroughening of textile surface before hydrophobisation with synthetic or natural low surface energy compounds. Materials used to create nano/micro scale surface roughness to textile include also TiO_2 (Xue et al, 2008; Kuruppu, 2021; Suryaprabha & Sethuraman, 2021), SiO_2 (Xue et al, 2008; Xue et al, 2009; Manatunga et al, 2016), ZnO (Fan et al, 2018; Gao et al, 2019; Lee et al. 2011; Ates & Unalan, 2012; Khosravi & Azizian, 2018), etc. Coating of nanostructures can be done by various methods, e.g., dip coating, solution immersion, dip-pad-dry-cure, spray coating, spin-coating, in-situ growth, etc. There are several biomaterials that have been used for lowering surface energy. Among them, fatty acids and their salts, for example stearic acid (Xue et al., 2008; Lee et al., 2011, Richard et al., 2013; Pan et al., 2012; Li & Guo, 2017; Suryaprabha & Sethuraman, 2017; Dong et al., 2019; Kundu et al., 2019), sodium stearate (Teli & Annaldewar, 2017; Lu et al., 2018), zinc stearate (Lu et al., 2018), copper stearate (Pan et al., 2019), calcium stearate, lauric acid (Fan et al., 2018), sodium laurate (Pan et al., 201; Liu et al., 2015), ammonium palmitate, cinnamic acid and myristic acid have been preferred. Other materials that have been used are natural waxes such as beewax or carnauba wax, cellulose oleoyl ester and cardanol- and eugenol- based benzoxazines (Shahid et al., 2022). The second step (low energy treatment) is usually carried out by dip coating or dip-pad-dry-cure (Shahid et al., 2022).
The second procedure is coating the textile substrates with pre-hydrophobised nanostructures (Shome et al., 2019; Gu et al., 2019; Rahman et al., 2021; Ivanova & Philipchenko, 2012). Compared to the first procedure this one takes less time, potentially costs less and is easier to apply in large scale applications. Bio-based superhydrophobic formulations that have been used until now contain cellulose oleoyl ester nanoparticles (Xiong et al., 2017), nano lauric acid copper (Zhang et al, 2019), beewax/lignin (Zhang et al., 2020), cationic starch/carnauba wax (Forsman et al., 2020), cellulose nanocrystals/chitin nanocrystals/chitosan (Yagoub et al., 2019), PLA/nanoclay (Gore & Kandasubramanian 2018), Chitosan/TiO_2 (Ren et al, 2020), cinnamic acid/myristic acid functionalised sepiolite nanoparticles (Razavi et al., 2019), etc. Among the various coating techniques employed, dip coating and spray coating are most widely used.
Most research achievements in the filed of bio-based production of hydrophobic textiles are still limited to laboratory-scale trials. Due to their low durability, the lack of standardised protocols and the lack of operational complexity, the processes described are not yet suitable for mass production. One of the most important remaining issues is how to develop long lasting hydrophobic textiles using biomaterial-based methods with well-defined procedures to meet the urgent market demand.

4. Current challenges and future perspective in the PFAS-free coating development

The search for PFAS-free alternatives for textile coatings is currently facing several challenges. A key challenge is the technical complexity of developing alternatives that match the performance characteristics of conventional PFAS coatings. Achieving a comparable level of functionality without compromising durability remains a major obstacle. Developing alternatives that match or exceed the performance of PFAS in water and oil repellency while maintaining durability is a complex task. Another technical

challenge isto take into account the wide range of substrates and fabrics used in the textile industry, as PFAS-free alternatives must be compatible with different materials. Another challenge is to ensure that these alternatives remain effective after multiple wash cycles and environmental conditions during wearing. In addition to closing the performance gap, it is particularly important for the textile industry to ensure a manufacturing process that is economically viable and suitable for mass production. Overcoming these multiple challenges will require interdisciplinary collaboration between researchers, manufacturers, regulators and markets to drive innovation, improve performance and facilitate the widespread adoption of PFAS-free alternatives in the textile industry.

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Invited lecture/Research

Design Optimization and Fatigue Evaluation of Wood Compo-site Gears

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Abstract:
A great deal of research in polymer gears has gained importance in the last decade. It is necessary to highlight the different polymer materials and fibers used for gears to meet the requirements of a particular drivetrain application. With the increasing need to recycle already used materials, there are trends towards the use of BIO-based materials that would allow recycling and reuse in secondary, less demanding parts or assemblies. To integrate these materials into a real mechanical part such as a gearbox, their mechanical, thermal, and tribological operational performances must be evaluated. In this study, life tests of wood-polymer composite gears were performed using High-Density Polyethylene (HDPE) reinforced with 20% spruce fibers and the same polymer matrix reinforced with 20% beech fibers. The wood-polymer composite gear was tested with a mating steel pinion. The study aimed to determine the life cycles to failure of wood-polymer composite gears, the temperatures generated in the gear pair contact, and the flank wear characteristics of both types of wood composite gears. The results show that HDPE with beech fibers lasts on average 15% longer compared to HDPE with spruce wood fibers. When analyzing the flank wear, the beech fibers proved to be more wear-resistant than the spruce fibers in the same polymer matrix. The analysis of the failure mechanisms shows that the crack propagation at the tooth root is slower in HDPE reinforced with beech fibers compared to HDPE with spruce fibers due to the better mechanical properties.

Keywords: High-density polyethylene; Wood; Fibers; Gears; Fatigue; Wear.

1. Introduction

1.1. Background of using polymer-based gears

Gear wheels produced from polymer materials have been used for power transmission for a long time. Initially, polymer-based gears were used in less demanding power transmission systems where they were not subjected to high loads and consequently generated excessive mechanical stresses and heat in the contact zone. Nowadays, they play an important role in various mechanical drive systems as they offer advantages over metal gears, in particular wear resistance, dry running capability, low weight and inertia, self-lubrication, improved noise, vibration, and harshness (NVH) behavior, etc.

Polymeric materials comprise a large group of plastics. The most used polymers for gears are thermoplastics. Thermoplastics consist of two main groups, namely crystalline and amorphous plastics. The most typical crystalline polymers are the following: Polyacetal - POM, Nylons - PA, Polyethylene - PE, and High-Density Polyethylene – HDPE, (Ehrenstein, 2001). Given the current environmental concerns regarding the green future, engineers and researchers have started to perform various mechanical, thermal, and fatigue tests on different BIO-based polymer materials for potential gear applications to evaluate their durability and tribological performance. One of the polymers that can be easily recycled is the polymer HDPE, as it has no branching and its structure is more densely packed, making HDPE a linear polymer. By adding natural wood fibers to the polymer matrix, the mechanical properties of the resulting composite (Wood Composite Polymer – WPC) can be improved in terms of flexural strength, which has a favorable effect on the transfer of dynamic loads during the gearing process.

The intensive process of rolling and sliding speeds generates high temperatures in the contact between the meshing gear pairs, which causes the material properties to deteriorate with running. A fatigue process leads to an increase in the number of cycles when a gear pair meshes, resulting in a deterioration of the mechanical properties, which causes high elongation of the component due to the viscoelastic behavior of the polymers. Therefore, it is crucial to predict the wear-fatigue behavior for selected polymer gear pairs. To obtain these results, researchers need to optimize the material combinations of gear pairs through laboratory tests in the form of gear pair durability tests and measurements of tooth flank wear of selected polymer or polymer composite materials.

Ezzahrae et al. (2022) tested three HDPE composite mixtures with a WF content of 40%, 50 %, and 60 % and investigated the density, flexural properties, hardness test, and thermal analysis. The results show that increasing the wood content from 40 % to 60 % increases the density and hardness of the WPCs. In addition, increasing the proportion of wood flour to 60% reduces the flexural strength, while the flexural modulus increases.

Koffi et al. (2021a) evaluated the performance of extruded HDPE test pipes reinforced with 10-30 wt.% together with an adhesion promoter. They concluded that the newly produced composites can reduce the deformation of wood-containing material compared to pure HDPE. Young's Modulus can also be increased in proportion to the filler content compared to pure HDPE.

Afrifah et al. (2009) investigated nano clay-reinforced HDPE as a matrix for wood-plastic composites. The experimental results showed that the flexural properties of HDPE/wood flour composites could be significantly improved by a suitable combination of bonding agent content and nano clay type in the composites.

Koffi et al. (2021b) characterized the impact strength, Izod impact strength, hardness, tensile strength, and modulus of elasticity of birch fiber-reinforced HDPE obtained by injection molding of test specimens. They found that the improvement in tensile strength by 19.7% of the fibers was above average.

Černe and Petkovšek (2022) developed a thermomechanical model that provides results that are consistent with experimental measurements of polymer gears operated with high-speed infrared thermography.

Blais and Toubal (2020) evaluated the mechanical behavior of high-density polyethylene (HDPE) reinforced with short natural fibers using a test rig designed to monitor the flexural fatigue properties of gear teeth at high cycles. Fatigue as a function of the number of cycles was modeled using S-N curves, damage indices, and a linearised Weibull distribution.

2. Materials and Methods

2.1 Production of wood polymer composites

The selection of raw materials was based on the representation of various segments of the polymer and wood processing industry in Slovenia and throughout Europe. For the polymer matrix, High-Density Polyethylene (HDPE) was chosen as 3 mm pellets.
To improve the mechanical properties of the pure HDPE, spruce, and beech fibres were used. The argument for the choice of reinforcement was that these tree species are among the most widely cultivated in Slovenian forests and Europe. The average particle size of the spruce fibers was 0.2 mm and that of the beech fibers was 0.24 mm. The density of the pure spruce fibers was 140 kg/m^3 and that of the beech fibers was 155 kg/m^3. The moisture content of the wood fibers was 7% (Hriberšek & Kulovec, 2023). The following material mixtures were produced as part of the research: the first composite - High-Density Polyethylene (HDPE) reinforced with 20% by weight of spruce fibers (SF) and the second composite – High-Density polyethylene reinforced with 20% by weight of beech fibers (BF). To improve the adhesion between the HDPE matrix and the wood fibers, suitable promoters for polymers such as polyethylene PE-g-MA grafted with maleic anhydride were used. The composite material was extruded and chopped into small pellets of 3 mm size.

2.2 Gear production

The production process for the WPC gears consisted of drying the WPC pellets and injection molding the round semi-finished parts using the Krauss Maffei KM 50/100 CX machine. After injection molding, internal turning was performed to manufacture the gear hole. Dry machining was carried out using an involute hob cutter on a Koepfer 200 CNC machine. The steel gears were manufactured from round 42CrMo4 + QT steel, which was hardened (Q) and tempered (T). The bars were cut to a predetermined width to obtain circular half-parts. Facing was used to obtain an appropriate width for the circular semi-finished products. Internal turning was used to produce bores. The gear profile was produced using an involute hob cutter on a Koepfer 200 CNC machine. After machining, burrs were removed from the edges of the steel gears. To ensure the prescribed class of quality of the gear profile according to (ISO 1328-1, 2013) all test gears were checked with the 3D coordinate measuring machine (Wenzel LH 54, 2024). Figure 1 presents the produced gears. Table 1 presents basic gear geometry. Both, the driving steel gear, and driven wood polymer composite gear have the same geometry.

Table 1. Important gear parameters.

	Steel and WPCs gear
Teeth number, z [-]	20
Gear width, b [mm]	6
Gear module, m_n [mm]	1
Pressure angle at normal section, α_{wt} [°]	20
Tip diameter, d_a [mm]	22
Reference diameter, d [mm]	20
Root diameter, d_f [mm]	17.5

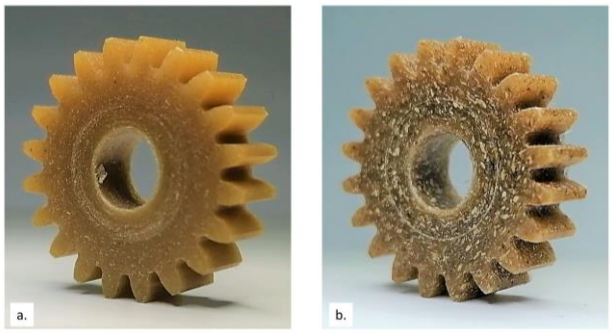

Figure 1. a. HDPE reinforced with 20% wt. spruce fibers and b. HDPE reinforced with 20% wt. beech fibers.

2.3 Testing procedures

The durability tests for polymer-based gears were conducted in a special test rig (see **Figure 1**). The test rig consisted of two 3-phase (4-pole) asynchronous squirrel cage electric motors with a frequency of 50 Hz and a power of 0.37 kW connected to two 1-phase frequency converters (power of 0.4 kW), the aim of which was to regulate the speed of gear 1 - driving and gear 2 – driven motors. The precision of setting the center distance of the gear pair was ensured by a linear rail with an accuracy of 0.001 mm. The required transmission of the rotary motion from the asynchronous motors was ensured by a belt with the appropriate transmission ratio on a pulley to the driving and driven shaft on which the gearboxes were mounted. The speeds of the individual motors were controlled by the corresponding frequency converter, with motor 1 acting as the drive source and motor 2 as the brakes source. The load acting on the shafts/gears was defined as the difference between the excitation frequency of motor 1 and the excitation frequency of motor 2. During the lifetime experiments of each gear pair, an average point temperature was measured and monitored in the meshing area. The thermal camera had a refresh rate of 50 Hz a display with a refresh rate of 20 Hz and a measurement area size of 2 x 2 mm. The device was connected to a PC on which the temperature distribution on the gears was visualized during a test. The gear pairs (steel/ HDPE 20% wt. spruce fibers and steel/ HDPE 20% wt. beech fibers) were tested under different torques (from 0.6 to 0.3 Nm), which were defined because the operation of a single gear pair had to reach from 100,000 cycles to several million cycles to characterize the fatigue life of each gear pair material. The speed of the gear pair was 1400 rpm and was tested at ambient temperature in the laboratory. **Figure 2a.** presents an experimental testing setup for durability testing of selected gear pairs and **Figure 2. b** presents an example of the tested gear pair.

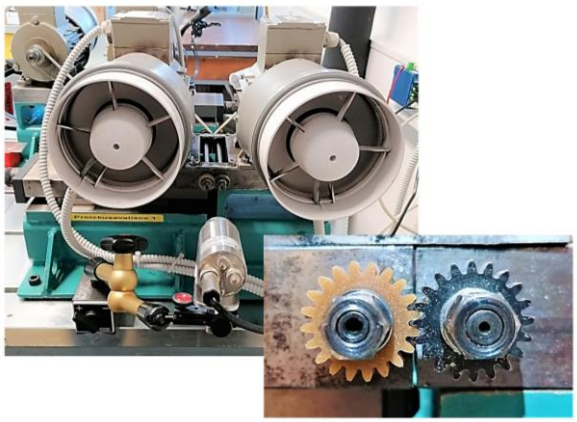

Figure 2. Experimental setup for durability gear pair testing.

During and after testing the durability of the gear pairs, wear measurements were carried out using an optical scanner. The measurement process was conducted at ten times magnification. The measuring range was 2 x 10 mm. The resolution of the measuring device

was set to 100 nm in the vertical direction and 4 μm in the lateral direction. The contrast of the monitored image was 1 and the exposure time of the measurements was 1.4 ms. **Figure 3a**. presents the optical 3D scanning process and **3b.** the scanned 3D profile of gear tooth.

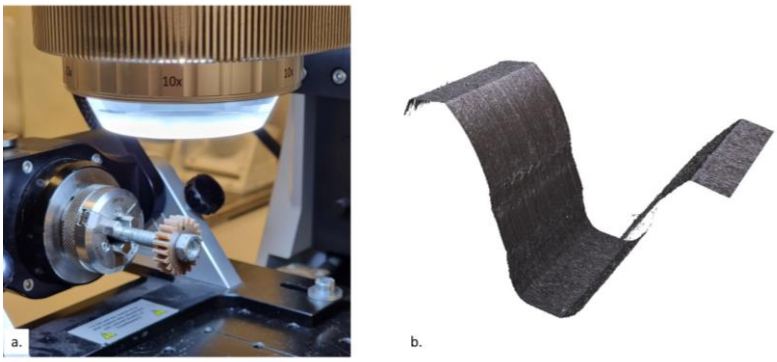

Figure 3. a. Optical scanning process of gear and b. 3D scanned profile of gear tooth.

The wear coefficients for each material were calculated using the calculation method cited in the VDI 2736 guideline. The VDI 2736 guideline defines a calculation method in which the abrasive wear of dry-running gears is evaluated. The wear coefficient k_w (10^{-6}/mm^3 Nm) can be calculated using the following equation (VDI 2736, 2016; Djebli et al., 2014),

$$k_w = \frac{W_m b_w z l_{Fl}}{2\pi T_d N_L H_V} \quad (1)$$

Where W_m is the average linear flank wear (in mm), l_{Fl} is the length of the contact line (in mm), T_d is torque (in Nm), N_L is the number of load cycles, and H_V is the degree of tooth loss.

3. Results

To accurately determine the lifespan of each WPC material in the infinite time range of fatigue strength (from 100,000 to 3 - 4 million load cycles), the following load levels were applied to each gear pairing (driving/driven) combination (steel/HDPE 20% spruce fibers - SF and steel/HDPE 20% beech fibers - BF): 0.3 Nm, 0.4 Nm, 0.5 Nm, and 0.6 Nm. Each experiment was repeated at least three times with the same load to obtain a satisfactory statistical validation of the process. The durability experiments were conducted at a room/laboratory temperature of approximately 24.0°C ± 0.5°C and an ambient pressure of 1013 hPa. During the tests, the average contact point temperature was measured for each experiment.

Figures 4, 5, 6, and **7** show the experimental results in the form of graphs of temperature (T) and load cycles (N) recorded during the tests with the infrared thermal camera. Each T (N) diagram consists of three characteristic parts. At the beginning of the test, a clear increase in the temperature gradient is observed. This is related to the deformation of the WPC tooth flanks and the increased wear rate caused by the harder steel driving wheel. This area is referred to as the running-in phase. In the next phase of gear running, the gear mesh temperature stabilizes with a constant temperature fluctuation, which defines a phase of linear wear that lasts a large part of the time of an experiment. In the last part of the running process, an increased temperature oscillation is observed, which is due to the deterioration of the cohesion between the bonds of the composite material, leading to a progression of cracks and increased flank wear, failing one or more teeth. In the tested gear pair made of steel/HDPE 20% BF, lower temperatures occurred in the meshing zone than in the HDPE 20% SF gears at the tested load levels of 0.6, 0.5, and 0.4 Nm. In addition, the HDPE 20% BF gears exhibited lower temperature fluctuations compared to the HDPE 20% SF gears. The reason for the lower temperatures and the associated temperature fluctuations may lie in the lower modulus of elasticity of the HDPE 20% SF material. This can be a reason for higher deformations of the teeth due to bending loads, which leads to a higher

spontaneous heat release in the contact between the gear pairs. If the heat is high enough to increase the temperature transition zone, the modulus of elasticity of this zone decreases significantly and the strength of the WPC material is reduced.

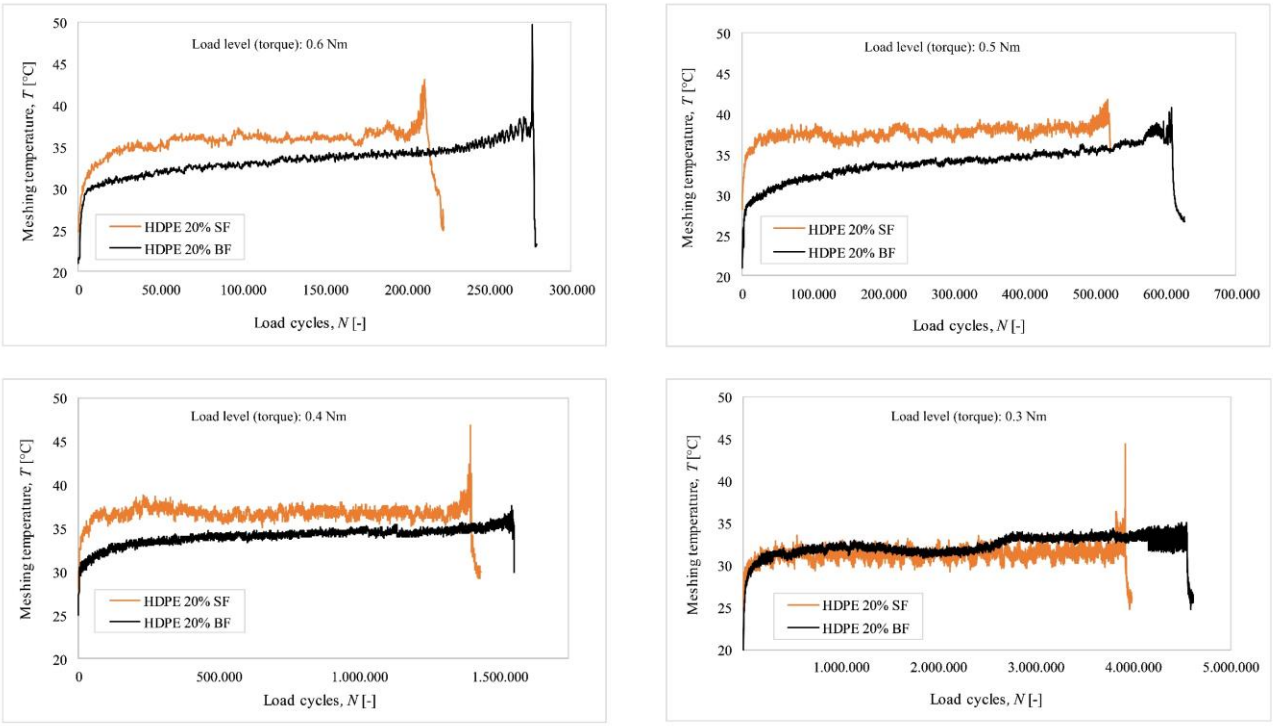

Figure 4. Comparison in durability testing results for observed gear pair combinations.

At the 0.6 Nm load level, the average difference in lifespan between the two WPC materials is 24%, while at the other three load levels, the difference is 10% in favor of the beech fibers. The results of the service life confirm that the composite material with beech fibers has a higher impact strength and consequently a better resistance to fatigue during the cross-linking process.

The conceptual procedure for the wear measurements was to perform each measurement just before the first tooth root fracture of each wood-polymer composite gear under the tested load levels. The wear of the flank profiles was analyzed in the 2D plane using a suitable calculation program at seven different gear diameters to obtain sufficient data to calculate the average linear wear of the flank profile. The wear coefficients of the materials were calculated based on the calculated average linear wear (**Figure 5**).

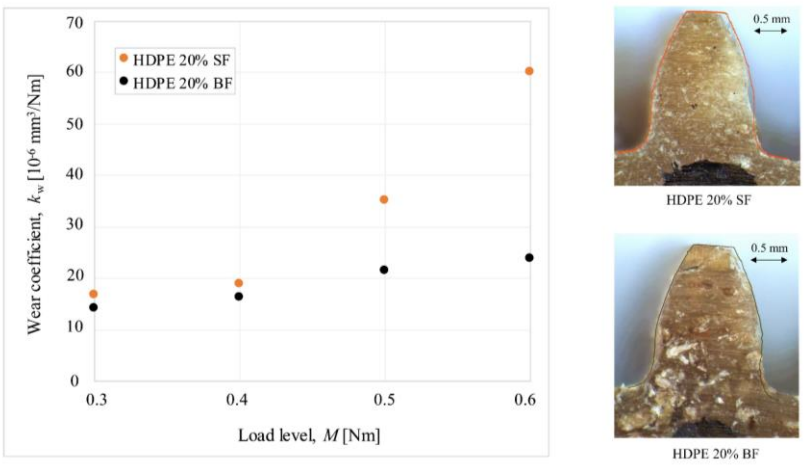

Figure 5. The dependence of wear coefficients on load level.

In **Figure 5**, At load levels of 0.3 and 0.4 Nm, the wear coefficients for both WPC materials are similar. At a load level of 0.5 Nm and even more clearly at 0.6 Nm, the wear coefficients for WPC with spruce fibers increase significantly compared to the wear coefficients for WPC with beech fibers. An important aspect when considering the wear properties of polymer-based materials is the mechanical properties of the polymer gear wheel when it is connected to a steel pinion. A higher modulus of elasticity can lead to better wear resistance of the flank profile, especially when considering materials with beech fibers compared to materials with spruce fibers. A lower modulus of elasticity could also have an indirect effect on the generation of higher temperatures in the meshing zone, which can also lead to higher wear of the gear, as can be seen in the case of gears with spruce fibers. The increased wear coefficient of the WPC with spruce fibers at 0.6 Nm can lead to a shorter lifespan of the test specimen.

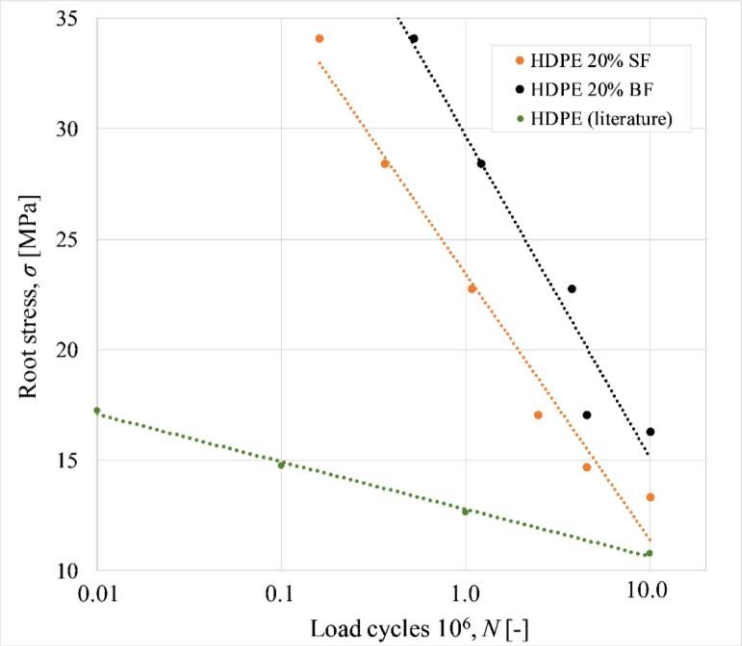

Figure 6. Stress-cycle (*S-N*) curves.

Based on the durability experiments and measured wear characteristics for both types of materials, fatigue life curves were modelled for both gear pair combinations. **Figure 6** presents the stress-cycle curve (*S-N* curve). To have a reference for the fatigue life of generic HDPE material, the S-N curve is added to the figure taken from the obtained literature. From the figure, it can be concluded a slighter better fatigue performance of HDPE reinforced with beech fibers due to better already described mechanical properties of the material and more resistant wear characteristics which prevent loss of material in the root and consequently shorter duration.

Figure 7 presents the failure mode of the observed wood composite gears. The failure mode is called tooth root fracture due to fatigue which is a consequence of the cyclic bending loads produced from steel driving gear synchronizing with meshing frequency. **Figure 7** presents the mentioned type of failure mode for each observed gear.

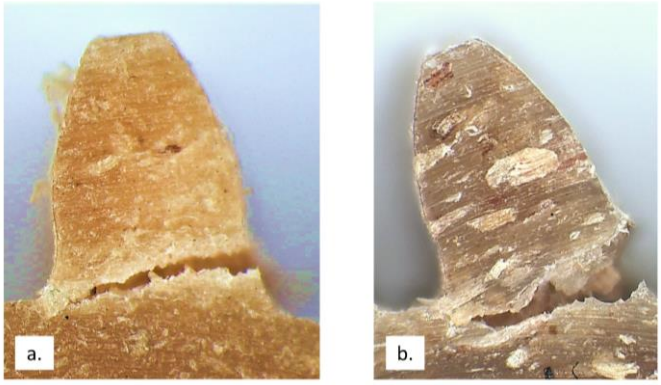

Figure 7. Failure mode: a. HDPE reinforced with 20% wt. spruce fibers and b. HDPE reinforced with 20% wt. beech fibers.

4. Discussion

When designing polymer-based gears, the main disadvantage is the lack of data on the fatigue strength of the various materials required for an accurate calculation for the selected gear application. This information cannot be found in the technical data sheets. Based on tests, it is possible to obtain data on the durability of gears, based on which the fatigue strength of the material can be calculated in the form of *S-N* lines.

This paper demonstrates the use of high-density polyethylene (HDPE) reinforced with equal weight percentages of natural spruce and beech wood fibers to produce gear components suitable for load-level power transmission applications, such as domestic drive mechanisms. As part of the research, blends of HDPE reinforced with 20% spruce fibers (SF) and HDPE reinforced with 20% beech fibers (BF) were produced to characterize and evaluate the performance of wood polymer composites (WPC). This could lead to the formation of a new material database in the field of material conversion data and the possibility of introducing materials into specific applications shortly, with the prospect of promoting sustainable green engineering in terms of reuse after the recycling process.

From the results of the experimental research and analysis, it can be concluded that the experiments show overall better operational properties of the gear pair with beech fibers compared to spruce fibers in the HDPE matrix. The composite material reinforced with beech fibers withstands the cyclic fatigue loads that occur during meshing better than the composite material reinforced with spruce fibers. The fatigue life achieved and wear rates for the two composites tested are comparable to the results found in the literature by Bravo et al. (2018) and Ghazali et al. (2017) in the field of testing BIO-based polyethylene. The calculated wear coefficients for the tested composites show satisfactory wear resistance depending on the applied loading conditions. The typical failure mode is tooth root fracture.

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

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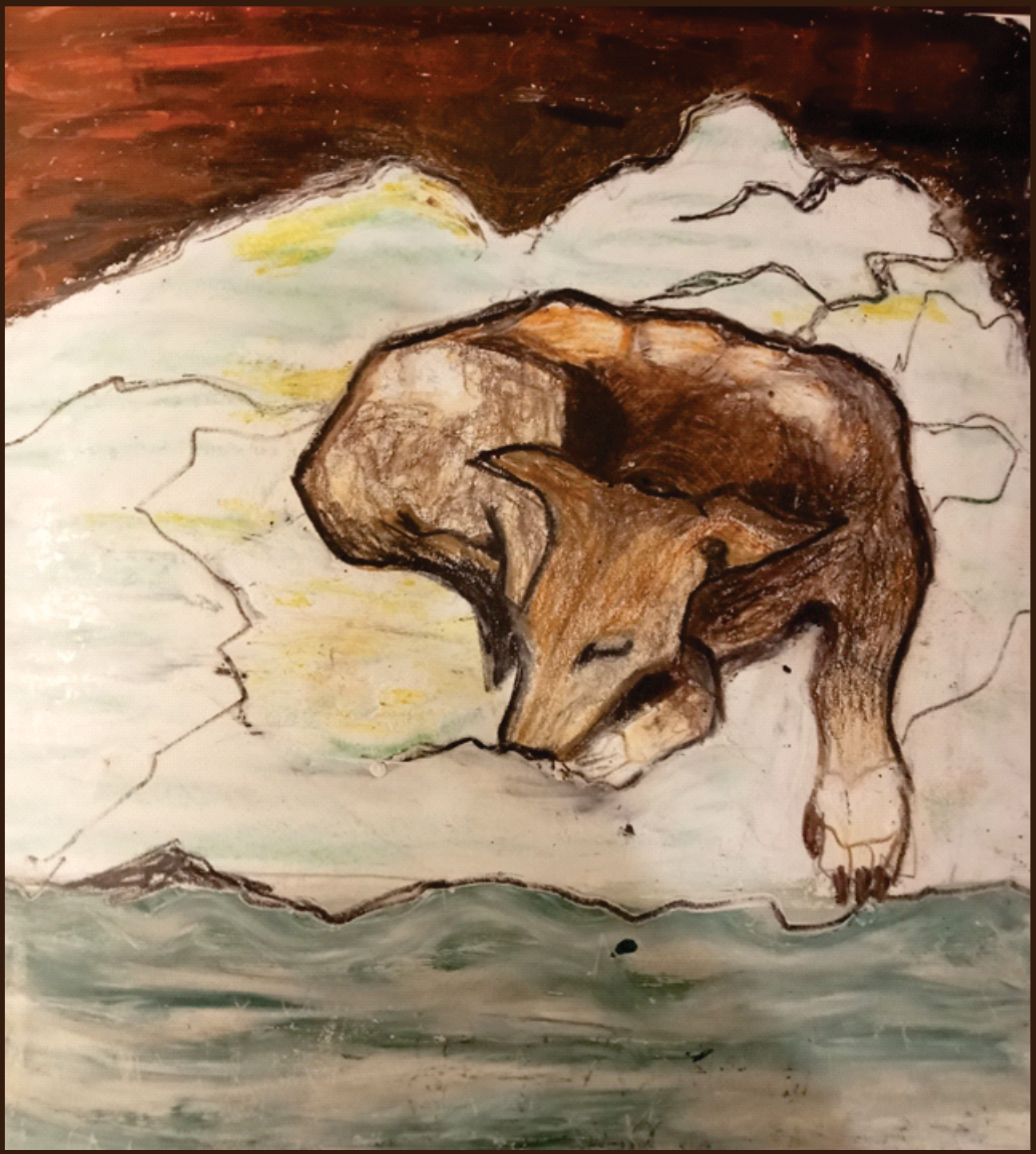

Invited lecture/Reaserch

Weathering Effects on Cellulose Acetate Microplastics from Discarded Cigarette Butts

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Abstract:
Cellulose acetate, commonly used in cigarette filters, poses environmental concerns due to its questionable (bio)degradability and prevalent presence in nature. This study compares the weathering effects on two types of cigarette filters: traditional and newer filters used in heated tobacco devices (HTP). Microplastics were derived from used cigarette parts and then subjected to artificial aging. Analytical techniques (Thermogravimetry-Differential Scanning Calorimetry TG/DSC, Fourier-Transform Infrared Spectroscopic Analysis (FTIR)) and loose bulk density measurements were employed pre- and post-aging cellulose acetate of both types of samples. Despite increasing evidence influencing European Union (EU) directives on tobacco product disposal, there's a lack of systematic analysis on the weathering impact, especially concerning the touted environmental benefits of newer filters. Results indicate decreased particle size in cellulose acetate filters post-aging. Variances were observed in thermal behavior, yet FTIR spectra remained unchanged.

Keywords: **C**ellulose acetate; Cigarette filters; Microplastics; Artificial weathering; UV radiation impact, Polymer degradation

1. Introduction

Microplastics pose a global threat to aquatic and terrestrial ecosystems. Annually, around 4.5 trillion cigarette filters contaminate various natural environments (WHO, accessed 30.11.2023). Tobacco products significantly impact the environment from cultivation to disposal, releasing microparticles, and nanoparticles, carbon, and heavy metals (Belzagui et al., 2021; Moerman & Potts, 2011; Zafeiridou et al., 2018) . Cigarette filters, primarily composed of plasticized cellulose acetate fibers, degrade into microplastics, further aggravating environmental pollution. These plastics face diverse conditions like rain, abrasion, salt, temperature fluctuations, and crucially, UV radiation, hastening their breakdown (Erdal and Hakkarainen, 2022; Felipe da Silva et al., 2023; Hon, 1977; Poppendieck et al., 2020; Vanapalli et al., 2023). Cellulose acetate fibers degrade notably under Ultra Violet (UV) light, with significant changes observed beyond 235 nm wavelengths (Hon, 1977). Studies on their degradation have examined diverse environments, including soil, compost, freshwater, seawater, and artificial seawater (Araújo et al., 2022; Yadav & Hakkarainen, 2022). Recognizing this, the World Health Organization and the EU Directive emphasize reducing the impact of plastics like cellulose acetate in tobacco products (Directive (EU) 2019/904, accessed 30.11. 2023; WHO, accessed 19.5. 2023). Recycling these filters remains challenging due to their slow hydrolysis into cellulose and acetic acid, posing environmental risks (De Fenzo et al., 2020). The newer tobacco products, incorporating poly(lactic acid) (PLA) polymer films alongside cellulose acetate, contribute additional environmental strain (Bonanomi et al., 2020). However, both PLA and cellulose acetate undergo slow biodegradation under environmental conditions (Bonanomi et al., 2020). This study explores the impact of UV and visible light, simulating natural sunlight, on cellulose acetate comparably gained from classic and newer cigarette butts (ISO 4892-2, 2013; Q-LAB, accessed 30.11. 2023).

2. Materials and methods

2.1. Materials

The research involved the collection of used cigarette filters to create two distinct forms of cellulose acetate (CA) microplastics in powder form. These variations included: (a) powder derived from filters of conventional cigarettes and (b) powder sourced from cigarette filters used in HTP. To ensure the purity of the samples, all filters were meticulously separated from the remaining cigarette components, such as the wrapping paper, and great care was taken to prevent any contamination from tobacco or ash.

In the case of HTP filters, the process involved removing the biodegradable, pleated poly(lactic acid) film, which acts as a cooling plug for aerosol cooling purposes, but this part of filters was not the subject of this study. Subsequently, the CA cigarette filters were individually ground using a ball mill to create loose powder for each of the filter types.

All used cigarette filters obtained for this study from regular/classic cigarettes belonged to the same brand, specifically BOSS classic cigarettes manufactured by Tobačna Ljubljana d.o.o., Slovenia. Filters from heat tobacco devices, known as HTP or commonly recognized as IQOS ("I Quit Ordinary Smoking" by Philip Morris International, Inc., USA), were obtained under the brand name HEETS. For clarity, the CA filter samples were designated as classic and HEETS, respectively.

2.2. Methods

The samples were first milled and analyzed using FTIR, TGA/DTA analysis, and loose bulk measurements, then they were exposed to weathering in a standardized chamber for accelerated aging and analyzed again.

2.3. Milling process for samples preparation

The collected and prepared materials were first ground in a laboratory mill Millmix 20 (Domel, Slovenia) to micro particles (**Figure 1**). Millmix 20 is a vibrating ball mill with two 50 mL grinding drums, in which the grinding of cigarette filters from CA took place in two stages. The filling mass for grinding cigarette filters was up to 1 g. Cigarette filters from CA were first ground for 1 minute with three grinding bodies, 8 mm in diameter and with a frequency of 30 Hz, so that the filter fibers were separated, then they were ground for another 3 minutes with grinding bodies, with a diameter

of 25 mm, also with a frequency of 30 Hz. After milling, we sifted samples through a 63 μm sieve raster to ensure the homogeneity of the sample.

Figure 1. Prepared cellulose acetate microplastics from used cigarette butts samples: from classic cigarette filters (left) and from HEETS filters (right).

2.4. Artificial weathering

Cigarette filters, ground into fine particles, were meticulously placed within glass petri dishes and shielded by a layer of quartz glass to safeguard against contamination or any potential sample loss during exposure. These milled filters underwent accelerated aging for a period of 1000 hours (equivalent to 42 days) inside a Q-SUN Xe-3 UV chamber, manufactured by Q-Lab in Bolton, UK. The chamber was equipped with three powerful 1800 W xenon lamps, specifically calibrated to emit wavelengths akin to natural sunlight.

Throughout the exposure duration, the ground cigarette filters underwent thorough daily mixing and homogenization to ensure uniformity and consistency in the aging process. The samples were subjected to irradiation at a power level of 60 W/m^2 while maintaining a chamber temperature of 38 °C, while the black standard was maintained at a temperature of 65 °C.

2.5. Fourier-Transform Infrared Spectroscopic Analysis (FTIR)

In order to determine differences in the used material before and after artificial weathering for the cellulose acetate filters, attenuated total reflection Fourier-transform infrared spectroscopy (FTIR) was carried out using a FTIR Spectrum Two spectrometer (PerkinElmer, Waltham, MA, USA). The spectra were recorded from 400 cm^{-1} to 4000 cm^{-1}, with an average of four scans taken at a resolution of 4 cm^{-1}.

2.6. Thermal analysis

We performed TGA/DSC analysis, which simultaneously measures the change in heat flow and the change in mass of the polymer material, depending on temperature, time and atmosphere. The degradation/disintegration temperature of the polymer material is determined by the first derivative of the TGA curve. In addition, endothermic or exothermic processes in the sample are simultaneously monitored during the analysis with the help of a temperature sensor, which affect changes in the heat flow and are determined with the help of the DSC signal. Thermal properties of artificially aged and non-aged used cigarette filters were determined by simultaneous TGA/DSC thermogravimetric analysis, on a Mettler Toledo TGA/DSC 3+ device. A 40 μL aluminum pot was used for the characterization. The testing of both types of cigarette filters was carried out in a nitrogen atmosphere, with an N_2 flow of 20.0 mL/min, in a temperature interval from 25 °C to 600 °C and a heating rate of 10 °C/min. At 600 °C, we switched from nitrogen to oxygen atmosphere, with an O_2 flow rate of 20 mL/min, and maintained this temperature for another 20 minutes.

2.7. Loose bulk density

The loose bulk density of the samples was determined by measuring the mass (using a Mettler Toledo AT201 analytical balance; Mettler Toledo, Switzerland) of a known volume of the container, without tapping, at room temperature (23 ± 2) °C (European Medicines Agency, 2010).

3. Results

3.1. FTIR analysis

Using FTIR spectroscopy (**Figure 2**), we compared the spectra of unaged CA cigarette filters (blue spectra) with artificially aged CA filters (red spectra) and observed an almost unchanged course of the curves after 1000 h of exposure to UV radiation for both samples: classic cigarette filters and HEETS filters. Absorption bands characteristic of the acetyl group in the CA structure appear at the same wavenumbers in all unaged and artificially aged CA cigarette filters. Even the intensity of the absorption bands remained almost exactly the same for CA cigarettes after UV exposure.

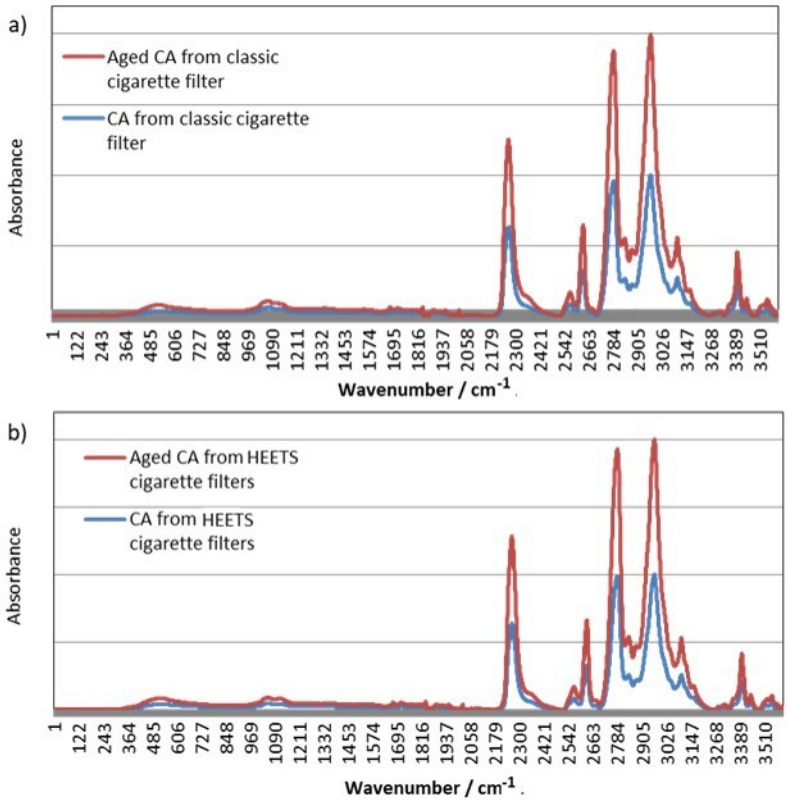

Figure 2. FTIR spectra of CA microplastics from used cigarette butts (a) from classic cigarette filters and (b) from HEETS filters.

3.2. Thermal analysis

The degradation of cigarette filters was assessed through simultaneous TGA/DSC analysis (**Figure 3**). In unaged CA cigarette filters, the initial mass loss commenced at a marginally lower temperature (Td1), coinciding with an endothermic peak around 138 °C. This peak is linked to the evaporation of absorbed moisture or volatile organic components trapped in the filter during smoking. Additionally, it corresponds to the decomposition of plasticizers present in the cigarette filter manufacturing process (De Fenzo et al., 2020). The absorbed water and volatile organic components in artificially aged CA cigarette filters were slightly reduced (-5.8 % for CA classic – Figure 3a and -8.6 % for CA HEETS – **Figure 3b**) due to their prior elimination through elevated temperature UV exposure for 1000 hours. Subsequently, used CA cigarette filters exhibited greater mass loss, attributable to washing and the release of chemical compounds, aligning with findings reported by other researchers (Joly & Coulis, 2017).

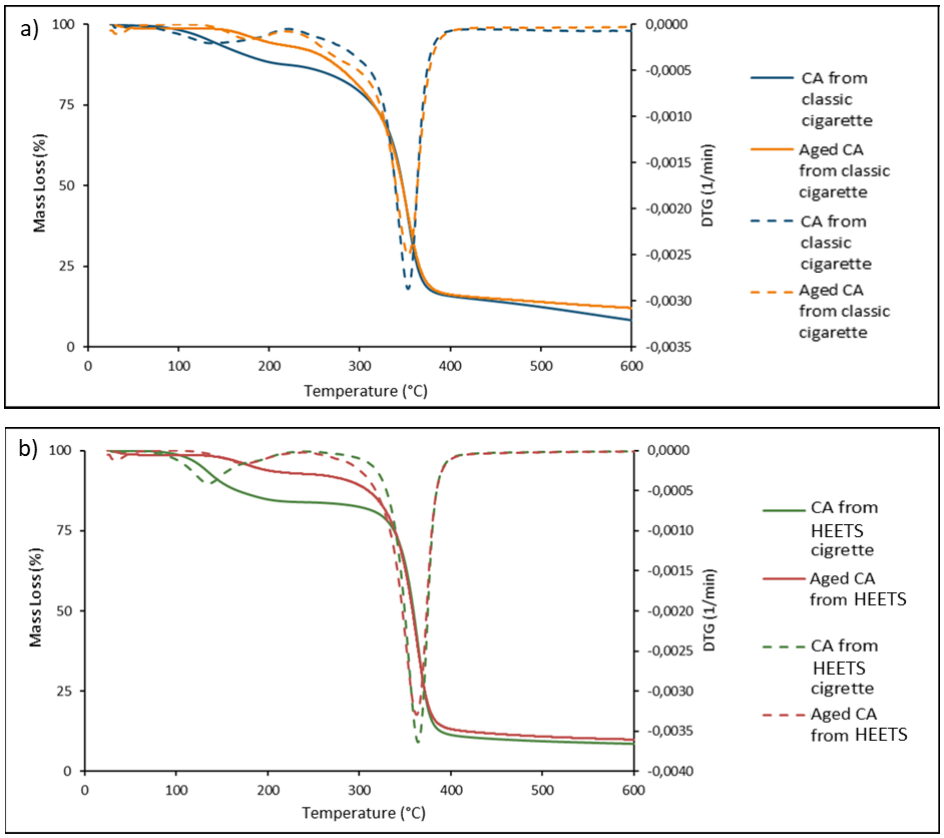

Figure 3. TGA/DTG analysis of unaged and artificially aged (a) CA classic and (b) CA HEETS cigarette filters.

Moreover, the degradation of CA in these filters occurred in a single step. This breakdown involves the degradation of sugars, lignin, pectin, and hemicellulose (Baker, 1987), alongside the deacetylation/depolymerization process of CA (De Fenzo et al., 2020), impacting the breakdown of glucosyl bonds (Barud et al., 2008). The organic CA component's decomposition temperature (Td2) in CA HEETS cigarette filters was approximately 367 °C, slightly higher compared to CA classic cigarette filters, which decomposed around 358 °C. This variance also manifests in the decay rate (ΔY), marginally higher in both unaged and artificially aged CA classic filters. This distinction might be linked to differences in filter composition, manufacturing processes of CA fibers, and maybe the method of using these filters.

The exothermic peak indicating the soot decomposition (Tds), observed in both unaged and artificially aged CA cigarettes, emerged at approximately 600 °C and is primarily influenced by carbon content. The heightened proportion of soot in artificially aged CA cigarette filters resulted from UV radiation exposure, impacting particle size reduction and an increase in their specific surface area. This effect was confirmed through loose bulk density measurements.

3.3. Loose bulk density

We conducted loose bulk density measurements on both unaged and artificially aged classic and HEETS cigarette filters (**Table 1**). Notably, all artificially aged CA cigarette filters exhibited a higher loose bulk density. After artificial aging, the loose bulk density of CA classic filters was increased by approximately 14 %, while for CA HEETS it was risen by about 9 %. Moreover, for all CA HEETS cigarette filters it was marginally higher than that of classic cigarette filters indicating the presence of smaller particles in the case of HEETS filters. This variance could potentially stem from differences in the

manufacturing process of the filters. However, it's plausible that the increased loose bulk density might also be linked to the distinct usage method between classic cigarettes, which burn, and CA HEETS. It might be associated with reduced filter contamination by chemicals or moisture. Such contamination typically causes particle adhesion, resulting in the formation of agglomerates.

Table 1. Results of average loose bulk density of unaged and artificially aged CA cigarette filters.

Samples	Non-aged	Aged (1000 h xenon chamber)	Changes in loose bulk density (%)
CA classic	0.21	0.24	14.3
CA HEETS	0.34	0.37	8.8

4. Discussion

We investigated the impact of UV light on the aging process of CA samples gained from cigarette filters. Analysis via FTIR spectra revealed no discernible differences between the spectra taken before exposing the samples to UV radiation and those obtained after controlled UV exposure. The observed trends are comparable to reports in the literature on the degradation of CA cigarette filters, where the authors also determined the unchanged chemical structure of microfibers from used CA cigarette filters after exposure to fresh water and natural sunlight for 18 months (Belzagui et al., 2021) and after exposure to 5 different environmental conditions for 720 days (Bonanomi et al., 2015).

However, complementary analyses including TGA/DSC evaluations and loose bulk density measurements showcased notable alterations in cellulose acetate samples derived from traditional cigarette filters versus those from HEETS filters.

Significantly, the exposure to UV light induced changes in the thermal stability of the samples and led to an increase in loose bulk density. These findings indicate evident physical transformations occurring during exposure to UV radiation.

5. Conclusion

Conclusively, our investigation demonstrates that UV light, an integral part of the solar spectrum, did not visibly induce chemical changes in cellulose acetate within a controlled 1000-hour exposure to accelerated UV radiation (approximation of sunlight). Therefore, it can be inferred that even discarded cigarette butts, which are exposed to sunlight containing UV radiation, do not undergo significant chemical alterations. Nonetheless, it's important to note that cigarette butts in the environment experience a variety of other influences, including abrasion, temperature fluctuations, and UV radiation, contributing to the breakdown of cigarette filters into microplastic particles. Intriguingly, these microplastics exhibit enhanced thermal stability when exposed to UV light.

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

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Invited lecture/Review

Digital education in the development of the company's human capital

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Abstract:
The company's human capital is its biggest asset, so its development is one of the essential areas of the company's development strategy. The concept of "human capital" has evolved for many years in connection with technological progress. And at each stage, human capital was a crucial element of business development. Today, we live and work in the era of digitalization, which has fundamentally changed all aspects of human life, including the approach to human capital development. An essential element in the development of human capital is the constant training of employees because, in the current conditions, the employee's knowledge, skills and competencies quickly become outdated, and, therefore, they need to be constantly updated. However, studying in the classical sense (attending classes, communicating with the lecturer, assignments, etc.) takes time and effort. It complicates human capital development under conditions of a high pace of activity. However, the situation has fundamentally changed in recent years due to the digitization of education. Today, digital education allows employees to study and work simultaneously, without prejudice to the quality of the activity results and the knowledge obtained. The article shows options for introducing digital education tools into the company's activities to expand human capital development opportunities. Attention is paid to the formation with the help of digital education tools of an individual training trajectory, which allows employees to increase the effectiveness of their training. It is considered employee competencies that can be developed and acquired due to digital education. The advantages and disadvantages of using such tools in the company's work are analysed.

Keywords: Human capital; Lifelong learning; Digital education; Competencies; Individual learning trajectory; Employee.

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1. Introduction

The company's human capital is its biggest asset, so the sustainable development of employees is one of the essential indicators of its success. It should be noted that the concept of "human capital" has evolved over the years in connection with technological progress, and at each stage, human capital has been a crucial element of business development. To date, we live and work in the era of digitalization, which has fundamentally changed all aspects of human life. For now, many work processes that were performed by humans have become digital and do not require human involvement. However, despite this, human capital remains the driving force of business development, so it needs to be studied to ensure its preservation and development.

The most effective way to develop human capital is education. Over the past decades, a new approach to its implementation has been formed in education, which has received the name lifelong learning. The idea of lifelong learning is associated with the half-life of knowledge concept, which Fritz Machlup proposed in the 20th century. The concept essence is that due to the rapid updating of information, some knowledge becomes irrelevant even before a person learns it. Accordingly, there are fields of activity in which the de-actualization of knowledge occurs very quickly, for example, the information technologies field. Under such conditions, knowledge needs constant updating; therefore, a person has to learn throughout life (Mariz-Péreza et al., 2012; Pasban & Nojedeh, 2016; Boon et al., 2018; Protasenko & Mygal, 2020; Protasenko et al., 2021; Wujarso et al., 2021; Ray et al., 2023).

In addition, the education of the 21st century uses new tools to ensure the learning process, which is manifested in the following. Firstly, education is not a universal program for everyone but an individualized system aimed at revealing a person's natural abilities and obtaining the knowledge he needs for further professional development. Secondly, Education is gradually becoming digital, which allows expanding opportunities for providing continuous education since digital education functions due to the use of digital technologies that work with the help of the Internet (Laal, 2011; Han & Shin I, 2016; Cefalo & Kazepov , 2018; Mygal & Protasenko, 2019; Alenezi, 2021; Gabriel et al., 2022; Haleem et al., 2022; Alenezi et al., 2023).

Therefore, the purpose is to study the current trends in digital education, to determine the employee priority competencies, and to consider possible ways of implementing digital education tools in the company's activity.

2. The role of education in human capital development

First of all, it is necessary to analyse the meaning of the concept of "human capital" to determine the relevant competencies of the employee and possible ways of implementing digital education tools in the company's activity. Today, human capital is a specific stock of health, knowledge, skills, abilities, and motivation formed and developed as a result of investments and accumulated by a person, which is purposefully used in one or another economic activity, contributes to the growth of labour productivity and, due to this, affects on the income growth of its owner, the profit of the enterprise and the national income (Grishnova, 2014).

Let's pay attention to several fundamental points arising from this definition:

firstly, human capital is a specific stock of health, knowledge, skills, abilities and motivations of the employee formed and developed as a result of the company's investments, and not a set of characteristics that the employee must possess at the time of hiring. Therefore, for the effective operation of human capital, it is necessary to develop it through investment in development and not just search for employees who meet a complex and long list of employer requirements;

secondly, the growth of human capital as a result of cost-effective investments in its development naturally leads to the growth of the company's income;

thirdly, the increase in the company's revenue due to the development of human capital stimulates further investment in it, which builds the basis for future income.

At the same time, investments in the development of human capital contribute not only to the development of a specific company but also stimulate socio-economic development by increasing the general level of well-being of the population (**Figure 1**).

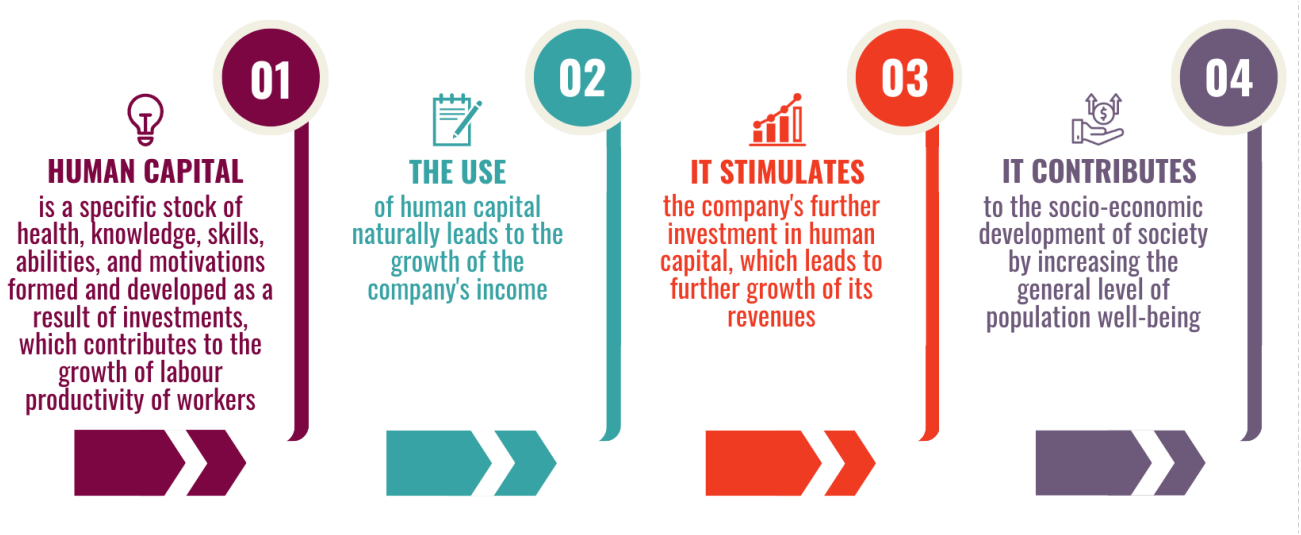

Figure 1. The role of human capital in the company and society development.

The next question is the competencies of a modern employee. As you know, an employee's qualification measure is his competence. It determines the employee's ability to perform his functions qualitatively and flawlessly in normal and extreme conditions, successfully master new knowledge and skills and quickly adapt to changing conditions. Practice shows that people with the same level of education work with different productivity and achieve different results. The very concept of employee competence allows us to identify the cause of these differences and determine the most effective direction of work with human capital.

The development of the employees' competencies takes place in acquiring the necessary knowledge, skills and abilities, which reveal a person's abilities and are embodied in the growth of his professional level and potential. Under modern conditions of hypercompetition, an employee must improve his qualification level throughout his life. Thus, the current stage of society's development shows a tendency towards rapid ageing of the knowledge and experience of employees, which is expressed in the lagging of personal knowledge and expertise from modern requirements for the profession. That is why human capital development costs in the world's leading companies make up to 10% of the salary fund.

So, what should be the competencies of a modern employee? The analysis of information on this issue showed that there are many such competencies since, to create a company development plan, it is necessary to form individual sets of competencies of employees that will ensure its development. However, among these competencies, the most in-demand ones can be singled out (**Figure 2**).

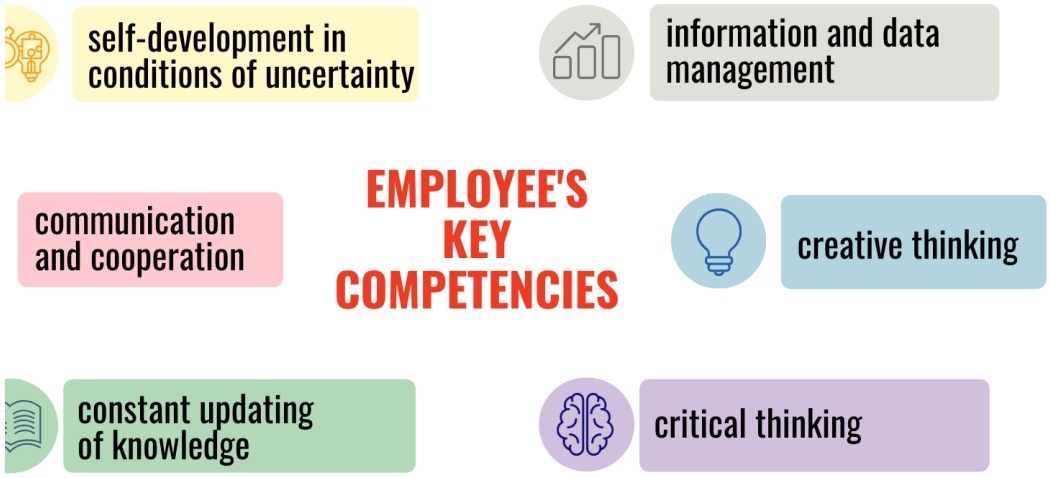

Figure 2. Employee's key competencies.

Let us describe these competencies:

1. Communication and cooperation involves a person's ability to use various digital tools to ensure interaction with other people and achieve set goals.

2. Self-development in conditions of uncertainty reflects a person's ability to self-study, set educational goals necessary for solving tasks, and choose ways to solve them, including using digital tools.

3. Creative thinking involves the ability to generate new ideas, rebuild known ways of solving problems, and create alternative options for actions to build more effective algorithms.

4. Information and data management describes the ability to find sources of information necessary for solving tasks and problems.

5. Critical thinking involves a person's ability to evaluate information and its reliability and draw logical conclusions based on incoming information and data.

6. Constant updating of knowledge includes expanding knowledge, improving skills and competencies.

In this way, human capital development is a work in advance since the employee must have competencies that not only meet the modern needs of society and the economy but also ahead of these needs. Only under this condition it is possible for the company to progress and maintain its competitiveness. The only way to ensure the continuous development of human capital is a lifelong learning (**Figure 3**).

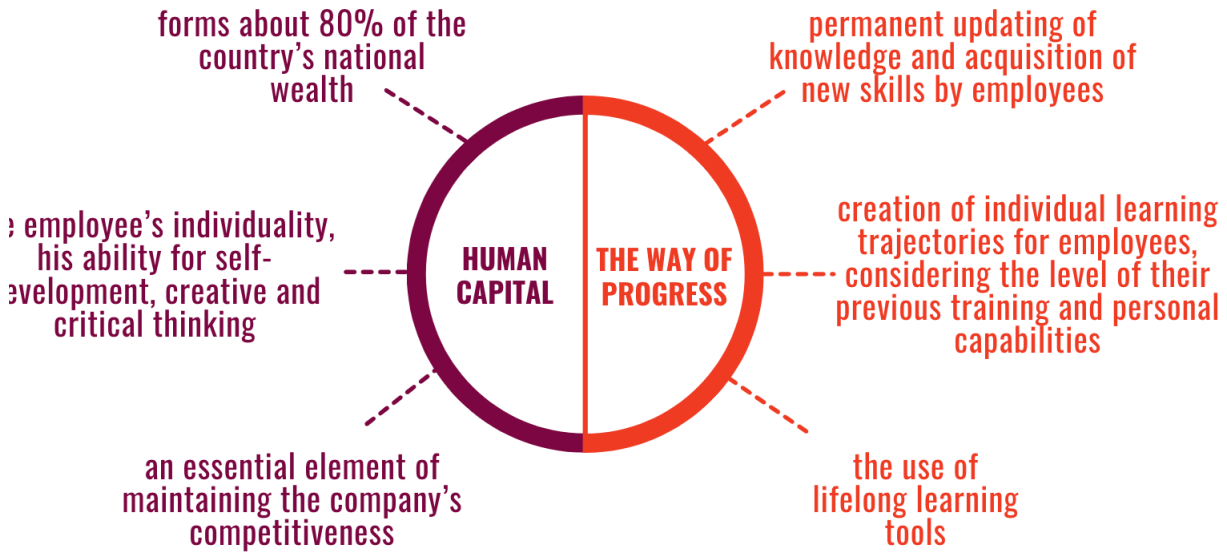

Figure 3. The role of lifelong learning in human capital development.

3. Digital education in providing lifelong learning

The introduction of lifelong learning for human capital development has led to the modernization of education. Following this, six principles of lifelong learning are defined (A Memorandum on Lifelong Learning):

1. New basic knowledge and skills for everyone. The goal is to guarantee universal continuous access to education for obtaining and updating the skills necessary for the inclusion of a person in the information society.
2. Increasing investment in human resources. The goal is to increase investment in human resources to raise the priority of human capital.
3. Innovative methods of teaching and learning. The goal is to develop new learning methodologies for the lifelong learning system. With the development of the information society, educational technologies are becoming more and more user-oriented. Learning methods in formal and informal education systems should be user-oriented, changing in the direction of personal motivation, critical thinking and learning ability.
4. A new evaluation system of received education. The goal is to change approaches to understanding and recognizing educational activity and its results, especially in informal and informal education. Recognition of such education will help increase motivation for continuous education.
5. Development of mentoring and consulting. The goal is to provide everyone with free access to information about educational opportunities and necessary consultations and recommendations throughout their lives.
6. Bringing education closer to the place of residence. The goal is to bring educational opportunities closer to the residence place of consumers with the help of a network of learning and consulting points and the use of information technologies.

The easiest way to ensure lifelong learning is through digital education tools. Currently, the digital transformation of education is a complex work on building an ecosystem of digital solutions in education, including the creation of a safe electronic educational environment, ensuring the necessary digital infrastructure of educational institutions, and increasing the level of digital competence.

The essential means of providing digital education is the learning management system (LMS). LMS is used to develop, manage and distribute online educational materials with shared access.

LMS is characterized by a convenient, flexible interface and functionality. It allows taking training to a qualitatively new level. LMS allows organising the learning process according

to the needs of a specific group of learners and tracks the success of learning by creating online courses available at any time and anywhere in the world where there is an Internet connection. At the same time, all training materials are stored in one place, and it is convenient to adapt and review them depending on the training goals and the type of company's activity.

In addition, the company can, according to its needs, purchase an LMS that is already working on the market and has user recommendations or develop its own. The company needs to analyse several factors to decide which option is better.

1. Determine the educational needs of the company. There are now a lot of learning management systems on the market for digital education services, so it is necessary to have a clear idea of what exactly the company needs from an LMS and how this system will help achieve the desired results. In addition, it is necessary to determine the target audience, namely: the student's age, the level of digital competence, the time of the classes, the duration of the courses, which digital devices are necessary for the educational process, etc.
2. Determine the requirements for the LMS system. The list of system requirements should be detailed to narrow down the search. Requirements can be grouped as follows (**Figure 4**).

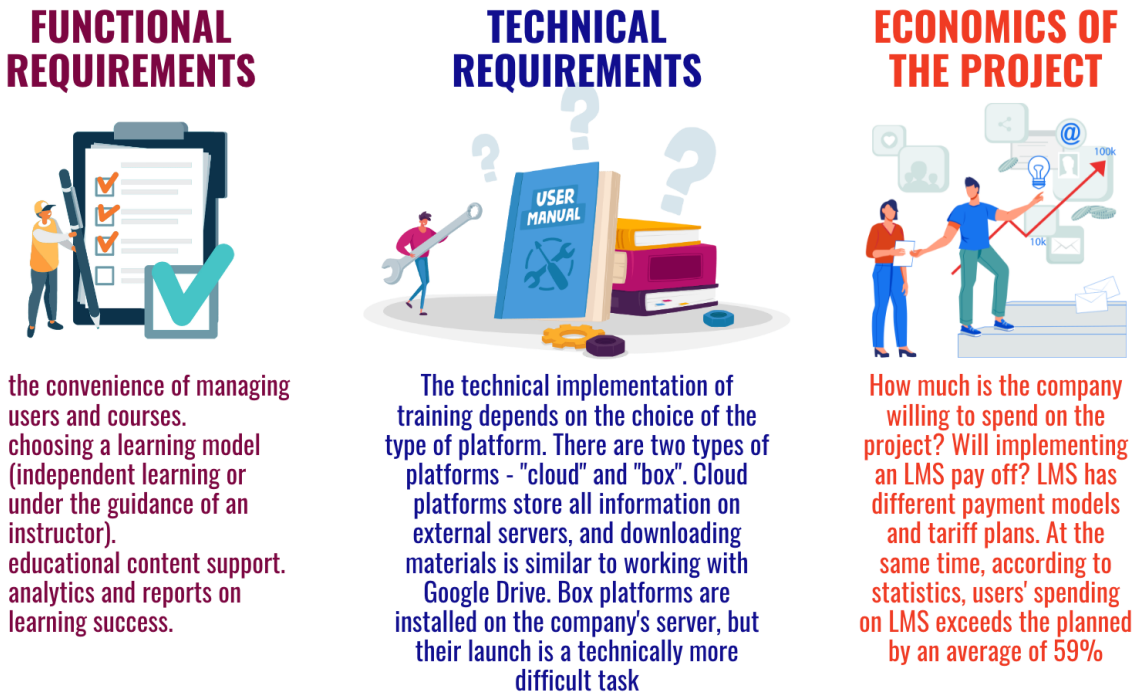

Figure 4. Key company's requirements for an LMS.

3. Is there an LMS that most fully and accurately meets the company's requirements? There are only two ways to find out. The first is to study developer websites, reviews, reports, and user recommendations. The second is to test the LMS in practice. Many developers provide access to a demo version. In this way, the company can evaluate the system's functionality without risking anything.

To improve the efficiency of human capital development, companies can use the principles of forming an individual learning trajectory along with digital education tools. However, the organization of training according to an individual learning trajectory requires a particular approach. This problem can be solved in two ways:

1. The first method is external assistance. In this case, the differentiation of training is carried out by specialists based on the results of the study of the individual abilities of the

employee. Accordingly, they differentiate the material for learning by the degree of complexity, orientation or other parameters.

2. The second way is independent determination. In this case, the employee builds the education trajectory independently, based on his needs and capabilities. In other words, each person can create his learning trajectory of mastering the necessary knowledge.

An individual learning trajectory is a personal way of realizing an employee's potential, which is formed considering his abilities, interests, needs, motivation, opportunities and experience. An individual learning trajectory is implemented through a free choice of types, forms and pace of study, educational courses and their level of complexity, methods and means of education (**Figure 5**).

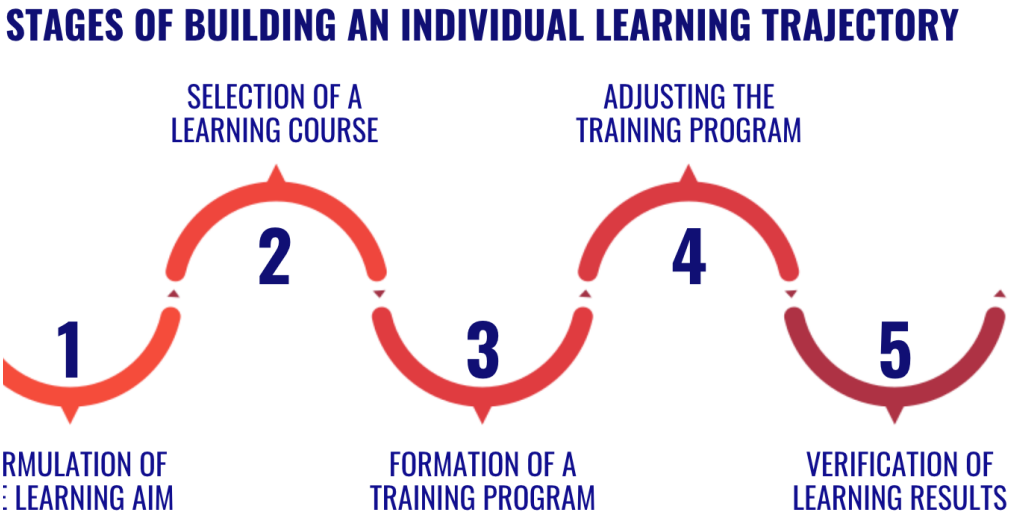

Figure 5. Stages of building an individual learning trajectory.

4. Conclusions

Therefore, the company's human capital is its biggest asset, so the development of employees is one of the key indicators of success. The lifelong learning concept allows the employee to constantly update knowledge and skills and remain relevant in the labour market. Such a trend led to an increase in interest among companies in the application of this concept for human capital development. However, this demand cannot be satisfied within the framework of traditional education, which is why the problem of incompatibility of the classical education system with the new needs of society arises. This situation contributes to the formation of a different approach to the organization of educational activities of employees - training that meets the interests and capabilities of employees at various levels and organically fits into their lifestyle. This approach is implemented with the help of digital education tools, which allows employees to minimize the time and effort spent on improving their qualifications. At the same time, individual learning trajectories can be used to increase the efficiency of knowledge and skills acquisition by employees.

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Invited lecture/Review

Strategies for Resilience in a Dynamic World: from VUCA to BANI

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Abstract:
This paper delves into the evolving landscape of business resilience, examining five conceptual frameworks shaping strategies for success in an era of unprecedented uncertainty. The concepts of SPOD-world, VUCA-world, BANI-world, RUPT-world, and TUNA-world are scrutinized to provide a comprehensive understanding of the challenges and opportunities they present.
SPOD-world, characterized by Stability, Predictability, Order, and Determinism, demands a strategy emphasizing efficiency and optimization. In contrast, VUCA-world, marked by Volatility, Uncertainty, Complexity, and Ambiguity, necessitates adaptive strategies focused on agility and rapid response. BANI-world, emphasizing Brittle, Anxious, Non-linear, and Incomprehensible dynamics, calls for strategies rooted in flexibility and constant adaptation. RUPT-world, shaped by Rapid, Unpredictable, Paradoxical, and Turbulent changes, requires strategies capable of managing crises and fostering resilience. TUNA-world, characterized by Transitory, Unstable, Novel, and Ambiguous factors, necessitates strategies that prioritize innovation and creative problem-solving.
Each conceptual framework is analyzed in terms of its implications for sustainability in business. The paper concludes by addressing the specific challenges faced by Ukrainian businesses in turbulent situations, recommending a strategy tailored to the unique context. To sustain the economy, Ukrainian businesses are advised to adopt a hybrid strategy combining adaptive measures for VUCA challenges, resilience-building for RUPT scenarios, and innovation-driven approaches for TUNA dynamics. This nuanced strategy aligns with the BANI-world framework, ensuring a comprehensive response to the multifaceted challenges of the modern business environment.

Keywords: SPOD-world, VUCA-world, BANI-world, RUPT-world, TUNA-world; dynamic environment, sustainability strategy

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1. Resilience Strategies in the Dynamic World

In today's world, the term "dynamic world" is vital in describing the environment we live in. Dynamism involves constant and rapid changes in technology, economy, socio-cultural spheres and other areas. This world is a complex network of interconnected factors where stability is rare and uncertainty and instability become the norm.

In a fast-changing and uncertain world, it's important to have resilience strategies in order to adapt successfully (Falke et.al, 2010). This helps us adapt quickly to change and uncertainty, and helps us respond effectively to challenges. Without such strategies, individuals, organizations, and businesses run the risk of becoming irrelevant and uncompetitive. Resilience demands strategic thinking (Koronis & Ponis, 2018), not just responding to changes. This way, we can create reserves to deal with unforeseen difficulties.

2. Static, Predictable, Ordered and Durable World (SPOD-world)

SPOD-world describes a world characterised by existing static conditions and an unsatisfactory level of change.

The main characteristics of a static world are the absence of significant changes or fluctuations in technology, economy and socio-cultural sphere. Stability, predictability, and good order are seen as key structural elements of the SPOD world (Chaliuk, 2022). Although this type of existence might appear attractive initially, it can lead to a lack of innovation and a slowdown in development over time.

The main problems in the SPOD-world are fixed research models, difficulty adapting to new conditions, and a lack of innovation. When the environment becomes over-stable, organisations and societies may face difficulties in addressing contemporary challenges and technological opportunities. This can slow down progress and leave the world more open to sudden changes that can destroy the existing system.

3. Volatility, Uncertainty, Complexity, and Ambiguity World (VUCA -world)

The concept of a VUCA world has emerged as a comprehensive framework for describing the challenges and uncertainties faced by organizations in the current global business and societal landscape.

Organizations must constantly adapt to volatile conditions, make decisions in the face of uncertainty (Sarkar, 2016), have a holistic understanding of complex systems, and navigate through ambiguity. These are the essential skills required to thrive in a VUCA world.

In the face of VUCA challenges, organizations must strategically address several issues:

Traditional long-term planning becomes difficult due to unforeseen events that can disrupt established trajectories.

The dynamic nature of the VUCA world demands leaders who foster adaptability and resilience, steering their teams through uncertainty and volatility. This involves not only embracing change but also fostering a culture that thrives on learning from challenges and responding proactively to unforeseen circumstances.

Innovation is essential, requiring a culture that encourages experimentation and embraces failure as a learning opportunity.

The interconnectedness of global markets introduces additional complexities, but organisations can confidently navigate these challenges by being mindful of geopolitical risks and trade dynamics.

In the VUCA world, organisations must rethink their approaches to leadership, strategy, and decision-making (Zakharov, 2022). They should embrace the dynamic nature of the environment and foster a culture of adaptability and innovation.

4. Rapid, Unpredictable, Paradoxical, and Tangled World (RUPT -world)

In the modern global landscape, the term RUPT (Center for Creative Leadership, 2019) world refers to an environment where change is a constant and essential aspect of the surroundings.

This change is driven by factors such as rapid progress, unforeseeable external influences, paradoxical situations, and intricate connections between different elements.

In a rapidly changing world, it is essential for organizations and individuals to be proactive and adaptable. Cultivating resilience and agility is necessary to surmount the challenges brought about by swift and unpredictable shifts (Bartone, 2017). To achieve this, it is crucial to create a culture of continuous learning, which involves a commitment to staying abreast of new trends and fostering an environment conducive to innovation. Collaboration and swift adaptation to changing circumstances are imperative for excelling in the ever-changing world of RUPT.

Perceiving change as an opportunity for progress rather than a setback is crucial. Companies that embrace this perspective position themselves to capitalize on the possibilities offered by the perpetual dynamism inherent in the RUPT world.

5. Turbulence, Uncertainty, Novelty, and Ambiguity World (TUNA -world)

In today's world, the concept of a TUNA world emphasises the importance of adaptability and flexibility in navigating our complex environment (Gordon, 2016).

Successful adaptation is not just advantageous but vital for coping with constant changes. Practical strategies for successful adaptation are necessary for success in this dynamic reality.

In an ever-changing world, it is imperative to continually engage in education and stay abreast of developments. The capacity to swiftly and decisively navigate uncertainties is a vital skill crucial for success in the contemporary landscape. Additionally, cultivating adaptability and a proactive approach to acquiring new knowledge enhances one's resilience and ensures a competitive edge in a dynamic environment.

In order to successfully navigate unfamiliar scenarios and seize nascent opportunities, it is imperative for both individuals and organizations to cultivate a culture that encourages innovative thinking (Carvalho et. al, 2020). Embracing creativity and forward-thinking not only enhances adaptability but also positions entities to thrive in dynamic environments.

Fostering collaborative relationships and networks not only elevates the effectiveness of collective problem-solving and increases overall adaptability but also establishes a foundation for shared knowledge and resource exchange. This interconnectedness further strengthens the capacity for innovation and resilience in the face of challenges.

Strengthening resilience is crucial for bouncing back from challenges and setbacks, creating a foundation for long-term success.

By embracing these principles and methods, both individuals and organizations can thrive in the ever-changing TUNA world. Adaptability is crucial for success in a constantly changing environment. The strategies presented here provide clear guidance for navigating the turbulence, uncertainty, novelty, and ambiguity that are inherent in our dynamic reality.

6. Brittle, Anxious, Nonlinear, and Incomprehensible World (BANI -world)

The BANI-world framework (Cascio, 2020) offers an integrated approach to understanding and navigating the challenges of this environment.

To succeed in our complex reality, it is imperative to adopt a sophisticated and integrated approach to resilience. The following principles can help individuals and organizations effectively navigate this complexity:

To make informed decisions, it is crucial to have a comprehensive understanding of the fragility of certain environmental aspects.

Offering training to individuals and teams, enhancing their skills to effectively respond to evolving circumstances, and nurturing a culture of adaptability holds significant importance (Salun et al., 2019).

Enhancing robustness in the face of unexpected challenges can be achieved by embracing diverse perspectives and establishing redundancy in systems.

Building teams with diverse skill sets and backgrounds fosters interdisciplinary collaboration and problem-solving (Douglas, 2021).

Establishing mechanisms for continuous feedback not only enables swift adjustments and enhancements but also promotes a culture of open communication and continuous improvement.

Implementing flexible resource allocation strategies not only aids in addressing dynamic and unpredictable circumstances but also enhances organizational resilience and responsiveness to change.

Developing metrics to assess and measure resilience allows for continuous improvement and adaptation.

By following these principles, individuals and organizations can adopt a nuanced and comprehensive approach to resilience. This enables them to effectively navigate the multifaceted challenges presented by the BANI world. These strategies serve as a roadmap for not only surviving but thriving in an environment characterized by complexity and uncertainty.

Table 1. Comparison of Basic Strategies for Resilience for the Dynamic Worlds

Dynamic world model	Basic Strategies for Resilience	Advantages	Disadvantages
SPOD-world	Long-term strategic planning is best based on standardised processes, stable business models, and a focus on stability and predictability in the internal environment. This approach will increase the probability of avoiding risks in a structured and controlled external environment.	An organized environment reduces anxiety and provides clear direction, leading to consistent and efficient operations through established routines.	Resistance to change, lack of adaptability, potential stagnation, limited innovation, difficulty in responding to disruptions, difficulty in dealing with unexpected events
VUCA-world	Short-term adaptive and flexible planning is based on the introduction of innovations. Sustainable leadership thinking and continuous updating of knowledge, skills, and abilities are necessary for this.	The ability to adapt quickly to changes through integrated problem-solving is crucial in developing flexible strategies and expediting decision-making.	Initial uncertainty and discomfort, potential information overload, resistance to ambiguity, risk of failure and setbacks, and the possibility of paralysis in the decision-making process.
RUPT-world	To develop plans for innovation in response to change, it is essential to accept change and have a proactive and adaptive attitude towards it. This requires a culture of continuous learning, teamwork, and collaboration.	The strategy will quickly adapt to progress, remain resilient to unforeseen challenges, and respond promptly to environmental factors and new market opportunities.	The high variability of information will cause potential instability, increased risks, and active resistance to change.
TUNA-world	The strategy promotes adaptability and flexibility by fostering a culture of innovative thinking, continuous learning, and sustainable networks of team collaboration.	This strategy effectively deals with the turbulence of both the external and internal environment by enhancing the skills and knowledge of employees. This enables them to exploit new market opportunities through collective problem-solving, which builds additional resilience.	Information overload leads to decision-making fatigue, resistance to learning, and discomfort with change.
BANI-world	To enhance adaptability and flexibility, we must cultivate a culture of ongoing improvement. This is achieved through implementing dynamic resource allocation and sustainable feedback mechanisms to evaluate sustainability indicators in a constantly changing environment.	This approach enhances system reliability and enables efficient response to dynamic challenges by facilitating prompt decision-making, followed by swift adjustments and improvements based on flexible resource management and performance metrics.	Complexity, high resource intensity, and potential redundancy can lead to resistance to change and difficulties in quantifying sustainability.

7. Comparison of Basic Strategies for Resilience for the Dynamic Worlds

The scientific debate currently focuses on researching adaptation and resilience strategies in dynamic environments (Kunzelmann & Rigotti, 2022; Douglas, 2021). Selecting and implementing strategies to increase resilience is crucial for developing effective approaches to managing change and instability in various spheres of activity. Analysing these issues

with confidence is key to success. Conducting a comparative analysis of basic sustainability strategies in dynamic worlds such as SPOD, VUCA, BANI, RUPT, and TUNA is an important step towards improving strategic management and addressing the challenges posed by uncertain and rapidly changing environments. The advantages and limitations of each strategy in the context of the respective dynamic world will be revealed through the analysis.

The comparison of basic resilience strategies across SPOD-world, VUCA-world, RUPT-world, TUNA-world, and BANI-world reveals the diverse approaches required to thrive in these dynamic environments. Each world presents unique challenges and opportunities, influencing the choice of strategies for building resilience.

Dynamic worlds pose a spectrum of challenges ranging from resistance to change and uncertainty to the need for continuous learning and adaptability. To ensure the future development of each dynamic world, it is crucial to embrace the advantages of chosen strategies while mitigating their associated disadvantages. Balancing and refining these strategies fosters resilience and adaptability in the face of ever-evolving environments.

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

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Invited lecture/Review

Features of the Formation of Cross-Cultural Communication Skills in IT Business

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Abstract:
Effective establishment of cross-cultural communication is a prerequisite for business success, regardless of the field in which it is organized or already exists. The issue appears especially relevant against the background of the rapid development of information technologies, digitalization, socialization, and globalization of business. Cross-cultural communication is a process of information exchange among representatives of different cultures, which aims to establish mutual understanding through the achievement of conformity of different culturally determined value systems. As the sector of information technology grows ever more complex, Information Technology (IT) professionals are called upon daily to explain technology applications to coworkers from other departments, managers, and customers. Building effective cross-cultural communication within an IT company and establishing the right communication links with stakeholders affect the effectiveness of management decisions both at the operational and strategic levels. Barriers to the formation of cross-cultural communication skills in the IT industry include IT professionals being used to working in isolation or functioning within tight-knit teams, differing work expectations, varying values and customs, and different levels of technological knowledge. Formation of cross-cultural communication skills should create a basis for effective establishment of communication in the development team, providing support for the common organization of the group work on the processing of general information and information linked to the project, creating conditions for collaborative software development, and promoting the formation and development of corporate culture in IT companies.

Keywords: Communication; Cross-cultural communication; Skills; IT business; IT services; IT team

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An established part of the modern world is globalization. Globalization means that there is an increase in the interaction and interdependence between different countries and regions of the world in the field of economy, social, politics, ecologic, and culture. The globalization has been accelerated by many factors, including the increased inter-national interconnectedness, the increased migration, both legal and illegal, particularly from less-developed to more-developed countries, and the ability of information and communication technology (Thomas & Inkson, 2017). In a general sense the process of globalization is forming an infinite world, when state borders turn into formalities on the path of displaced both factors and results of processes of human management of the mankind.
Despite the talk of de-globalization (Qu & Yang, 2023; Salavrakos, 2023), the globalization index shows a growing trend (**Figure 1**). Moreover, the WTO report (World Trade Report 2023, 2023) contends that international trade continues to thrive and points to the expansion of digital services trade. And, the global IT services market size is expected to grow at a compound annual growth rate (CAGR) of 9.7% from 2023 to 2030 (IT Services Market Size And Share Analysis Report, 2030).

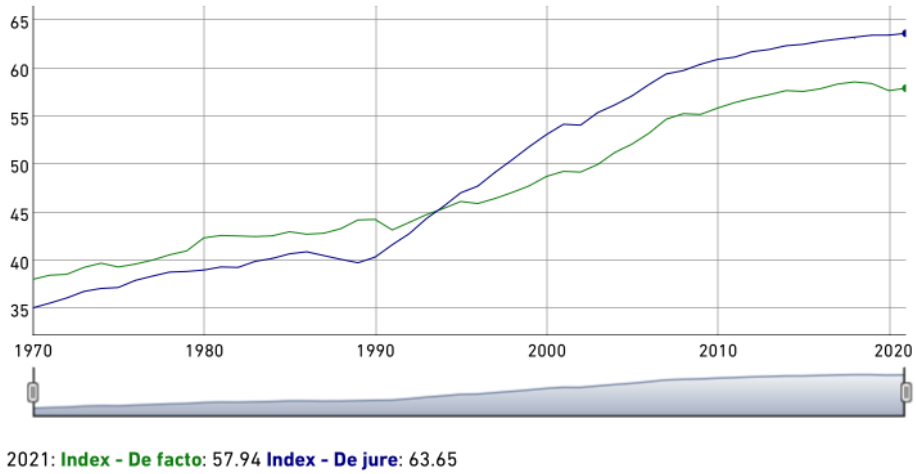

Figure 1. The dynamics of the globalization index (KOF Globalisation Index, 2023).

Definitely, globalization creates significant advantages for the IT business. The main ones are easier access to new sales markets, the possibility of attracting highly qualified specialists from around the world, acceleration of technology transfer, etc. At the same time, it also presents several problems and challenges: increases the risks of infringement of intellectual property rights; intensifies competition both in the sales market and in the labor market, creates threats to cyber security, and increases the problems of cultural, ethical and legal compliance. The problem of establishing effective cross-cultural communication is especially acute for the IT sector. Cross-cultural communication is a process of information exchange among representatives of different cultures, which aims to establish mutual understanding through the achievement of conformity of different culturally determined value systems (Sahadevan & Sumangala, 2021).
Cross-cultural communication is of paramount significance in the IT industry, driven by globalization, multinational workforces, the need for effective collaboration, and the importance of building strong client relationships. It plays a pivotal role in problem-solving, innovation, and adaptation to global markets. Moreover, it influences talent acquisition and retention while fostering cultural sensitivity, making it a vital skill for success in this dynamic and competitive field. That's why the significance of cross-cultural communication skills in the IT sector has grown fast. Effective cross-cultural communication is not just a desirable skill; it's a necessity for success in this dynamic and ever-evolving field.
The goal of research is to investigate and understand the specific aspects, challenges, and effective tactics involved in developing cross-cultural communication skills within the IT industry. Moreover, the publication aims to furnish an information, insights, and guidance, pivotal in enhancing the communicative competencies of individuals, enterprises,

and information technology professionals. This enhancement is particularly crucial for navigating and excelling in cross-cultural interactions, thereby facilitating efficacious collaboration and operational success within the intricate of a globalized environment.
The IT industry is continuously growing in complexity, requiring IT professionals to regularly communicate the utilization of technologies to colleagues from various departments, managers, customers, and interested parties. It is unquestionable that the development of efficient cross-cultural communication within an IT organization and the establishment of appropriate communication channels with stakeholders significantly impact the effectiveness of managerial decisions, both at the operational and strategic levels. Barriers to the development of cross-cultural communication skills in the IT business are multifaceted and can pose significant challenges for IT professionals. These obstacles often arise from the unique dynamics and characteristics of the IT field:

1. Isolated work: Information Technology professionals frequently exhibit a proclivity for solitary labor or working within tightly-knit teams of similarly specialized confreres. The shift towards an environment characterized by global dimensions necessitates a significant adaptation towards a paradigm that is inherently more collaborative and heterogeneous. This transition poses a substantial challenge for individuals who are habituated to autonomous activities. The demands for heightened intercultural interaction and diverse team dynamics require a recalibration of both professional and interpersonal skill sets in globalized professional contexts.
2. Divergent Occupational Expectations: Distinct cultural milieus exhibit variegated norms and anticipations concerning professional temporal allocation, the equilibrium between vocational and personal life, and methodologies employed in problem resolution. The process of maneuvering through these dissimilarities and achieving a concordance of expectations creates multifaceted challenges and may require compromise and flexibility.
3. Different Cultural Values and Customs: Cultural diversity often means that people have different values, norms, and traditions. These differences can be seen in workplace behavior, ways of communication, and how decisions are made. Therefore, IT professionals need to be really aware of and sensitive to these varied cultural aspects. Understanding and respecting these differences is crucial for effective communication across cultures, especially in today's global work environment.
4. Heterogeneity in Technological Proficiency of Knowledge: Professionals in the field of Information Technology may frequently interface with clients or collaborators possessing a different spectrum of expertise in technological domains. The task of articulating intricate technical concepts to stakeholders with primary proficiency in technological matters presents a substantial challenge. This task necessitates the cultivation of a communicative skill set that is adept at converting complex information into comprehensible and accessible formats, thereby bridging the knowledge gap and facilitating effective knowledge transfer.
5. Language Impediments: Disparities in language present a notable obstacle within the realm of cross-cultural communication. Professionals engaged in the Information Technology sector frequently employ a lexicon replete with technical jargon and specific terminologies, which might pose comprehension challenges for individuals who are not native speakers of English or those hailing from diverse linguistic origins. This linguistic divide necessitates an increased focus on the clarity and adaptability of language use, ensuring that communication remains accessible and comprehensible.

Forming cross-cultural communication skills in the IT business involves a combination of methods and strategies that individuals and organizations can employ. Developing cross-cultural communication skills should establish a foundation for facilitating effective communication within the development team. In the world of IT development, teams often consist of professionals from various cultural backgrounds. The ability to communicate effectively and consider cultural differences contributes to seamless and productive teamwork.

Drawing upon the scientific work of N. A. Dlugunovych and Yu. V. Forkun (Длугунович and Форкун, 2017), it is pertinent to acknowledge that the cultivation of intercultural communication competencies necessitates the facilitation of collaborative group endeavors. Such collaborative frameworks should encompass the assimilation and synthesis of information related to distinct project-oriented objectives. This approach underscores the importance of integrating diverse perspectives and expertise in the collective processing of information, thereby enriching the scope and depth of intercultural communicative practice. It involves the processing of shared information distributed among teams and related to the project. Efficient collaboration and resource-sharing improve project productivity. The advancement of cross-cultural communicative proficiency is imperative in fostering an environment that is optimally suited for collaborative endeavors in software development. Within the domain of software engineering, the act of collaboration with colleagues from an array of cultural backgrounds is a basis for the genesis of innovation and the appearance of creative problem-solving ways. The capacity to amalgamate and synergize ideas emanating from a plethora of cultural vantage points not only augments the value of software products but also significantly propels the velocity of product development cycles. This multicultural approach to software development is instrumental in enhancing both the quality and efficiency of technological innovations.

Furthermore, the facilitation of cross-cultural communication plays a pivotal role in cultivating an environment that is propitious for the establishment and nurturing of corporate culture within Information Technology enterprises. Painstaking consideration of cultural subtleties is instrumental in the development of an internal organizational philosophy that not only promotes harmonious collaboration but also bolsters the morale of the workforce. This approach is critical in engendering a cohesive and productive corporate atmosphere, thereby enhancing both employee satisfaction and operational efficiency.

The improver of cross-cultural communicative competencies within an IT company necessitates a synergistic blend of both individual initiative and organizational strategy. It is imperative to embark upon a detailed exploration of the methodologies and approaches that can be adopted by IT professionals, as well as the enterprise entity in its entirety, to augment these essential skills. IT-professionals and the company as a whole can take a number of steps to improve these skills.

For IT specialists, it will be useful to increase their cultural awareness, cross-cultural learning, language skills in the context of IT business, cultural sensitivity, and practice active listening during cross-cultural interactions. They should cultivate empathy for colleagues from different cultures, seek feedback from colleagues of different cultures about their communication styles, and seek mentoring from colleagues who have experience in cross-cultural communication.

Developing cross-cultural communication skills in an IT company requires a company-wide commitment to diversity, cultural sensitivity, and ongoing training. When the organization as a whole actively works to enhance these skills, it can lead to more productive and harmonious cross-cultural interactions within the IT industry. The main steps for forming cross-cultural communication skills for companies are: offering cross-cultural training programs to all employees; implementing diversity and inclusion initiatives that promote cultural diversity within the workplace; establishing clear communication channels for employees to report any issues related to cultural misunderstandings or conflicts; providing access to cultural resource materials, such as books, documentaries, and online resources, that employees can use to expand their cultural knowledge; implementing cultural competency assessments or surveys to gauge employees' understanding of cross-cultural communication; encouraging cultural diversity in leadership positions to reflect the company's commitment to inclusivity; scheduling regular check-ins with international teams or colleagues to ensure that communication is effective and to address any issues promptly.

Conclusion

In conclusion, the pivotal role of cross-cultural communicative skills within the Information Technology sector, particularly in the context of an increasingly globalized milieu, cannot be overstated. The successful navigation of this complex, international landscape

necessitates a concerted and dual-faceted approach, encompassing both individual endeavors and organizational strategies. The acquisition and refinement of these cross-cultural communication skills are imperative for IT professionals, as they facilitate effective engagement with representatives of different cultures. This, in turn, improves productivity and sparks innovation, while simultaneously addressing the multifarious challenges inherent in the globalized business. Such competencies are integral to the sustenance and advancement of the IT industry in its pursuit of global interconnectedness and technological progress.

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Invited lecture/Review

Digital Transformations: Trends and Perspectives Worldwide

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Abstract:
An analysis of the structure of keywords that form the concept of digital transformation was carried out using the VOSviewer software based on the generalization of information from journals that are indexed in the Web of Science and Scopus scientific metric databases. The author's vision of the concept of "digital transformations" is presented. The growth trends of the digital transformation market are considered. The main characteristics of the digital transformation market are considered: key drivers, restraints, opportunities, and challenges are highlighted. The analysis of states by the level of implementation of digital transformation is presented. The volume of global spending for implementing digital transformation with a forecast for the future has been studied. The dimensions of the global e-learning market by segment are considered. The main gains from the implementation of the digital model are presented. The understanding of digital business from the managers' point of view is considered. The most developed industries in the context of digital transformation have been identified.
The relationship between the levels of environmental responsibility of business and digital transformation has been established. The leading technologies of digital transformation are analyzed. Consider the impact of digital transformation on human capital and the workforce. The level of digital transformation in Ukraine, features of its implementation, further prospects, and difficulties are provided. Strategies for intensifying the digital transformation of business and society in Ukraine are proposed.

Keywords: digital transformation; global spending; environmental responsibility; human capital; global e-learning market.

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1. An analysis of the structure of keywords «digital transformation»

The analysis of the concept of digital transformation should begin with a consideration of the set of keywords that form this concept, the relationship of which is presented in **Figure 1**. The frequency of send concepts and their general connection, which are presented most often among the keywords of the structure of this category, are given in **Figure 2**. As we can see, this concept is most often associated with metadata, digital technologies, and digital storage. The first block of the most frequently used words ends with Industry 4.0. An essential role in this category is played by the immediate person and, in parallel with it, the concept of artificial intelligence, which, by the number of mentions, almost doubles the human factor. The concept of mathematical transformations should be added to the same cluster of keywords. The next block of concepts combines e-learning, Internet of Things decision-making, and digitalization.

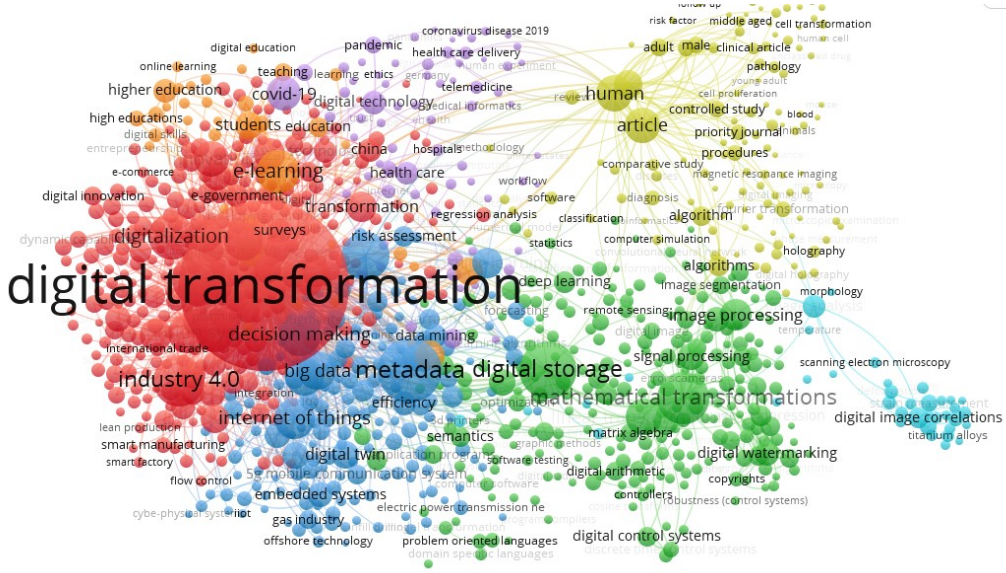

Figure 1. The structure of the concept of digital transformations by key words (built in VOSviewer).

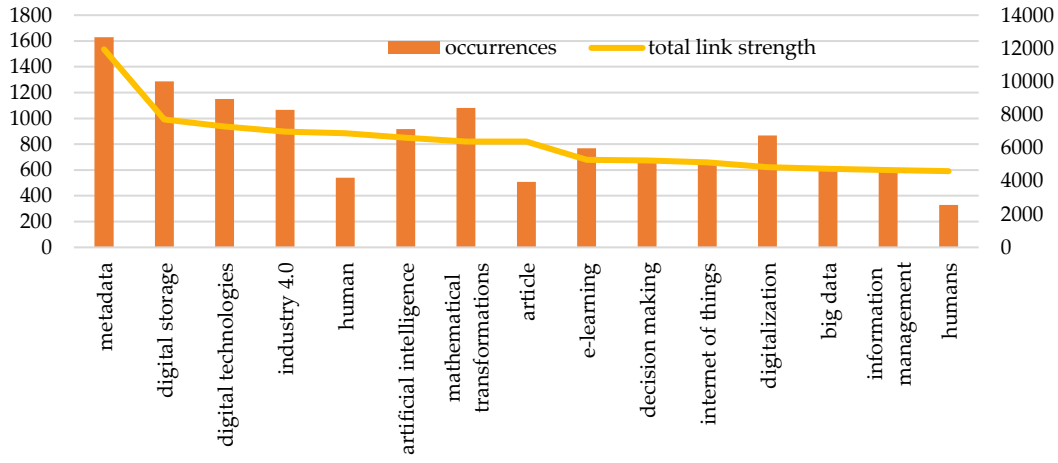

Figure 2. Selected keyword's analysis (from VOSviewer).

Well, the last block combines big data management and information that includes the human factor. The human factor in this category plays a rather important role, but not the main one. The digital transformation category is firmly associated with digital technologies, digitalization, digital preservation, artificial intelligence, mathematical transformations, and modern achievements in this field, represented by the Internet of Things and artificial intelligence. It is worth noting that e-learning is included in the group of concepts, which indicates the urgency of solving issues of sustainable competence formation in this field for further deepening the penetration of digital technologies in all areas of human life, which indicates the urgency of solving issues of sustainable competence formation in this field for further deepening the penetration of digital technologies in all areas of human life, which are actually united by the complex concept of digital transformation.

The generalized concept of digital transformation in various aspects of social life allows us to establish that it is considered a cultural, organizational, operational change in the structures of the industry or the entire ecosystem through the integration of digital technologies, processes and competencies at all levels of functioning (Digital transformation), the evolution of relations and in society under the influence of implementation information and communication technologies (Tymoshenko & Shabanova, 2021; Kruhlov, 2021), improving business by improving relationships with various stakeholder groups through digital technologies to change the culture and mindset of the organization and accelerate the development of technology (Holovachov & Nifatova, 2021; Novak, 2021), the influence of the state on society as a whole and its institutions, including business for implementation of information and communication technologies (Marchenko, 2019).

We see that the fundamental concept of digital transformation is most closely related to such categories as:

- From the point of view of scientists, the concept of digital transformation is a process of evolution of economic, social, industrial, technical, and technological relations in society caused by the development of information and communication technologies.
- From a business point of view, this is a mechanism for changing business models to improve the efficiency of its operation.
- About the state, this is the saturation of the physical world with electronic and digital devices, means, systems, and the establishment of electronic communication exchange between them.
- From the public's point of view, this is a new paradigm for the development of life processes, the basis of which are digital technologies.

In general, we can identify the concept of digital transformation as the process of building new systems of interaction between various participants in social relations, socio-economic systems of states and the world as a whole, where the main elements of interaction are information and communication technologies and achievements of the modern digital world, including artificial intelligence, the Internet of Things, big data, In cooperation with the key characteristics of Industry 4, within which the complete automation of activities, the construction of cyber-physical systems, and the implementation of Internet technologies for the interaction of personnel and machines are foreseen. Digital transformations include creating added value in business and the interaction of the state, society, and business in a new digital communication format to improve the interaction of a fundamentally new ecosystem of cooperation in the socio-economic system.

Digital transformations should be understood as the comprehensive and large-scale implementation of modern digital technologies, which are oriented towards the final specific consumer, considering the nature of the interaction of various stakeholders in business. These transformations involve new procedures for implementing classical business processes using software, a variety of new electronic devices, and information and communication technologies.

2. The characteristics of the digital transformation market

The volume of the digital transformation market in 2022 was $594.4 billion, in 2023 this market grew to $ 695.5 billion, and the forecast until 2030 is $3144.91. Global spending on digital transformation technologies and services worldwide (2017 to 2026) (Fig. 3.)

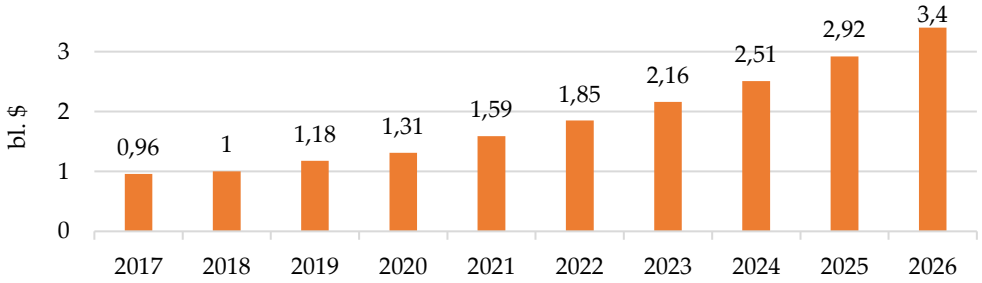

Figure 3. Global spending on digital transformation technologies and services worldwide.

The global e-learning market size was worth around USD 194.25 billion in 2022 and is predicted to grow to around USD 545.38 billion by 2030 with a compound annual growth rate (CAGR) of roughly 13.81% between 2023 and 2030. It is worth noting that the global e-learning market will only benefit from growing investments in the development of employees. Adapting employees to changing business conditions is critical to business survival and gaining competitive digital advantages. Currently, American companies spend more than $100 billion on employee development. E-learning consists of the lack of a living environment for engaging students in the process, which can be solved using modern e-learning tools: interactive whiteboards, virtual reality (VR) & augmented reality (AR) systems, extensive and comprehensive learning management systems (LMS), artificial intelligence (AI) powered tools (E-Learning Market, 2023). Obstacles to the further implementation of e-learning are the need for more access for all interested parties due to the lack of devices and robust Internet networks. In the competitive market, there will be a deepening of interaction between significant competitors to enter world markets and further deepen integration.

Priority strategies, management processes, and solutions that CIOs plan for 2023: 1. Cybersecurity and risk management; 2. Digital government and digital services; 3. Readying workforce for the future; 4. Legacy modernization; 5. Identity and access management; 6. Cloud services; 7. Consolidation/Optimization of operations; 8. Data and information management; 9. Broadband and wireless connectivity; 10. Customer relationship management (Top 10 policy, 2023). The leading technologies of digital transformation include mobile technology, internet of things (IoT), robotics, artificial intelligence and machine learning, augmented reality, big data and real-time analytics, digital twin, API-based integrations, robotic process automation (RPA), cloud-based technology (Top 10 Digital Transformation Technologies, 2023). These technologies are characterized by high complexity and cost for their implementation. Accordingly, in parallel with the convergence of various socio-economic systems during the digital transformation, the digital gap in the context of ultra-sophisticated technologies will only grow due to the need for more investment fun.

3. The main characteristics of the digital transformation market can be considered as key drivers, restraints, opportunities, and challenges

Let's consider the qualitative characteristics of the digital transformation market.

3.1. Drivers. One of the drivers of increasing these processes is the increase in the use of mobile phones and applications. Mobile devices allow you to quickly access the Internet, be constantly connected, and receive various digital services anywhere at any time. This has changed how people interact and use information and services, creating new business models and organizational opportunities. Mobile devices provide tools and applications that increase productivity and efficiency. Employees can be more productive with the ability to access business applications and manage tasks.

3.2. Key obstacles. Key obstacles to the development of digital transformation are the need for more protection of private data and compliance with security. The need to process a large amount of data creates difficulties in ensuring its security. One of the risks remains unauthorized access and inappropriate use of personal information among consumers, state authorities, and businesses directly.

3.3. Growth opportunities. Growth opportunities are the introduction of digital services and technologies. Digital transformations create significant opportunities for businesses in the context of revolutionary changes in their operations by introducing digital tools and technologies. Implementation of such innovative solutions as data storage, artificial intelligence, analysis of large volumes of data, and company automation can activate processes, increase efficiency, and achieve new competitive advantages. Digital tools enable soft collaboration. Digital tools enable soft collaboration, remote work, and constant access to real-time data, inspiring employees to work more productively from anywhere. In the future, digital transformations will open the door to improved consumer experiences through personalized interaction, faster response to requests, and individual solutions.

3.4. Challenges. Challenges for digital transformation are legacy in the form of inefficient systems and outdated technologies. Many organizations still use old systems that were implemented many years ago. These technologies are often characterized by a need for more compatibility with modern technologies, which prevents integration into new digital solutions. Upgrading or replacing these legacy systems requires careful planning, significant investment, and potential disruption to current operations.

The impact of Digital Transformation on human capital consists of the formation of new fundamental competencies that employees will require. The need for constant learning, self-improvement, and compliance with the principles of digital security remains critical due to the widespread use of personal and commercial information in various sources, applications, and databases.

4. The level of digital transformation in Ukraine further prospects, difficulties are strategies for intensifying

The problem of digital transformation in Ukraine is the immaturity of digital culture and the perception of digitalization of business. For a long time, it has been essential to understand that digitalization can be implemented through the development of companies and individuals, confirming the integrity of e-learning. Difficulties still need to be addressed with the access of the entire population to a high-quality Internet network and the use of modern applications. It also inhibits the implementation of the concept of an inclusive economy as one that creates equal opportunities for the participation of every citizen in the creation of added value. Ukraine can boast of significant successes in digital transformation, such as the widely popular application "*Дія*", which acts as a full-fledged substitute for the vital identity documents of a citizen in Ukraine and creates opportunities for him to conduct full-fledged activities. Ukraine has also achieved significant success in the digitalization of banking services. Most leading banks have mobile applications that allow you to carry out virtually all financial transactions through a mobile application. In state-building, digital transformation can be represented by successes in public procurement, which has been carried out for many years through electronic transparent platforms.

Investing in the development of Industry 4.0 technologies, which requires the development of a modern innovation ecosystem that ensures the implementation and development of these technologies in business activities, is a promising strategy for deepening the digital transformation processes of education.

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

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Invited lecture/Research

Innovative Approaches in Teaching Business Courses: Start-Up Simulation in the Change Management course; students' experience

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Abstract:
Simulation is an experiential learning process that speed-ups learning process and make it more interesting and practical. According to Hakeem (2001), when students are involved in different activities of experiential learning, they better understand the subject in comparison with students who just have lectures-only classes. Moreover, experiential learning raises critical thinking abilities and self-directed learning (Kreber, 2001). In business schools simulations have been used to manage trading skills (Truscott et al., 2000) and business ventures development (Goosen et al., 2001). For the Change Management undergraduate course of the Business School there was offered a simulation "Start-Up Wars" that was developed by one of the USA start-ups. This simulation was created especially for colleges and universities students with a goal to introduce students with the real stories of different start-ups and practice making decisions on a daily routine with a goal, according to the scenario, to survive for the first ninety days. Each new day brought a new challenge. By solving everyday entrepreneur's tasks students have experienced and applied their knowledge into practice in different directions such as strategic thinking, marketing, decisions making, used their managerial and leadership skills, teamwork, planning, and financial modeling. After the simulation students provided the feedback on their experience and met through online session with the developers of the simulation where students shared their experience, ideas and general understanding of the activity. Overall, students provided positive feedback about conducting simulations in the learning process and raised their interest in using business simulations as class activities. Students highlighted the beneficial and practical component of simulations in understanding the business world.

Keywords: simulation, start-up, change management, experiential learning, innovative approach, students, business school.

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1. Introduction

The purpose of the current article is to share about innovative approaches in teaching business courses for undergraduate students in order to make the learning process more interesting, informative and practical.

The modern tendencies in teaching process and innovative technologies require instructors to think about new methods in teaching to attract students to the learning process. One of the innovative approaches are business simulations with different scenarios where students could apply their knowledge, creative and analytical thinking, decision making skills, try to think as real businessmen in a real business situations taking into account the market challenges. So, using such business simulation was the main goal.

The course chosen for this activity is a 'Change Management' course of one of the business schools in Kazakhstan – Kazakh-British Technical University. The majority of students are on their third and fourth year of study, majors are Finance, Marketing, Management and Accounting specializations.

Change management course is the course where students learn how to manage changes in the organization, how to make different types of analysis and make decisions that allow to achieve the main goal of the company – make improvements in different directions. Using simulations in the course of Change management is a very interesting experience as students do different things, they change their usual process of learning. Changes in learning, personal life, in decision making are the main concept of the course.

According to Aldrich (2003), simulation is the experiential learning process that speeds-up learning process. It enhances students' learning experiences by providing them with hands-on, experiential opportunities to apply theoretical concepts in a simulated business environment (Aldrich, 2003). Billimoria (2000) suggests that educatrs should lead new innovatibe approaches in teaching and create 'value innovation' for students (Billimora, 2000). The literature on this topic highlights several benefits, challenges, and effective practices associated with integrating simulations and business games into business education (Doyle & Brown, 2000; Kolb, 1984).

2. Methods

The Simulation approach for business courses was chosen because of several benefits for students (Thompson & Stappenbeck, 1995; Fripp, 1993).

First of all, it is innovative and newest way of delivering knowledge and this gives students absolutely another way of learning and understanding their knowledge through their own experience by making decisions based on real time situations. So, it allows students to apply theoretical knowledge in a realistic business context, enhances their understanding of complex concepts.

Second, such experience actively engages students in the learning process. The interactive nature of simulations encourages participation, collaboration, and critical thinking.

Third, simulations help students develop a range of skills, including decision-making, problem-solving, strategic thinking and teamwork.

Fourth, the application-oriented nature of simulations enables students to bridge the gap between theory and practice. It reinforces theoretical concepts by immersing students in scenarios that mirror real business challenges. According to Kreber (2001), simulations and experiential exercises foster students' learning and, especially, 'critical thinking ability' skill.

Fifth, simulations provide a risk-free environment for students to experiment with different strategies and decisions. This allows them to learn from failures and successes without real-world consequences (Kolb, 1984; Thompson & Stappenbeck, 1995). In the simulation that we used with students in the class there were given three trials to start and run a business. All students failed in the first and second trials. If students make the analysis and understand the challenges, they usually start earning some profit in the third trial and survive.

Sixth, the use of simulations often increases students' motivation and interest in the subject matter. The dynamic and competitive nature of business games can inherently engaging.

By the results of the semester, one student opened its own business and started earning. In the last day of the class, he declared that the simulation tended him to think about a

new opportunity to open a business and he opened drop shipping business taking into account the experience and knowledge that he got during the practice. Moreover, he was the only one student who successfully played the simulation from the first trial and showed great results with a big profit.

3. Results and Discussion

The outcome and results of using simulations during the class activities are based on students' feedback.

Moreover, after playing the simulation there was conducted a separate session with the developers of the simulation. During such live session online students shared their experience and asked questions, they also provided feedback about some challenges that they have experienced during the practice and recommendations what could be improved, what was unclear and some other useful thoughts and ideas. As the developers of "Startup Wars" simulation shared on their website: "Startup Wars is truly a platform built "For Students, By Students" (Available from: https://www.startupwars.com/our-mission/)

Using such activities as simulations showed students interest in the learning process, raised their engagement in study, discussions and cases analysis, participation in different class's activities raised, students became more friendly, they started experiment more. The students' absenteeism decreased, they started actively participate in other different activities in the class.

About the Start-Up Simulation design. There are Four different types of start-ups:

- Mobile App developing
- Coffee-shop
- Hand-made shop
- T-Shirt company

According to the scenario there were given 90 days to survive; three trials (in order to improve the performance).

Daily decision were made on different issues: marketing, business model, human resources, and finance. Three levels: basic, intermediate, advanced were included. Moreover, different resources were provided for educators and students (tutorials, visuals for instructor for analysis and discussion). Student's results were shown when the student bankrupted.

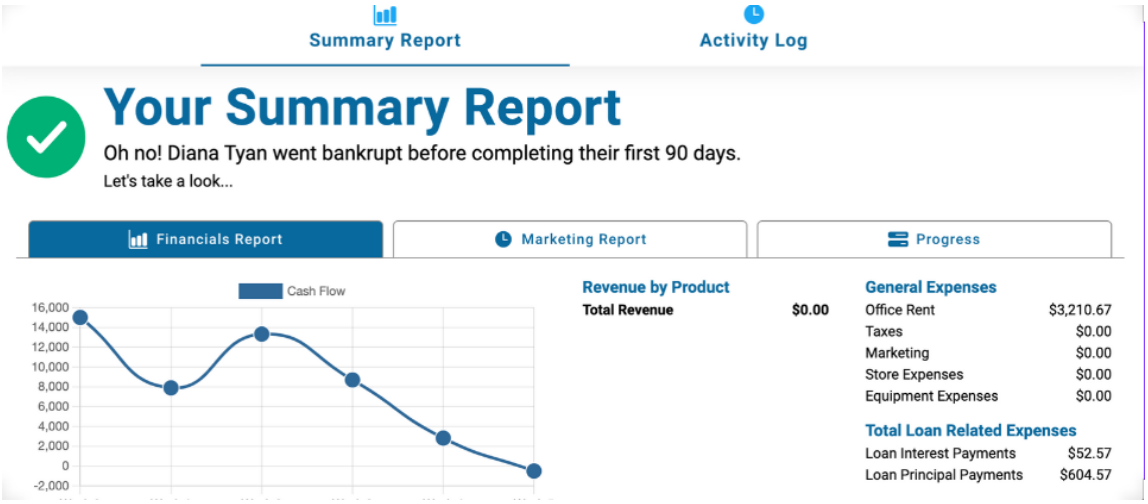

Figure 1. Student's progress day after day: what was done, completed, what decision was made.

Another student successfully survived during first ninety days, and some goals were achieved. For example, the student established brand reputation, generated revenue with a crafting company (from USD 1,000.00 to USD 5,000.00 that is a good result).

It shows that student can plan, delegate and distribute the resurces. This was one of the successful cases when the student successfully completed first ninety days and managed

revenue (he has achieved USD 80,546.00) (**Figure 2**). The Report also provided a detailed distribution of the finances (**Figure 3**).

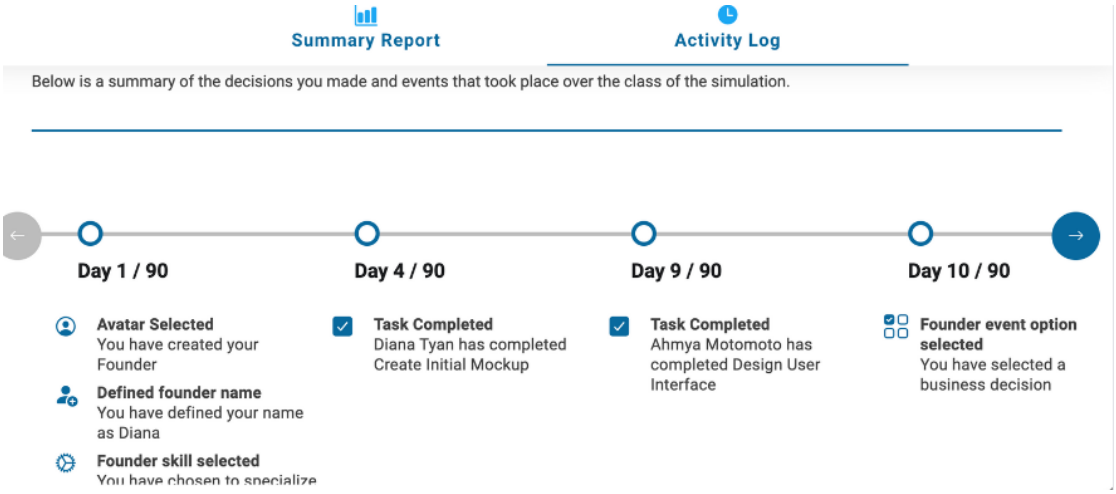

Figure 2. Student's milestones reported.

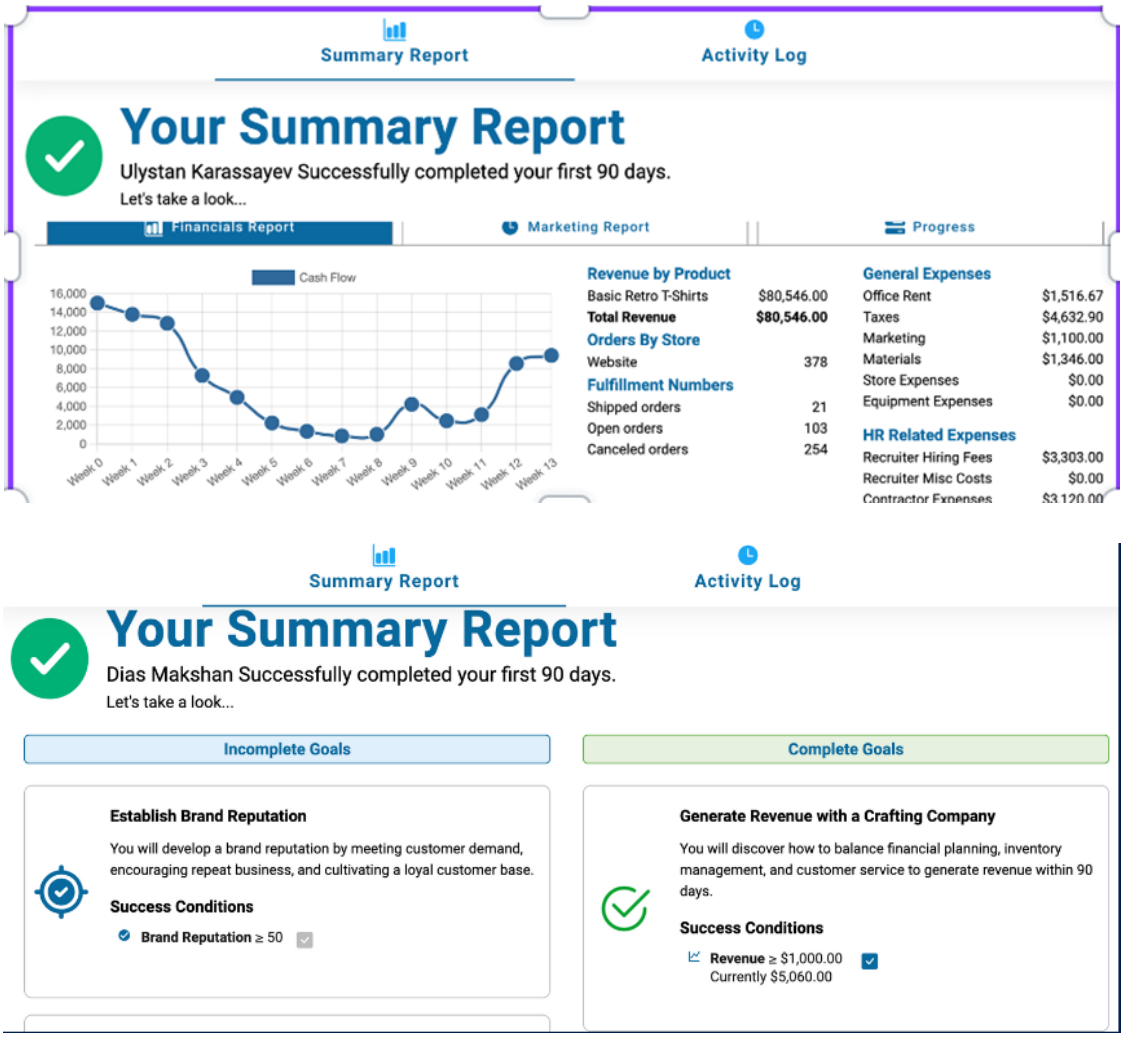

Figure 3. Reports on the successful students' progress.

Moreover, very interesting part is students' feedback about the experience and playing the simulation. Here is one of the feedbacks: "This startup game was fun and learning experience"; "The business simulation game was fun and educational experience"; "A rare chance to put myself in the position of business owner and make important decisions in a supervised but real setting"; "There are some important lessons that I learned from experience:

1. Realistic Scenario: The simulation produced a business setting that closely mirrored the difficulties encountered in real-world situations.
2. Decision-Making: it was hard to make decisions in many areas, such as marketing and production.
3. Learning from Failure: This opportunity enabled me to develop my soft and hard skills. The gained knowledge really helps me."

Another student shared: "STARTUP WARS is an exciting and innovative experience that skillfully combines elements of strategy, business and competition. This game offers players a unique opportunity to immerse themselves in the world of startups, allowing them to experience the joys and challenges of starting and running their own business."

There also was a critical review of personal experience, such as: "So, overall the idea of the programme is great and I really liked it although my entrepreneurship skills and financial decisions made me bankrupt. In my opinion, using the programme in class is great idea, it is interesting to start, it is a courage to continue, get bigger, achieve goals and make a lot of money. Also, at the end of the journey you can make some decisions and get ideas about your strengths and weaknesses. So, now I know that most of the time going for the risk and not having clear picture of financial part of the business is my error, my weaknesses."

It is really interesting to see how students played, experienced and what finally they said or thought about such experience. I am very grateful for such experience provided by the developers of this Start Up Wars. Hope to continue cooperation in the future to give students more opportunity to practice and learn new skills through such interesting activity.

4. Conclusion

'Start Up Wars' as simulation is a very useful experience that challenges the students and makes the learning process more interesting and various. It gives opportunity to students to think differently, make tough decision, to understand about business processes, also change something (e.g., to be more confident). The literature on using simulations in the learning process emphasizes the transformative impact of experiential learning on student outcomes. While challenges exist, effective implementation strategies and a commitment to leveraging simulations as tools for active engagement contribute significantly to the educational experience. Continuous research in this area seeks to refine and expand the use of simulations across diverse educational contexts. Moreover, there is a strong impact on students outcomes:

- Improved retention: studies suggest that students who engage in simulation-based learning may retain information better compared to traditional instructional methods.
- Enhanced critical thinking: simulations have been associated with improvements in critical thinking skills as students actively analyze and respond to complex scenarios.
- Better decision-making skills: exposure to decision-making in simulated environments contributes to the development of better decision-making skills among students.
- Increased motivation: simulations are often linked to increased student motivation and interest in the subject matter, leading to more active participation in the learning process.

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Review

Economic and Financial Analysis of Artificial Intelligence's Impact on Law and Legal Profession

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Abstract:
Chat Generative Pre-trained Transformer (ChatGPT) and other Large Language Models (LLMs) present a significant disruption to the legal profession. Their ability to generate text in a way that closely resembles human writing and the nature of their training which equips them with large amounts of data present significant competition to individual lawyers and an opportunity for large increase in their productivity. They also mean changes on firm level. Technology firms have already entered the market, offering legal LLMs and other solutions to augment the work of lawyers and other legal experts. However, due to the nature of the legal profession, proprietary solutions are probably preferred over the long term. Those require significant capital expenditures by law firms, which are currently restricted by legal obstacles to outside capital being invested in law firms. Bar association rules will probably have to be relaxed to maintain competitiveness with firms from countries with looser regulation.

Keywords: large language models, LLMs, law, lawyers, bar association, investment, finance.

1. Introduction

It has been more than a year since Open Artificial Intelligence (OpenAI) unveiled ChatGPT and enabled free use of the model during the research preview phase (OpenAI, 2022). ChatGPT was, until then, the most significant contact between artificial intelligence and the public. It is a model which the public can use with added value (Marr, 2023). ChatGPT is not only available to researchers and academics, but to the general public, in a way that was easy, intuitive and free to use (Roose, 2022). Its consequent popularity was unprecedented. In 5 days since its official launch on 30th November 2022 (OpenAI, 2022), ChatGPT had more than 1 million users (Marr, 2023), and in two months since launch, it became the "fastest-growing consumer application in history" (Hu, 2023) with 100 million monthly active users (Hu, 2023; The Economist, 2023b)[1]. In comparison, it took TikTok 9 months to reach the same number of monthly active users (Gordon, 2022). The use of ChatGPT was not limited to personal use but quickly became a valuable tool for professionals (Zinkula & Mok, 2024; Brower, 2023; Mancini, 2023) with OpenAI already offering enterprise solutions of its product (OpenAI, 2023c). Specifically, ChatGPT is useful for jobs that require dealing with long texts, either writing or reading them (Brower, 2023) and is expected to significantly impact labour market in those industries (Eloundou et al., 2023; Zinkula & Mok, 2024; UBS, 2023) – with law and legal industry being the prime candidate for disruption. ChatGPT has in the last year left a significant mark on law, both from academic and business perspectives, and promises to significantly change law and legal industry as we know it.

We discuss economic and financial impact of artificial intelligence on legal industry. In the 1st part, a general overview of technology and some applications to legal profession are presented. The 2nd part focuses on selected financial and economic changes that ChatGPT has had and is expected to have on the legal profession.

2. ChatGPT and Large Language Models (LLMs)

ChatGPT and its new and improved version, GPT-4, are Generative Pre-trained Transformers (GPTs) and are both members of the large language models family (Eloundou et al., 2023). Large language models, or LLMs, are statistical models, which use probability to generate text (Roose, 2022; Wu et al., 2023) that is practically indistinguishable of human writing (Wu *et al.*, 2023) – on which they were trained. Indeed, the most recent of OpenAI's LLMs, the GPT-4, has passed the Bar Exam, an entry barrier to becoming a lawyer, scoring in the 90th Percentile (OpenAI, 2023b). While GPT-4 is already a multimodal LLM, which means its inputs can include not only text, but also voice and images (Heaven, 2023; OpenAI, 2023a; OpenAI, 2023b), we primarily focus on the written output of the model(s), which are the most relevant to the legal profession.

A common misconception when talking about LLMs is that the models know and understand human language and can understand their own output (Brower, 2023; McKinsey, 2023). That is (probably) not the case – as explained above, they are merely complex statistical models without any intrinsic understanding of the underlying material (Titus, 2024). The huge reams of data on which LLMs are trained (Knight, 2023; The Economist, 2023a; The Economist, 2023d) make this possible, but also extremely costly and time consuming. By OpenAI estimates, training the newest GPT-4 cost more than $100m (Knight, 2023).

Given the functioning of LLMs and the statistical nature of their methods, the inputs human users provide is very important (Cowen & Tabarrok, 2023) and has become an art in and of itself– and has led to even OpenAI providing guidelines for ChatGPT usage (OpenAI, n.d.). The output can only be as good as the input is.

An obvious consequence of LLMs is that they tend to hallucinate. Hallucinations are factual errors in the output generated by LLMs. Hallucinations occur because LLMs are incapable of evaluating their output as true or false, but merely print out the best prediction they can, given the input. LLMs do not understand the meaning behind words and text (Wu *et al.*, 2023). Thus, ChatGPT's answers should be taken *cum grano salis* and critically

[1] Meta's Threads have since beaten its record in just 5 days (Paul and Sriram, 2023; Nolan, 2023), however, ChatGPT's success is much more notable as majority of Threads users created profile through Instagram, another one of Meta's social networks (Chowdhury, 2022; Nolan, 2023).

evaluated. The critical analysis part and our touch with reality is still an area where humans have the advantage, despite LLMs overtaking most of us when it comes to grammar, ability to express ourselves in a coherent manner and the overall language ability.

3. Financial and economic impact of LLMs on law and legal industry

This tendency to hallucinate has proven to be a professional embarrassment and quite costly to some lawyers (The Economist, 2023c; Weiser, 2023; Verma and Oremus, 2023). However, that has not discouraged exploring potential applications of LLMs and more broadly artificial intelligence to the legal profession. Indeed, the largest international law offices have invested significantly into either developing their own LLMs or adapting current ones for use in law (Skolnik, 2023). These law firms are joined by new competitors, mostly from technology sector, which hope to disrupt the industry (see for example Thomson Reuters, n.d.; Casetext, n.d.; Robin AI, n.d.; Harvey, n.d.; and others).

Generally, there are three ways law firms can invest in proprietary development of LLMs or LLMs' adaptation to the needs of the legal profession. They can, firstly, commission the development of LLMs, either in-house or by contracting the work to certain service providers, in which case the LLMs are the property of the law firm[2], secondly, they can create subsidiaries which focus on LLM development and adaptation for use in law, and lastly, they can invest or purchase the companies developing the LLMs (Armour & Sako, 2020).

All of the aforementioned strategies, however, require significant capital and long-term investment horizon, both of which are incompatible with the current structure and regulations of law firms across the world. Specifically, law firms cannot have investors who are not themselves lawyers or members of the Bar Association regulating the profession in the country. That severely limits the ability to attract outside capital needed to develop LLMs. That leaves just internal sources of capital, namely capital from partners, which can come either from retained earnings or new investments from current partners. Both financing options are generally incompatible with incentives of most lawyers, as the investment will only pay off over the longer term, when the majority of current partners will not be there to benefit from them (Armour & Sako, 2020). Furthermore, the LLMs or other assistants tend to benefit young and inexperienced workers the most, while older workers, see a lower increase in productivity (Brown 2023), further disincentivising investments.

Some jurisdictions, such as United Kingdom, have more relaxed rules which enable law firms to attract outside capital, and take advantage of different corporate structures that enable better risk management of such LLM development projects (Armour and Sako, 2020). Developing LLMs in-house or at least for their own use is very important to law firms due to the confidential nature of their business and the ability to retain competitive advantage by keeping certain legal solutions their competitors have not yet thought of confidential. The privacy concerns of law offices will probably discourage cooperation between LLM service providers, i.e. LLM models developed by technology companies and offered to law firms on a subscription, pay-as-you-go or otherwise basis, until the privacy concern can be allayed. The aforementioned reasons suggest that over long term, law firms primarily stationed in jurisdictions with looser restrictions, will have a significant competitive advantage over the firms located in other jurisdictions. That will probably lead to loosening of law firms' regulations elsewhere or the aforementioned firms (gradually) acquiring the smaller firms operating in those jurisdictions.

4. Conclusion

The implications of LLM revolution on legal industry will probably be extensive and comprehensive. LLMs could mean to law what tractors meant to farmers. However, only the future will show their true effect. Whatever it will be, it will most likely be gradual, and part of general evolution of the industry. The investment horizon of law firms should therefore lengthen and rules regarding possible inflows of outside capital held by non-lawyers relaxed if law firms are to stay competitive.

[2] This approach is different than for example subscribing or otherwise paying for the use of software developed and intellectually protected by a technology company or competitor. The aproaches mentioned in the body are proprietary solutions of the law firm itself, or solutions that acording to intellectual property rights belong to the law firm in question.

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Invited lecture/Research

OSINT-Technologies: Applications and Challenges in the Digital Age

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Abstract:
The article examines the importance and application of open source intelligence technologies (OSINT) in today's digital age. OSINT is defined as the systematic process of gathering and analysing information from open sources to draw informed conclusions and make effective decisions. The application of OSINT-technologies is becoming an integral part of the strategic analytical process in areas such as cyber security, intelligence, law enforcement, intelligence and public safety.
The main focus of the study is a specific aspect of this broad spectrum – the OSINT Framework, which is a set of tools and resources for collecting and analysing information from open sources on the Internet. Using this framework allows us to study, monitor and analyse a variety of data to identify patterns, trends and potential threats.
The article defines the purpose of scientific research and in-depth analysis of OSINT, in particular the OSINT Framework, with the aim of understanding its structure, functionality and effectiveness in solving tasks in the field of intelligence, cyber security and data analysis.
The overall state of OSINT-technologies is defined by a wide variety of tools, including social networks, geospatial analysis, textual and visual information analysis, data mining platforms, and ethnographic analysis. The use of scientific methods and technologies in this area allows you to optimize the processes of data collection and analysis, providing more insights for making informed decisions.

Keywords: OSINT; OSINT Framework; Open source; Data collection and analysis

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1. Introduction

1.1. Background

In the modern digital era, characterized by the rapid development of information technologies and mass access to the Internet, OSINT technologies (open sources of information) occupy a decisive place in the context of gathering, analysing and using information. OSINT is defined as the systematic process of collecting and analysing information available in open sources in order to draw informed conclusions and make effective decisions. Gradually increasing in importance in numerous fields such as cyber security, intelligence, law enforcement, intelligence and public safety, OSINT technologies are becoming an integral part of the strategic analytical process. Thanks to the wide range of tools and methodologies they include, these technologies allow the study, monitoring and analysis of large volumes of information to identify patterns, trends and potential threats.
However, given the multifaceted nature and variety of OSINT tools, our study focuses on a specific aspect of this broad spectrum—the OSINT Framework. The OSINT Framework is a set of tools and resources aimed at collecting and analysing information from open sources on the Internet. Using this framework provides an opportunity to study, monitor and analyse a variety of data to identify patterns, trends and potential threats.

1.2. Scope of the article

The purpose of this article is to conduct a scientific study and in-depth analysis of open source intelligence tools known as OSINT. Focusing specifically on the OSINT Framework, the authors seek to uncover and explore the various aspects of this tool in the context of today's digital age. The main goal is to understand the structure, functionality and capabilities of this framework, as well as to evaluate its effectiveness in solving various tasks in the field of intelligence, cyber security and data analysis. The study is aimed at identifying the advantages and limitations of using OSINT tools, as well as providing science-based recommendations for their optimal use in various application scenarios.

1.3. Current state of OSINT- Technologies

Open source intelligence (OSINT) is a field that studies and uses open sources to obtain and analyse information that is available to general public. The primary goal of OSINT is to gain an understanding of a situation or object by processing and analysing public information. The development of technology and the increase in the amount of available information create wide opportunities for the use of OSINT in various areas.

Social networks and media:

Analysis of social media platforms: Includes activity monitoring, interactions, community analysis and key person identification.

Media monitoring: Monitoring news and content from various sources to get a complete picture of the situation.

Geospatial analysis:

Use of geodata: Involvement of satellite images, geographic information systems and other sources to determine the location of objects and study their spatial relationships.

Analysis of textual and visual information:

Natural Language Processing (NLP): Using NLP algorithms to analyse textual information, identify connections and emotions.

Computer Vision: Using computer vision technologies to analyse visual content such as photos and videos.

Data analysis platforms: Maltego,

Recorded Future: Specialized tools for integrating and analysing diverse information from diverse sources.

Ethnographic analysis:

Sociocultural learning: Understanding behavioural patterns and cultural differences to better contextualize the information received.

These tools are used in various fields, including security, intelligence, business intelligence, and others. The application of scientific methods and technologies in OSINT allows to optimise the processes of data collection and analysis, providing more insights for making informed decisions.

2. Methods

The article uses a multifaceted approach to achieving the goal. The research uses both theoretical and empirical methods, combining a comprehensive analysis of specialized literature with practical research. The theoretical base involves an in-depth study of the existing literature related to the use of OSINT technologies.

3. Results

The OSINT Framework is essentially a set of various tools and methods used to collect and scrutinize information available in the public domain (OSINT, 2023). This methodology aims to offer a structured and methodical way of sourcing and analysing data from diverse channels, including the internet, social media platforms, government records, and more.
Its purpose is to assist individuals and organizations in methodically and effectively analysis of information from open sources. The framework organizes digital resources into multiple categories, such as searching for individual profiles, analysing networks and others.
Each of these categories comprises specific online resources suitable for OSINT activities. For instance, the category dedicated to searching for people includes resources for finding details about individuals through social media profiles, online directories, and other similar platforms.
Similarly, the category for network analysis provides resources to gather details about digital infrastructures like IP addresses and subdomains. Utilizing this OSINT Methodology enables users to fully leverage the advantages of OSINT and stay vigilant against potential security threats (**Figure 1)**.

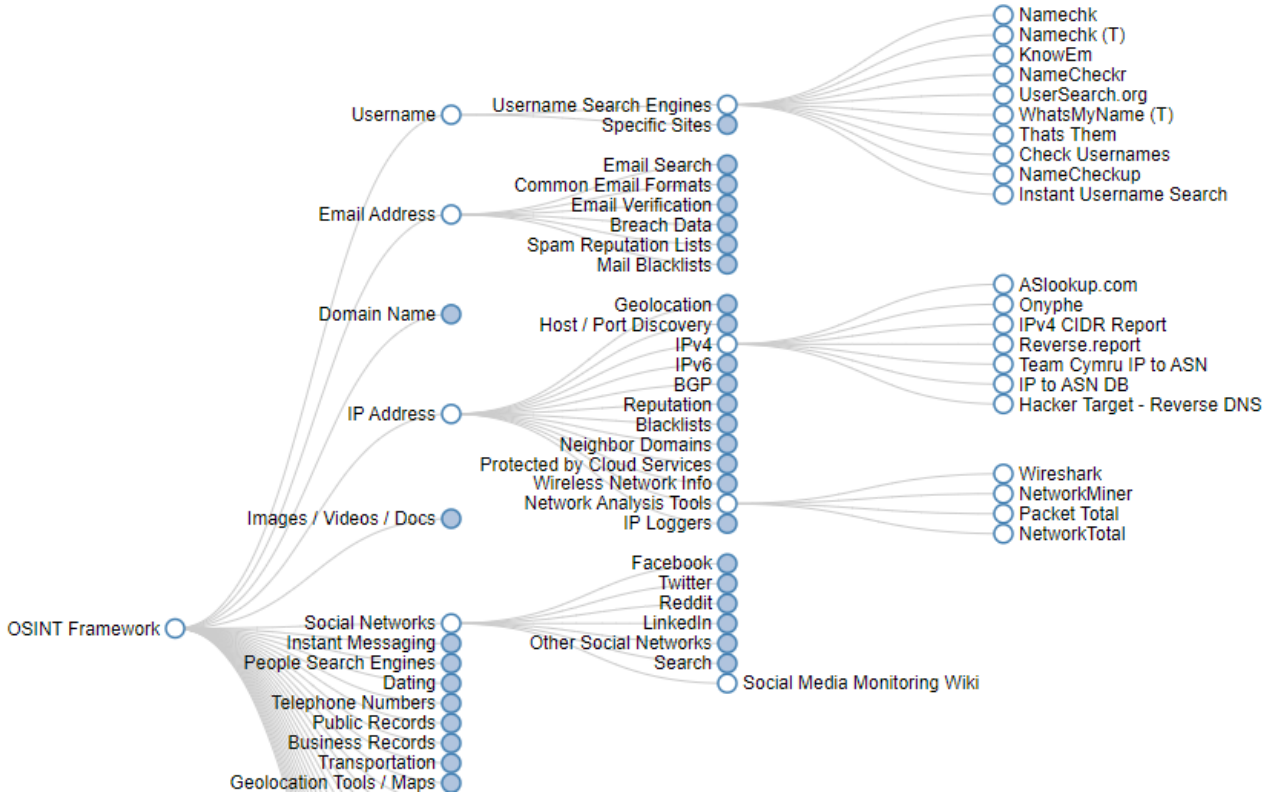

Figure 1. An example of programs and applications in the structure of the OSINT Framework.

4. Discussion

In summary, the article highlights the crucial role of open source intelligence technologies (OSINT) in today's digital landscape, with a specific focus on the OSINT Framework. This framework, comprising various tools, proves instrumental in systematically gathering and analysing information from open sources. The study emphasizes the versatility of OSINT tools, spanning social networks, geospatial analysis, textual and visual information analysis, and more.

Through a blend of theoretical and empirical methods, the research underscores the OSINT Framework's structured approach to data collection, offering specific categories for tasks such as individual profile searches and network analysis. The framework enhances the efficiency of OSINT activities, enabling users to extract valuable insights and stay vigilant against potential security threats. Overall, the article contributes to a deeper understanding of OSINT technologies, providing science-based recommendations for their effective application in intelligence, cyber security, and data analysis.

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Invited lecture/Research

Innovative Approaches to Language Learning through the Lens of Use Artificial Intelligence

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Abstract:
The article explores the question of innovative approaches to language learning through the lens of leveraging artificial intelligence (AI) capabilities, using the English language as an example. Therefore, the aim of this article is to highlight the possibilities of using AI in language learning, with a specific focus on the English language.
The research task involves analyzing some of the latest publications on this topic and identifying their common trends. Among them, special emphasis is placed on individualizing the learning process, the broad potential of text, audio, and visual material generation by AI, and the shift in the role of the teacher towards that of a guide and advisor.
In addition, the paper provides statistical data regarding the positive experiences of using AI capabilities among 75 high school students at a school in Astana, Kazakhstan.
The article also discusses the advantages and disadvantages of using AI in English language learning. As a result of the conducted research, it was found that the utilization of AI capabilities in language learning is an integral part of innovative approaches in this field.

Keywords: language learning; artificial intelligence; innovative approaches; didactics.

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1. Introduction

The emergence of AI stands out as a highly notable occurrence in recent scientific history. Presenting a wide field of potential applications in the realm of education demonstrates a distinct advantage in promoting increased student involvement in the learning process. An illustration of this is the autonomous acquisition of language skills facilitated by chatbots, audio, and video content generators. This method allows for significant adaptability in language learning, considering the unique attributes of each participant in the educational domain. As a result, the multitude of inventive approaches in language learning through the lens of use AI constitutes an expansive domain necessitating comprehensive and prolonged scrutiny.

Nevertheless, despite its numerous benefits, the application of this technology to language learning requires various enhancements. A crucial lingering inquiry revolves around whether AI can truly replace a live teacher. Currently, its functionalities act as a supplementary element to innovative language learning methodologies. All of this leads to a new phase of reevaluation, or in other words, the emergence of a new scientific paradigm in education (Choudhary et al., 2022).

It is worth noting that the most significant impact is experienced by the new generation, often referred to as individuals born in the age of digital technologies, or "Generation Z" (Pikhart, 2020). Therefore, it is evident that the application of the capabilities of digital technologies, especially AI, in language learning sessions allows for a closer alignment with their understanding of the educational environment.

However, there are numerous debates about the appropriateness of using the capabilities of AI (Barrett & Pack, 2023), which introduces a large number of innovations into the process of learning a foreign language. Therefore, the goal of this research is to examine the features of using innovative approaches in language learning through the lens of AI utilization.

2. Methods

The abstract-analytical method was employed as a method for identifying trends on the subject of this research. On one hand, the analysis highlighted the key turning points in recent research regarding the use of AI innovations in language learning. On the other hand, the abstract method allowed for the identification of connecting links among the analyzed studies in this direction.

Also, a survey method was utilized for the collection of statistical data.

In addition to the aforementioned methods, the observation method was employed. It facilitated the delineation of the boundaries of existing possibilities and drawbacks in using innovative approaches in language learning through the lens of AI utilization.

3. Results

Among the advantages of using AI in language learning, the following are particularly notable:

1. Individualization of the language learning process. Students have the opportunity to practice study materials at a convenient time and place (Kushmar et al., 2022). They can choose the study material that interests them at the moment. AI allows learners to be more pragmatic in selecting topics for study, enabling them to plan their learning based on their knowledge and gaps (Ermağan & Ermağan, 2022). All four language skills can be improved without the presence of a live teacher (Ali, 2020). Students have the flexibility to choose the pace of their learning.
2. Integration of Text, Audio, and Visual Materials. AI enables the seamless combination of textual, audio, and visual educational materials into a unified logical structure without requiring significant effort from the teacher (Nur Fitria, 2021). This frees up a considerable amount of time and energy for the teacher to enhance their professional competencies.
3. Changing the Role of the Teacher. The use of AI shifts the role of the teacher to that of an advisor or guide in the world of foreign language learning (Celik et al., 2022).

However, there are significant drawbacks to the use of AI, including its limited ability to process information accurately, the high cost of more advanced program versions (Nalbant, 2021), and the inability to fully replace a live teacher.

To understand how the use of AI in language learning sessions would be perceived by students and to gauge the level of interest in the lessons, a series of sessions were conducted among 75 students in grades 8-11 at a school in Astana, Kazakhstan. The following applications were used during the sessions: AI Chat, Grammarly (Schmidt & Strasser, 2022), Bing, Chat GPT (Rospigliosi, 2023), Bing Image Creator, Stable Diffusion, Lexica Aperture, and AI Studious. For example, to memorize vocabulary related to the lesson's topic, students entered a word or combination of words, receiving a ready-made image or video fragment. The sessions involved activities to practice speaking and writing skills, such as formulating questions correctly to obtain the desired answer. To reinforce grammar, students were tasked with creating a video lesson by generating text and video on the studied topic using AI. Upon completion of the sessions there was a survey that indicated a high level of satisfaction. The obtained data is presented in **Table 1**.

Table 1. Tabulated statistics depicting the satisfaction levels with the learning outcomes.

№	Student satisfaction level	№	Student satisfaction level	№	Student satisfaction level	№	Student satisfaction level	№	Student satisfaction level
1	83.5%	16	89.9%	31	83.1%	46	88.4%	61	84.7%
2	86.8%	17	80.1%	32	91.4%	47	83.3%	62	85.5%
3	84.4%	18	80.8%	33	76.0%	48	94.7%	63	81.2%
4	92.8%	19	91.1%	34	92.0%	49	94.1%	64	81.0%
5	74.6%	20	77.7%	35	82.1%	50	90.4%	65	79.2%
6	77.1%	21	94.9%	36	77.5%	51	93.3%	66	76.8%
7	94.2%	22	72.5%	37	85.3%	52	88.3%	67	80.0%
8	84.2%	23	84.5%	38	88.4%	53	79.9%	68	86.4%
9	85.7%	24	72.5%	39	92.0%	54	94.3%	69	78.7%
10	74.0%	25	74.7%	40	87.1%	55	80.0%	70	90.8%
11	83.9%	26	74.5%	41	76.9%	56	92.4%	71	90.6%
12	86.3%	27	89.9%	42	86.2%	57	85.0%	72	80.01%
13	73.1%	28	81.0%	43	72.5%	58	89.8%	73	86.0%
14	90.2%	29	82.9%	44	94.8%	59	80.0%	74	93.2%
15	85.0%	30	94.2%	45	76.4%	60	91.7%	75	86.2%

4. Discussion

Among 75 students who took part in the survey, the satisfaction levels were between 72.5% and 94.9%. The computed mean stands at 84.86%.

Thus, the satisfaction level of students with the conducted sessions utilizing AI capabilities showed positive results. This serves as another small piece of evidence in favor of the necessity of incorporating its capabilities in language learning.

However, the following drawbacks were observed. Due to the generation of unsatisfactory quality responses by some AI-based systems, students experienced a decline in their initial positive attitude toward the session. For example, when entering a word or combination of words, AI generated images partially or with unsatisfactorily quality or that had illogical content. This prolonged the process of obtaining answers and required additional efforts from the students.

Another significant drawback is that without a live teacher shaping the curriculum and combining the capabilities of AI, students were unable to independently generate study materials and progress in language learning. Because of lacking sufficient knowledge and experience, students could not determine the correct direction of their learning journey

(Rebolledo Font de laVall & Gonzales, 2023). Therefore, despite the fact that AI is capable of generating a vast amount of information, it still cannot fully replace a live teacher (Parab, 2020).

5. Conclusion

The creation of AI is undoubtedly one of the most significant developments in the world of science in recent times. Unveiling a vast range of possibilities for its use in education provides a clear advantage in fostering greater student engagement in the learning process. The ability for autonomous language learning through the use of chatbots, audio and video content generators are just a few examples. It offers considerable flexibility in language learning, taking into account the individual characteristics of each participant of the educational process. Therefore, the diversity of innovative approaches in language learning through the lens of AI is a very extensive area that requires thorough and prolonged study.

However, despite all its advantages, the use of this technology in language learning still requires numerous refinements. One of the main questions that remains is that AI is not yet capable of replacing a live teacher. Therefore, today, its capabilities serve as a complement to innovative language learning approaches.

Thus, the application of innovative approaches in language learning through the capabilities of AI demonstrates positive results in practice and raises numerous theoretical questions, thereby necessitating further research in this direction.

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

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Research

Music in the Life and Works of Boris Leonidovich Pasternak

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Abstract:

This article describes how the life and works of Boris Pasternak were strongly connected with music. Pasternak began his artistic career as a musician, namely a pianist and student of composition. His interest in music was contributed by his acquaintance with the composer Alexander Scriabin, who made a strong impression on the young Pasternak and inspired him to music. Pasternak devoted several chapters to Scriabin in his short prose *Safe Conduct* and *Essay in Autobiography*. A few years later, when Pasternak gave up music, his sense of the musicality of the verse remained in his poetry. Even in his prose work, we can find several passages that mention music or musicians. Pasternak uses musical examples to describe the outer and inner world of his literary heroes. This article focuses on Pasternak's prose work, especially on the novel *Doctor Zhivago* and the autobiographical works *Safe Conduct* and *Essay in Autobiography*, but also gives a very few examples from his rich poetry.

Keywords: Boris Pasternak, Doctor Zhivago, Scriabin, Musicality of the verse.

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1. Introduction

1.1. Artistic environment

The data on life and work of the Russian writer and poet Boris Leonidovich Pasternak (1890-1960) can found in the foreword within (Pasternak, 1986). Pasternak began his artistic career as a musician. He studied composition and played the piano, but then gave up music and began to study philosophy, only then finding his calling in literature. He was born into an artistic Jewish family, where his mother was the pianist Rosa Kaufmann, a student of Anton Rubinstein, and his father was the painter Leonid Pasternak, a professor at the Moscow School of Fine Arts and a friend of Tolstoy, Rilke and Scriabin, among others. The four-year-old Pasternak had the opportunity to attend a home concert in honor of Leo Tolstoy's visit, which was performed by his mother pianist and professors cellist and violinist from the Moscow Conservatory.[1]

Among many others, also the painter Levitan and the composers Rachmaninov and Scriabin visited the Pasternaks' home. So Pasternak grew up in an artistic environment and knew from an early age that he would be involved in art himself, but he did not immediately know which kind of art he would devote his life to.

After deciding to give up music, he studied philosophy in Moscow and in Germany, dropping that too and eventually graduating in law.

Pasternak wrote around five hundred poems and some prose works. He wrote his most famous work, the novel Doctor Zhivago, in 1955. It was published two years later in an Italian translation by the publisher Feltrinelli in Milan, as he could not publish it in his homeland. The editors of the Soviet magazine 'Novi Mir' demanded content changes, but later completely rejected them. In 1958, Pasternak was awarded the Nobel Prize for Literature, but he decided to refuse it due to the hostility against him in Russia.

The music for David Lean's famous film 'Doctor Zhivago' from 1965, based on the novel and later awarded five Oscars, was written by the French composer Maurice Jarre, the most famous of which is 'Lara's theme' (or 'Lara's song'), originally written for orchestra with balalaika.

1.2. Influences

Pasternak was greatly inspired by the poet Alexander Blok. He highly appreciated, among others, Vladimir Mayakovsky, Anna Akhmatova, Marina Tsvetayeva and of course Russian classics, like Dostoevsky, which he also expressed with the words of Yuri Andreyevich Zhivago, the protagonist of his only novel: *"I've always liked Mayakovsky. He is a sort of continuation of Dostoevsky. Or rather, he is a Dostoevsky character writing lyrical poems - one of his young rebels, »The Raw Youth« or Hyppolite*[2] *or Raskolnikov. What an all-devouring poetic energy! And his way of saying a thing once or for all […]"* (Pasternak, 1958, p. 149).

2. Music in Pasternak's prose

2.1. Music genres

In 1956, Pasternak wrote in his *Essay in Autobiography*: *»I have just finished my chief and most important work, the only one of which I am not ashamed and for which I take full responsibility, a novel in prose with a section in verse, Doctor Zhivago. The poems scattered over the past years of my life and collected in the present book are steps preparatory to the novel. (Pasternak 1919*[3]*, p. 119). It seems that the description of the literary creation of the protagonist, doctor Zhivago, is an autobiographical fact with which the writer marked his own artistic path: »Yura had a good mind and was an excellent writer. Ever since his schooldays he had dreamed of composing a book about life which would contain, like buried explosives, the most striking things he had so far seen and thought about. But he was too young to write such a book; instead, he wrote poetry. He was like a*

[1] He describes this event in the *Safe Conduct* and states that they probably performed Tchaikovsky's trio (Pasternak, 1980, p. 125).

[2] In the Slovenian translation, the comma is missing between the words Ippolit and Raskolnikov, so it looks as if Raskolnikov's name was Hypollite (he was actually Rodion). These are two different people: Ippolit Terentyev, the sick young nihilist from the novel *The Idiot*, and Rodion Raskolnikov from *Crime and Punishment*.

[3] Strangely, the date quoted in the book is wrong. Pasternak had finished *Doctor Zhivago* much latter (1955) and *The Essay in Auto biography* in 1957.

painter who was always making sketches for a big canvas he had in mind." (Pasternak, 1958, p. 107).

The novel *Doctor Zhivago* begins with a description of the funeral of Zhivago's mother. On it, a song 'Rest Eternal' ('Вечная память') had been sung, which the translater incorrectly put into Slovenian as 'Blagor mu, ki si spočije' (this is a grave poem of Davorin Jenko, set to the text by Fran Cegnar) and 'The souls of the righteous' ('Со духи праведных'). Maybe the translater wanted to bring those songs closer to the Slovenian environment. Even in the work Essay in Autobiography, where Pasternak describes how young people carried the coffin with the dead writer Tolstoy at the Astapovo station, where he died, to the train, they were singing the song 'Eternal memory'. That one is again incorrectly stated in the Slovenian translation (Pasternak, 1980, p. 151).

In the novel *Doctor Zhivago*, there is a passage where Lara dreams that she is dead, and on earth they sing the gypsy romance 'Распошёл', which Pasternak quotes with the verse 'Black eyes and white breast' ('Чёрные очи и белая грудь') and a Russian folk song 'Masha must not go to the river' ('Не велят Маше за реченьку ходит') (p. 81). The first poem is a gypsy romance of the "Kursk's nightingale", that was the famous pre-war gypsy singer Varja Panina[4] (Fedunina, 2011, p. 48). The choice of songs or their lyrics coincide with the mental experience of the heroine. In the next chapter (Pasternak, 1958, p. 81), Pasternak writes about the "inner music" in Lara's life, which is like a kind of connection with religion: *"Lara was not religious. She did not believe in ritual. But sometimes, to be able to bear life, she needed the accompaniment of an inner music. She could not always compose such a music for herself. That music was God's word of life, and it was to weep over it that she went to church."* (idem). Pasternak also mentions a church psalm, which was sung in the church in Lara's presence (Pasternak, 1958, p. 82).

Two songs that were sung at the Lara's wedding with Pasha Antipov, are also mentioned. One woman sang 'The Vineyard' with the double refrain "God give you love and concord" and the song "Undo the braid, scatter the fair hair." ('Расплетайся трубчата коса, рассыпайтесь русы волоса'. (Pasternak, 1958, p. 156). 'The Vineyard' is originally called 'Vine Blooms in the Garden' ('Виноград в саду цветет'), this is an old Russian wedding song.

Another allegedly old Russian song is mentioned in the novel, which the protagonist did not know. He thought that it was a possible to be an improvisation, when he heard it from the mouth of a folk witch who used to cast a spell on livestock. Its text is given in its entirety. (Pasternak, 1958, pp. 578-579).

Using the metaphor of water behind a dam, Pasternak artistically describes the characteristics of Russian folk songs. He says that the Russian song *"is like water held back by a dam. It looks as if it were still and were no longer flowing, but in its depths it is ceaselessly rushing through the sluice gates and the stillness of its surface is deceptive. By every possible means, by repetitions and similes, the song slows down the gradual unfolding of its theme. Then at some point it suddenly reveals itself and astounds us. That is how the song's sorrowing spirit comes to expression. The song is an insane attempt to stop time by means of its words."* (Pasternak, 1958, p. 578). Among the text of the novel, he also quotes part of a song sung by workers in the factories in the Urals,[5] where Zhivago's family moved, but it is basically a miners' song: *"Goodbye, main office, / Goodbye, shaft and mine. / The master's bread is stale to me / And I am sick of drinking water. / A swan is swimming past the shores, / He makes furrows in the water. / […]"* (Pasternak, 1958, p. 428). Pasternak must have heard this song during his two-year stay in the Urals before the revolution, where he worked in the administration of a military factory, and then mentioned it in his novel four decades later.

The author mentions another type of song from the Urals, namely *"the indecent song about the silly old woman Sentetiurikha, which was well known throughout the Urals, came into her mind,*

[4] Varvara Panina inspired with her talent as well other Russian artists, such as the poet Alexander Blok and the painter Konstantin Korovin (Panina V, 2023).

[5] Pasternak lived in the village of Vsevolodo-Vilva in the Perm region, which is said to be the prototype for Varikina from the novel. He was also a few times in Perm, which he knew very well, and this city became the prototype for the novel city of Yuriatin (Krasnov Tour Agency, 2023).

but only the first two lines could be quoted: "Sentetiurikha sold her cart / And bought a balalaika..." (Pasternak, 1958, p. 500).

Pasternak also mentions Georgian folk music in Tbilisi, in that time named Tiflis, namely the drums that beat the rhythm of the Caucasian fast dance of the lezginka[6] and *"the goat-like bleating of bagpipes,[7] and the sound of other instruments."* (Pasternak, 1919, p. 111). The writer visited Georgia twice, in 1931 and 1933, and translated Georgian poets in addition to, among others, Goethe, Heine and Shakespeare.

In relation to classical music, the writer mentions an interest in music in a family of intellectuals who were *»cultivated, hospitable, and great connoisseurs and lovers of music. They often held receptions and evenings of chamber music at which piano trios, violin sonatas, and string quartets were performed.« In January 1906, they had one of their regular chamber events. »There was to be a first performance of a violin sonata by a young composer, a pupil of Taneiev's, and a trio by Tchaikovsky.«* (Pasternak, 1958, p. 90) The performing pianist was César Cui's nephew. *»The sonata was known to be dry, labored, and boring. The performance confirmed this belief, and the work turned out to be terribly long as well.«* (Pasternak 1958, p. 93).

2.2. *Descriptions of different sounds*

It is interesting how Pasternak described the cries of babies in the maternity wards in purely musical terms, as if he were describing a piece of music: *»"Wa, wa," yelled the babies all on one note, almost impassively, without feeling, as if it were all in the day's work. Only one voice stood out from the others. It was also yelling "wa, wa," and it did not express any more suffering than the rest, but it was deeper and seemed to shout less out of duty than with a deliberate, sullen hostility. […] For some reason he imagined that the voice he had singled out was that of his son; perhaps it was because this particular cry had its own character and seemed to foreshadow the future personality and destiny of a particular human being; it had its own soundcoloring"* (Pasternak, 1958, p. 272).

The description of sounds in the novel is sometimes associated with music. For example, this is how he describes the soundscape in the forest: *"The wood echoed to the hoarse ringing of other saws; somewhere, very far away, a nightingale was trying out its voice, and at longer intervals a blackbird whistled as if blowing dust[8] out of a flute."* (Pasternak, 1958, p. 383).

In another passage of the novel, Pasternak, through the words or diary notes of Doctor Zhivago, thinks about bird song, namely the song of the nightingale, and analyzes it a bit. About nightingales, he writes that he *"wondered at the difference between their song and that of all other birds, at the sudden jump, without transitions, that nature makes to the richness and uniqueness of their trills. Such variety and power and resonance! Turgenev somewhere describes these whistling, fluting modulations. There were two phrases that stood out particularly. One was a luxurious, greedily repetitive tiokh-tiokh-tiokh, in response to which the vegetation, all covered with dew, trembled with delight. The other was in two syllables, grave, imploring, an appeal or a warning: 'Wake up! Wake up!'"*(Pasternak, 1958, p. 458).

2.3 Music as a metaphor

At the end of the novel, Pasternak compares the love between Zhivago and Lara to music: *"Oh, what a love it was, utterly free, unique, like nothing else on earth! Their thoughts were like other people's songs."* (Pasternak, 1958, p. 799). Lara and Zhivago were so coordinated, united in perfect harmony and beauty like singing.

He also describes Lara's emotional farewell to the dead Zhivago with the help of music: *"And now she took her leave of him, addressing him in the direct language of everyday life. Her speech, though lively and informal, was not down-to-earth. Like the choruses and monologues of ancient tragedies, like the language of poetry or music, or any other conventional mode of expression, its logic was not rational but emotional."* (Pasternak, 1958, p. 800).

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6 Lezginka is a dance of the Lezgin people, a North Caucasian ethnic group with their own language (Wikipedia).

7 In the Slovene translation, the traducer left the word »bagpipes« in Russian language (»volinka«), which is not right. Etymologically, those bagpipes come from the Volyn region of Ukraine. They are made of goat skin, which is why Pasternak writes about "goat bleating".

8 In the sense that he has to uncork the flute and blows it. It is interesting that he associates birdsong with the flute, which is also a common phenomenon in the classical repertoire for the flute.

In the autobiographical prose work *Safe Conduct*, he also describes his unhappy love using musical terms: *"My craving for that one last farewell which would have laid waste to everything was still unassuaged. It was like a craving for a grand cadence such as might shake a great piece of music to the very roots and be strong enough to wrench it right out at last by the heave of that final harmony."* (Pasternak, 1958, p. 207).

In the *Safe Conduct* he also describes an evening in Venice[9] with an outdoor concert. He characterizes the fast and furious walking of female walkers: *"allegro irato, was a strange response to the black vibrancy of the firework display between the white furrows of flaming diamonds."* (Pasternak, 1959, p. 240). Venice was full of sounds, and at night the writer was awakened by a *"guitar arpeggio"* (Pasternak, 1959, p. 241).

Pasternak uses some musical examples to describe cities and nature. This is what he writes about the German city of Marburg, where he went to study philosophy: *"And up on the hill Marburg would break into operatic light."* (Pasternak, 1959, p. 197). He describes sounds in nature completely musically, even with the definition of an interval: *"Till morning came, the roar of the sluice waters maintaned unchanged the deafening note they had assumed as night descended. At the interval of a third, the air-fending whine of the sawmills sang seconds to the bullocks at the slaughter-house. Every now and then something broke, something flared up, something snorted steam, something was overtuned. Something writhed as it drew crimsoned smoke over itself."* (Pasternak, 1959, p. 198-199).

About Berlin, which he visited with his parents in 1906, he writes that it was full of Russians, including the late Romantic composer and pianist Vladimir Rebikov, who played his 'Christmas Tree' for acquaintances (Pasternak, 1919, p. 60). The mentioned music is essentially a 'Valzer op. 21' from the musical drama 'Christmas Tree', which at that time was being performed with great success from Berlin to Perm (website of the Mariinsky Theater).

3. Pasternak's poetry

Intensive involvement in music in his youth and great enthusiasm for musical art left a strong influence in Pasternak's poetry. This influence is most visible in the "vocality and vocal structure of the verse" (Vera Brnčič, afterword in: Pasternak, 1986, p. 9). Russian musicologist Mihail Kazinik wrote about Pasternak's poetry that his excellent musical knowledge contributed to the poet's musicality of verses, which in Russian poetry is comparable only to Pushkin, and with them Pasternak managed to express the musical ideas of Chopin, Beethoven and Scriabin (Kazinik, 2015, p. 504).

Pasternak himself wrote that this musicality is *"not a matter of acoustics or of harmonising vowels and consonants as such, but of relating sound to meaning"* (Pasternak, 1919, p. 68), as considered his poet colleague Andrei Beli. Beli was thinking about poetry in a rather scientific, theoretical way. He conducted a course of practical lessons in Russian classical iambic verse and *"in discussion with his students used statistics to explain its rhythmic figures and variations"* (idem).

Pasternak also described this curiosity of the poet's work in the novel *Doctor Zhivago*, where the protagonist doctor also writes poems. *"After two or three stanzas and several images by which he himself was struck, his work took possession of him and he felt the approach of what is called inspiration. At such moments the relation of the forces that determine artistic creation is, as it were, reversed. The dominant thing is no longer the state of mind the artist seeks to express but the language in which he wants to express it. Language, the home and receptacle of beauty and meaning, itself begins to think and speak for man and turns wholly into music, not in terms of sonority but in terms of the impetuousness and power of its inward flow. In the following, he writes how the poet tried different meters and found a parallel with music in his poetry: "At first he used a broad, spacious pentameter. The regularity of the rhythm, independent of the meaning and inherent in the meter itself, annoyed him by its doggerel artificiality. He gave up the pompous meter and the caesura and cut down the lines to four beats, as you cut out useless words in prose. […] The task was now more difficult but more engaging. The result was livelier but still too verbose. He forced himself to even shorter lines. Now the words were crammed in their trimeters […] the right words to fill the short lines came, prompted by the measure. Things scarcely named in the lines*

[9] Pasternak traveled to Italy, to Venice and Florence, in 1912 and wrote enthusiastically about them in the *Safe Conduct*.

evoked concrete images. He heard the horse's hoofs ringing on the surface of the poem, as you hear the ambling of a horse in one of Chopin's ballads." (Pasternak, 1958, p. 704). The poems written by the protagonist are also part of the novel. The mentioned poem, written by Zhivago during his last days in company with Lara, contains the motif of the victory of good over evil. The title of this song is 'Fairy Tale', which features a knight on a horse who kills a dragon and thus saves a girl.

Pasternak also mentions Chopin's music in several of his poems. For example, in the song 'A Midsummer Night's Dream' from the cycle Themes and Variations (1916-1922), he mentions Chopin's etudes (Pasternak, 1991, p. 65).

Pasternak compares everything sublime, such as beauty, love and poetry to music. About poetry, in the poem entitled 'Definition of Poetry', he says: *"It is Figaro like hot hail hurled / From the flutes on the wet flower bed."* (Poemhunter.com - The World's Poetry Archive, p. 45). He also wrote a poem entitled 'Music', in which he mentions Chopin, Wagner's 'Valkyrie' and Tchaikovsky's opera 'Paolo and Francesca'.

In the poem 'Winter Nears' Pasternak writes: *"A silvered hazel October. Pewter glow since frost began. Autumn twilight, of Chekhov, Tchaikovsky, and Levitan."* (Poemhunter.com - The World's Poetry Archive, p. 167). When Pasternak quotes Tchaikovsky in these verses, he has in mind his composition 'October' from the cycle 'The Seasons'. As noted by the Russian musicologist Mihail Kazinik, in this composition we find all the characteristics of Tchaikovsky, who was mostly sadly melancholic, but also had joyful moments (Kazinik, 2020).

Among Pasternak's poems, there are several that mention music and musicians or have a purely musical title (for example: 'Paganini's Violin', "Beethoven's bust" in the poem 'Definition of the Creative Art', Tchaikovsky in 'Winter Nears', or a poem that mentions Brahms: '*Sometime at a concert hall, in recollection': "Sometime at a concert hall, in recollection, / A Brahms intermezzo will wound me-I'll start" ('Годами когда-нибудь в зале концертной / Мне Брамса сыграют')*.

As noted by the Russian politician, literary critic and translator Mark Slonim, Pasternak's poetry can only be roughly translated into English due to its complexity and specific combination of image, music and meaning (quoted from the 'Poetry Foundation' website). In recent times, Pasternak's poetry has mainly been translated into Slovene language by a poet Tone Pavček. Pasternak's prose was translated by Janko Moder, who is also the author of the first Slovenian translation of *Doctor Zhivago* from 1967. Since then, several reprints have been published, but the translation would need updating, as well as the translations of his short prose, which were published in 1980. This this fact is also confirmed by the previously mentioned examples of inadequate translation solutions related to music: in the *Essay in Autobiography*, there is a Russian instead of Slovene word for bagpipes, and the song 'Blagor mu', which appears in the novel *Doctor Zhivago* and in the *Essay in Autobiography*, is in fact a Russian obituary poem 'Eternal Memory'.

4. Pasternak as a composer and his acquaintance with Scriabin

Pasternak was intensively involved in music for six years, from the age of thirteen to nineteen. His composition teachers were Reinhold M. Glière and Joel D. Engel, under whose guidance he prepared for the exam at the Moscow Conservatory. His idol who had the greatest influence on him was Alexander Scriabin, who was a friend of Pasternak's family.[10] With Pasternak's father, Leonid Osipovich, Scriabin *"argued about good and evil and life and art, he attacked Tolstoy and preached Nietzsche's superman and amoralism. They agreed only in their conception of the essence and problems of craftsmanship, in everything else they differed."* (Pasternak, 1919, p. 44). Pasternak writes that he was twelve years old[11] at the time and did not understand their disputes, but Scriabin conquered him *"by the freshness of his mind."* He *"worshipped him."* (Pasternak 1919, p. 45). Pasternak closely followed Scriabin's compositional activity *"of his middle period, roughly between his third and fifth sonatas."* (Pasternak, 1919, p. 49).

[10] The Pasternaks and Scriabins were neighbors in their summer cottages (dachas) in 1903; the two families met that year. Pasternak writes that the melodies of Scriabin's 'Third Symphony' and 'Divine Poem', played on the piano, spread throughout the forest where they were vacationing (Pasternak, 1919, p. 42).

[11] At that time (in 1903), Pasternak was already thirteen years old, as he was born on February 10, 1890.

Pasternak was musically talented and Scriabin praised[12] his compositions and encouraged him; he writes about this in more detail in the *Safe Conduct* and in the *Essay in Autobiography*. Pasternak loved music deeply, but he doubted about his talent, and since he had no absolute pitch, he gave up music completely, gradually stopped playing the piano, and then even stopped going to concerts and avoided meeting musicians. In his *Essay in Autobiography* he writes: *"For six years I had lived for music. Now I tore it up and flung it from me as you throw away your dearest treasure."* (Pasternak, 1919, p. 48). At the time of his transition from music to literature, he was a member of a circle called "Serdarda" and at the beginning of their evenings, when the guests were arriving, on the piano he *"improvised musical sketches of each of them."* (Pasternak, 1919, p. 66). The opera singer Guryev from Saratov, whom the writer remembers in the same autobiographical work, also participated in this circle: *"he had a deep, gentle, powerful voice and the art of bringing out every tonal and dramatic subtlety of anything he sang."* (Pasternak, 1919, p. 65).

Pasternak wrote about Scriabin in his prose works *Safe Conduct* and *Essay in Autobiography*. In *Safe Conduct*, he mentions, among other things, how Scriabin came to Pasternak's family before he left for Italy. At that time he had dinner with them and played the piano (Pasternak, 1959, p. 170). In another place (in the *Essay in Autobiography*, Pasternak 1980, p. 46), it is written that Scriabin travelled to Switzerland for six years at that time. According to the Scriabin Association website, Scriabin lived outside of Russia between 1904 and 1909. He moved to Switzerland, and in 1905 he resided with his second wife[13] in Bogliasco, near Genoa, Italy. Upon his return to Russia, concerts of his compositions were immediately organized, namely in St. Petersburg and in Moscow at the Conservatory. The Moscow concert evening at the Conservatory and the performance of 'Ecstasy' are mentioned by Pasternak in the *Safe Conduct*. After Scriabin's return from abroad, the young Pasternak took his composition to him for evaluation. At that time, Scriabin *"talked — about the harm of improvisation, and of when, with what purpose and how to compose. As models of the simplicity at which I should always aim he offered his latest sonatas, famous for their brain-racking character. Examples of the complexity to be condemned he drew from the most commonplace sentimental romances."* (Pasternak, 1959, p. 176). At that time, the composer advised Pasternak to enroll in philosophy instead of law, where he had planned to study, and Pasternak followed his advice, enrolled in philosophy studies in Germany, and then abandoned this too and devoted himself to literary creation.

According to the website of Boris Tarakanov's sheet music archive, two of Pasternak's compositions have survived, namely two preludes and a piano sonata.

5. Conclusion

The role of music in Pasternak's life was highly important for his work. His poetry is characterized by a musical perfection of the verses, and the musical examples themselves play an important role in describing the atmosphere. Even in Pasternak's prose we can find many examples that relate to music and musicians. A special part of his prose are the passages where he describes his meetings with the composer Scriabin and the influence that the composer's strong personality had on the teenage Pasternak. These short chapters are also a valuable resource from a musicological perspective.

[12] Pasternak also mentions that Scriabin was self-centered, but he was very benevolent towards the almost child Pasternak. Otherwise, pianist Arthur Rubinstein in his autobiography *My Many Years* wrote an interesting anecdote in connection with Scriabin's opinion about the work of his composer colleagues. During Rubinstein's youthful stay in Paris, Scriabin came to the French capital to play his compositions. The composer invited the pianist, his great admirer, to tea and cakes at the Café de la Paix and asked him during the conversation which was his favorite composer. Rubinstein immediately replied that it was Brahms. Scriabin "slammed his fist on the table." "What, what?" he shouted. "How can you love this terrible composer and me at the same time? When I was your age, I was passionate about Chopin. Later I became a bit of a Wagnerian, but now I can only admire Scriabin!' And he grabbed his hat and stormed out of the cafe, full of rage, leaving me stunned by this scene and the bill I had to pay." (Rubinstein, 1983, pp. 168-169).

[13] Pasternak was also a friend of the Russian pianist Heinrich Neuhaus, whose wife Zinaida divorced him after meeting Pasternak and married Pasternak in 1934. Zinaida Neuhaus Pasternak thus became Pasternak's second wife.

From examples of references to music in prose, we learn, among other things, which compositions were in fashion at that time, which folk songs Pasternak heard in the Urals, because they were probably widespread, and how the writer skilfully used purely musical terms to describe the sounds of the environment and musical metaphors to describe non-musical phenomena. For him, music was a metonymy for harmony, moderation, beauty and love.

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