SOCRATIC LECTURES

11TH INTERNATIONAL SYMPOSIUM, LJUBLJANA, 8. JUNE 2024
PEER REVIEWED PROCEEDINGS

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11TH SOCRATIC LECTURES 8.6.2024

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The members of the Organizing Committee of 11th Socratic Lectures:
Veronika Kralj-Iglič, Yelena Istileulova, Anna Romolo.

Program of the Symposium Socratic Lectures, June 8, 2024, 15:00 – 18:00 (Ljubljana time)

15.00 - 15.05: Veronika Kralj-Iglič: Welcome to the Z-STEAM activities and Socratic lectures on line https://uni-lj-si.zoom.us/j/98434774673

15.05 -15.30: Plenary lecture: Jelena Danilović-Luković: Application of electron microscopy in research of bioremediation of heavy metals by microalgae

Scientific sections

Section 1: Biophysics and Biomechanics, organized by **Veronika Kralj-Iglič**

15:45 - 16:00: Matic Kolar, Vane Antolič: The role of bone stock in total hip replacement

16.00 - 16:15: Kovačić Borut, Slokar Tanja: A less invasive Legg-Calve-Perthes Disease treatment

16:15 - 16:30: Fallabela Petra, Večerić - Haler Željka: Arginine vasopressin resistance (AVP-R)

16:40 - 16:55: Arko Matevž: Characterization of cellular nanoparticles from equine milk and colostrum

16:55 - 17:10: Marija Ipavec: Experience with different types of above knee prostheses

17:10 - 17:25: Boštjan Korenjak: Determination of number density and size of extracellular particles directly in diluted blood

17:25 - 17:40: Laure Bar, Marta Lavrič: Quartz crystal microbalance with dissipation monitoring: A method for studying biomimetic membranes

Section 2 : Navigating Uncertainty in Education: Equipping for Life-Long Learning and Arts Skills, organized by Yelena Istileulova https://uni-lj-si.zoom.us/j/98434774673

15:45 - 16:15 Bakhyt Adrysheva: The Inner Healer: Exploring the Potential of Archetypal Art Therapy in School Counselling

16:15 - 16:35: Rosakebia Estela Mendoza: Cultivating longetivity through storytelling: The silver passport case study

16:35 - 16:55: Marko Jeran: The research work of youth as inspiration for their career choices and as a basis for the longevity of knowledge

16:55 - 17:15: Maria Salun: MOOCs and their contribution to non-formal learning in the realities of Ukrainian business education

17:15 - 17:35: Yelena Istileulova, Larisa Mukhamedjanova: Life-long learning skills: from the foresight to the past

17.40-18.00 Honorary lecture for all participants https://uni-lj-si.zoom.us/j/98434774673:

Balas Valentina, Rad Dana: Analyzing AI Integration in Education

Editorial

11th Socratic Lectures took place online. It featured a plenary lecture by Jelena Danilović Luković, two scientific sessions (12 lectures), a honorary lecture by Balas Valentina, Rad Dana and a poster session. The plenary lecture entitled "Application of electron microscopy in research of bioremediation of heavy metals by microalgae" focused on microalgae, the underappreciated majority. It was an important contribution to the awareness of the necessity of their wellbeing and understanding of the One health principle. Section 1 entitled "Biophysics and Biomechanics" and Section 2 entitled "Navigating Uncertainty in Education: Equipping for Life-Long Learning and Arts Skills" were focused on students who were actively included in the lectures. Honorary lecture entitled "Analyzing AI Integration in Education" tackled artificial intelligence that is of wide interest. Socratic lectures are embedded into the Z-STEAM activities (Science, Technology, Engineering, Art, Mathematics) and Arts and Health principles. 11th Socratic Lectures were another event promoting excellence in science and dedication of students. It will be remembered with gratitude to all who generously donated their contributions.

Veronika Kralj-Iglič

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Case report

Arginine Vasopressin Resistance (AVP-R)

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Abstract:

Arginine vasopressin resistance (AVP-R) previously known as nephrogenic diabetes insipidus is a rare disorder characterised with large fluid output due to resistance to arginine vasopressin in kidneys. It can be caused by different etiologies, including hereditary causes. In diagnosis we must determine the reason for polyuria (vasopressin deficiency, resistance, or primary polydipsia). Treatment is mostly symptomatic with adequate water consumption in combination with low-salt and low-protein diet. The main drugs used to treat AVP-R are thiazide diuretics, non-steroidal anti-inflammatory drugs (NSAIDs) and amiloride. In the article we present an illustrative clinical case.

Keywords: Arginine vasopressin resistance (AVP-R), polyuria, thiazide diuretics, non-steroidal anti-inflammatory drugs (NSAIDs), amiloride, acute kidney impairment

1. Introduction

Arginine vasopressin resistance, previously known as nephrogenic diabetes insipidus (till 2022 when endocrinologists decided to change the name (Hui et al., 2024)) is a disorder that results in an inability to concentrate urine due to failure of the kidneys to respond to antidiuretic hormone (Kavanagh and Uy, 2019).

In its broader sense, it can occur quite frequently when the concentration ability of the kidneys is decreased due to acute or chronic kidney disease. In the narrow sense, it is a very rare disorder characterized with polyuria (>50 mL/kg), dilute urine (osmolality <300 mOsm/L), and increased thirst with the intake of up to 20 L/day fluid intake (Hui et al., 2024).

2. Epidemiology

AVP-R is far less prevalent than arginine vasopressin deficiency (AVP-D, previously called central diabetes insipidus), which is estimated to be present in 1 of 25,000 individuals. Congenital AVP-R represents less than 10% of all conditions previously known as diabetes insipidus.(Christ-Crain & Gaisl, 2021)

In congenital AVP-R the X-linked hereditary pattern due to mutations in the AVPR2 gene accounts in 90% of cases and occurs with a frequency of 4–8/1 million male live births. For the remaining cases of congenital nephrogenic diabetes insipidus (DI), autosomal recessive and dominant hereditary patterns due to mutations in the aquaporin - 2 (AQP2) gene are responsible.(Christ-Crain & Gaisl, 2021).

3. Pathophysiology

Under normal circumstances, vasopressin is secreted from the pituitary gland when osmolarity rises above 280-290 mOsm/kg. It is then bound to vasopresin 2 receptor (V2R) in the distal tubule of the nephron, which stimulates a signaling cascade that leads to the insertion of AQP2 channels on the apical side and enables water reabsorption (Kavanagh and Uy, 2019). In AVP-R, there is a defect in the signaling pathway for urine concentration in distal tubule. Congenital AVP-R is a result of mutation in AVPR2 (dysfunction of the V2R receptor) or AQP2 genes (dysfunction of aquaporin 2 water channels) (Kavanagh & Uy, 2019).

4. Etiology

AVP-R can be caused by:
- Medications - predominately lithium which can cause irreversible state after long term use (Garofeanu et al., 2005)
- Hypercalcemia (Rosen et al., 1990)
- Hypokalemia (Marples et al., 1996)
- Kidney disease:
  - Autosomal dominant polycystic kidney disease (Valenti & Tamma, 2021)
  - Chronic kidney disease(Tannen et al.,1969)
  - Infiltrating lesions (Christ-Crain & Gaisl, 2021)
  - Sjögren's syndrome (Patel, 2021)
- Urinary tract obstruction (Carpenter et al., 2018; Frokiaer et al., 1996)
- Hereditary (Kavanagh & Uy, 2019)

5. Clinical manifestation

The most important clinical symptoms are polyuria and polydipsia. People with AVP-R are prone to dehydration and hypernatremia due to extreme urine secretion (Kavanagh and Uy, 2019).

If the disorder is congenital, it usually manifests in the first year of life. Infants may refuse milk, prefer water, may vomit and fail to thrive (Lopez-Garcia et al., 2020).

6. Diagnosis

It must first be determined whether polyuria is present, which is characterized by a urine output of more than 3 L/day or more than 40 to 50 mL/kg/day in an adult (Christ-Crain et al., 2021a). This can be determined with a 24-hour urine collection. There are several age-

dependent limits for children. The osmolarity of the urine is then measured, which must be less than 300 mOsm/kg or between 300and 600 mOsm/kg if the calculated total daily solute excretion is less than 1000 mOsm (Christ-Crain et al., 2021a).

Once the inability to concentrate urine has been established, it should be determined if there is vasopressin deficiency, resistance, or primary polydipsia (Christ-Crain et al., 2021a). To differentiate, a water restriction test and an evaluation of the response to desmopressin should be performed. If AVP-R is detected, it is important to determine the etiology, which may be based on the patient's medical history (medication use, known kidney disease, family history) or laboratory findings (electrolyte disturbances).

The measurement of copeptin can also help with the diagnosis. Copeptin is formed from the precursor protein pre–pro-vasopressin together with AVP and neurophysin II. It has been shown that Copeptin reflects the AVP concentration (Christ-Crain et al., 2021a). As it is more stable than AVP, measurements are easier in clinical practice (Christ-Crain et al., 2021a). In children, genetic testing is performed (Milano et al., 2017).

7. Treatment

There is no specific treatment for AVP-R (Milano et al., 2017). Symptomatic treatment includes adequate fluid intake in combination with a low-salt and low-protein diet to minimize mandatory water excretion (Christ-Crain et al., 2021a). The main drugs used to treat AVP-R are thiazide diuretics, non-steroidal anti-inflammatory drugs (NSAIDs) and amiloride, which are used individually or in combination (Christ-Crain et al., 2021a). Thiazide diuretics are effective in reducing urine output when combined with a very low sodium diet (Christ-Crain et al., 2021a). Potassium-sparing agents such as amiloride may have an additional effect with thiazide diuretics via a mechanism probably related to inhibition of thiazide-induced potassium loss (Christ-Crain et al., 2021a). Ibuprofen and indomethacin improve urinary concentrations in AVP-R patients, where urinary excretion can be reduced by 25-50% (Milano et al., 2017). However, a small study found no significant effect of ibuprofen on urinary excretion of urine osmolarity (Libber et al., 1986). In children with genetic AVP-R, early symptomatic treatment is crucial to prevent developmental disorders caused by hypernatremia and dehydration (Lopez-Garcia et al., 2020).

8. Presentation of an illustrative clinical case

A 43-year old man was admitted to the nephrology department due to an acute kidney impairment with anasarca.

He had seen a pediatric endocrinologist regularly throughout his childhood, and the treatment of choice was symptomatic with regulated hydration. He drank about 20 liters of water per day and urinated about 18 liters of fluid per day. He never had problems with electrolyte imbalance, including a normal serum sodium in the normal range, and his kidney function was normal (i.e. last normal serum creatinine before admission was 78 μmol/l). He has also been diagnosed with type 2 diabetes mellitus and arterial hypertension in the last 10 years. His brother also had a congenital form of AVP-R and his daughter had Abernethy syndrome.

The current medical problem began about a month before hospitalization when he sustained a muscle injury to his leg for which he was prescribed ibuprofen (400 mg three times a day) for pain relief. Due to his work as a butcher, he was unable to rest and took the medication a maximum of three times a day as prescribed, and if the pain was too severe, he took one or two more tablets. After about a week, he noticed increasing swelling and reduced urine output, so he instinctively reduced his water intake by half. At the end of the month, he noticed that he was suffering from a persistent cough and shortness of breath. By restricting his water intake, he lost 3 kg, but the edema was still present, his urine output dropped to less than 10% of his usual daily urine output (though by strict definition he was never oliguric, i.e. he had a daily output of more than 400 ml) and he felt extremely tired. He went to the emergency room, where laboratory results showed severely impaired kidney function with severe hyponatremia, whereupon he was admitted to the hospital. The laboratory findings after admission showed hyponatremia with Na concentration 111 mmol/l and a deterioration in kidney function with an increase in creatinine up to 700 mcmol/l. The urine sediment was unremarkable. We decided to

perform a kidney biopsy, which revealed signs of diffuse acute tubular damage and diabetic nephropathy. The kidney biopsy suggested drug- or ischemia-induced tubular damage.

Fluid restriction and a loop diuretic were initiated, after which diuresis gradually improved and polyuria gradually returned. During this time, the sodium concentration gradually increased into the normal range. The patient lost a total of 20 kg of water in 4 days. On the day of discharge, diuresis was about 9 liters per day with a normal and stable sodium concentration and a creatinine level of 320 μmol/l. The patient was satisfied with the fluid loss and the disappearance of symptoms.

This report discusses a multifactorial Acute Kidney Injury (AKI) in a patient with a congenital AVP-R.

The presented case is an indication of the strong effect of NSAIDs on urinary excretion in patients with congenital AVP-R. The case is very illustrative because of the contrast between the extreme polyuria in the compensated state and the severely reduced urine output in the phase of AKI, which, however, could not be so obvious as the patient rarely reached the criteria for oliguria. It is known that NSAIDs can reduce urine output by 25% to 50% in these patients (Milano et al., 2017).

The reason for the deterioration of kidney function in the present case could be an overdose of NSAIDs that caused acute kidney injury with a reduction in urine output. With the reduced urine output and maintaining the normal fluid intake of 20 L per day, the patient entered the volume overload phase. As a result, his hypertension became out of control and he developed congestion and mild pulmonary edema. The mild hyponatremia was probably due to hypervolemia. Fortunately AKI resolved with symptomatic medical treatment without the need for dialysis and eventually with fool recovery of kidney function.

This case emphasizes the importance of careful monitoring when prescribing new drugs for patients with specific conditions such as congenital AVP-R. NSAIDs, thiazides and potassium-sparing diuretics can reduce urine output in patients with congenital AVP-R (Milano et al., 2017), which can be counterintuitive in some cases. Patients with AVP-R, while accustomed to regulating increased urine output, are not accustomed to regulating fluid intake when urine output is suddenly reduced. When they are prescribed a new medication or start a new diet, they should always be advised to monitor their body weight and reassess fluid intake if they suddenly gain weight.

9. Conclusion

AVP-R is a quite rare disease, and its characteristics may pose many challenges when treating patients who have it. Firstly, there is an enormous required fluid intake and fluid waste which have to be accounted for, and secondly, commonly prescribed drugs such as NSAIDs and thiazide diuretics can impose additional challenges as they interfere with water balance.

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

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Review

Fluorescence and Fluorescein as Pivotal Tools in Cancer Diagnosis and Therapy

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Abstract:
Fluorescent dyes have become important tools in various scientific and medical fields due to their unique capabilities for visualizing and analysing complex biological systems. This review focuses on the pivotal role of fluorescein and its derivatives in cancer diagnosis and therapy. Fluorescein, initially synthesized in the 19ᵗʰ century, has evolved into a crucial diagnostic and research tool, particularly valued for its intense fluorescence and versatility. In medical practice, fluorescein is used in high-contrast imaging techniques such as fluorescein angiography and fluorescence-guided surgery, significantly enhancing the detection and treatment of tumours, especially in oncology. This review covers the properties and applications of fluorescein, including its derivatives such as eosin and fluorescein isothiocyanate (FITC), in various medical and non-medical contexts. Special attention is given to the innovative use of these dyes in paediatrics cancer diagnostics and surgery, highlighting their potential to improve patient outcomes. The review also explores the broader applications of fluorescein dyes in chemistry, forensics, and environmental science, underscoring their ability to detect and visualize substances at very low concentrations. As research continues to advance, the scope and efficacy of fluorescent dyes are expected to expand, offering new possibilities for scientific disoveryes and medical advancements.

Keywords: Fluorescent dyes, Fluorescein, Cancer diagnostics, Paediatric oncology, High-contrast imaging, Tumor delineation

1. Introduction

Fluorescent dyes have become an indispensable tool in a variety of scientific and medical fields, providing unique capabilities for visualizing and analyzing complex biological systems. Fluorescent molecules including fluorescein absorb light at a shorter wavelength and emit light at a higher wavelength. This difference in wavelength allows observers to cast light from a source, filter this source light out, and then observe only the emitted light. As non-fluorescent molecules do not emit light of higher wavelengths at areas where the fluorescent molecules are localized, high contrast with respect to the darker areas is formed. Among such dyes, fluorescein and its derivatives stand out for their intense fluorescence and versatility (**Figure 1**). Originally synthesized in the 19th century, fluorescein has evolved into a crucial diagnostic and research tool, particularly in medical applications such as ophthalmology, oncology, and histology (Jun et al., 2020).

In medical practice, fluorescein is used for its ability to provide high-contrast images, making it invaluable in procedures such as fluorescein angiography and fluorescence-guided surgery. These applications are particularly significant in the field of oncology, where precise imaging can greatly enhance the detection and treatment of tumors. Paediatric oncology, in particular, has benefited from the use of fluorescent dyes, enabling more accurate tumor localization and minimizing damage to healthy tissues during surgical procedures (He et al., 2017).
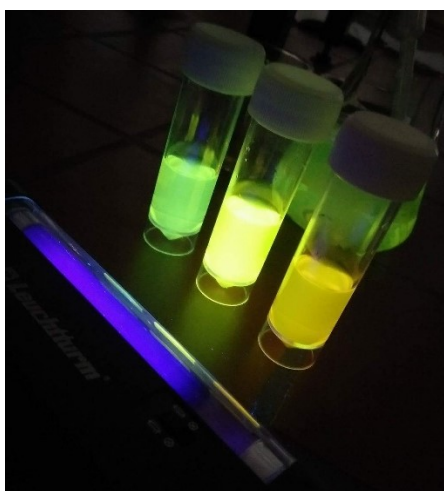
Figure 1. Fluorescence of fluorescein dyes under UV-illumination (Photo: Marko Jeran).

Besides in medicine, fluorescein dyes play critical roles in various scientific disciplines, including chemistry, forensics, and environmental science. Their ability to detect and visualize different substances at very low picomolar concentrations makes them a powerful tool for research and diagnostics (Bell, 2009; Jeran et al., 2020; Jeran et al., 2019). The following sections will cover the properties and applications of fluorescein, including its derivatives such as eosin and fluorescein isothiocyanate (FITC), and their usage in different medical and non-medical contexts. Special attention will be given to the innovative use of these dyes in paediatric cancer diagnostics and surgery, highlighting their potential to improve patient outcomes. As research continues to advance, the scope and efficacy of fluorescent dyes are expected to expand, offering new possibilities for scientific discovery and medical advancements (Le Guern et al., 2020).

2. Fluorescein and the use of fluorescent dyes

In fields such as medicine, forensics, and chemistry, several different fluorescent dyes are widely used, the vast majority of which are fluorescein-based (**Figure 2**). Fluorescein is a crystalline solid organic fluorophore of a yellow-orange color (National Center for Biotechnology Information, 2024). When exposed to light, it has an absorption maxima at 460 nm and emits a greenish-yellow fluorescence with an emission maxima at 515 nm

(Sigma, n. d.). This organic dye fluoresces so intensely that it can be detected even at dilutions of 1:50,000,000 (Britannica, 2018). Fluorescein is also known as D&C Yellow #7, and it was first synthesized by Adolf von Bayer in 1871, from phthalic anhydride and resorcinol. Fluorescein is poorly soluble in water and many other organic solvents, but its sodium salt easily dissolves in water (American Chemical Society, 2013). Its aqueous solutions are sensitive to prolonged exposure to light due to the degradation process (Martínek et al., 2023). Its toxicity is low (National Center for Biotechnology Information, 2024). It is also widely used as a marker in medical and biological applications, especially as a probe for localizing tumor tissues (Pothen and Parmar, 2023). Due to the strong intensity of its fluorescence, it was the first dye used in 1962 to color the Chicago River green on St Patrick's Day (American Chemical Society, 2013).
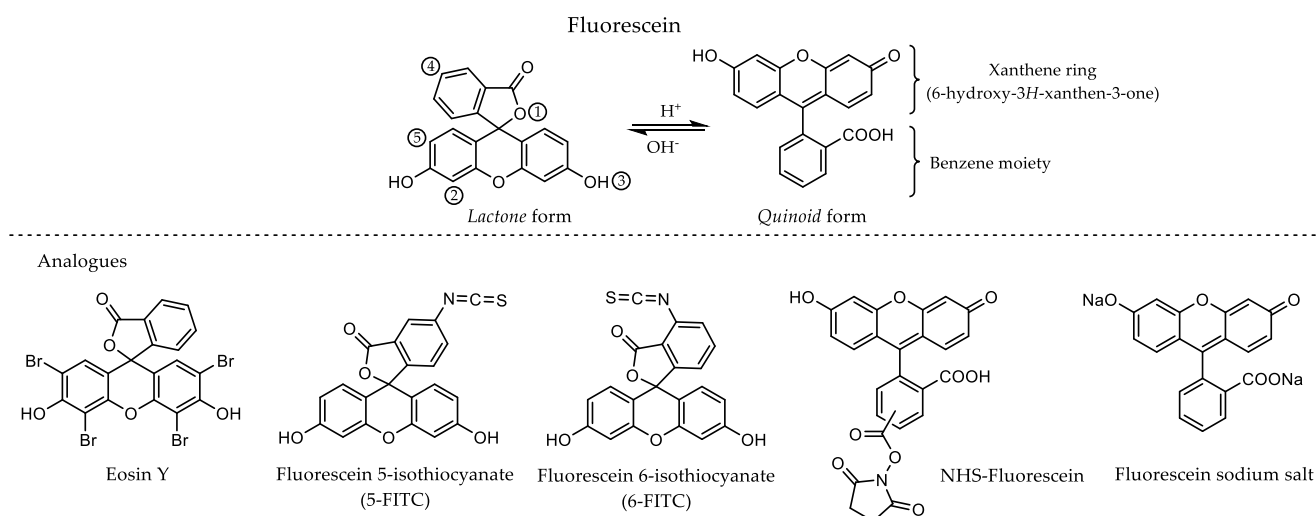
Figure 2. Fluorescein in two different forms, sites for modifications and its relevant analogues.

Fluorescein is on the World Health Organization's list of essential medicines (World Health Organization, 2019). It is a diagnostic contrast agent used mainly in various ophthalmological procedures, such as checking for possible corneal or blood vessel abnormalities. The dye produces an excellent contrast, which can be used to determine if any external corneas, epithelial keratitis, herpes simplex keratitis, or corneal foreign bodies are present. Such lesions can be detected by applying a few drops of fluorescein to the surface of the eye before the examination. Side effects of using the drug may include temporary blurred vision and discoloration of soft contact lenses (Pothen and Parmar, 2023).

Fluorescein is also used in fluorescein angiography, which is a special eye examination using fluorescein. Before the examination, a few drops of mydriatire are instilled into the eyes to dilate the pupils, and then the fluorescein is injected intravenously (through a vein in the arm). The dye then travels with the blood throughout the body and over time reaches the vessels of the retina and choroid. If the background of the eye is healthy, the blood vessels will be coloured and the dye will not come out of them, but if the vessels are damaged, the dye will either emerge or it will be unevenly retained in a certain area. Possible side effects that may occur are transient nausea, allergic reactions (rare), hematoma at the puncture site (General Hospital Celje, 2022). In a study by Huang and co-workers (2016), researchers performed aqueous angiography with fluorescein in bovine eyes. The goal of this study was to examine ventricular angiography using real-time imaging modality of the ventricular outflow tract in cow's eyes using two tracers that had different molecular characteristics. Either fluorescein or indocyanine green solution was

injected. The study showed segments of angiographic outflow patterns using fluorescein (Huang et al., 2016).

Fluorescein dyes are also used in bioimaging, where the fluorescence emitted by fluorescein provides good insight into the identification of non-diseased tissues, tumor-affected tissues or histological markers. Fluorescence can greatly assist the surgeon in removing or repairing the damaged or pathological tissues. The range of application of a fluorescein dye can vary from a bundle of nerves, to a single blood vessel, to tissue abnormalities and even at the molecular level (Pothen and Parmar, 2023). Dyes can also be a conjugated species or a biomarker, where fluorescent microscopy enables the identification of microorganisms or cellular components, such as proteins in immunohistological staining. In the enzyme-linked immunosorbent assay (ELISA), fluorescein acts as fluorescence emitting agent conjugated to secondary antibodies (Pothen and Parmar, 2023).

3. Common Fluorescein Derivatives and Their Usage

3.1. Eosin

Eosin is a derivative of brominated fluorescein (Agar Scientific, n. d.). It is often used in laboratory microscopy as a reddish dye to mark collagen, cytoplasm, muscle fibers, lymphocytes and bacteria (Clinic Sciences, n. d.). Several different types of eosin are commercially available, but the most widely used is eosin Y, which is soluble in both water and alcohol. A 0.5 or 1.0 % aqueous solution is commonly used as a cytoplasmic cell staining agent and, with the addition of thymol crystals, it is used to inhibit fungal growth. Staining with eosin can be strong after fixation with mercury, which can cause difficulties in achieving adequate differentiation (Science Direct, n. d).

For histological studies of paraffin sections, the technique of staining with eosin in combination with hematoxylin has been used for decades. In the staining process, hematoxylin is used to stain anionic components such as RNA and DNA, while eosin dye marks cationic proteins and binds to the phenolic and carboxyl groups of arginine, lysine, histidine and tryptophan residues. Eosin has been used as a selective marker for elastic fibres, muscle cells and mitotic spindle fibres but it is also a fluorescent pH indicator. This dye has also been used as a diagnostic tool to quantify liver damage resulting from hepatitis. The fluorescence pattern of eosin was assessed in a model of liver injury. They concluded that the fluorescence varies according to the health status of the tissue and the dye can be further used to help diagnose and quantify the severity of different liver diseases (Ali et al., 2017).

3.2. Fluorescein Isothiocyanate (FITC)

Fluorescein isothiocyanate (FITC) is one of the most commonly used derivatives of fluorescein. It is used to mark proteins, antibodies, peptides, hormones, amine-modified oligonucleotides, and other amine-containing molecules. FITC is also used to detect compounds that can then be observed using a fluorescence microscope (Rožman, 2012). Its use is also possible in flow cytometry (LS Bio, 2022). At the bottom of the ring, an isothiocyanate functional group ($-N{=}C{=}S$) replaces the hydrogen ($-H$) atom. This isothiocyanate group is the part of the molecule that is amine reactive (LS Bio, 2022). The isothiocyanate group on FITC reacts with primary amines to form covalent thiourea bonds that will bind the fluorescein to the biomolecule. The isothiocyanate group is reactive towards any nucleophilic site, but the dye will selectively react with *N*-terminal amines, due to these bonds being much more stable. FITC is often found as a mixture of its two isomers, namely, fluorescein 5-isothiocyanate (5-FITC) and fluorescein 6-isothiocyanate (6-FITC) (Takai et al., 2011).

In acidic environments where the pH value is less than 2, fluorescein isothiocyanate is found in cationic form. At pH value of about 3.3, it is generally in a neutral form, but when it is present in an environment where pH value is about 5.5, it can be found in the monoanion form. In basic conditions where the pH value is greater than 8, FITC can be found in the dianionic form (Casanovas et al., 2008; Rožman, 2012). In 2013, a study

evaluated the apparent permeability coefficients of model agents (fluorescently labeled FITC-dextran, rhodamine 123 and enalaprilat) in different parts of the isolated rat intestine *in vitro* using the method of bilateral Sweetana-Grass diffusion cells. For the FITC-dextran permeability, the apparent permeability coefficients in absorptive compared to the eliminator direction were not statistically significantly different. No differences between the groups or within a single part of the intestine could be detected. The latter thus indicates a comparable process of transport of the paracellular marker FITC-dextran in both directions, regardless of the part of the intestine (Šenica, 2013).

3.3. 5/6-Carboxyfluorescein succinimidyl ester, mixed isomer (NHS-fluorescein)

NHS-fluorescein is also amine-reactive and has a wide range of applications. It is most commonly used for antibody labeling, in fluorescence microscopy, immunofluorescence-based assays (*e.g.*, ELISA, Western blotting) and in flow cytometry. Unlike FITC, NHS-fluorescein is more specific for primary amines in the presence of the other nucleophiles and has a more stable binding after labelling (Thermo Fisher Scientific, n. d).

NHS-fluorescein is activated with the functional group *N*-hydroxy-succinimidyl-ester (NHS ester). Compared to FITC, the NHS ester derivative exhibits greater specificity for primary amines in the presence of other nucleophiles and results in more stable binding after labeling. Pierce amine-reactive fluorescein dyes are mixtures of isomers with reactive groups at the 5- and 6-positions of the lower ring. The properties of these isomers are indistinguishable in terms of excitation and emission spectra, and there is no need to isolate a specific isomer for protein applications (Thermo Fisher Scientific, n. d).

3.4. Fluorescein sodium salt

The sodium salt is the most common commercially used fluorescein (Mascen Labs, n. d.). The compound is also known by many other names such as resorcinolphthalein, naphthalene, uranine, acid yellow 73, D&C yellow #8 and yellow 8. The dye can be detected at dilutions up to 1:40,000,000 due to its strength (Jacobs, 1992). After injection into the bloodstream, about 80% of the dye binds to plasma proteins, mainly albumin. Then it is metabolized in the liver and kidneys, and is excreted from the body after 24 to 36 hours (Olson & Mandava, 2006). Sodium fluorescein is soluble in aqueous alkaline solutions and fluoresces under cobalt blue filtered light at 465 to 490 nm, emitting green color in the wavelength range of 520 to 530 nm. It is frequently used in opthamology in fluorescein angiography for eye examinations, in bioimaging, and as a biomarker. It is also an indispensable tool in vascular neurosurgery where it helps to track and assess blood flow (Mascen Labs, n. d.).

Sodium fluorescein was first used in 1948 to identify various brain tumors (Moore et al., 1948). Since then, its use and that of other fluorescent markers, particularly in the resection of glioblastoma multiforme, has been reported in the literature. Nevertheless, it has not been presented as an adjuvant in the resection of skull base lesions (da Silva et al., 2010).

Cerebral metastases occur in a large proportion of intracranial tumors and research tells us that the incidence of cerebral metastases is increasing. Resection has been shown to be the most effective treatment method for patients, but bright light surgery (BLS) is usually not sufficient to achieve resection. The research into the use of fluorescence guided surgery (FGS) for brain tumors has only recently started to develop. Sodium fluorescein can be used as a dye in FGS as it is effective in staining tumors such as glioblastoma, malignant melanoma and lymphoma (Xiao et al., 2018).

4. Fluorescein Sodium and its Uses in Cancer Medicine

4.1. Brain tumours

The clinical application of sodium fluorescein has already been extensively documented in diagnosing and finding brain tumors (Moore et al., 1950). Brain tumor develop from abnormal cell growth in the brain or nearby region including the nerves, pituitary glands, pineal gland, and membranes surrounding the brain. Primary brain tumors are those starting in the brain, while secondary tumors refer to its spread to other parts of the body, also known as metastatic brain tumors (Mayo Clinic, 2023). Prior to undergoing craniotomy where a section of the skull is temporarily removed to access the brain, patients

receive an injection of sodium fluorescein (Moore et al., 1950). During surgery, the brain cortex is examined under UV light, revealing yellow-green fluorescence in superficial tumors, which aids in their identification and localization of tumor boundaries (Moore et al., 1950). For deeper tumors, brain needles are used to obtain biopsy material, which is then examined for fluorescence (Moore et al., 1950). The presence or absence of fluorescence of the tissue is a reliable indicator of whether the tissue is abnormal or not (Moore et al., 1950). Even though the tumor is not directly probed, its proximity can be inferred by the fluorescence of adjacent biopsy material.

Among patients suspected of having brain tumors, those with fluorescent tumors were confirmed to have tumors upon examination, while those without fluorescence did not (Moore et al., 1950). However, limitations exist, including benign and slow-growing tumors that do not fluoresce (Moore et al., 1950). Fluorescein's utility in identifying tumor tissue is not exclusive and can also be seen in lesions that disrupt the blood-brain barrier mechanism, such as brain edema, abscess capsules, and traumatized tissue (Schebesch et al., 2016; Moore et al., 1950). Metastatic tumors consistently show bright fluorescence, even in cases with significant tumor necrosis (Moore et al., 1950).

4.2. Breast cancer brain metastasis

Fluorescein sodium applications can also be found in breast cancer brain metastasis (BCBM) surgery (Xiao et al., 2018). Breast carcinoma stands as the most common cancer type and a leading cause of cancer-related death among women (Farahani et al., 2023). Exposure to too much estrogen potentially contributes to DNA damage and genetic changes, responsible for breast cancer progression (Farahani et al., 2023). Research over the years has proven that mutations in genes BRCA1 and BRCA2, which are believed to function as tumor suppressor genes, can cause the development of breast and ovarian cancer (Casaubon et al., 2023). Metastases are responsible for 90% of cancer-related deaths and are a major cause of fatality in breast cancer (Farahani et al., 2023). The brain, axillary lymph nodes, bones, lungs, and liver are the main sites where breast cancer spreads to. Breast cancer cells (BCCs) can spread to the brain by invading their surroundings thus migrating through circulatory system and lastly multiplying inside the brain, therefore developing brain metastases (Farahani et al., 2023).

A study by Xiao et al. (2018) was conducted between May 2012 and June 2016 and it was carried out in 38 patients, who were clinically and pathologically diagnosed with breast cancer brain metastasis. Their objective was evaluating fluorescence-guided surgery as opposed to standard neurosurgical procedure. The patients were divided into two groups. Group 1, which underwent fluorescein-guided surgery, and Group 2, which underwent standard microsurgery using bright light surgery, which typically fails short in complete removal of cerebral metastases. Therefore Group 1 was intravenously injected with 5 mg/kg of fluorescein sodium following allergy testing and prior to general anesthesia, while Group 2 was not administered with fluorescein sodium. The study evaluated surgical outcomes in patients with BCBM who underwent fluorescein-guided surgery compared to standard microsurgery. Results showed that fluorescein-guided surgery yielded better results by enhancing the tumor visibility and had a more notable impact on patients' performance status as opposed to those who underwent standard microsurgery. Additionally, the overall survival after treatment of cerebral metastases (CMs) was slightly higher in patients who received fluorescein compared to those who did not, making fluorescein-guided surgery a secure and feasible approach for resecting BCBM (Xiao et al., 2018).

4.3. Gastric cancer

Stomach (gastric) cancer originates in cells which line the stomach, with the most common stomach cancer being adenocarcinoma, which originates in the mucus-producing cells located in the innermost lining of the stomach (National Cancer Institute, n. d.). In 2002 Bhunchet and co-workers (2002) did a study examining a new method for detecting gastric cancer using fluorescein electronic endoscopy, which is used for detecting early-stage gastric cancer. The study was conducted in 16 patients diagnosed with early-stage gastric cancer (using white light endoscopy and chromoendoscopy), who underwent fluorescein electronic endoscopy with fluorescein sodium before surgery. Subsequently, thorough

histopathologic evaluation was conducted on the resection specimens. Around 10 seconds following intravenous injection of fluorescein, fluorescence was noted and rapidly spread across the inner gastric surface. Early-stage gastric cancers with more abundant stroma than the surrounding normal mucosa displayed significantly stronger fluorescence as opposed to early-stage gastric cancer with less stroma, which displayed weaker fluorescence. Stronger fluorescence intensity was also present in undifferentiated early-stage gastric cancers, characterized by widened stroma due to malignant invasion. The boundaries of early-stage gastric cancers were distinct and clearly observed in all instances. During surgery, some accompanying lesions were also detected, which were previously unnoticed by routine endoscopy (Bhunchet et al., 2002).

4.4. Mouth (oral) cancer

Mouth (oral) cancer covers cancers that form in various parts of the mouth and is categorized within head and neck cancers. Treatment approaches for oral cancers are often similar to those for other cancers in this group. The primary site for mouth cancers typically originates in the flat, thin cells known as squamous cells, which line the interior of the mouth and lips (Mayo Clinic, 2024). A study was conducted in 2020 by Qaiser with co-workers (2020) involving 100 individuals displaying with 42 oral potentially malignant disorders (OPMDs), 40 oral squamous cell carcinoma (OSCC) and 18 controls. The control group consisted of people with inflammatory conditions like pericoronitis and benign fibrous polyps. It was also the first study to document the role of fluorescein in detecting oral cancer and OPMD. The patients underwent a clinical oral visual examination using white light to document the location, characteristics, and extent of the lesion. They were examined under blue light in a darkened room for autofluorescence, and any autofluorescence exhibiting lesion was excluded from the study. Later, sodium fluorescein was topically applied on the lesion and its surroundings, mouth was rinsed and checked for fluorescence from and around the lesion. The researchers indicated that fluorescein offers a swift and sensitive approach to identifying potentially malignant disorders (OPMD) and oral squamous cell carcinoma (OSCC), as it is capable of distinguishing between non-dysplasia and dysplasia/malignant lesions of the oral cavity (Qaiser et al., 2020).

4.5. Bladder cancer

Bladder cancer is still the 10ᵗʰ most common cancer in the world according to data from 2020, and is more prevelant in male than female populations (World Cancer Research Fund International, 2020). While abstaining from smoking, avoiding exposure to metalworking fluids, aromatic amines, and polyaromatic hydrocarbons and such decreases the risk of bladder cancer, proper screening and early medical intervention can help slow the spread and increase the chance of positive therapeutic outcome for bladder cancer treatment (World Cancer Research Fund International, 2020). Early detection of bladder cancer is of upmost importance and this can be done by using fluorescence methods. We can focus on either endogenous or exogenous fluorescence techniques for early detection of *in situ* carcinoma of the bladder. Endogenous fluorescence, also known as autofluorescence, is a phenomenon where diagnostic information is extracted from the fluorescence emitted by tissue fluorophores. The primary contributors to tissue autofluorescence include the reduced form of nicotinamide adenine dinucleotide (NADH) and collagen, particularly in *in vivo* conditions. Utilizing ultraviolet (UV) light, a comparison can be drawn between cancerous and healthy tissue (D'Hallewin et al., 2002). In individuals with bladder cancer, alterations in autofluorescence of healthy tissue occur due to heightened blood absorption, increased thickness of epithelial cells, and diminished excitation of collagen and NADH (D'Hallewin et al., 2002). Due to these changes, fluorescence intensities from carcinoma *in situ* (CIS) fall between those seen in normal tissue and papillary transitional cell carcinoma (TCC), a type of bladder cancer that grows outward from the inner lining of the bladder (D'Hallewin et al., 2002). To distinguish between normal tissue and tumorous lesions *in vivo* fluorescence intensities at two crucial wavelengths should be compared and statistically significant distinction can be made between normal tissue and tumorous lesions (D'Hallewin et al., 2002). The true challenge lies in figuring out the threshold value that separates normal epithelium from tumorous epithelium for more exact diagnosis.

While autofluorescence techniques can be used for detection of bladder cancer, it is not the most reliable, as it is influenced by factors like blood absorption and epithelial thickness. In the case of carcinoma *in situ*, such cancer can occur without visible thickened epithelium (D'Hallewin et al., 2002).

Exogenous fluorescence involves intravesical instillation of fluorophores followed by visible light excitation (D'Hallewin et al., 2002). The fluorophores used are derived from photodynamic therapy (PDT), which works on a straightforward principle. A photosensitizing drug is administered via diverse ways and begins accumulating within tumor while sparing normal tissue. Afterwards, light is employed to activate the sensitizer, leading to tumor necrosis while minimizing harm to surrounding healthy tissue. The concept of photodynamic therapy dates to 1900 when Raab and von Tappeiner described it using acridine and visible light (Raab, 1900; von Tappeiner, 1990). Policard (1924) then utilized the characteristic brick-red fluorescence of porphyrins for tumor detection. In 1942, Auler and Banzer first documented the photodynamic activity of porphyrins, with Figge and co-workers (1948) next outlining their tumor-specific localization. Schwartz and co-workers (1955) later improved this with a derivative of hematoporphyrin, enhancing its ability to localize within tumors. Kelly and Snell (1976) were the first to publish findings on using hematoporphyrins for fluorescence-guided diagnosis of bladder carcinoma *in situ* in human tissue samples. They observed bright red fluorescence in carcinoma *in situ*, dysplasia, and exophytic tumors in cystectomy specimens, while none was present in normal mucosa (D'Hallewin et al., 2002).

The concept of photodynamic diagnosis (PDD) centres on the interaction between a fluorochrome (a fluorescent dye with a strong attachment to tumorous cells) and light of a precise wavelength. Upon absorbing the light, the fluorochrome emits light at a longer wavelength, generating fluorescence which is what PDD capitalizes on to pinpoint potential abnormal tissue. The observed fluorescence can originate either from autofluorescence or from added fluorochromes. 5-Aminolevulinic acid (ALA) (**Figure 3**) has been explored since the early 1990s by researchers for its potential in fluorescent detection of urothelial cancer (Steinbach et al., 1995). ALA, a precursor in the heme biosynthesis pathway, triggers the buildup of fluorescent endogenous porphyrins, particularly protoporphyrin IX (PPIX), in epithelial tissues. PPIX serves as the key metabolite for fluorescent detection, with an excitation spectrum of 400 nm. Following intravesical administration of ALA, there is a specific accumulation of PPIX in urothelial cancer cells. This accumulation results in a striking colour contrast between red-fluorescing malignant lesions and the non-fluorescing normal mucosa, which typically appears in back-scattered blue light (Zaak et al., 2005).
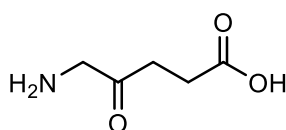
Figure 3. 5-aminolevulinic acid (ALA).

5. Fluorescence Used in Surgery

Before operating on cancer-ridden patients, preoperative imaging techniques like computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) are used, yet the rate of surgical margin positivity has not improved in all three, as it remains between 15–60% across all types of cancers (Zheng et al., 2019). This is largely due to the subjective nature of visual inspection and palpation by surgeons, and the time-consuming process of intraoperative histopathological analysis (Zheng et al., 2019). Fluorescence-guided surgery (FGS) offers an objective and straightforward approach to define tumor margins, providing real-time imaging during surgery (Zheng et al., 2019). FGS is less expensive and easier to operate compared to traditional imaging methods and is growing in popularity (Zheng et al., 2019). FGS relies on two key components: a fluorescence probe and an imaging device. The fluorescence probe, usually

an organic molecule, needs to be able to absorb light at a specific wavelength and emit it at a longer wavelength. For FGS applications, the probe must accumulate in cancerous tissues and only a few probes are FDA - approved. Most commonly used in clinical studies are indocyanine green (ICG), methylene blue (MB), fluorescein sodium, and ALA. Of those four, ICG is the one used most often (Zheng et al., 2019). Its excitation and emission wavelengths are outside the range of most tissue autofluorescence (Zheng et al., 2019). Fluorescein sodium and MB are used less often, and ALA is unique because it is not inherently fluorescent (Zheng et al., 2019). FGS has been used to treat various cancers, such as head and neck, breast, lung, esophagus, gastric, colorectal, anal, prostate, penile cancers, hepatocellular carcinoma and melanoma (Zheng et al., 2019).

6. Fluorescent Dyes in Paediatric Cancer for Diagnosis and Surgery

The application of fluorescent dyes in paediatric cancer has emerged as a groundbreaking approach in oncology, providing critical advancements in diagnostics and treatment (Goldstein et al., 2021; Abdelhafeez et al., 2021). Fluorescent dyes, such as indocyanine green (ICG) and fluorescein, have been extensively researched for their capabilities in enhancing the visualization of cancerous tissues. These dyes exhibit unique properties that allow them to selectively accumulate in tumor cells, which can then be illuminated under specific wavelengths of light to create a vivid contrast between malignant and healthy tissues (Abdelhafeez et al., 2021). This enhanced imaging capability is particularly beneficial during surgical procedures, where it aids surgeons in accurately delineating tumor margins, thereby increasing the precision of tumor excision and reducing the risk of residual disease (Rijs et al., 2021).

Beyond surgical applications, fluorescent dyes have also shown promise in diagnostic imaging. They enhance the detection sensitivity of various imaging modalities, such as fluorescence-guided endoscopy and intraoperative imaging, facilitating earlier and more accurate diagnosis of paediatric cancers. This early detection is essential for initiating timely treatment and improving prognosis (Weiser et al., 2013).

In pediatric oncology, where the preservation of healthy tissue is paramount for the child's growth and development, the precision offered by fluorescent dye-guided surgery is invaluable. The real-time feedback provided by these dyes ensures that surgeons can achieve more complete resections while minimizing damage to surrounding healthy tissues. This is crucial in maintaining the functional integrity of vital structures and improving postoperative outcomes (Te Velde et al., 2010).

Moreover, the field has seen significant advancements with the development of targeted fluorescent probes. These probes are conjugated with antibodies, peptides, or other ligands that bind specifically to tumor-associated antigens or receptors, thereby increasing the specificity and sensitivity of tumor detection. Such targeted approaches enable the precise localization of tumors at a molecular level, which is particularly beneficial in complex cases where tumors are not easily distinguishable from normal tissues (Bertacca et al., 2023).

The integration of fluorescent dyes in paediatric oncology represents a significant leap forward in cancer management. These technologies not only enhance the accuracy and efficacy of surgical interventions but also open new avenues for non-invasive diagnostics and personalized treatment strategies. As research progresses, it is anticipated that the role of fluorescent dyes will continue to expand, offering even more sophisticated tools for tackling paediatric cancers and improving the survival rate and quality of life of young patients (Goldstein et al., 2021).

7. Other Nonmedical applications of Fluorescein

Fluorescein and its derivates can also be used for detection and optical imaging as important fluorescent probes, for which fluorescein derivates are created by introducing aldehyde groups or ester groups onto the fluorescein xanthene ring and benzene moiety. Such derivates can form complexes with analytes, resulting in colour changes and alterations in fluorescence intensity due to their high activity (Yan et al., 2017). In both aqueous solutions and living cells, fluorescein probes can be utilized to detect a range of metal ions including enviromental contaminants including copper, zinc, mercury, gold, silver, palladium, iron, magnesium, cadmium, and lead (Yan et al., 2017). Fluorescein

probes are modified across the five positions (**Figure 2**) and when merged with metal ions, a change of colour and fluorescent intensity can occur. Fluorescein probes can be modified through Schiff base and esterification reactions, providing binding sites for anions (Yan et al., 2017). When these probes react with anions, the unstable bonds within the probe-anion complex result in either fluorescence enhancement or quenching. Fluorescein probes can detect anions like hypochlorite, sulfide, nitrate radical, fluoride ions, and thiocyanate (Yan et al., 2017). There are also multifunctional probes, whose advantage is detecting both anions and cations. To detect small molecules, fluorescein probes' lipophilic properties were better enhanced by esterification and alkylation in site 3 (Yan et al., 2017). When small molecules encounter fluorescein probes, they break ester bonds present in the probes, which restores the π-conjugation structure in probes and further leads to changes in fluorescence emission (Yan et al., 2017). Fluorescein probes have the capability to detect a range of small molecules including amino acids, nitric oxide, hydrogen sulfide, reactive oxygen species, hydrazine, phosphate, and adenosine triphosphate (ATP) (Yan et al., 2017). Another use for fluorescein probes is the detection of enzymes. When enzymes hydrolyse fluorescein's phenolic groups connected to an organism's organic compounds, fluorescence recovery is observed (Yan et al., 2017).

8. Conclusion

The application of fluorescein and its derivatives has notably advanced diagnostic and therapeutic modalities, particularly within oncology. These fluorescent dyes provide essential high-contrast imaging and precise visualization capabilities, which are critical for accurate tumor detection and surgical excision. Their use in procedures such as fluorescein angiography and fluorescence-guided surgery underscores their significant role in contemporary medical practice. In paediatric oncology, the precision afforded by these dyes in delineating tumor margins is paramount, minimizing collateral damage to healthy tissues and thereby improving patient outcomes.

Furthermore, fluorescein's utility extends into environmental science and forensic analysis, where its high sensitivity facilitates the detection of trace substances. While the versatility and efficacy of fluorescein are well-documented, it is important to acknowledge the inherent limitations and potential adverse effects associated with its use. Ongoing research and advancements in fluorescent dye technology are anticipated to broaden their application spectrum, fostering new avenues for scientific discovery and clinical innovation.

In conclusion, fluorescein and its derivatives represent indispensable tools in both clinical and research domains. Their continued development and judicious application hold substantial potential for enhancing diagnostic accuracy, therapeutic efficacy, and contributions to diverse scientific fields. As the discipline advances, the innovative and careful utilization of fluorescent dyes is expected to yield significant improvements in both scientific research and medical practice.

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Research

Critical Analysis of Obstetric Interventions: Perspectives from the ARRIVE Trial and Subsequent Findings in Slovenia

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Abstract:
The quest for optimal maternal and neonatal outcomes in midwifery and obstetrics revolves around the timing of labor induction, a subject of ongoing debate. The ARRIVE trial (A Randomized Trial of Induction Versus Expectant Management) furthered this discourse by comparing labor induction with expectant management, revealing reduced cesarean section rates and improved perinatal outcomes with induction. Concerns arose regarding the generalizability and potential biases of the ARRIVE trial's findings. European studies questioned the applicability of its findings to their populations, given demographic disparities. Despite varied results on cesarean section rates, Cochrane reviews affirmed induction's positive impact on perinatal outcomes. However, recent studies indicated increased risk for cesarean delivery for low-risk nulliparous women undergoing induction. Following the publication of the ARRIVE trial, obstetric practices worldwide experienced a notable shift towards earlier inductions. However, our own study, conducted across all 14 Slovenian hospitals, indicates a rise in labor induction rates that does not correspond with the timeline of the ARRIVE trial's publication. Specifically, the trend of inducing labor in women whose labor started spontaneously in Slovenia is concerning, suggesting a trend towards medicalization of labor. Considering the complexities surrounding the ARRIVE trial, recommendations suggest a balanced approach. Healthcare providers should offer comprehensive information, including trial findings and limitations, empowering women to make personalized decisions. This patient-centered approach ensures optimal outcomes while acknowledging the nuances of individual circumstances.

Keywords: Labor induction, Low-risk pregnancy, Gynecology and obstetrics, ARRIVE trial, Healthcare, Slovenia

1. Introduction

1.1. Navigating timing of labor induction

The practice of midwifery and obstetrics is marked by continual quest for optimal maternal and neonatal outcomes, driven by evolving clinical guidelines, seminal research endeavors and shifting paradigms. Central to this discourse is the appropriate timing of labor induction. Over the past decades, significant efforts have been directed towards elucidating the ideal gestational age for labor induction, with a particular focus on mitigating risks associated with elective inductions and promoting optimal fetal development and maternal health.

In 2009, the American College of Obstetricians and Gynecologists (ACOG) issued a statement advocating against labor induction before 39 weeks of gestation in the absence of medical indications. This guideline aimed to minimize the risks associated with elective inductions and promote optimal fetal development and maternal health (ACOG practice). Furthermore, in 2007, the March of Dimes launched the "Healthy Babies are Worth the Wait" campaign. This initiative sought to educate both women and healthcare professionals about the neurological benefits of childbirth occurring after 39 weeks of gestation for the fetus (Healthy Babies are Worth the Wait). By disseminating information on the importance of allowing pregnancies to reach full term, the campaign aimed to reduce the incidence of elective inductions and promote better health outcomes for newborns (James-Conterelli & Kennedy, 2023).

1.2. A Randomized Trial of Induction Versus Expectant Management (ARRIVE trial)

In the year 2018, a seminal study was published, which continues to be cited by scholars engaged in discourse pertaining to the induction of labour beyond the 39th week of gestation (Grobman et al., 2018). This study, now known worldwide as the ARRIVE trial (A Randomized Trial of Induction Versus Expectant Management), marked a departure from conventional inquiries by eschewing the comparison of induced labour against spontaneous labour, opting instead to contrast labour induction with expectant management – a decision-making process commonplace in obstetric practice (Grobman et al., 2018; Walker et al., 2016).

2. Methods

This retrospective cohort study utilized data extracted from the National Informational Perinatal System (NIPS), encompassing comprehensive records from all 14 hospitals across Slovenia. The study population comprised all women admitted for labor between 2002 and 2022, ensuring a representative sample across various clinical presentations. The original data is included in the appendices (**Appendix A**, **Appendix B**).

The study aimed to classify labor cases according to a modified Robson classification system based on a set of specific criteria (Rossen et al., 2017). The Robson classification system is a widely accepted method for categorizing women into groups based on characteristics that affect the likelihood of cesarean section. The criteria and classification methods applied in this study are as follows:

Group 1: Women were assigned to Robson group 1 if they were carrying a singleton fetus in cephalic presentation, at term, had spontaneous labor, and were primiparous.

Group 2: Women were assigned to Robson group 2 if they were carrying a singleton fetus in cephalic presentation, at term, had labor induced, and were primiparous. This group also includes elective cesarean sections; if these are excluded, it is referred to as group 2A.

Group 3: Women were assigned to Robson group 3 if they were carrying a singleton fetus in cephalic presentation, at term, had spontaneous labor, and were multiparous without a previous cesarean section.

Group 4: Women were assigned to Robson group 4 if they were carrying a singleton fetus in cephalic presentation, at term, had labor induced, and were multiparous without a previous cesarean section. This group also includes elective cesarean sections; if these are excluded, it is referred to as group 4A.

Group 5: Women were assigned to Robson group 5 if they were carrying a singleton fetus in cephalic presentation, at term, were multiparous with a previous cesarean section, regardless of whether labor was spontaneous or induced.

Group 6: Women were assigned to Robson group 6 if they were carrying a singleton fetus in breech presentation and were primiparous, regardless of gestational age or whether labor was spontaneous or induced.

Group 7: Women were assigned to Robson group 7 if they were carrying a singleton fetus in breech presentation and were multiparous, regardless of gestational age or whether labor was spontaneous or induced.

Group 8: Women were assigned to Robson group 8 if they were carrying multiple fetuses.

Group 9: Women were assigned to Robson group 9 if they had a fetus in transverse lie.

Group 10: Women were assigned to Robson group 10 if they did not fit into the previous categories and had a preterm birth.

Each woman's data was evaluated according to these criteria, and they were assigned to the appropriate Robson group accordingly. This classification allowed for the standardized comparison of labor induction outcomes across different subgroups.

3. The ARRIVE trial and its generalisability

The study cohort in the ARRIVE trial comprised 6096 eligible women, randomly allocated into two distinct groups: those subjected to labour induction and those assigned to expectant management. Analysis revealed a significant reduction in cesarean deliveries among the labor induction group compared to expectant management, alongside improved perinatal outcomes, with adverse events occurring less frequently, specifically 4.3%, as opposed to the higher incidence of 5.4% recorded within the expectant management cohort (Grobman et al., 2018).

The findings of this investigation portend implications for clinical practice and policy formulation. Specifically, they suggest that discouraging elective labor induction among low-risk nulliparous women at 39 weeks may not effectively reduce population-level cesarean section rates (Grobman et al., 2018). On a different note, James-Conterelli and Kennedy highlighted alternative strategies to reduce cesarean deliveries beyond labor induction. They emphasized the importance of considering other factors and interventions that may impact birth outcomes (James-Conterelli and Kennedy, 2023).

James-Conterelli and Kennedy (2023) provided insightful critiques in their article, highlighting several notable counterpoints regarding the ARRIVE trial. Firstly, they underscored the absence of data regarding differences in cesarean section rates among the 41 hospitals involved in the study. This omission is particularly relevant as it is known that a woman's likelihood of experiencing a spontaneous vaginal birth may be diminished in hospitals with high cesarean section rates. Additionally, the authors raised concerns about the relatively low enrollment in the study, suggesting that women who chose to participate may have held more favorable attitudes towards induction, potentially biasing the results. They also noted the absence of mention regarding the use of continuous electronic fetal monitoring, which has been associated with higher cesarean delivery rates among low-risk women (James-Conterelli and Kennedy, 2023).

An important argument put forth by the authors is the observation that the cesarean rate for the expectant management cohort in the ARRIVE trial was 22%, notably lower than the average national rates in the United States (James-Conterelli and Kennedy, 2023). This raises questions about the generalisability of the trial's findings to broader population contexts and underscores the need for further investigation into the factors influencing cesarean delivery rates (Carmichael and Snowden, 2019).

It is crucial to acknowledge that the ARRIVE trial was conducted exclusively on the American population. Consequently, there arises a pertinent question regarding the generalizability of the trial's findings and the applicability of suggested policies to countries outside the United States. European nations, in particular, exhibit distinct population characteristics and socio-cultural environments compared to the United States. Factors such as the percentage of obese women, average childbearing age, and overall sociodemographic landscape vary significantly between European countries and the United States. Notably, European women tend to be older on average compared to their

American counterparts, yet they exhibit lower rates of obesity (Facchinetti et al., 2022). Additionally, neonatal outcomes in European countries are often superior to those observed in the United States (Facchinetti et al., 2022). Given these disparities, the authors of the study assert that the potential benefits of elective labor induction at 39 weeks, as indicated by the ARRIVE trial, may not be readily applicable to low-risk women in most European countries (Facchinetti et al., 2022).

Stock et al. (2012) conducted a population-based cohort study, reporting no significant difference in cesarean birth rates between labor induction and expectant management groups. Nevertheless, they also reported that women in the labor induction cohort exhibited a decreased likelihood of perinatal death in comparison to those who underwent expectant management. However, akin to the ARRIVE trial, the generalizability of the data is limited. Additionally, the study did not differentiate between nulliparous and parous women, despite evidence indicating distinct cesarean section rates among these demographic categories (Stock et al., 2012).

In 2018, a Cochrane review was published, clearly affirming the positive effects of labor induction on both the baby and the rate of cesarean sections (Middleton et al., 2018). The review highlighted a reduction in perinatal deaths and a lower incidence of cesarean sections in the induction group, irrespective of the timing of induction or the state of the cervix (Middleton et al., 2018). Grobman et al. (2018) observed that labor induction at 39 weeks in low-risk nulliparous women was significantly associated with a lower caesarean delivery rate but not reduced frequency of adverse perinatal outcomes. Mishanina et al. (2014) confirmed the benefits of labor induction in reducing the risk of cesarean delivery in both term and post-term gestations. However, Butler et al. (2024) identified that low-risk, nulliparous women whose labor was induced between 38 and 41 completed weeks of gestation exhibited a higher likelihood of requiring an unplanned cesarean section compared to those who underwent expectant management.

4. Changes in obstetrical practice following the ARRIVE trial

Following the conclusion of the ARRIVE trial, numerous obstetric departments encountered challenges in adapting their clinical protocols. A study in May 2022, published in the American Journal of Obstetrics and Gynecology, evaluated the impact of the ARRIVE trial on obstetric practices and perinatal outcomes (Gilroy et al., 2022). The study endeavored to compare obstetric practices and adverse perinatal outcomes between pre-ARRIVE and post-ARRIVE cohorts. Notably, individuals in the post-ARRIVE group exhibited a higher propensity for labor induction and a reduced likelihood of delivering beyond 39+6 weeks of gestation. Echoing the findings of the ARRIVE trial, these individuals also demonstrated a diminished incidence of cesarean deliveries relative to their counterparts in the pre-ARRIVE cohorts (Gilroy et al., 2022).

Moreover, subsequent to the ARRIVE trial, a conspicuous increase in both labor inductions and deliveries preceding the 39 + 6 weeks gestational mark occurred, surpassing the incremental rates observed prior to the trial (Gilroy et al., 2022). However, in contrast to the findings of the ARRIVE trial, the aforementioned study revealed a heightened occurrence of immediate assisted ventilation and prolonged assisted ventilation (beyond 6 hours) among neonates in the post-ARRIVE cohort. Additionally, neonates within this cohort demonstrated an elevated probability of presenting with a 5-minute Apgar score below 3. Nonetheless, it is paramount to emphasize that significant pre-existing upward trends had been discerned prior to the dissemination of the ARRIVE trial findings, particularly evident in the utilization of immediate assisted ventilation and prolonged assisted ventilation exceeding 6 hours. Furthermore, there was a notable escalation in Intensive Care Unit (ICU) admissions in 2019, alongside an increased demand for blood transfusions. It is noteworthy that the latter exhibited a pre-existing upward trajectory, whereas ICU admissions did not display such a trend. Despite the unforeseen surpassing of projected values for immediate ventilation requirements in 2019, it is worth noting that both maternal blood transfusions and prolonged neonatal ventilation would have registered higher frequencies in the same year had the pre-ARRIVE trends persisted (Gilroy et al., 2022).

It has been well-established that a significant portion of women express a preference for labor induction over expectant management once they reach post-term gestational stages (Heimstad et al., 2007). However, following the ARRIVE trial, a pertinent inquiry arose: do these preferences extend to labor induction prior to the due date? Gallagher et al. (2020) sought to address this query by investigating women's attitudes towards labor induction for maternal or fetal indications, and whether their stance shifts in the absence of such indications. The study revealed overwhelmingly positive responses from participants regarding labor induction for maternal or fetal reasons. Conversely, when there were no medical indications, slightly fewer than half of the surveyed women expressed interest in labor induction before their due date (Gallagher et al., 2020).

The study highlighted the pivotal role of women's knowledge concerning labor induction. Despite exhibiting good awareness of the techniques employed for labor induction, only 27% of participants were informed about one of the primary findings of the ARRIVE trial: that labor induction reduces the risk of cesarean delivery. Moreover, nearly half of the surveyed women harbored concerns that labor induction could potentially harm their baby, a misconception refuted by the ARRIVE trial, which found no significant disparities in adverse perinatal outcomes between the labor induction and expectant management cohorts (Gallagher et al., 2020).

5.   Results

An examination of labor onsets in Slovenia over the period from 2002 to 2022 yielded notable findings. Analysis of the data reveals an upward trend in the percentage of labor inductions, including elective cesarean sections, accompanied by a corresponding decrease in spontaneous labor onset. As illustrated in **Napaka! Vira sklicevanja ni bilo mogoče najti.**, the increase in labor inductions commenced prior to the publication of the ARRIVE trial in 2018, suggesting that this trial alone cannot be attributed as a significant factor influencing the observed trend.
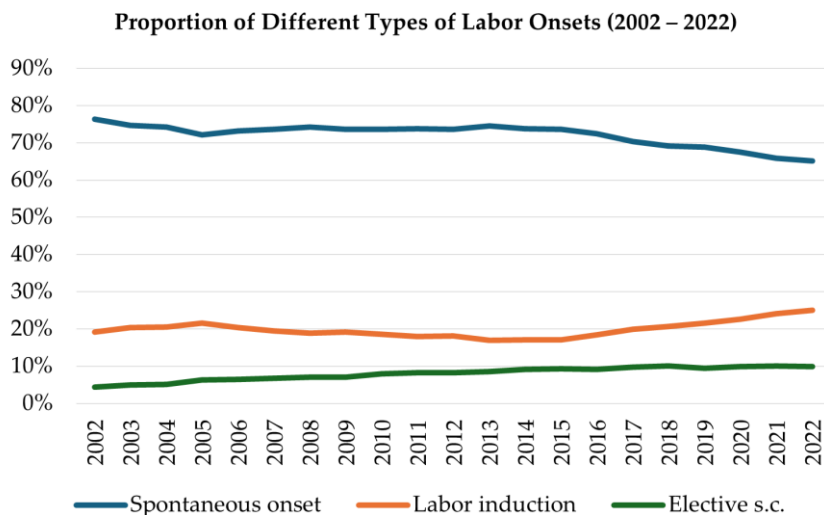
Figure 1. Proportion of different types of labor onsets in Slovenia during the years 2002–2022.

An examination of the distribution of labor inductions in Slovenia across different Robson groups over a 20-year period, as observed in **Napaka! Vira sklicevanja ni bilo mogoče najti.**, reveals a relatively stable overall pattern. There are no significant fluctuations in the percentage of inductions among the various Robson groups. Labor inductions are most commonly performed in women whose labor started spontaneously, specifically in Robson Group 1 (nulliparous, single cephalic, ≥37 weeks, in spontaneous labor) and Robson Group 3 (multiparous, single cephalic, ≥37 weeks, in spontaneous labor). Although the rate of inductions in these two groups has been gradually decreasing, they continue to constitute the largest proportion of labor inductions. The increase in inductions observed

in Robson Group 5 (multiparous with a single cephalic pregnancy, ≥37 weeks, with at least one previous cesarean section) can likely be attributed to the rising number of cesarean sections, resulting in a higher number of women falling into this category.
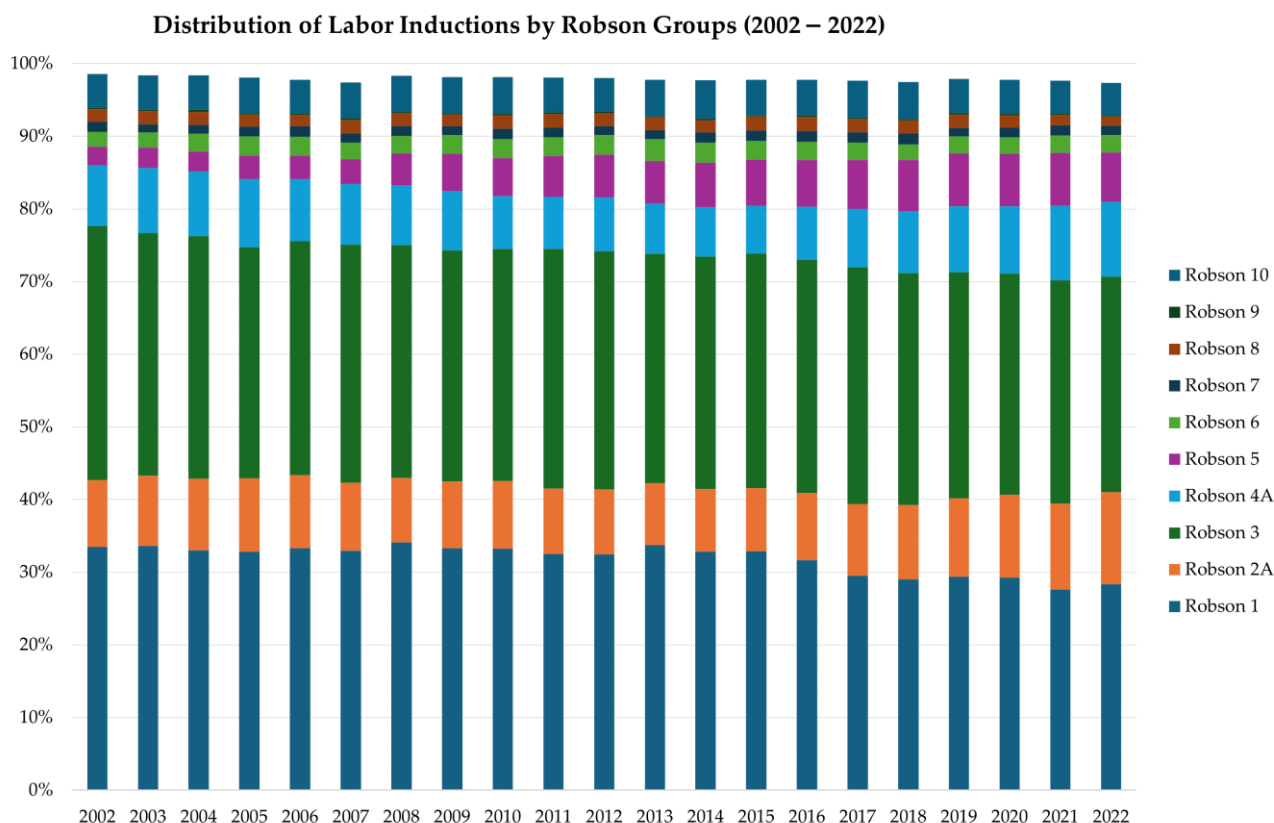
Figure 2. Distribution of Labor Inductions in Slovenia by Robson Groups between 2002 and 2022.

The predominance of labor inductions in Robson Groups 1 and 3 is concerning, as it suggests a trend towards the medicalization of labor in Slovenia. This observation raises critical questions regarding the pre-induction protocols and whether there is sufficient consistency and persistence in employing non-pharmacological and non-invasive techniques to facilitate the continuation of labor. Before proceeding with labor induction, it is imperative to rigorously and systematically incorporate methods such as changing birth positions, which have been demonstrated to enhance the strength of contractions.

6.   Conclusion

Considering the various critiques and complexities surrounding the ARRIVE trial, the recommendations put forth by reputable organizations such as the American College of Obstetricians and Gynecologists (ACOG), the Society for Maternal-Fetal Medicine (SMFM), and the American College of Nurse-Midwives (ACNM) appear to offer a prudent approach. It is advised that the results of the ARRIVE trial not be uncritically adopted as universal guidelines (Carmichael and Snowden, 2019; James-Conterelli and Kennedy, 2023). The significance of education and informed consent regarding both labor induction and expectant management cannot be overstated in enabling women to make informed decisions about their pregnancies.

Our study identifies a notable rise in labor induction rates over the period examined, although the timeline does not align with the publication of the ARRIVE trial. Therefore, any direct attribution of this increase to the ARRIVE trial cannot be made. Nonetheless, the observed trend of increasing labor induction rates raises significant questions regarding the implications and thresholds of such practices. The trend towards labor inductions in Robson Groups 1 and 3 in Slovenia raises concerns about the medicalization

of labor, emphasizing the importance of ensuring vigorous pre-induction protocols that prioritize non-pharmacological and non-invasive approaches to support natural labor progression.

Healthcare providers should exercise discretion and present laboring women with comprehensive information, including the findings of the ARRIVE trial along with its limitations. By providing transparent and balanced discussions, midwives can empower women to make informed decisions tailored to their individual circumstances. This approach ensures that women are actively engaged in the decision-making process regarding their labor induction, thus promoting woman-centered care and optimal maternal and neonatal outcomes.

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

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Appendix A: Types of labor onsets in Slovenia during the years 2002 – 2022

Table 1. Types of labor onsets in Slovenia during the years 2002 – 2022.

			Spontaneous	Induction	Elective s.c.	Total
Year	2002	Count	13254	3334	759	17347
		%	76,40	19,20	4,40	100,00
	2003	Count	12633	3441	831	16905
		%	74,70	20,40	4,90	100,00
	2004	Count	13107	3618	904	17629
		%	74,30	20,50	5,10	100,00
	2005	Count	12918	3841	1128	17887
		%	72,20	21,50	6,30	100,00
	2006	Count	13663	3787	1211	18661
		%	73,20	20,30	6,50	100,00
	2007	Count	14427	3816	1341	19584
		%	73,70	19,50	6,80	100,00
	2008	Count	15997	4041	1507	21545
		%	74,20	18,80	7,00	100,00
	2009	Count	15826	4127	1530	21483
		%	73,70	19,20	7,10	100,00
	2010	Count	16199	4067	1735	22001
		%	73,60	18,50	7,90	100,00
	2011	Count	15907	3876	1771	21554
		%	73,80	18,00	8,20	100,00
	2012	Count	15821	3889	1776	21486
		%	73,60	18,10	8,30	100,00
	2013	Count	15374	3489	1772	20635
		%	74,50	16,90	8,60	100,00
	2014	Count	15221	3512	1878	20611
		%	73,80	17,00	9,10	100,00
	2015	Count	14749	3397	1857	20003
		%	73,70	17,00	9,30	100,00
	2016	Count	14357	3635	1814	19806
		%	72,50	18,40	9,20	100,00
	2017	Count	13885	3928	1913	19726
		%	70,40	19,90	9,70	100,00
	2018	Count	13236	3959	1941	19136
		%	69,20	20,70	10,10	100,00
	2019	Count	13051	4104	1810	18965
		%	68,80	21,60	9,50	100,00
	2020	Count	12481	4179	1829	18489
		%	67,50	22,60	9,90	100,00
	2021	Count	12384	4523	1880	18787
		%	65,90	24,10	10,00	100,00
	2022	Count	11394	4369	1734	17497
		%	65,10	25,00	9,90	100,00

Appendix B: Labor inductions by Robson Groups in Slovenia between 2002 and 2022

Table 2. Labor inductions by Robson Groups in Slovenia between 2002 and 2022.

		Missing data	Robson 1	Robson 2A	Robson 3	Robson 4A	Robson 5	Robson 6	Robson 7	Robson 8	Robson 9	Robson 10	El. s.c. 2	El. s.c. 4	Total
	2002	1	5809	1600	6061	1446	446	359	239	303	28	804	105	146	17347
	2003	1	5681	1637	5646	1513	475	353	191	308	33	800	103	164	16905
	2004	1	5824	1733	5885	1566	488	439	213	314	46	835	129	156	17629
	2005	2	5867	1813	5683	1679	589	469	239	298	34	868	144	202	17887
	2006	14	6213	1878	6015	1588	612	477	270	286	40	864	182	222	18661
	2007	5	6454	1837	6418	1632	666	450	247	369	46	954	203	303	19584
	2008	3	7353	1908	6901	1770	957	511	292	387	37	1066	181	179	21545
	2009	3	7154	1978	6834	1746	1113	545	262	343	38	1074	217	176	21483
	2010	2	7320	2044	7020	1608	1146	581	304	411	53	1106	222	184	22001
	2011	4	7012	1935	7103	1554	1208	561	293	394	44	1035	210	201	21554
Year	2012	4	6979	1920	7047	1588	1264	582	264	381	50	991	212	204	21486
	2013	3	6966	1755	6515	1419	1226	612	254	363	32	1034	195	261	20635
	2014	0	6761	1787	6586	1407	1265	561	295	347	45	1082	190	285	20611
	2015	0	6575	1738	6466	1309	1281	508	279	391	28	982	186	260	20003
	2016	2	6272	1837	6355	1445	1270	500	292	387	37	966	195	248	19806
	2017	4	5820	1954	6423	1589	1326	471	282	355	41	1005	179	277	19726
	2018	0	5554	1958	6104	1636	1352	411	284	339	37	971	197	293	19136
	2019	1	5570	2049	5904	1716	1390	441	223	346	47	885	157	236	18965
	2020	1	5411	2113	5629	1708	1334	417	258	301	46	864	158	249	18489
	2021	1	5189	2219	5778	1928	1366	447	268	263	41	852	143	292	18787
	2022	1	4956	2222	5188	1800	1197	420	219	223	38	772	158	303	17497

Research

Cigarette Smoking and Use of Electronic Cigarettes and Heated Tobacco Products among Pre-School Teachers in Slovenia

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Abstract:

Introduction: Smoking and use of new tobacco and connected products usually start at an early age, and a person remains addicted for life. Therefore, it is important that healthy role models are present in one's life, especially in the early years when children and young people are the most vulnerable. Pre-school teachers are one of the crucial role models for children, and it is important that they provide a healthy example. The objective of this paper is to present data on smoking/use and intention to quit smoking/use of cigarettes, electronic cigarettes (e-cigarettes), and heated tobacco products (HTPs) in this occupational group and state of infringements of the ban on smoking/use of these products within the kindergarten and on its functional land.

Methods: We conducted a cross-sectional survey on a convenience sample of pre-school teachers and assistant pre-school teachers from all regions of Slovenia in 2022 and 2023. We used a web questionnaire, including demographic questions, questions on smoking/use of the products, intentions to quit smoking/use, and detection of infringements of the current ban on smoking/use.

Results: 1367 pre-school and assistant pre-school teachers participated in the study. Almost one fifth (18%) of the respondents were current smokers, and less than one tenth were users of e-cigarettes (5%) and HTPs (7%). There is a low percentage of pre-school teachers who intend quit smoking or using e-cigarettes or HTPs in near future (next 30 days) - 9% of current smokers, 16% of current users of e-cigarettes and 6% of HTPs users. App. every fifth respondent detected infringement of smoke-free restrictions inside the kindergarten or on its functional land.

Conclusions: As they are important role models to children, there should be more focus on raising awareness and promoting cessation among pre-school and assistant pre-school teachers. Emphasis should be given to maximum compliance with the ban on smoking/use of these products within the kindergarten and on its functional land.

Keywords: pre-school teachers; smoking; smoking cessation; electronic cigarettes; heated tobacco products; initiation

1.   **Introduction**

1.1. Tobacco smoking and use of e-cigarettes and HTPs

Tobacco use represents a major risk for cardiovascular and respiratory diseases, over 20 different types or subtypes of cancer, and many other health problems (WHO, 2023). Every year, more than 8 million people die from tobacco use, including approximately 1.3 million non-smokers who are exposed to second-hand tobacco smoke (WHO, 2023). Nearly half of all children breathe air polluted by tobacco smoke, and 65,000 children die each year due to illnesses related to second-hand smoke (WHO, 2023). An estimated 37 million children aged 13-15 years use tobacco (WHO, 2024). In high-income countries, socio-economic inequalities in smoking prevalence as well as exposure to second-hand tobacco smoke (and in health) are obvious – people with lower education and lower income are more likely to smoke and be exposed to second-hand tobacco smoke (WHO, 2019; Kim et al. 2023).

In Slovenia, every fifth adult (18-74 years of age) and almost every seventh 15-year-old smokes. Tobacco is the second most common risk factor for death and the leading risk factor for lost years of healthy life (Koprivnikar et al., 2021). 60 people die from disease caused by tobacco every week, 23% of them before the age of 70 (Koprivnikar et al., 2021). Almost one in six deaths (15%) among the population aged 30 and over is attributable to tobacco (Koprivnikar et al., 2021). In Slovenia, tobacco causes more death per year than accidents (including traffic accidents), alcohol, illicit drugs, suicides and AIDS altogether (Koprivnikar et al., 2021).

HTPs contain tobacco and expose users to toxic emissions, many of which cause cancer and are harmful to health (WHO, 2023). E-cigarettes do not contain tobacco and may or may not contain nicotine, but are harmful to health and undoubtedly unsafe (WHO, 2023). However, it is too early to provide a clear answer on the long-term impact of HTPs and/or e-cigarette use (WHO, 2023; Koprivnikar and Farkaš Lainščak, 2023). In the World Health Organization European Region, e-cigarettes are more popular among young than conventional cigarettes, with 32% 15-year-olds reporting e-cigarette use at some point and 20% in the last 30 days (WHO, 2024). Also there is increasing evidence that exposure to HTPs and e-cigarettes' smoke can have a harmful impact on human health (WHO, 2020; Simonavicius et al., 2019; Amalia et al., 2023).

Different types of tobacco and nicotine products have appeared in Slovenia over the last decades. They are mainly used by young people and their use is increasing (Koprivnikar et al., 2021). Preliminary results from show that use the of e-cigarettes is increasing among adolescents in Slovenia, while a decline in cigarette smoking prevalence is no longer detected (NIJZ, 2024).

1.2. Role models and smoking initiation

Today, young children spend many hours at kindergarten every day. They are daily in contact and high interaction with pre-school teachers whose health behaviours likely have an important and lasting effect on the general health behaviour and future lives of the children. Additionally, pre-school teachers can promote healthy behaviour among children and their parents (Hoffmann et al., 2013; Kawalkar et al., 2023) and are primarily role models for children and their parents due to their qualification to work with children and pedagogical skills (Radha et al., 2020). To our knowledge, there are no published studies in English or Slovene on the association between pre-school teachers' smoking or use of related products and the smoking initiation of children/adolescents. Most research addresses influence of teachers on smoking uptake among adolescents, since adolescence and early adulthood is the time when most people start smoking (Koprivnikar and Korošec, 2015; Escario and Wilkinson, 2018). One of the fundamental principles for smoking prevalence prevention is reducing the smoking initiation rates, in which school teachers play an important role (Arguvanli and Sungur, 2013). Reducing smoking among teachers results in reduced smoking initiation among students (Roohafza et al., 2013; Paek et al., 2013).

1.3. Visibility of smoking and association with smoking initiation

The pre-school's social environment is a key place where children create and reinforce their attitudes, beliefs, and behaviour (as reported for school environment from Lovato et al., 2007). There is limited research that examining if/how school teachers' smoking or using of related products is affecting their students. Positive association between teacher smoking on school grounds and student smoking behaviour has already been reported (Zhang et al.; 2014; Roohafza et al., 2013; Nikaj and Chaloupka, 2016). E-cigarettes are specially designed to attract young children (e.g. child-friendly flavours, designs from cartoon characters and sleek designs) (WHO, 2024) therefore it is very important that children come in contact with them as rarely as possible. One of the ways to provide that is to prohibit smoking and the use of e-cigarettes and use of HTPs inside the kindergarten and on its functional land. - in Slovenia this is banned by law (Zakon o omejevanju uporabe tobačnih in povezanih izdelkov – ZOUTPI (Uradni list RS, št. 9/17, 29/17, 31/24)). Smoking and use of e-cigarettes and HTPs inside the kindergarten or on its functional land is not only bad example for children, but can also be harmful because of the exposure of children to second-hand smoke (WHO, 2021). Violations of the Restriction on the Use of Tobacco and Related Products Act was commonly detected in Slovenia (Koprivnikar et al., 2021) and it is important for kindergartens to respect restrictions and take actions if they detected infringements.

The objective of this paper is to present data on the prevalence of cigarette smoking and use of the e-cigarettes and use of HTPs among pre-school teachers in Slovenia, their intention to quit smoking or using e-cigarettes/HTPs and the state of infringements of smoke-free restrictions inside the kindergarten or on its functional land by age groups and education of the respondents.

2. Methods

This cross-sectional survey was planned and carried out within the target research program entitled »Key aspects in the field of tobacco and related products, with an emphasis on inequalities, awareness raising and proposals for tobacco and nicotine free Slovenia« (target research programmes are scientific and research programmes financed by the Slovenian Research and Innovation Agency – ARIS and the Ministry of Health of the Republic of Slovenia). The survey was carried out with the help of colleagues working on the program Health in kindergarten within the National institute of public health. Within the program "Health in Kindergarten", active from 2006, activities that preserve and strengthen health in the kindergarten environment are implemented.

The web-based questionnaire contained demographic questions (gender, age, education, position in the kindergarten) and questions on the use of cigarettes, HTPs and e-cigarettes, intentions to quit, and the detection of infringement of smoke-free legislation in the kindergarten or on its functional land. Questions on cigarette smoking or use of e-cigarettes and HTPs included ever smoking/use (smoked/used the product at least once in a lifetime); current smoking/use (current smoking/use of the product, daily or occasionally); daily smoking/use (the current daily smoking/use of the product); occasional smoking/use (current occasional smoking/use of the product, i.e. less often than every day). The current users of these products were asked about intention to quit smoking/using e-cigarettes and HTPs. Answers are represented as percentage of those who do not intend to quit (does not think about it, does not intent to quit), percentage of those who intend to quit soon – in the next 30 days and those who intend to quit sometime in the future (intend to quit in the next 6 months, intend to quit sometime in the future). Detected infringement of smoke-free restrictions inside the kindergarten or on its functional land were reported as percentage of the respondents who detected infringement (combined answers: detected infringements only on the functional land of the kindergarten, detected infringements only inside the kindergarten, detected infringements on functional land of the kindergarten and inside the kindergarten), the percentage of those who did not detect infringements and percentage of those who do not know whether they detected it or not.

The invitation to participate in the survey was sent to all kindergartens in their respective regions by regional coordinators of the programme Health in kindergarten. The survey

took place from 3. 5. to 24. 5. 2022 in regional unit Novo mesto, and from 13. 2. to 7. 3. 2023 in all other regional units. With this survey we thus covered all regions of Slovenia. Data from all regions were then joined together for the analysis.

For the analysis, three age groups were created: 20–34 years, 35–49 years and over 50 years. Respondents were divided into three educational groups: secondary education or less (lower or secondary vocational education, secondary vocational education, general education or less), tertiary education (professional higher education (including 1st Bologna degree), more than tertiary education (including 2nd Bologna degree), specialisation, master's degree, doctorate).

Data were analysed with the IBM SPSS 25 program (SPSS Inc., Chicago, IL, USA). To analyse the associations between the selected variables, we used the chi-square test (χ2). The two-tailed test was used to test for differences in answer proportions between groups within the same socio-demographic variable (e.g. proportion of current smokers by age group – comparisons between all different pairs of age groups). A statistical significance value of $p < 0.050$ was considered in all cases. Percentages for certain groups may differ due to rounding to decimal places (e.g. the percentage may exceed or be less than 100%). The possibility of fragmenting the data into more numerous groups is limited by the sample size.

3. Results

3.1. Characteristics of the respondents

1367 Slovenian pre-school teachers and assistant pre-school teachers (reported as one group of respondents: pre-school teachers) aged 18─64 years participated in the survey. They represent 10.8% of all pre-school teachers employed in kindergartens in 2022/2023 (total number was 12,593, according to the Statistical Office of the Republic of Slovenia (SURS, 2023)). We expected the sample to be predominantly female, which was confirmed. As the sample is 97% female, we did not split the results by sex. By age, 24.8% of respondents were in the age group 20–34 years, 50.7% in the age group 35–49 years (50.7%) and 24.5% were over 50 years. Regarding educational groups, 36.4% of respondents had secondary education or less, 16.4% tertiary education and 47.2%, more than tertiary education.

3.2. Cigarette smoking and use of e-cigarettes and HTPs

The highest percentage of respondents were ever, current, and everyday cigarette smokers of the respondents, while the use of e-cigarettes or HTPs is less common (**Table 1**). There are significant differences in current cigarette smoking by education, ever and current use of e-cigarettes by age, ever, current, and daily use of HTPs by age, and daily use of HTPs by education ($p<0.050$).

Table 1. Percentage of ever, current, and daily cigarette smokers/users of e-cigarettes or HTPs among pre-school teachers in Slovenia.

			Ever smokers/users (%)	Current smokers/users (%)	Daily smokers/users (%)
Cigarettes		Total (n = 1364)	55.9	18.0	12.0
	Age	20–34 years	44.3	15.9	9.2
		35–49 years	44.8	17.8	11.8
		50+ years	41.8	20.1	14.5
	Education	Secondary or less	58.4	22.2	14.1
		Tertiary	55.8	18.4	13.0
		More than tertiary	54.7	14.9	9.6
E-cigarettes		Total (n = 1364)	11.0	5.3	1.5
	Age	20–34 years	20.4	5.8	1.5
		35–49 years	9.7	2.2	1.3
		50+ years	4.3	1.2	0.9
	Education	Secondary or less	11.6	3.9	2.5
		Tertiary	10.7	3.1	1.3
		More than tertiary	10.9	2.5	0.8
HTPs		Total (n = 1364)	11.1	6.6	4.5
	Age	20–34 years	18.5	10.0	6.4
		35–49 years	10.1	5.7	4.3
		50+ years	5.2	4.3	2.5
	Education	Secondary or less	12.8	8.3	6.6
		Tertiary	11.2	6.7	3.6
		More than tertiary	9.9	5.4	3.4

3.3. Intention to quit cigarette smoking, use of e-cigarettes or HTPs
There are high percentages of respondents who do not intend to quit smoking cigarettes or using e-cigarettes or HTPs (**Table 2**). The percentage of those who intend to quit smoking or using e-cigarettes and HTPs in the next 30 days is low. There are significant differences in intentions to quit cigarette smoking by age ($p<0.050$).

Table 2. Percentage of pre-school teachers (current smokers or users of e-cigarettes or HTPs) in Slovenia who intend/not intend to quit cigarette smoking/use of e-cigarettes or HTPs.

			Do not intend to quit (%)	Intend to quit in the next 30 days (%)	Intend to quit in the future (%)
Cigarettes	Total (n = 267)		38.2	9.4	52.4
	Age	20–34 years	50.0	10.0	40.0
		35–49 years	37.5	6.3	56.3
		50+ years	27.5	14.5	58.0
	Education	Secondary or less	38.8	10.3	50.9
		Tertiary	34.0	6.4	59.6
		More than tertiary	40.2	9.8	50.0
E-cigarettes	Total (n = 63)		60.3	15.9	23.8
	Age	20–34 years	62.5	20.8	16.7
		35–49 years	54.2	12.5	33.3
		50+ years	70.0	0.0	30.0
	Education	Secondary or less	55.6	18.5	25.9
		Tertiary	90.9	–	9.1
		More than tertiary	52.0	20.0	28.0
HTPs	Total (n = 103)		47.6	5.8	46.6
	Age	20–34 years	45.7	8.6	45.7
		35–49 years	45.5	2.3	52.3
		50+ years	44.4	5.6	50.0
	Education	Secondary or less	50.0	6.3	43.8
		Tertiary	52.9	–	47.1
		More than tertiary	42.1	7.9	50.0

– no occurrence of an event

3.4. Detection of infringements of smoke-free restrictions inside the kindergarten or on its functional land

Almost every fifth respondent detected infringement of smoke-free restrictions inside the kindergarten or on its functional land. There are significant differences in detection of smoke-free restrictions infringement by education (**Table 3**).

Table 3. Percentage of pre-school teachers who detected infringement of smoke-free restrictions inside the kindergarten or on its functional land.

		Infringement was detected (%)	Infringement was not detected (%)	Doesn't know (%)
Total (n = 1357)		17.1	72.3	10.6
Age	20–34 years	14.6	76.9	8.5
	35–49 years	18.2	70.6	11.3
	50+ years	16.6	72.5	10.9
Education	Secondary or less	14.9	74.7	10.5
	Tertiary	18.4	71.3	10.3
	More than tertiary	18.1	71.8	10.1

4. Discussion

Our study reveals that cigarette smoking, use of e-cigarettes and HTPs among pre-school teachers in Slovenia are prevalent at levels similar to the general population, albeit somewhat lower; compared to a panel survey of the general adult Slovenian population aged 18-74 years (Koprivnikar et al., 2023), our survey shows a 5 percentage point lower prevalence of cigarette smokers (18% in our survey vs. 23% in the general population). Similarly, there is a 2 percentage point lower prevalence of e-cigarettes use (5% in our survey vs. 7% in the general population), and a negligible 1 percentage point difference in HTPs use (7% in our survey vs. 6% in general population). Comparable trends have been observed in Germany, where cigarette smoking prevalence among pre-school teachers is lower than in the general population (Hoffmann et al., 2013), whereas in Japan, smoking prevalence among pre-school teachers mirrors that of the general population (Ohida et al., 2000).

In our study, younger pre-school teachers exhibit higher rates of current and ever use of e-cigarettes, as well as ever, current, and daily use of HTPs, aligning with findings from other Slovenian surveys indicating that e-cigarettes and HTPs are more popular among youth (Koprivnikar et al. 2023). This pattern is consistent across other European countries as well (WHO, 2024). Additionally, higher percentages of current cigarette smokers and daily HTPs users are observed among pre-school teachers with lower education levels, mirroring findings in the general population of Slovenia (Koprivnikar et al., 2023) and other European countries (WHO, 2019; Kim et al., 2023).

Given the pre-school teachers and teachers are role models for children and adolescents, it is crucial to minimize the prevalence of smoking and use of related products among them. According to the Social Ecological Model (Stokols,1992) and Social Learning Theory (Bandura, 1977), children often emulate behaviour of their adult role models and opinion leaders – roles that pre-school teachers undoubtedly fulfill for children. Previous literature suggests that state and school campaigns can raise awareness among teachers about the association between their smoking behaviour and student behaviour, as well as assist with smoking cessation efforts (Escario & Wilkinson, 2018), which may also apply to pre-school teachers. Although Slovenia currently lacks a cessation program specifically tailored for pre-school teachers, widely available and cost-free cessation programs for tobacco and nicotine products exist in Health Centers across the country (NIJZ, 2023). Our findings indicate that approximately one in ten respondents intend to quit smoking in the next 30 days,

with a slightly higher percentage (16%) intending to quit e-cigarette use, and a lower percentage (6%) intending to quit HTPs use. These figures underscore the need for targeted smoking cessation initiatives among pre-school teachers. Our study also highlights that the intention to quit cigarettes is lower among younger pre-school teachers, whereas we did not detect significant age-based differences for e-cigarettes and HTPs. Among all product categories, the highest percentage of pre-school teachers intending to quit cigarette smoking in the near future (next 30 days) is found among those aged 50 years and older, consistent with other reported data suggesting that older individuals are more likely to attempt to quit smoking, often due to experiencing health consequences associated with smoking (Fahey et al., 2023).

Nearly one-fifth (17%) of interviewed pre-school teachers reported detecting infringements of smoke-free restrictions within the kindergarten or on its functional land (**Table 3**). Previous research indicates that when teachers smoke on school grounds, students may perceive school tobacco policies as less restrictive (Lovato et al., 2007), potentially leading students to disregard or challenge these rules (Piontek et al., 2008). Comprehensive and enforced tobacco-free school policies are shown to correlate with lower rates of adolescent smoking (Lovato et al., 2007; Paek et al., 2013), maintaining an environment where neither teachers, staff, nor students use tobacco products (Goldstein et al., 2003). Adolescents are more likely to perceive smoking as unacceptable when exposed less to smoking, as facilitated by a comprehensive tobacco-free environment (Stephens & English, 2002). This principle is applicable to kindergarten environments and pre-school children as well. The extent of the association between teacher smoking and student smoking depends on the visibility of teacher smoking, and may even exceed that of parent smoking and child smoking (Escario & Wilkinson, 2018). While research on similar effects of e-cigarette and HTP use by pre-school teachers is not available, comparable effects could be anticipated. As part of the "Health in Kindergarten" program, two lectures were included in the education program for pre-school teachers, one on new tobacco and nicotine products (including e-cigarettes and HTPs), and the other on the risks of second-hand smoke exposure. These lectures were conducted by expert from the National Institute of Public Health, underscoring the importance of creating tobacco- and smoke-free kindergarten environments to pre-school teachers.

Our study is the first of its kind among this occupational group in Slovenia, however it is not without limitations. Self-reported data in our study may contribute to under-reporting and socially desirable responses, potentially leading to underestimations of use or other measured variables. Another significant limitation is participation bias, which could also result in underestimations. Nonetheless, our findings provide valuable insights into addressing tobacco smoking, e-cigarette use, and HTPs use among pre-school teachers, especially considering the scarcity of similar studies.

5.   Conclusions

Pre-school teachers are important role models for children and it is important that they give them a healthy example. Among the interviewed pre-school teachers, almost a fifth are current smokers, and every twentieth current user of e-cigarettes or HTPs. It is important that percentages of users are as low as possible; therefore, it would be important that pre-school teachers are invited to participate more intensively in the cessation programmes taking place in Slovenia and additional activities for increasing awareness and promoting cessation could also be planned within the program "Health in Kindergarten". Compliance with smoke-free restrictions inside the kindergarten or on its functional land is important, since the pre-school's social environment is a key place where children create and reinforce their attitudes, beliefs, and behaviour, but still almost every fifth respondent reported detection of restriction infringement. Pre-school teachers should be aware of the importance of compliance with smoke-free restrictions and kindergartens should strictly act in case of detecting infringements. Protecting young children from exposure to tobacco and related products results in denormalization of smoking and use of other tobacco and related products and therefore contributes to the prevention of initiation of use among them in the future.

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Research

Soft Hand Exoskeleton with Three-Dimensional Printed Soft Pneumatic Actuators

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Abstract:

The hand is an extremely complex organ, so restoring its function presents a challenge. There are various types of orthoses prescribed for impairments. Hand exoskeletons are intended to improve the function. They consist of a static base and dynamic components. The latter include actuators, most often rigid ones, such as electric motors. While the systems they employ are powerful and precise within a limited range of motion, they have numerous shortcomings, which can be circumvented by incorporating insights from soft robotics into orthosis design. Soft actuators can be manufactured, typically inspired by muscles. Their operation depends on supplied energy, design geometry, and material properties. Due to their characteristics, they are suitable for numerous applications across different fields, including orthotics and prosthetics. We developed a prototype orthosis consisting of a static base and a functional part, incorporating dynamic components. The static base was assembled from 3D-printed components. The functional part includes cable pulls, 3D-printed soft pneumatic actuators and a compressor. The most important dynamic components are the actuators. We printed various models and tested them. They achieve different maximum forces and contractions, with their operation influenced by several factors. The resulting hand exoskeleton has many characteristics of a good medical device; however, it has several, albeit solvable, shortcomings. Both the static base and the actuators need improvement, as the latter are not yet powerful enough for practical use, but adequately demonstrate the orthosis's operating principle.

Keywords: Orthotics; Hand exoskeleton; Soft robotics; Soft pneumatic actuators; 3D printing

1. Introduction

1.1. Anatomy and biomechanics of the hand

The human hand is an exceptionally complex organ with a wide range of functionalities. It serves as a tool for interaction with the environment, aimed not only at physical survival but also at social participation. Coordinated hand movements are essential for grasping and manipulating objects, which is fundamental for performing all daily activities (Bos et al., 2016; Hlebš, 2019). Impaired hand function can significantly reduce an individual's quality of life (Križnar et al., 2019), which is why orthotists and prosthetists seek for possible solutions to restore it.

The hand is a very compact structure and biomechanically one of the most complex biological systems (Duncan et al., 2013; Du Plessis et al., 2021; Hlebš, 2019). It is the most movable segment of the upper limb. It has many degrees of freedom (DOF), 21 in total, allowing for many movements within a wide range of motion (ROM) (Du Plessis et al., 2021). The hand can be placed in various positions. Functional ones - grips - can be divided into two groups: working or firm and precise or fine (Hlebš, 2019). Movements are performed and coordinated by muscles that are functionally interconnected, so the position of the wrist affects the performance of finger muscles and thus hand function. A slight dorsal flexion of the wrist (20°-30°) prevents the finger flexors from acting on the wrist, thereby increasing and stabilizing grip strength (Coppard & Lohman, 2015; Hlebš, 2019).

1.2. Pathologies

The functions of the upper limb are much more complex than those of the lower limb, making it harder to fully restore them in case of various impairments (Du Plessis et al., 2021). Common causes of hand impairments include central nervous system disorders, peripheral nerve damage, congenital defects, arthritic changes, burns, tendon injuries, and other injuries such as sports injuries. An orthosis is usually needed to optimize function (Križnar et al., 2019).

1.3. Hand orthoses

In Europe, hand orthoses are commonly classified by function into three groups: static, passive dynamic, and active dynamic. Static orthoses are prescribed for immobilization, maintaining position, and correcting deformities; passive dynamic orthoses prevent or reduce contractures and exercise muscles; active dynamic orthoses improve upper limb function—enabling grasp, release, performing specific tasks, or improving upper limb positioning in space (Ortar and Burgar, 2001).

The most used hand orthoses are static orthoses (Coppard & Lohman, 2015). They are composed solely of static elements (Ortar and Burgar, 2001). Dynamic orthoses consist of static elements with added dynamic elements, such as elastic bands or spring wires, assisting the patient by regulating the range of motion and the plane in which the movement is executed. These are called passive dynamic orthoses (Hsu et al., 2008; Pervez and Nagrare, 2022). When dynamic elements include actuators, these are active dynamic orthoses, also known as exoskeletons (Pervez and Nagrare, 2022). They can be defined as wearable robotic devices that help the user perform specific movements (Du Plessis et al., 2021).

1.4. Actuator

Robotic devices are programmable systems designed to perform various tasks. They must have the following subsystems: a drive unit, sensors, actuators, a body or mechanism for transmitting forces or torques, and a controller (Chen et al., 2017). An actuator is a device that can activate or drive a mechanism by drawing energy from a generator and supplying it to another device. The general principle of an actuator is the conversion of any type of energy into mechanical energy, resulting in movement. Examples of actuators include electric motors and pneumatic pistons (Chen et al., 2017; Whitesides, 2018).

Traditional robotic systems are made of rigid, mechanically inflexible materials and are driven by equally rigid electric actuators. They move at high speeds and consequently generate large forces. The downside is that such systems are often rigid in movement,

heavy, and energy intensive. These characteristics make them potentially dangerous during human interaction. Additionally, achieving complex movements requires technologically advanced control systems. Based on their transportability, robotic systems can be either tethered or untethered (Whitesides, 2018). The former are more prevalent. They include non-portable drive units (Du Plessis et al., 2021). Systems using rigid actuators are powerful and usually precise but only within a limited range of motion. This makes them less effective in adapting to various environmental conditions and different users. The field of soft robotics is currently undergoing development to overcome these limitations (Pan et al., 2021).

1.5. Soft robotics

Soft robotics is a rapidly evolving field (Higueras-Ruiz et al., 2022; Whitesides, 2018; Xavier et al., 2022), still in its relatively early development stages (Whitesides, 2018). The inspiration for designing systems is drawn from nature, particularly biological models of various organisms like starfish, worms, snakes, fish, and human muscles (Whitesides, 2018; Xavier et al., 2022). Bio-inspired design is based on the realization that nature offers solutions to problems that existing engineering methods still find challenging. Soft robotics emulates nature by incorporating soft and elastic materials into systems (Majidi, 2018). These materials allow the creation of soft, highly deformable, and adaptable (compliant) components, including actuators (Pagoli et al., 2022). Hydrogels, electroactive polymers, and elastomers are used for their fabrication (Xavier et al., 2022). Often, silicones are utilized due to their favorable properties (Pagoli et al., 2022). The elastic modules of these materials are comparable to those of soft biological materials, including human tissues (Pan et al., 2021).
By leveraging the advantages of soft, adaptable materials, the need for complex actuation and control systems is avoided. The result is simpler task execution, lower production costs, and a more affordable final product. The material properties also lead to greater impact resistance, durability concerning the degree and number of deformations, and light weight of the actuators without sacrificing power. Most importantly, they enable safe interaction between humans and devices (Pan et al., 2021; Whitesides, 2018; Xavier et al., 2022).

1.6. Soft actuators

Soft actuators are types of actuators that are made from flexible or otherwise adaptable materials. Their operation is movement as a result of supplied energy, their physical shape, and the material from which they are made. They can move axially, radially, in a twisting motion, bending, or in various combinations of these movements (Higueras-Ruiz et al., 2022). Based on their mode of operation, several types exist, with soft pneumatic actuators being among the most commonly used. They can use either positive or negative pressure for operation. In recent years, there has been significant progress in this field (Pagoli et al., 2022). The production of more sophisticated soft actuators has been enabled primarily by advanced additive technologies, initially used for printing molds in which liquid elastomeric material was poured, hardened, and retained the shape of the mold. Nowadays, more direct printing of actuators is increasingly used. Common technologies include Fused Deposition Modelling (FDM), Direct Ink Writing (DIW), and Stereolithography (SLA) (Wallin et al., 2018).

1.7. Applications of soft pneumatic actuators

Soft pneumatic actuators are suitable for numerous applications in various fields due to their properties, including industrial and service robotics, rehabilitation and other biomedical sectors (Kalita et al., 2022). Their use is particularly beneficial in environments that are highly dynamic and sensitive to physical interaction (Xavier et al., 2022). Interest is also growing in developing wearable soft rehabilitation and supportive robotic devices, where soft actuators are being incorporated into orthotic designs (Cianchetti et al., 2018; Kaviri et al., 2023).

1.8. Hand exoskeletons

The term "exoskeleton" refers to the hard outer structure or shell of some animals that supports their body. Similarly, hand exoskeletons are designed to fit on the dorsal, lateral, or

(less often) palmar sides of the hand and fingers. In rehabilitation they are frequently used to restore motor functions in patients with hand impairments or to provide haptic feedback in virtual reality environments (Kaviri et al., 2023; Du Plessis et al., 2021).

Based on the rigidity of their components, exoskeletons can be divided into rigid, soft, and hybrid types, which are combinations of both (Du Plessis et al., 2021; Noronha & Accoto, 2021; Pérez Vidal et al., 2021). The mechanism of action for rigid exoskeletons involves transmitting forces or torques to the joints through rigid mechanical structures. There are various designs (Du Plessis et al., 2021). Most of rigid exoskeletons are bulky, massive, rigid in operation, and often tethered (Pervez & Nagrare, 2022). Additionally, their complex structure extends the donning time. Due to their rigid construction, they do not optimally conform to the shape of the hand, limiting the range of motion and potentially causing pressure-related injuries. Another major issue is the enormous cost of such systems (Gorgey, 2018). In contrast, soft hand exoskeletons are a promising trend in the field (Du Plessis et al., 2021).

1.9. Soft hand exoskeletons

Soft hand exoskeletons are generally designed like gloves with added flexible elements that transmit forces and torques to the finger joints. These elements can be various forms of pulling or pushing wires or cables (tendon-driven gloves) or continuous structures, such as soft pneumatic actuators (jointless structures). This allows for the creation of comfortable, compact, and lightweight exoskeletons. However, the main challenge is the electrical cables or air tubes that connect the exoskeleton to the drive unit, which is often not portable (Du Plessis et al., 2021). Recently, underactuated devices, which use fewer actuators to support multiple movements, have become prevalent. Typically, hand exoskeletons assist with finger flexion, but far fewer are designed primarily to support finger extension (Chen et al., 2021).

Most such devices are still in the research phase. Many are in early development stages, some are being tested on healthy individuals, a few are undergoing limited clinical trials, and very few are commercially available and used in clinical practice (Noronha & Accoto, 2021).

2. Material and Methods

2.1. Prototype development of a soft hand exoskeleton

We developed a prototype soft hand exoskeleton to assist with finger extension using 3D-printed soft pneumatic actuators. It consists of a static base and dynamic components, which make up the functional part of the orthosis. For the static base, we assembled an exoskeleton from 3D-printed components. The functional part of the orthosis includes cable pulls, 3D-printed soft pneumatic actuators and a compressor as an energy generator. The principle of operation is as follows: the compressor is activated, the pressure inside the actuator increases, the actuator contracts and pulls the cables, which extends the fingers. We tried to make the orthosis lightweight, comfortable, safe, easy to put on, use and maintain, untethered, relatively easy to make, accessible, adaptable in its function - in the sense that it is suitable for use with various pathologies and multiple patients and, if possible, adjustable in correction. The ultimate goal was a working orthosis with its practical use. Note: The term "exoskeleton" is used for "active dynamic orthosis", but sometimes it means "static base." The context clarifies the meaning.

2.2.1. Static base of the orthosis

In the computer-aided design (CAD) software program Autodesk Fusion 360 (2023) (Version 2.0.16490, Autodesk, Inc., San Francisco, California), we designed the components of the exoskeleton and printed them on a FDM 3D printer, the Creality CR-10 Smart Pro (Shenzhen Creality 3D Technology Co., Ltd., China). For the printing of softer parts of the static base, we used thermoplastic polyurethane (TPU), more specifically material varioShore TPU (colorFabb, Belfeld, Netherlands). This material has the advantageous property of foaming differently at various temperatures, allowing the printing of lightweight, soft, and comfortable components with minimal material usage. We adjusted the hardness of the components by changing the thickness or the number of layers during printing. We

aimed to print components soft enough not to hinder the preserved movement of the hand, while still providing a sufficiently firm support for the dynamic components of the exoskeleton. We printed the guides with a harder material, TPU 95A (colorFabb, Belfeld, Netherlands). The printing process took about 22 hours (excluding modelling and drying). The assembly of the exoskeleton followed, where we glued and mechanically connected the parts.

The exoskeleton is mainly composed of 3D-printed components, with only a few additional essential parts, such as common Velcro straps for closing and attaching the orthosis to the hand (**Figure 1**). At the fingertips, we created distal attachments for the cables (or wires) by inserting regular rivets into the holes of the guides. These were also used to form attachment points for the cables (or wires) at the top of the actuator. We didn't use any specific materials, but commercially available straps, rivets, cotton cords and universal secondary glue (you can find them in every store), because they are sufficient to demonstrate the operating principle of the orthosis.
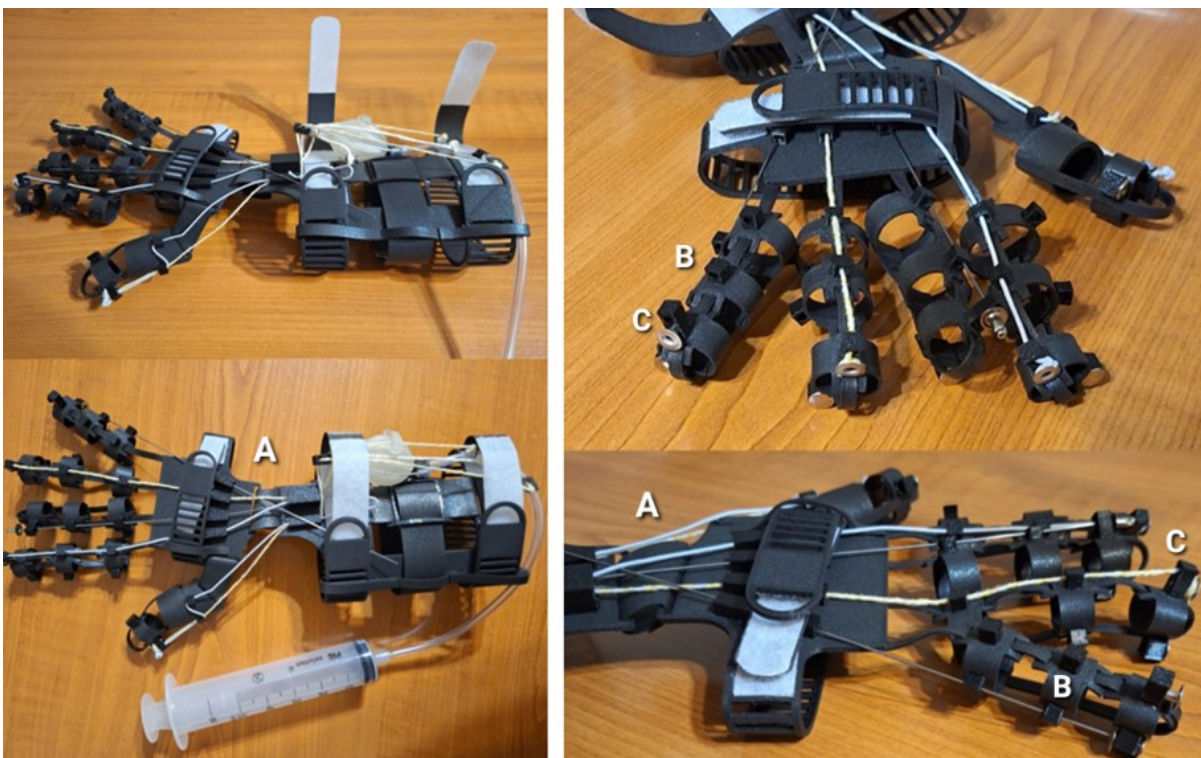

Figure 1. Final static base of the exoskeleton, with already added all tested options for dynamic components (left). The distal part of the orthosis is zoomed in to show the guides and cables (right). Letters indicate the following elements: **A**) the wrist joint, **B**) a guide and **C**) a distal cable attachment.

The static base consists of two main parts. More rigid components do not extend over the wrist joint and finger joints, being interrupted and connected by softer linking elements at these points. The first, proximal part covers the forearm area, including two fixation straps and a longitudinally adjustable strap for attaching the actuators. The second, distal part extends over the back of the hand, with four straps representing the connecting elements onto which the finger thimbles are placed. The strap passes through the thimbles on the dorsal side of the finger, bends distally across the middle of the finger pad, and runs along the palmar side of the finger to the most proximal thimble. This design allows for adjustability of the thimbles` positions, simply moving them up or down the connecting strap. Distally, at the fingertips, it forms a loop preventing longitudinal slipping of the thimbles under pulling force, while also facilitating easier removal of the exoskeleton after use. The finger pad is not compressed or completely covered, so sensory and proprioceptive feedback is not significantly reduced. Unlike the other fingers, the thumb is its own active subunit. We simplified the carpometacarpal (CMC) joint of the thumb, combining it with the metacarpophalangeal (MCP) joint and making it from a 1-millimeter strip in the shape of

a trapezoid, trying to achieve an anatomically appropriate angle (with the second finger), without hindering movement, especially opposition. The two main parts of the orthosis are connected at the wrist joint, made from a 1-millimeter trapezoidal strip, glued into a cylindrical shape at the ends. The result is a joint considering minor anatomical ulnar deviation, not hindering wrist movement in abduction and adduction. It does not restrict dorsal flexion and only minimally restricts volar flexion (**Figure 1 - A**). The exoskeleton is designed to be compatible with other wrist orthoses.

2.2.2. Dynamic components of the orthosis – cables
On the completed softer static base, we attached stiffer guides (**Figure 1 - B**). These are small blocks with a hole in the middle, except for the distal cable attachments, which have an additional identical guide on top, providing some vertical force component to further prevent thimble slippage (**Figure 1 - C**). The basic force generated by the actuator is a pulling force, so we prefer the idea of the exoskeleton working in pulling, but we also tried and added guides for the potential use of pushing wires to extend the fingers. These were placed on the lateral side of the fingers, one on each thimble. They were attached lower than the joint pivots (**Figure 1**).
Cables run through the guides along the fingers, attached at both ends. One end is attached to the distal attachment on the dorsal side of the fingertips and the other to the actuator. We tested various options for the latter, as seen in **Figure 1**. The final attachment on the actuator is made from the same guide as those on the thimbles (**Figure 2**).
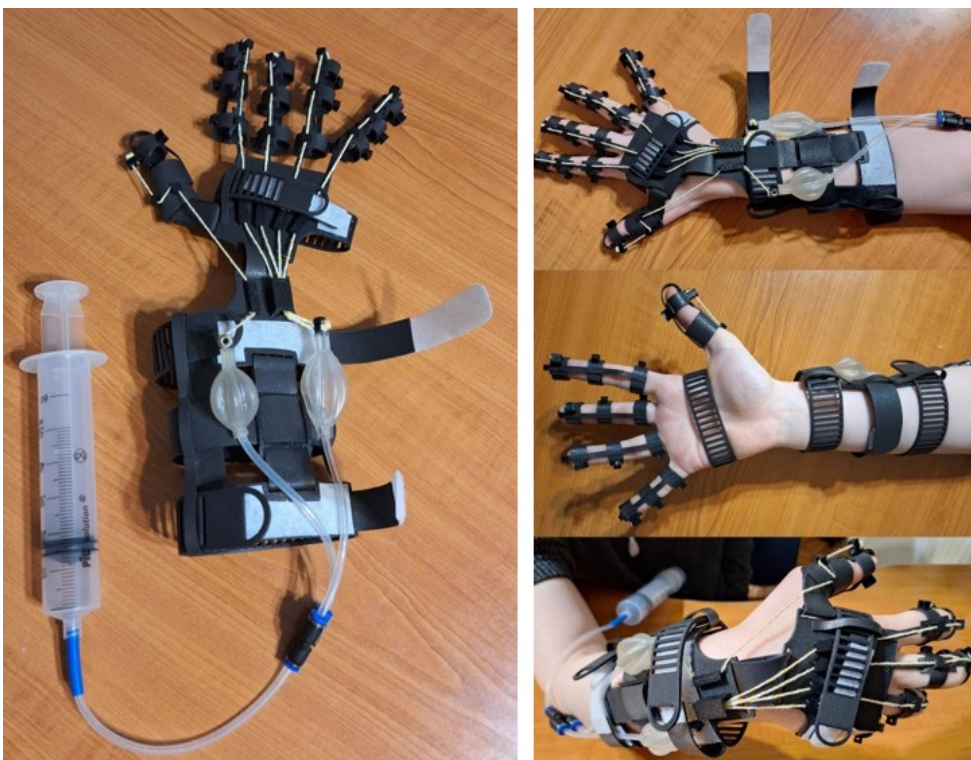

Figure 2. Final prototype of soft hand exoskeleton with 3D printed soft pneumatic actuators for assisting extension of all five fingers.

2.2.3. Dynamic components of the orthosis – actuators and energy generator
Actuators play a central role in the orthosis's function. Based on our practical needs and literature review, we decided to create soft pneumatic actuators that operate through linear motion. The pneumatic actuators were made by using 3D printing, inspired by designs from Sparrman et al. (2021) and De Pascali et al. (2022). The main difference in operation is that designs by Sparman et al. (2021) elongate with increased pressure, while designs by De Pascali et al. (2022) contract. Initially, we tried those by Sparman et al. (2021), but they were not effective, so we focused on those inspired by De Pascali et al. (2022). We designed

various similar actuator models and printed test samples using VarioShore TPU (white actuators) (**Figure 3 - A**) and TPU A85 (black actuators) (**Figure 3 - B**). The larger ones (4 cm in height) performed slightly better (**Figure 3 - C**), but the smaller ones (2 cm and 3 cm) were more suitable for hand orthosis applications (**Figure 3 - D**). While the material softness affected performance, the relationship was not proportional to filament hardness – there was no noticeable difference between softer and harder actuators (not shown).

We then printed the actuators by a SLA printer (Elegoo Mars 3) using flexible resin with Shore hardness A43 - Liqcreate Elastomer – X (Liqcreate, Utrecht, Netherlands) (**Figure 3**). After printing they were cleaned in isopropanol and UV cured at 60°C for 30 minutes. The printed models tended to tear along the layers at higher pressures, especially larger ones and those with thinner walls. Despite post-processing, they felt slightly sticky. Based on those facts, quality concerns are rising. The actuator operates by inflating (increasing in diameter) and contracting (linear movement) under air pressure, creating a pulling force that pulls the cables and extends the fingers. For pressure generation, we initially used a 50-ml syringe, later switching to a small electric compressor. All types of models are shown in **Figure 3**.
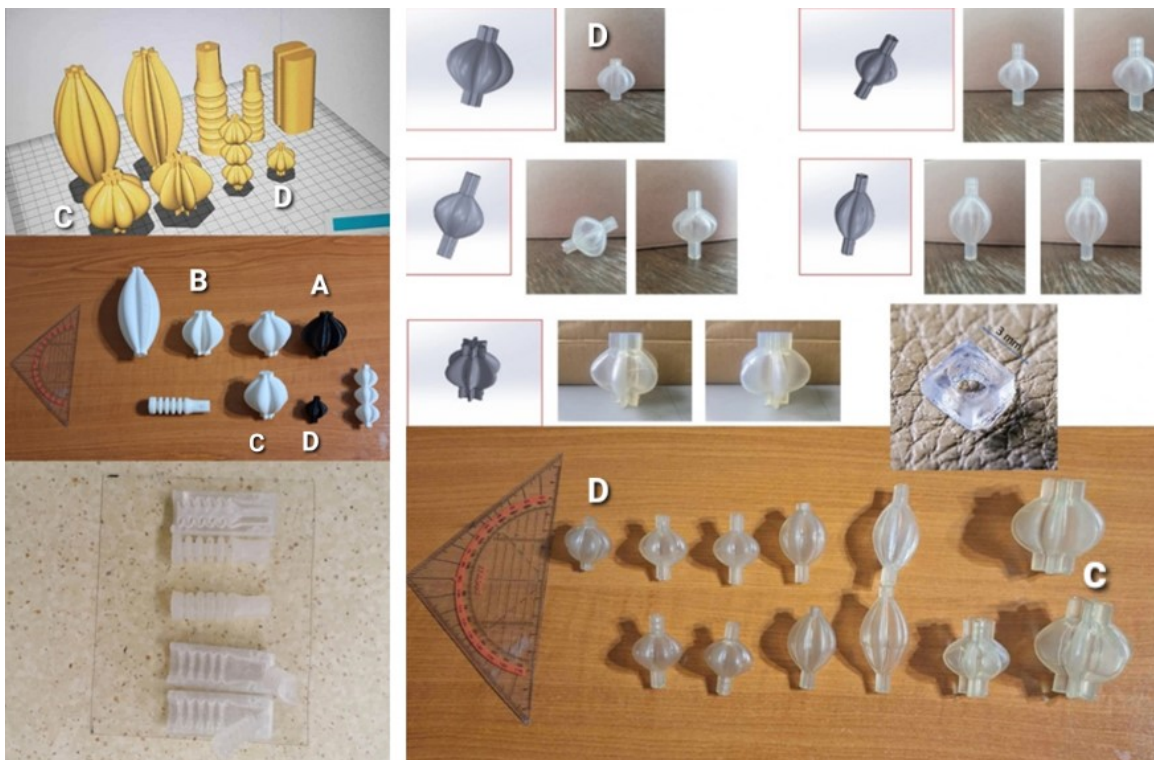

Figure 3. Different actuator models printed on FDM (left) and SLA (right) printer. **A**) an actuator printed with TPU A85, **B**) an actuator printed with TPU VarioShore, **C**) a large actuator and **D**) a small actuator.

We used two such actuators for the orthosis's operation: one for thumb extension and one for the other fingers extension. Although the goal was to achieve separate triggering and thus independent thumb extension from the other fingers, for demonstration purposes, both actuators were connected to the compressor to work synchronously. The compressor activates simply by pressing a switch, which suffices for demonstrating the operation.

3.   Results

Most of the information on the exoskeleton's structure and its components is already presented in the *Material and Methods*, as this article is primarily about the manufacturing process.

The actuators were tested before use to observe their response to increasing pressure. The tested actuators varied in rib (pleats) count, rib depth, length, wall thickness, and UV exposure time during printing, resulting in different maximum forces at various pressures and contraction sizes (**Table 1**).

Table 1. Test results of the top six printed actuators.

Sample	Number of ribs	Depth of the ribs [mm]	Thickness of the wall [mm]	Length of AFP [mm]	Total length [mm]	Exposure time [s]	Pressure [mbar]	Max force [N]	Contraction [mm]
T_2_2	8	5	0.8	30	50	48	320	4	3.7
T_4_1	8	4	0.8	40	60	64	120	3.5	7.3
T_3_3	8	5	1	40	60	64	110	3.1	7.6
T_2_1	6	6	0.8	40	60	64	388	2.2	7.1
T_6_1	6	4	0.8	30	50	48	150	2.1	3.4
T_2_3	6	6	0.8	40	60	64	388	2	5

Sample means tested actuator. Ribs are longitudinal pleats. AFP means actuator`s functional part and it is the part that changes its shape under the pressure. Total length incorporates the length of its neck. Length is also referred to as height in the text above. Exposure time means time of UV- exposure within the process of printing. Pressure means the pressure required for actuator to achieve its maximal force. Max force is the maximum pulling force produced by the actuator when the pressure is increased and contraction is the amplitude or linear displacement by which the actuator contracts when the pressure is increased.

We noted two common behaviours: a slight delay in activation with increasing pressure and a transition from contraction to expansion beyond a certain pressure, causing movement in the opposite direction (**Figure 4**).
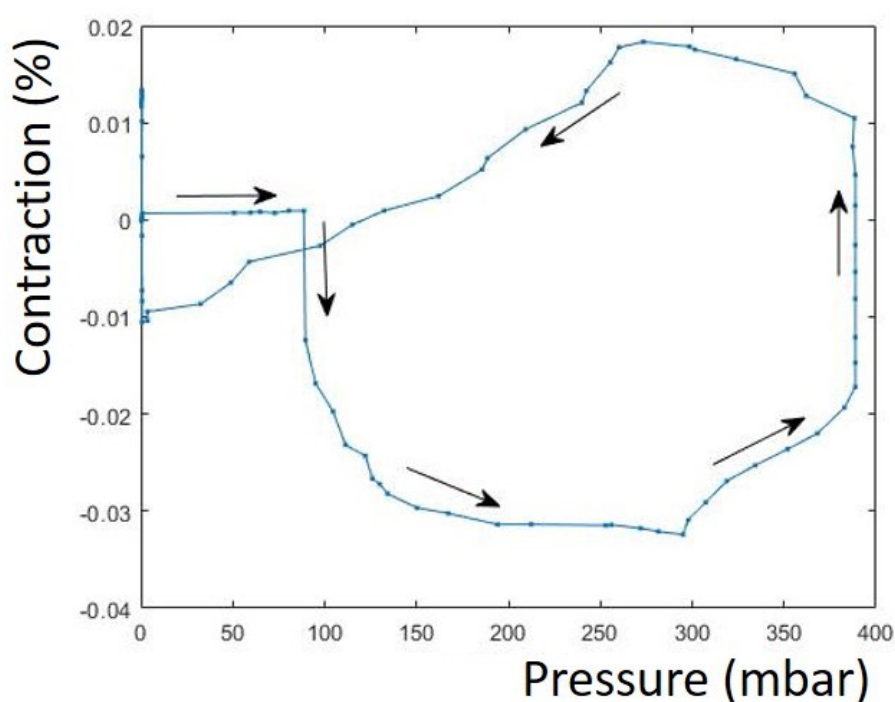

Figure 4. Relative actuator contraction on the ordinate axis in dependence on pressure (mbar) (on the abscissa axis).

The printed actuators do not yet generate sufficient force for practical use (maximum 4 N). Similarly, their contraction (linear movement) is generally insufficient (maximum 7.6 mm). The contraction size depends not only on force but also on the actuator's design, particularly its size, suggesting further research into the optimal design for greater movement. The pressure at which actuators achieve maximum force also depends on design, their geometrical structure, specifically rib depth. We confirmed that actuators` performance is not solely affected by material softness, which they are made of. They are functional within a pressure range of approximately 100 to 300 mbar. Below 100 mbar, there is no movement; within this range, actuators contract, but near 300 mbar, they begin to inflate, causing reverse movement. The graph above shows testing of a 2-cm actuator, which contracts by 0.032 or 3.2%, equivalent to approximately 6 mm (**Figure 3**).

A good ratio between actuator mass and generated force was achieved. Several good models generating forces from 2 to 4 N and weighing less than 1 g to a maximum of 3 g, were obtained, favoring force over weight. We also aimed to keep the actuators as small as possible, as size is a crucial factor in this sort of application.

The presented preliminary results demonstrate the actuators' and orthosis's operating mechanism. Actuator testing and optimization remain an active research area at the Faculty of Mechanical Engineering, University of Ljubljana.

The overall result of our work can be seen in **Figure 4.**

4. Discussion

The hand is a very complex structure, making its functional restoration challenging. Simple hand orthoses and also more complex ones made of rigid materials often do not meet functional needs. We recognize the potential advantages of incorporating soft components into the design of medical devices. Thus, we attempted to create an active dynamic hand orthosis from soft materials, which uses soft actuators to operate.

We present a prototype soft hand exoskeleton with 3D-printed soft pneumatic actuators (**Figure 2**). Also the static base components were 3D printed. The exoskeleton is suitable for use in various pathologies, somewhat size-adjustable, and correctively adaptable, being compatible with other wrist orthoses. It is soft, lightweight, comfortable, and breathable, minimally hindering sensory and proprioceptive feedback and preserving hand movement. It does not generate excessive forces or cause sudden movements and harmful torques on finger joints, making it safe. However, the device is still in the early research phase. It is a prototype with several shortcomings and conposed of improvised materials. The design is not optimal, with fitting still largely influenced by accurate dimension measurements. Precise measurements of the thimble circumferences, length and width of the surface on the dorsal side of the hand and the length of the connecting finger straps are required for perfect fitting of the device. The exoskeleton itself does not perform direct correction, meaning that additional orthosis is needed for the purpose of correcting position of the wrist or any other joint, required for example with spastic patients. The principle of "pulling" cables could be replaced by "pushing", but a more compact static base would be required. As better explained: with a pulley, the force produced by the actuator (and its contraction) could be reversed and thus finger extension achieved, not by pulling, but by pushing the cables. Pneumatics is not an optimal energy source, the exoskeleton also does not yet include a sensible control mode. The quality of the actuators is questionable. They are not yet powerful enough, generating too little force and too little movement, respectively contraction. However, useful conclusions about what affects their performance have been achieved. Actuators could also be replaced by pneumatic bending actuators, or by actuators of a completely different type. The device does not meet mandatory standards, we have not tested its applicability and it is therefore not yet suitable for clinical use.

We have demonstrated the potential use of additive technologies for the manufacture of medical devices and presented an example of the implementation of soft robotics solutions in the field of orthotics and prosthetics.

5. Conclusions

Creativity and inovative ideas are the driving force behind development, which is essential if we want to achieve ever better solutions for patients that will improve their quality of life. This was the main reason we attempted to develop a new type of active dynamic hand orthosis. The resulting hand exoskeleton is suitable for use in various pathologies, adjustable to some extent in size and adaptable in correction. It is soft, lightweight, comfortable, and breathable. It does not overly impede sensory perception and proprioception, nor does it restrict retained hand movement. It does not generate excessive forces or abrupt movements, nor does it cause harmful torques on finger joints, making it safe for use. Nevertheless, it is only a prototype and thus has several, albeit solvable, shortcomings. We believe indiviidual components hold promise, so additional research must be invested to arrive to in-practice-usable hand exoskeleton.

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

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Research

Standard Operating Procedure for One - Spin Individualized Therapeutic Plasma Based on a Mathematical Model and Test Spin

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Abstract:
Standard operating procedure (SOP) for one-spin centrifugation of blood to prepare plasma is presented. The aim of the procedure is to prepare plasma with highest content of platelets and extracellular vesicles (EVs) from an individual blood sample. As sedimenting erythrocytes push plasma that carries platelets and EVs in the opposite direction, the optimal time is defined at the point when the lower bound of the ascending plasma meets the upper bound of the sedimenting erythrocytes. This process is described by a mathematical model which has one adjustable parameter. The parameter is estimated by a test spin at low centripetal acceleration of the centrifuge rotor and short centrifugation time. By using the mathematical model and the estimated parameter, the optimal time of centrifugation at chosen centripetal acceleration of the centrifuge rotor can be set. In the presented contribution we describe the protocole and state the elements needed for safe preparation of optimized and individualized plasma intended for regeneration.

Keywords: Therapeutic plasma, Platelet rich plasma, Platelet and extracellular vesicles rich plasma; Regeneration; Extracellular vesicles; Extracellular particles; Exosomes; Sedimentation of erythrocytes

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1. Definitions
EVs: extracellular vesicles
PVRP: plasma rich with platelets and extracellular vesicles
SOP: standard operating procedure
QMS: Quality Management System

2. Background
Blood plasma that is rich with platelets and extracellular vesicles (PVRP) is widely used in clinical practice in various fields of medicine for regeneration and healing (Troha et al., 2023). The gold standard method for preparation of PVRP is sedimentation of erythrocytes in blood samples taken into tubes with anticoagulant, due to a force upon the particles (e.g. graviation or centrifugal force; **Figure 1**). Sedimentation of erythrocytes in blood is subjected to direct interactions between blood constituents and causes an increase of the plasma platelet concentration above its baseline level in whole blood (Božič et al., 2022). The preparation is made from patient's own blood following a procedure that is straightforward and easy to perform and can be administred directly to the damaged area. With autologous application there is low risk of complications such as infections or immune rejection (De Pascale et al., 2015). Initially, healing capacity of plasma preparations was attributed to platelets; it was found that activated platelets release different growth and inflammation factors to the extracellular millieu, which have been regarded as vectors of the healing process (Etulain et al., 2018). Later on, other features were considered, i.e. platelet membrane receptors, their effects on the immunomodulatory actions of the innate and adaptive immune system, effects of leukocytes and of mesenchymal stem cells (reviewed in Everts al., 2020), indicating complex interactions

between cells in the healing process. Recent developments in the field of biology and medicine have outlined nano-sized (ranging from 20 nm-1000 nm) membrane-enclosed cellular fragments (extracellular vesicles – EVs) which are abundant in preparations from processed plasma (Šuštar et al., 2011a,b; Božič et al., 2020) as important mediators of intercellular communication
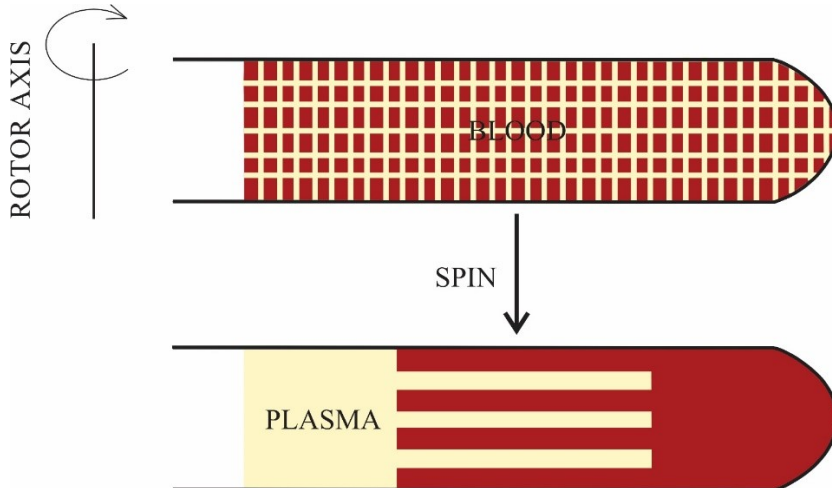

Figure 1. Preparation of PVRP by one-spin protocol. Blood with anticoagulant is centrifuged (Spin) to separate erythrocytes (pellet) from plasma (supernatant). The yellowish area denotes plasma and the red area denotes packed erythrocytes. Stripes indicate formation of channels during sedimentation of erythrocytes.

Plasma preparation was named »platelet- rich plasma« due to elevated platelet concentration. However, more recent results indicate that also EVs are abundant in plasma (Božič et al., 2022) so here we refer to the preparation as "platelet and EV – rich plasma" (PVRP). Both platelets and EVs seem to be important bioactive substances in plasma and the presented SOP is based on the desire to obtain plasma with maximal possible number density of platelets, EVs and concentration of growth factors. For the definition of the SOP, we chose the model identified in the Quality Management System (QMS) for EV studies developed within the H2020 Fet-Open project VES4US (Liguori and Kisslinger, 2021).

3. Purpose, Scope and Applicability

Despite pervasive use and extensive clinical and basic science study, many important questions regarding plasma preparation remain unanswered. Therapy with plasma - derived preparations appeared ineffective – in about 20 to 40 percent of cases, depending on the injury and lack of standardization in preparation and dosage renders results difficult to interpret (Middleton et al., 2012). The presence and the role of EVs in PVRP are largely unknown. As the effects of the processing on the samples are multiple and complex, better understanding of the mechanisms taking place during plasma processing is urgently needed to define the key parameters in preparation protocols and thereby develop a better controlled and more effective therapeutic preparation.

The purpose of the SOP is to focus on the parameters of the sedimentation (the force that pulls the particles, the time of acting of the force, and the physical properties of the sample).

The scope of the SOP is to define the plasma preparation with highest yield of platelets, EVs and growth factors, starting from the point where the sample is transported to the laboratory in the vacutubes to the point when plasma leaves the centrifuge still staying in the vacutubes.

The applicability of the SOP is mainly targeted to plasma preparations for regeneration of tympanoplasty postoperative wounds, however, they can be used in many fields of medicine, for regeneration of different tissues, such as skin, fibrotic and neural tissues. It is being used in otorhinolaryngology (Steiner et al., 2022), where plasma preparations are especially effective in otology and skull-base surgery. Based on research conducted to date, it was shown that PVRP improves and accelerates healing of damaged tissues (Vozel et al., 2021a, Vozel et al., 2021b) and reduces likelihood of postoperative inflammation (Bielecki

et al., 2007). The therapeutic challenge considered is inflammation of the middle ear due to low regenerative capacity of the tissue. Surgical treatment of the inflammation of the middle ear aims at removing the destructive inflammatory process (ie ablative treatment) and restoring the physiological mechanism of sound wave transmission to the inner ear (ie reconstructive treatment - tympanoplasty). The success rate of tympanoplasty reported in the literature ranges between 75% and 98% (Bayram et al., 2020). Thus, post-operative healing presents a problem that has not yet been satisfactorily solved.

4. Health and safety warning
Working with blood and preparations from blood presents in general a risk for infection of the staff or anyone that comes in contact with the material. The hazards and safety measures regarding working with blood and its products are thoroughly described in Guidance on Working Safely with Human Blood and Plasma https://www.sgul.ac.uk/about/our-professional-services/safety-health-and-environment/documents/guidance-on-working-safely-with-human-blood-or-plasma.pdf. These risks and the relative preventive and corrective measures should be carefully

acknowledged and taken into account by the staff. Vacutubes are used, so blood stays within the tubes during the processing considered in this SOP. This diminishes the risk of infection, however, the probability of mistakes or spills must be considered. Gloves, caps, clothes and footware protection should be used to protect the staff as well as the sample from infection. Dispose of used material should be in accordance with applicable laws and good research and laboratory practices.

Besides material, the hazards may derive from using the centrifuge. The hazards and safety measures regarding the use of the centrifuge are thoroughly described in Centrifuge Safety https://ehs.stanford.edu/reference/centrifuge-safety. They include mechanical failure of the centrifuge and dispersion of aerosols in case of spillout. **Mechanical failure** can cause ejection of pieces of metal, or the production of sparks and heat derived from friction. In preparation of PVRP, centrifugal pull is moderate (up to cca 1500 g, where g is the Earth gravity constant) and the rotor should be loaded in a balanced way to minimize vibrations causing sparks and local hot spots. **Dispersion of aerosol** from blood into the centrifuge chamber or into the room can occur in case of damaged or poorly closed tubes or if centrifuge is opened before they have settled.

5. Cautions
Dispose of used material and equipment should be in accordance with applicable laws and good research and laboratory practices. The centrifuge should be fixed to the working surface, regularly maintained and lubricated. For the correct use of the centrifuge, the rotor should be loaded in a balanced way, with centrifuge tubes adequately balanced, and centrifuge speed should be increased gradually. Possibility of aeration of the room should be provided. Chamber and rotor should be cleaned and desinfected before and after use. An electronic or paper register for user reservations should be provided. Written operating procedures of the centrifuge should be at hand available for the users.

6. Personnel Qualifications / Responsibilities
Blood sampling should be performed by a trained nurse. Staff who uses SOP should acknowledge the hazards connected to blood and its products and the hazards connected to operation of the centrifuge. The staff should undergo training on how to handle blood samples, use the centrifuge and apply SOP.

7. Materials, Equipment and Supplies
Blood samples taken into evacuated closed tubes with anticoagulant derive from hospital facilities. They should be gently (avoiding shaking or cooling) transported to the laboratory in a room temperature - saving container and processed immediately. The samples should be used as soon as possible.
Devices: Centrifuge Centric 400R with swinging rotor RS4/100 (Domel, Železniki, Slovenia).
Other equipment: A ruler to measure the dimensions of the centrifuge rotor and the sample tube and of the length of the plasma column after the Test spin.

8. Computer Hardware and Software
Use of the mathematical model (as described below) is key in SOP. Hardware: Office PC; software: Microsoft Excel; data saving and sharing: Cloud and/or Drive documents.

9. Mathematical Model of Plasma Formation
Mathematical model of the first generation was introduced in Božič et al. (2022) and of the second generation considering the effect of hematocrit on the sedimentation in Berry and Kralj-Iglič (2023). In this SOP we follow the general course of the previous models. For clarity we present again the basic equations (1) – (13) from (Berry and Kralj-Iglič, 2023) describing movement of erythrocytes. The model applies above the region of the forming sediment where the motion of erythrocytes is not hindered by direct interactions between each other and with the tube walls. Upon centrifugal acceleration, erythrocytes move from a chosen slice (denoted by index i in **Figure 2**) into the one which is closer to the bottom of the tube (denoted by index $i+1$ in **Figure 2**). At the same time the slice receives the erythrocytes from the slice above – the one closer to the rotor axis (i -1, **Figure 2**).
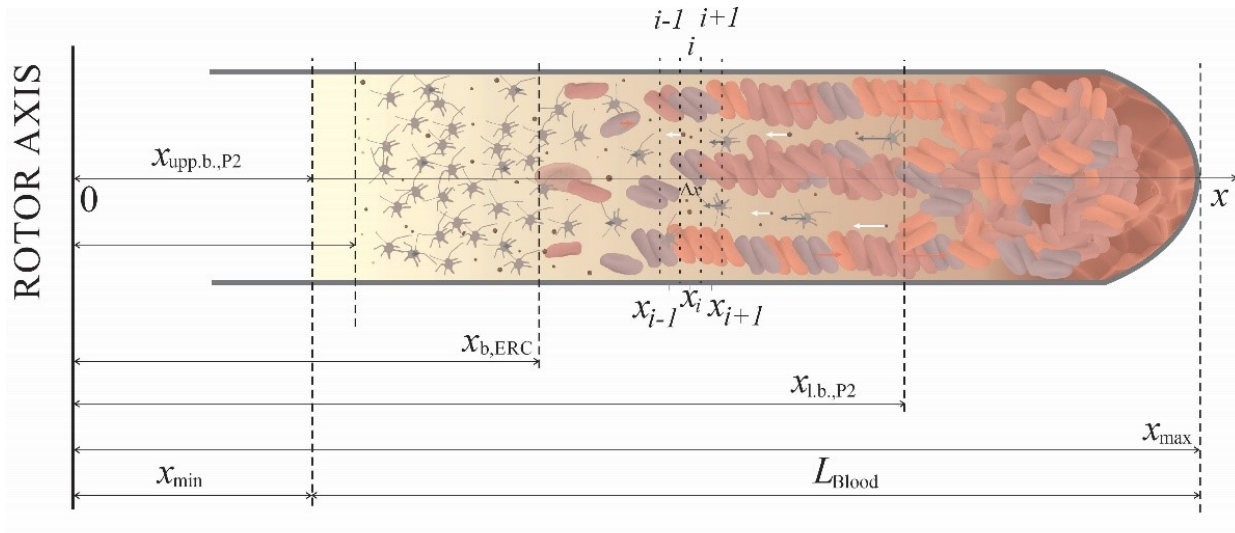

Figure 2. Modeling of the movement of blood cells during centrifugation. The sample is imagined as divided in thin slices with uniform properties.

The change of the number of erythrocytes in the i-th slice N_i with time t is

$$dN_i/dt = c_{i-1}\, S\, u_{i-1}(t) - c_i\, S\, u_i(t) , \tag{1}$$

where c_i is the number density (concentration) of particles, S is the cross section of the tube and u_i is the velocity of the particles in the i-th slice at the time t.
Following Božič et al. (2022) and Berry and Kralj-Iglič et al. (2023) it is assumed that the concentration and the velocity of erythrocytes change only slightly from one slice to another, so that the expansion can be used to approximate the changes,

$$c_i - c_{i-1} = dc_i/dx\, \Delta x \tag{2}$$

$$u_i - u_{i-1} = du_i/dx\, \Delta x . \tag{3}$$

Neglecting the terms quadratic in Δx and performing the limit $\Delta x \to dx$ yields after rearrangement of Eqs. (1) – (3) a differential equation

$$dc(x,t)/dt = - (dc(x,t)/dx\; u(x,t) + du(x,t)/dx\; c(x,t)) . \tag{4}$$

It is assumed that the velocity of the platelets is proportional to the centripetal acceleration which is expressed by a multiplicity of the Earth gravity constant X

$$u(x) = \omega \, X \, x \qquad . \tag{5}$$

where ω is a constant characteristic for the sample. It follows from Eqs. (4) and (5) that

$$dc/dt = -\omega \, X \, d(cx)/dx \qquad . \tag{6}$$

By using dimensionless quantities: γ - concentration of erythrocytes divided by its initial value c_0,

$$\gamma(x,t) = c(x,t)/c_0 \qquad , \tag{7}$$

ξ - distance divided by the maximal length from rotor axis to the bottom of the tube, x_{max}

$$\xi = x/x_{max} \qquad , \tag{8}$$

and relative sedimentation time τ - time multiplied by ωX

$$\tau = \omega \, X \, t \qquad , \tag{9}$$

the Eq. (6) is given in a dimensionless form

$$d\gamma_P(\xi,\tau)/d\tau = -d(\gamma(\xi,\tau)\xi)/d\xi \quad . \tag{10}$$

An ansatz solution is

$$\gamma(\xi,\tau_P) = C \exp(-\tau) + D \exp(-\tau/2) \, \xi^{-1/2} \qquad , \tag{11}$$

where C and D are constants. We chose D = 0 (Berry and Kralj-Iglič, 2023). The initial condition

$$\gamma_P(0) = 1 \tag{12}$$

implies that C = 1 and the relative number density of erythrocytes is therefore

$$\gamma(\xi,\tau) = \exp(-\tau) \qquad . \tag{13}$$

The erythrocytes that were at the top of the sample are forming a boundary which is moving towards the bottom with the velocity $dx_{b,ERC}/dt$. According to Eq.(5)

$$dx_{b,ERC}/dt = u(x) = \omega \, X \, x \qquad . \tag{14}$$

Using dimensionless expressions (8) and (9), Eq. (14) transforms into

$$d\xi_{b,ERC}/d\tau = \xi_{b,ERC} \tag{15}$$

with the solution

$$\xi_{b,ERC} = \xi_{min} \exp(\tau) \qquad . \tag{16}$$

Here $\xi_{min} = x_{min}/x_{max}$ is the dimensionless distance of the surface level of the sample from the rotor axis.
The measurable quantity is the length of the plasma column formed after a certain sedimentation time $L_{plasma} = x_{max} \, l_{plasma}$, where

$$l_{plasma} = \xi_{b,ERC} - \xi_{min} \quad . \tag{17}$$

Using Eqs. (16) and (17) yields the dependence of the dimensionless length of the plasma column on dimensionless time

$$l_{plasma} = \xi_{min} \left(\exp(\tau) - 1 \right) \qquad . \tag{18}$$

Inserting the measurable quantities by using Eqs.(8) and (9) yields the dependence of the length of the plasma on time

$$L_{plasma} = x_{min} \left(\exp(\omega X t) - 1 \right) \qquad . \tag{19}$$

Measurement of L_{plasma}, x_{min} and t following a Test spin is used to determine the unknown parameter ω which is characteristic for the sample. By some rearrangement of Eq. (19) we get

$$\omega = \ln(1 + L_{plasma}/x_{min})/X\,t \tag{20}$$

where we insert the measured values $L_{plasma} = L_{plasma,TEST}$, X_{TEST} and $t = t_{TEST}$.

Within the model, it is assumed that the settling erythrocytes push plasma that carries platelets and EVs in the opposite direction (Božič et al., 2022). The optimal centrifuge setting is defined by the condition that the lower bound of the plasma $\xi_{l.b.\ PLASMA}$ given by

$$\xi_{l.b.\ PLASMA} = \xi_{max} \exp(-\tau) \tag{21}$$

meets the upper bound of the erythrocytes $\xi_{b.ERC}$ (Eq.(16)),

$$\xi_{max} \exp(-\tau) = \xi_{min} \exp(\tau) \qquad . \tag{22}$$

After rearrangement of Eq.(22) we obtain

$$\tau_{OPT} = \ln(x_{max}/x_{min})^{1/2} \tag{23}$$

which yields with insertion of the measurable quantities (Eqs.(8) and (9)) and the parameter ω (Eq. (20)) the expression

$$X_{OPT}\,t_{OPT} = X_{TEST}\,t_{TEST}\,\ln(x_{max}/x_{min})^{1/2}/\ln(1 + L_{plasma,TEST}/x_{min}) \qquad . \tag{24}$$

10. Step by Step Procedure

10.1. *Measurement of x_{max} and x_{min}*

Assuming horisontal position during the spin, the distance from the rotor axis to the bottom of the tube x_{max} and the distance from the rotor axis to the level of the blood x_{min} (**Figure 2**) are measured by the ruler.

10.2. *Test Spin and Measurement of L_{TEST}*

The purpose of this step is to estimate the physical properties of the blood comprised in the parameter ω in Eq. (5). One tube containing blood is placed into the centrifuge rotor and centrifuged mildly (e.g. at lowest possible centripetal acceleration for 5 minutes). In this time a small amount of plasma is expected to form (cca 1 cm). The length of the test plasma column

$$L_{plasma,TEST} = x_{b,ERC,TEST} - x_{min} \tag{25}$$

is measured by the ruler (**Figure 3**).

10.3. *Determination of Centrifuge Setting*

The product $X_{OPT}\,t_{OPT}$ is calculated by using Eq.(24),

$$X_{OPT}\,t_{OPT} = X_{TEST}\,t_{TEST}\,\ln(x_{max}/x_{min})^{1/2}/\ln(1 + L_{plasma,TEST}/x_{min}) \qquad . \tag{26}$$

The expression should be inserted in a convenient software, e.g. Microsoft Excel, to minimize possibility of mistakes and minimize the time blood is waiting. In the model, the centripetal acceleration of the centrifuge rotor and the time of centrifugation stay linked which means that it is arbitrary to chose one of the two parameters and adjust the other accordingly. It should however be taken into consideration that the centrifuge must accelerate to reach the required centripetal acceleration and decelerate to stop and that the centripetal acceleration affects the angle of the tubes with respect to the horizontal if the swinging rotor is used. These issues have not yet been elaborated by experiments.
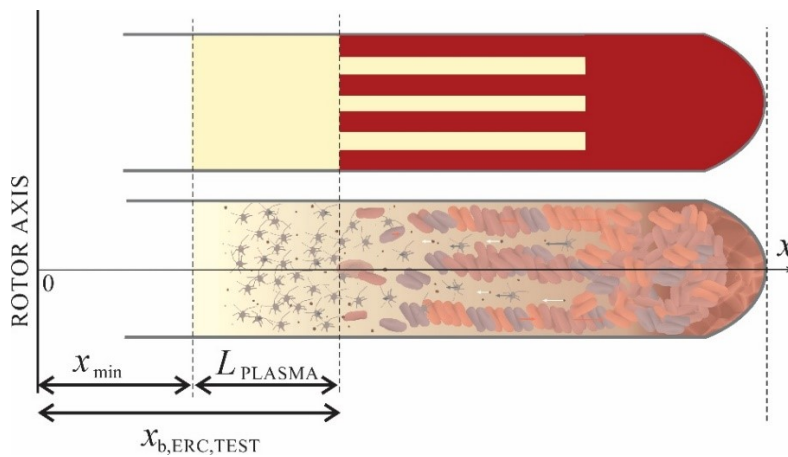

Figure 3. Measurement of the distance between the rotor axis and the bottom of the tube in the horizontal position of the tube with respect to the rotor axis x_{max}, the distance from the rotor axis to the level of the blood x_{min} and the length of the plasma column $L_{plasma,TEST} = x_{b,ERC,TEST} - x_{min}$ (Eq. (25)). The swinging buckett should be placed in the horizontal position to measure x_{min} and x_{max}.

10.4. Plasma preparation
Time and centripetal acceleration of the centrifuge are set by Eq. (24) and the blood in the tubes is centrifuged. If there are multiple tubes of blood acquired from the patient, the setting should be done as given below. The test tube should be centrifuged together with other tubes and blood in the test tube should not be re-suspended. This will somewhat deplete the plasma of the test tube with platelets. If the test tube is the only tube acquired then the time of the test spin t_{TEST} should be subtracted from t_{OPT}.

$$t = t_{OPT} \text{ (for multiple tubes) or } t = t_{OPT} - t_{TEST} \text{ (for a single tube)} \tag{27}$$

10.5. Data acquisition
We report on processing of 6 samples from 2 donors according to the above SOP (**Table 1**).

Table 1. Comparison between optimal length of the plasma column L_{opt} predicted by the mathematical model and length of the plasma column measured in the experiment L_{exp}.

Donor	X	x_{min} (mm)	x_{max} (mm)	$\omega \times 10^{-5}$ (min^{-1})	L_{test} (mm)	t_{opt} (min)	L_{opt} (mm)	L_{exp} (mm)
1	300	95	142	4.08	6	17	21	15
1	300	96.5	142	4.35	6.5	15	20	14
1	300	100	145	4.82	7.5	13	20	13
2	300	95	142	9.77	15	7	21	15
2	300	96.5	142	9.03	14	7	20	18
2	300	102	145	8.57	14	7	20	15

Also shown are centrifuge centripetal acceleration setting (multiplicity of g) X, distance from the centrifuge rotor axis and the level of the blood sample x_{min} , distance from the centrifuge rotor axis and the bottom of the tube x_{max}, adjustable model parameter ω, length of the plasma column after the test spin L_{test} and optimal time of the spin estimated by the mathematical model t_{opt}. From Berry and Kralj-Iglič (2023).

Blood was donated by two of the authors (a female aged 64 years and a male aged 22 years, with no record of disease). 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 tubes with trisodium citrate (BD Vacutainers, 367714A, Becton Dickinson, Franklin Lakes, NJ, USA) were used. Blood was processed fresh within 1 hour of sampling. While waiting to be centrifuged, the samples were gently mixed on a carousel at room temperature. The test spin was performed at 300 g, 5 minutes. Blood was centrifuged in the tubes in which it was sampled at 18 °C and 300g in the Centric 400R centrifuge (Domel, Železniki, Slovenia) with rotor RS4/100. The results are shown in Table 1. It can be seen that the model somewhat underestimated the length of the optimal plasma column. In donor 1 the estimated proportion of the column length was 69% while in donor 2 it was 79%. In both cases, X_{OPT} t_{OPT} was not overestimated which is positive. Namely too long or too strong sedimentation depletes plasma of platelets which is not desired. On the other hand, eventhough the volume of plasma does not reach the optimal, the plasma remains rich in platelets and EVs.

10.6. Troubleshooting
- In the test spin, the plasma does not form. The centripetal acceleration and/or the time of centrifugation should be increased and Test spin performed again.
- In the test spin, all erythrocytes reach the level of the hematocrit. If there are multiple tubes available, the Test spin should be performed with a new tube and chosing shorter time of centrifugation. If only one tube is available, plasma should be used as formed in the Test spin. Do not resuspend the sample and spin again.

11. Data and records management
All the experimental details are recorded within the lab journal carefully. All raw as well as treated data is stored in electronic form with physical backup for a minimum of 10 years after data generation.

12. Waste management
There is no waste in this SOP as regards the samples. Protective gloves, caps, clothes and footware shields used by the staff should be disposed in biological sanitary wastes, according with applicable laws and good research laboratory practices. Damaged samples should be disposed in yellow plastic containers for hazardous materials.

13. Related protocols or SOPS
There are many reported protocols for preparation of PVRP (reviewed for example by Dhurat and Sukesh, (2014), Chahla et al., (2017), Miranda et al., (2019)). However, the distinction of our protocole is in the modeling of an individual sample.

14. Quality control and quality assurance section
14.1 Instrument calibration
Not applicable.

14.2 Critical processes parameters and checkpoints
The test time and centripetal acceleration of the centrifuge are critical parameters. In particular, their product should not be too high. As the physical properties of the sample are not known at the time of the decision, especially in patients with higher erythrocyte sedimentation rate, setting the Xt_{OPT} too high may deplete plasma of platelets already at the test spin and no additional spin can be performed.
The temperature during the processing is also important. As the processing times are rather short, the rotor should be thermally pre-equilibrated to the desired temperature.
Quality control of plasma preparations should be continuously performed by collecting data on the volume and content of plasma in correlation with the measurement of hematocrit (proportion of the volume of the erythrocytes in blood) and blood cells count available for the individual patients.

Conclusions

Mathematical models, guidelines and SOPs are extremely useful tools for optimization and standardization of the procedures, enhancing reproducibility of the results, minimizing risks and failures, and therefore increasing experimental efficacy and efficiency (Bongiovanni et al., 2015; Digilio et al., 2016; Mancinelli et al., 2019; 2021, Mascia et al., 2021; Holmann et al., 2022). Guidelines and SOPs should be periodically monitored and eventually revised, to be constantly updated and ameliorated, according to scientific progress and research requirements (Digilio et al., 2016). The single tools are even more efficent if they are inserted in the context of a total QMS, as long as it is customised for research purposes and needs without curtailing the freedom of researchers (Liguori and Kisslinger 2020; 2022).

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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Research

Characterization of Extracellular Particles from Equine Milk and Colostrum

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Abstract:

Extracellular particles (EPs) from mare (equine) milk and colostrum were investigated. The samples were taken from a Posavje mare on days 1, 3 and 7 after the parturition. Number density n and hydrodynamic diameter D_h of EPs were measured by interferometric light microscopy (ILM). Higher number density of nano - sized EPs were found in skimmed milk when compared to the whole milk on days 1, 3 and 7 after the parturition, however the difference was statistically significant only on the day 7 after the parturition. The average D_h was statistically significantly lower in the whole milk than in the skimmed milk at day 7 after the parturition.

Keywords: Extracellular vesicles; Light scattering; Vesicle characterization; Mare; Colostrum; Milk; Nanovesicles

1. Introduction

1.1. Mare udder, lactation, colostrum and milk

Colostrum (the first milk secretion after the parturition) and milk are being produced in the mammary glands of a female mammals (Reiter and Reed, 2023). There are two halves of the mare udder. In each half, there are two separate mammary gland complexes one in front and the other behind. Each of the two separate mammary complexes has its own separate teat cistern and teat canal. Each of both teats has therefore two openings, one in front and the other behind (Dzidic, 2003).

Lactation is energetically very demanding for a mare. It may last for over a year in the wild. It is highest 30 to 60 days after foaling, when daily milk production can range from 12 to 15 liters (Morresey, 2012). Both, colostrum and milk are essential for the foal's survival. The foal's early growth, development, and immune function depend on colostrum. Colostrum provides the newborn foal with essential nutrients as amino acids, bioactive proteins, immunological factors, and antioxidants (Reiter and Reed, 2023).

The transfer of passive immunity occurs via colostrum which is critical for the proper development of the immune system in the foal (Reiter and Reed, 2023). After the parturition, the immune system of the foal is very immature because the equine placenta does not enable the permeability to proteins like immunoglobulins (Reiter and Reed, 2023). Foals are born without immunoglobulins in circulation. Therefore, immunoglobulins from the colostrum are critical for the protection of the neonate against the pathogens from the environment and therefore help the development of the immune system. Approximately 60% of the protein in the colostrum are immunoglobulins (Reiter and Reed, 2023). The predominant immunoglobulin in equine colostrum is immunoglobulin G (IgG), followed by IgA occurring at a lower concentration (Reiter and Reed, 2023). Approximately 12 hours after the parturition the colostrum immunoglobulin concentration decreases (Pasquini et al., 2005). The foal's intestine enables the absorption of the immunoglobulins only first 6 to 12 hours of life (Reiter and Redde, 2023).

Colostrum turns to milk in approximately 2 days after the parturition, but the transition from colostrum to mature milk is gradual and takes several weeks (Reiter and Reed, 2023). The mature milk contains nutrients for the foal and non-nutritive bioactive factors (Reiter and Reed, 2023). Due to limited fat and carbohydrate content, equine milk contains less energy in comparison to bovine or human milk (Reiter and Reed, 2023). However, mature equine milk contains more crude proteins than human milk but less than bovine milk (Malacarne et al., 2002).

Figure 1. Posavje mare with the foal.

2. Material and Methods

2.1. Milk Sampling

Colostrum (milk collected on the day 1 after the parturition) or milk was milked by hand from a Posavje (breed) mare (**Figure 1**) on days 1, 3 and 7 of the lactation. Colostrum or 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, the whole milk was centrifuged at 300 g for 15 min. The centrifugation was after that repeated once again. The cream was removed from the top using a pipette with the tip shortened for 2 mm by scissors. The tubes with colostrum and milk are shown in **Figure 2**.
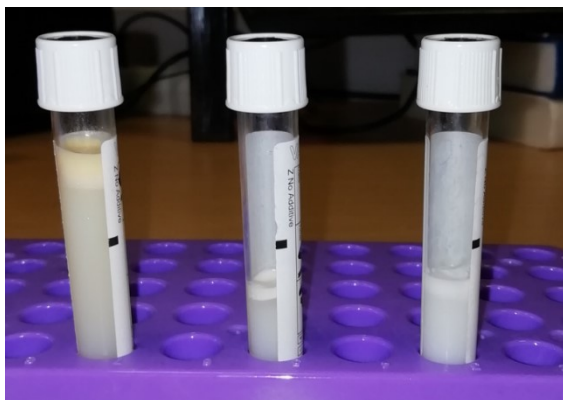

Figure 2. The tubes with mare colostrum and milk. From left to right: The tube on the left contains mare colostrum milked on the day of parturition, the middle tube contains mare milk from the third day after the parturition and the tube on the right contains mare milk milked on the seventh day after the parturition. On the top of the samples the cream layer is clearly visible. Colostrum has a yellow hue unlike the white milk.

2.2. Interferometric Light Microscopy (ILM)

The average hydrodynamic diameter (D_h) and the number density of small particles in milk (n) were determined by ILM using Videodrop (Myriade, Paris, France) as described previously (Romolo et al., 2022). Before measurement the milk was diluted 100 × by saline for injections (Braun, Melsungen, Germany). 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. Briefly, the interference pattern between incident and scattered light forms contrasting black and white spots which were recognized as particles and their respective positions were determined. Counting the number of particles in a defined volume (of the order of 15 pL) yields the number density of EPs. D_h was determined by following the position of EPs with time and assuming Brownian motion of EPs. Assuming that the diffusion coefficient D of EPs is proportional to the mean square displacement d of the EP between two consecutive frames taken in the time interval Δt, $<d^2(\Delta t)> = <4D\ \Delta t>$, D_h was estimated by using the Stokes-Einstein relation $D_h = kT/3\pi\eta D$, where k is the Boltzmann constant and T is temperature. 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 sofware. The value p = 0.05 was taken as a threshold for statistical significance.

3. Results

Higher concentration of nano - sized particles was found in skimmed milk when compared to the whole milk on days 1, 3 and 7 after the parturition, however differences were statistically significant only on day 7 **(Figure 3. A)**. The differences between average D_h of the particles were within the error of the method **(Figure 3. B)**.
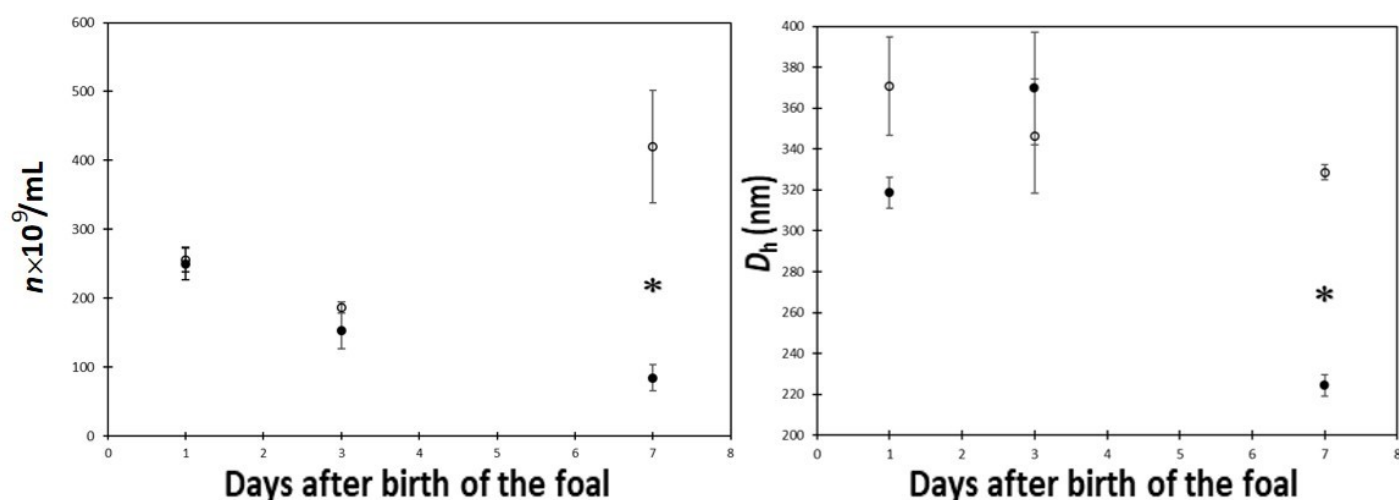
Figure 3. A: average number density n and B: average hydrodynamic diameter D_h of EPs in the mare whole (full circles) and skimmed (empty circles) milk. The error bars represent standard deviations. Asterisk (*) represents statistically significant difference between the whole and the skimmed milk results on day 7.

4. Discussion

Higher concentration of nano - sized particles was found in skimmed milk when compared to the whole milk on days 1, 3 and 7 after the parturition, however differences were statistically significant only on day 7. D_h was smaller on day 7 than in days 1 and 3, the differences were statistically significant.

In our previous work, higher concentration of micro and nano-sized particles was found in skimmed mature equine and bovine milk measured by interferometric light microscopy and flow cytometry when compared to the mature whole milk and the differences were statistically significant (Arko et al., 2024), which is in agreement with our present results. Higher number density of EPs in skimmed milk could be explained by removal of larger particles with the cream.

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

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

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Review

Assessment of Extracellular Particles Directly in Plasma. A review

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Abstract:

Extracellular nanoparticles (EPs) in blood have been amply studied since their biological role was indicated more than 50 years ago. Among the various types of nano-sized blood constituents, the studies outlined lipoproteins, immune complexes and extracellular particles. Efforts have been invested to separate or separately assess different types of particles. Recently, much attention has been devoted to extracellular vesicles (EVs), however, experimental evidence indicates that the methods used for their harvesting and assessment importantly influence the content of the samples whereas the information on characteristics of the samples that could reflect the clinical status of the sample donor may be altered. Recent developments of the characterization methods point to the possibility of assessing nano-sized EPs directly in plasma and blood, thereby avoiding artefacts caused by processing. In this contribution, we consider the assessment of nano-sized EPs from blood and provide evidence supporting the direct assessment of EPs in plasma.

Keywords: Extracellular particles from blood; Extracellular particles from plasma; Extracellular vesicles from blood: Extracellular vesicles from plasma; Interferometric light microscopy; Dynamic light scattering

1. Extracellular particles (EPs) and their applications

EPs are mediators of intercellular communication by exchanging the material and information within cellular fragments. EPs travel to the recipient cell where EP cargo (including different types of RNAs) can be internalized. EPs are the subject of increasing interest as they are considered potential indicators of the body's response to the disease and effectors in systemic metabolic regulation. EPs have been isolated from diverse body fluids, including blood, urine, saliva, breast milk, amniotic fluid, ascites, cerebrospinal fluid, bile, and semen (Yanez Mo et al., 2015). Here we focus on EPs from blood.

2. Biological significance of blood and plasma extracellular particles

Blood and blood-derived products such as plasma contain cells, molecules, ions, molecular aggregates such as lipid and protein complexes, and nano-sized EPs. It was observed that the coagulant activity of stored citrated plasma increased with time in the interval of hours (Hougie, 1955), and that platelet-free plasma and serum generate thrombin on recalcification, which was affected by prior high-speed centrifugation of the plasma (Chargaff and West, 1946; O'Brien, 1955). The coagulant activity of the platelet-poor blood products was attributed to platelet-derived EPs (Wolf, 1967). The pioneering work of Wolf (1967) showed that EPs have biological effects which implied their potential roles in diagnostics and therapy. Platelets and their fragments were suggested to have also regenerative roles in plasma (Uršič et al., 2014, Troha et al., 2023). Wolf (1976) further reported on isolating platelet-derived EPs and presented electron microscope images of the isolates and images showing budding of activated platelets. Since then, many studies on blood EPs have been performed, and knowledge of EP morphology, physicochemical properties and specific biological effects is highly warranted.

The International Society for Extracellular Vesicles (ISEV) is addressing nomenclature, methods for harvesting and characterization of EPs and functional studies of various biological samples to strengthen the approach to challenges connected to EPs and facilitate communication between scientists working in this multidisciplinary field. ISEV has recently published an update on minimal information for studies of extracellular vesicles (Welsh et al., 2024). The origin, nature, and features of EPs are diverse, and many different names have been used in the literature, referring to their size, origin (prostasomes, oncosomes), proposed functions (calcifying matrix vesicles, argosomes, tolerosomes), or presence outside the cells (prefix exo or ecto: ectosomes, exosomes, exovesicles, exosome-like vesicles)(Colombo et al., 2014). As regards nomination, the recommendations have been suggested by Welsh et al., (2024). Blood – derived EPs are one of the focus fields of ISEV. The ISEV Blood EV Task Force created the Minimal Information for Blood EV Research (MIBlood-EV), a tool to record and report information about pre-analytical protocols used for plasma and serum preparation as well as assays used to assess the quality of these preparations (Lucien et al., 2023). Harvesting of EPs from blood and blood-derived products is not the scope of this work and will not be considered; however, the methods follow those for harvesting of EPs in other biological samples (Welsh et al., 2024).

3. Challenges with assessment of EPs from blood-derived products

Blood contains high concentrations of soluble proteins such as serum albumin, immunoglobulins and fibrinogen, as well as aggregates such as lipid-protein and ribonucleoprotein aggregates. These proteins and aggregates may be associated with EPs released from cells (Welsh et al., 2024). The mechanisms of formation of such complexes are unclear, and to our best knowledge there is no method available that could separate all lipoproteins from EPs. As it is not possible to separate all lipoproteins from EVs, a combination of methods that exploit different physical and biochemical properties is recommended (Welsh et al., 2024). It was emphasized that the reported EV number density in blood plasma spans six orders of magnitude depending on the measurement method (Johnsen et al., 2019).

4. Methods for assessment of number density and size of EPs in biological samples

We briefly describe some widely used methods for the assessment of EPs: dynamic light scattering, nanoparticle tracking analysis, flow cytometry, electron microscopy, and super-

resolution imaging technologies (Wu et al., 2024 and the references therein) and interfero-
metric light microscopy: a method recently introduced into the field of EPs (Romolo et al.,
2022, Sausset et al., 2023).

4.1. Microscopy of samples containing cells and EPs

Due to the limitation of light diffraction phenomena, the resolution of an optical micro-
scope is about half of the light wavelength. Sub-micron sized particles should therefore be
observed using electron microscopy or upgraded optical microscopy. Electron micros-
copy, with sufficient resolution, can characterize the morphology and size of a single EP
as well as populations of EPs (Wu et al., 2024). Various methods are applied: scanning
electron microscopy (SEM), transmission electron microscopy (TEM), cryogenic scanning
electron microscopy (cryo SEM), atomic force microscopy (AFM) and super-resolution im-
aging technology. It is considered that the typical extracellular vesicle (EV) morphology,
assessed through negative staining TEM, is a cup-shape, however, further details are not
yet clear (Jung and Mun, 2018). In isolates from plasma, different shapes of EVs were ob-
served by SEM (Junkar et al., 2009; Mrvar-Brečko et al., 2010). These shapes were compared
with the vesicle shapes on a larger scale, and explained by considering the minimization
of the membrane free energy (Kralj-Iglič et al., 2020). It was concluded that EVs can attain
a variely of shapes according to the composition of their membrane and the surrounding
solution (Kralj-Iglič et al., 2020). SEM is currently the best technique to give insight into the
3D shape of EVs, however, as in this technique the object is the metallic replica of the sam-
ple, it cannot reveal the ultrastructure of the particles. A limitation of SEM is that it can be
subject to artefacts due to the demanding preparation of the sample. Samples are stained
by heavy metal and they undergo drying to remove water, during which EPs may be de-
stroyed or transformed. The drying process shrinks EPs, affecting their size and shape. The
ultrastructure of EPs can be observed in ultrathin sectioned and negative or immuno -
stained samples imaged by TEM (Jung and Mun, 2018) and cryoTEM (Brisson et al., 2017).
Cryo TEM requires samples without larger particles (e.g. platelets or other blood cells).
The samples are therefore subjected to filtering before imaging. In AFM, the presence of
sub-micron sized particles can be detected (Junkar et al., 2009). The resolution allows only
for rough appearance of EPs and diversity of shapes obtained by SEM cannot be observed.
The resolution of super-resolution imaging technology is below 200 nm and is therefore
appropriate for observing EPs (Wu et al., 2024).

4.2. Dynamic light scattering (DLS)

DLS is an optical analysis method for measuring the size and distribution of submicron
particles (Sitar et al., 2014; Stetefeld et al., 2016). The Brownian motion of the particles
causes change in the light scattering signal, which is represented by a correlation function
from which the diffusion velocity and particle distribution are obtained. Because of the
higher motion rate of small particles, the intensity fluctuation is larger and there is a pro-
nounced decline in the correlation curve. The size of the particles is estimated based on the
assumption that the particles are undergoing Brownian motion. Taking that the particles
are spherical, the Stokes - Einstein equation is used to determine hydrodynamic diameter
D_h.

4.3. Nanoparticle tracking analysis (NTA)

In NTA the particles are irradiated by the laser light and intensity of the light scattered
from the sample is detected by a high-speed camera (Vestad et al., 2017). Also, in NTA, the
Stokes - Einstein equation is used to determine D_h.

4.4. Flow cytometry (FCM)

In flow cytometry, a flux of single particles is established and illuminated by a laser light.
The light scattered in forward and side directions is detected, with each detected event
considered as one particle. If the particles in the sample emit light, it can be acquired by a
detector array. FCM is therefore convenient for counting autofluorescent particles and par-
ticles dyed by fluorescent dyes. Thus, specific gene or protein expression and enzyme ac-
tivity can be specifically measured by different channels. Scatters of light from particles

reflect the size and number density of cells or particles and can also be estimated by comparing the scatter diagrams with those of standardized beads. However, scattering on inorganic material beads reflects the properties of EPs only to a certain extent.

4.5. Tunable Resistive Pulse Sensing (TRPS)
In TRPS, the suspension of particles is mixed with the electrolyte, and the flow is established through a nanopore chip with a specific aperture between two electrodes. The change of the resistance to the current between the electrodes is detected by creating a pulse signal. The intensity and frequency of the signal are related to the number density and size of EPs (Maas et al., 2014; 2017; Wu et al., 2024). The limitation of TRPS is that the pore is sensitive and prone to block while the blood derived samples may contain cell – sized particles. It is therefore necessary to effectively remove such particles from the sample e.g. by filtration.

4.6. Interferometric light microscopy (ILM)
In ILM, the sample is illuminated by LED light, and the light scattered on the particle is imaged by a bright-field microscope objective and allowed to interfere with the incoming light. The image is recorded by a complementary metal-oxide-semiconductor high-resolution high-speed camera. Interference enhances the information in the scattered light. The obtained pattern, which includes contrasting black and white spots, is recognized as a particle, and its position in the sample is assessed. The diffusion coefficient of the motion of the particle is taken to be proportional to the mean square displacement of the particle between two consecutive frames taken and the hydrodynamic diameter is estimated by using the Stokes–Einstein formula.

4.7. Limitations of DLS, NTA and ILM
All three methods assume that the EPs are free to move subject to thermal motion and use Stokes – Einstein formula to determine hydrodynamic diameter of the particles. However, the motion of the particles can be hindered due to presence of molecules, filaments or other EPs if the suspension is too dense. In this way the instrument underestimates the mobility of the particles and overestimates their hydrodynamic diameter. Also, the viscosity of the sample is estimated by using viscosity of water which can - albeit diluted - be a poor estimation as regards blood or plasma. These methods detect a large number of EPs, however, they cannot reveal the identity of the particles. Also, they have different limits of detection so the results pertaining to the same sample may differ.

5. Assessment of EPs in isolates from plasma
The majority of studies on EPs from plasma aim at isolation and purification of EVs. Commonly used methods are (ultra)centrifugation, size exclusion chromatography, (ultra)filtration, field-flow fractionation (FFF), asymmetric flow FFF, free-flow electrophoresis, precipitation and affinity methods that capture EVs based on their surface charge or molecular composition (Welsh et al., 2024). Published work in the recent years has highlighted that it is very difficult to obtain a pure sample of EVs from blood plasma or serum because several types of lipoproteins contaminate the EV samples (Johnsen et al., 2019). Recently, a laboratory-built nano-flow cytometer was used to analyze the quality and efficiency assessment of EVs isolated from plasma obtained by five widely used commercial isolation kits and compared with the traditional differential ultracentrifugation (Tian et al., 2019). Two to four orders of magnitude higher particle concentrations were observed for EV preparations from platelet-free plasma (PFP) by kits when compared with the EV preparation by UC, yet the purity was much lower. Meanwhile, the particle size distribution profiles of EV preparations by kits closely resembled those of PFP whereas the EV preparation by UC showed a broader size distribution at relatively large particle size. During sampling and harvesting, samples are subjected to mechanical, thermal and chemical stress (Štukelj et al., 2014; 2017), which may considerably affect the morphology, number density and composition of EPs (Šuštar et al., 2011a,b, Božič et al., 2020) and these processes are different in different methods applied.

Božič et al. (2020) have shown that the material in the plasma – derived samples is being transformed throughout the centrifugation processing. By assessment with FSC, the side

scattered light correlated with changes of centripetal acceleration of the centrifuge rotor and of the temperature (Božič et al., 2020). During centrifugation, cells and their fragments are subjected to shear forces that are maximal at the tube wall. This can lead to cell fragmentation, resulting in the formation of EPs and EVs. A high amount of EPs/EVs can be produced from cells that were not efficiently removed in the previous steps of isolation. Also, overly aggressive processing can be detrimental for EPs. Visualization of the isolate from plasma obtained by differential ultracentrifugation at 100,000 g for 8h was of unrecognisable structure (Božič et al., 2020). The results of Božič et al. (2020) provided evidence in favour of the hypothesis that the majority of the isolated particles from plasma are produced after blood sampling and are fragments of residual cells in the samples (Šuštar et al., 2011a).

The aspect of standardization of the procedures is believed to improve repeatability and accuracy of results. Diehl et al. (2023) have suggested a protocol for the isolation and quantification of plasma EVs, including isolation of vesicles by automated size exclusion chromatography and quantification by TRPS. This workflow optimizes reproducibility by minimizing variations in processing, handling, and storage of EVs (Diehl et al., 2023). However, attempts to standardize the procedures are limited, as it remains unclear which parameters critically influence the process and because the mechanisms of EPs formation are not yet adequately understood.

6. Assessment of EPs directly in plasma or blood

Yet another aspect would be to avoid processing of samples as much as possible. Lawrie et al. (2008; 2009) have applied DLS to assess EPs directly in diluted plasma. Božič et al. (2019) have also used DLS to study EPs in blood plasma and isolates from the blood plasma of 3 healthy donors. Later, the method was applied to samples of plasma, peritoneal fluid, and peritoneal washing of patients with ovarian cancer (Kogej et al., 2021). Recently, ILM was used to assess the number density of EPs and their hydrodynamic diameter in different EV samples (Romolo et al., 2022; Sausset et al., 2023) including diluted plasma (Berry et al., 2024). Berry et al. (2024) measured EPs in plasma samples from multiple species—canine, equine, and human. By reporting on the analysis of 250 samples of the diluted plasma, Berry et al. (2024) showed that that high-throughput measurement of the number density and size of EPs in plasma is feasible. Hydrodynamic diameters in samples from all three species were confined to the interval between 130 nm and 200 nm (Berry et al., 2024). The advantage of ILM over DLS is that preparation of the sample for assessment with ILM does not require filtration of the sample, which is necessary for DLS. Namely, larger particles scatter light much more than smaller ones, and their presence renders smaller particles indetectable by DLS. On the other hand, ILM has a detection limit at cca 80 nm, while DLS can detect also smaller particles.

Results from Berry et al. (2024) indicate the possibility to assess EPs directly in diluted blood, which would additionally diminish a possibility of artefacts due to sample processing. Namely, to obtain plasma, erythrocytes are sedimented, usually by centrifugation. Although the centripetal acceleration of the centrifuge rotor required to produce plasma is moderate, fragmentation of cells is likely to occure.

ILM is a promising method for detecting EPs in different biological samples. It was hitherto used to detect viruses (Boccara et al., 2016; Roose-Amsaleg et al., 2017; Yurdakul et al., 2020, Turkki et al., 2021), phages (Boccara et al., 2016, Sausset et al., 2023), liposomes (Romolo et al., 2022; Spasovski et al., 2024) and EPs (Sabbagh et al., 2021; Romolo et al., 2022; Jeran et al., 2023; Berry et al., 2024). Furthermore, interferometric NTA offers possibility of even better resolution downto smaller EP sizes (Kashkanova et al., 2022).

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

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Research

Quartz Crystal Microbalance with Dissipation Monitoring: A Method for Studying Biomimetic Membranes

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Abstract:
The human body contains a few trillions of cells of different types and functions, which are crucial for living. The study of cell membranes is important for understanding biological events and developing new medicines. As cell membranes are very complex, researchers often create and study simple models, referred to as biomimetic membranes, in order to understand their behavior and properties. In this paper, we briefly present quartz crystal microbalance with dissipation monitoring (QCMD) as a surface-sensitive technique to study biomimetic membranes. By measuring changes in the oscillation frequency and dissipation energy of quartz crystal sensors, QCMD can monitor in real time molecular events occurring at the quartz sensor-sample interface, such as formation of supported layers, changes in thickness and structural properties, as well as biomolecular interactions. We present a concise description of the basic QCMD principles, followed by a few examples on the adsorption of model membranes, namely, solid-supported lipid bilayers and vesicles, as well as on studying lipid phase transitions.

Keywords: supported lipid bilayers, supported lipid vesicles, phase transitions, quartz crystal microbalance with dissipation monitoring (QCMD)

1.   Introduction

Phospholipids are amphiphilic biomolecules composed of a hydrophilic head and hydrophobic chains, thus, within an aqueous environment they assemble to create bilayers, vesicles or micelles. Phospholipid bilayers form the fundamental infrastructure of the membranes enclosing the biological cells and the contained organelles. The proteins, carbohydrates, as well as the other types of lipids which are embedded in the bilayers, make the structure and composition of the latter very complex, and modify the membrane properties (e.g., fluidity), or cell functions (e.g., transport of substances). The various parameters are thus hard to investigate independently. For that reason, model systems of lower complexity, such as solid-supported lipid bilayers (SLBs) or solid-supported lipid vesicles (SLVs) are utilized and are easy to prepare.

Quartz crystal microbalance with dissipation monitoring (QCMD) is an advantageous technique to study supported lipid systems since it can monitor in the liquid state and in real time the formation of lipid layers with a high sensitivity (mass detection of a few ng per cm^2). In addition, QCMD does not require any labelling which could influence the lipid membrane organization, contrary to other techniques such as nuclear magnetic resonance or fluorescence microscopy.

2.   Quartz crystal microbalance with dissipation monitoring

QCMD is an acoustic device working on the inverse piezoelectric effect: the application of an alternating AC voltage on an AT-cut quartz crystal used as sensor leads to its oscillatory motion. A standing wave is generated when the applied voltage matches the crystal resonance frequency f (typically of 5 to 10 MHz) or its odd overtones n (generally, n = 3 to 11). When the voltage is switched off, the oscillation decays exponentially. From the cyclic deformation of the quartz sensor, two parameters are recorded upon the adsorption of molecules: the frequency changes $\Delta f/n$, related to the adsorbed hydrated mass on the surface, i.e. the mass of the molecules and water trapped ($\Delta f/n$ decrease denotes a mass increase), and the dissipation energy loss ΔD, related to the viscoelastic properties of the supported layer (ΔD increases with viscosity) (Reviakine et al., 2011). Different coatings can be formed on the sensor surface to match the particular needs of each experiment, among them SiO_2, TiO_2, Al_2O_3 and Au. Prior to experiments, all sensors are thoroughly cleaned following standard protocols (chemical cleaning, exposure to UV light), to remove any impurities.

Our QCMD setup (Q-Sense Analyser, Biolin Scientific, Sweden) uses four modules (sensor chambers) enabling simultaneous experiments, variable flow rates of liquid samples (typically 50 to 100 µl/min for SLBs and SLVs studies). The temperature can be changed in the range from 15 °C to 60 °C using a Peltier element. Performing temperature ramps is useful to detect lipid phase transitions in adsorbed lipid films (SLBs, SLVs).

After presenting the formation of a SLB and a SLV on quartz sensors, we will introduce some examples how the QCMD is used for studying biomimetic membranes. We will first show the kinetics of adsorbing unilamellar vesicles with three different sizes, as well as multilamellar ones. Afterwards, we will demonstrate how to detect phase transitions of a lipid vesicle layer.

3.   Results

The formation of SLBs or SLVs can be easily followed by QCMD via the frequency or dissipation changes when lipid vesicles are injected on surfaces. Vesicles are prepared by a well-defined, standard protocol: lipid monomers are first dissolved in chloroform and dried out slowly under an inert gas flow to obtain a homogeneous lipid film. The film is then hydrated with buffer, generating multilamellar vesicles, which are in turn extruded to become unilamellar with a defined diameter.

A typical QCMD experiment is structured as follows: first, a buffer injection is performed to create a baseline, then lipid vesicles are injected until reaching a stable signal plateau,

and a buffer-rinsing step follows. The obtainment of SLV or SLB depends on several parameters, among them: the vesicle composition (transition temperature of lipids, bending modulus) and size, the sensor surface energy, and the adsorption temperature (Bar et al., 2023). In **Figure 1**, a solution of 1,2-dipalmitoylphosphatidylcholine (DPPC) vesicles of 50nm diameter was injected on two sensors with different surface coverage, namely, Au and SiO2.
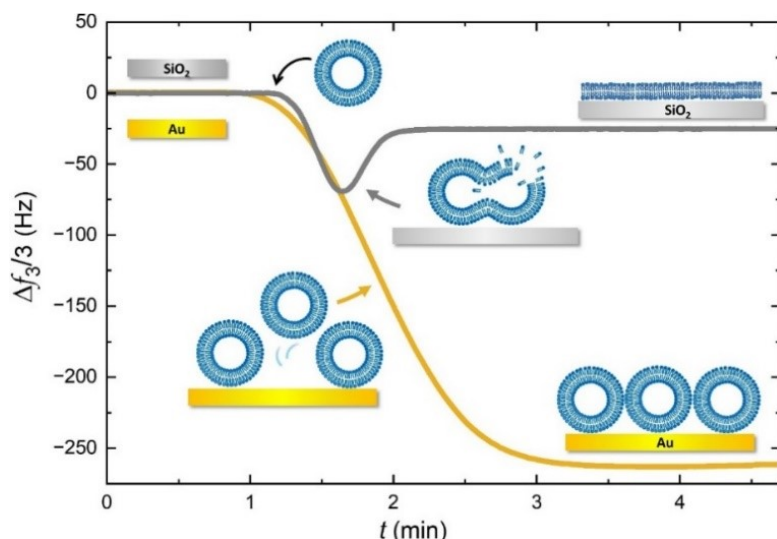

Figure 1: Frequency changes obtained while adsorbing DPPC lipid vesicles (0.5 mg/ml in Hepes buffer) at 50μl/min on Au (at 50°C) and SiO₂ (at 20°C), giving rise to the formation of a SLV and a SLB respectively.

When adsorbed on Au substrate at a temperature T below the DPPC main transition temperature ($T_m \approx 42.5°C$), the lipid is in the gel phase (quite rigid) and the adhesion energy is not strong enough to break the vesicles. They simply adsorb and form a homogeneous SLV. This is probed by a continuous drop in frequency until the sensor surface is fully covered with vesicles, i.e., when it reaches a plateau. If the adsorption is done at 50°C ($T > T_m$, DPPC being in the liquid phase) on SiO₂ substrate (strong surface energy), then vesicle breaking can occur. We observe first a $\Delta f_n/n$ decrease due to vesicle adsorption. Once a certain quantity is adsorbed (maximum negative peak value), vesicles fuse and break, releasing the trapped water, with a mass loss displayed by a $\Delta f_n/n$ increase. A final value of -25 Hz is typical for a homogeneous lipid bilayer (Reviakine et al., 2011).

The observation of the kinetics of vesicle adsorption, as well as the plateau values, provide extra information about the lipid behavior. In **Figure 2**, we compare the adsorption of DPPC vesicles on Au-coated quartz sensors as a function of their size. We have used multilamellar (in μm range), and unilamellar vesicles extruded through membrane filters with pore sizes of 200 nm, 100 nm and 50 nm. The slopes of frequency shifts reveal that the mass gain is fastest for the smallest 50 nm vesicles, providing first a full coverage of the sensor surface, i.e., the frequency shift reaching the plateau value. The bigger the vesicles (going from 50 nm, to 100 nm, 200 nm to multilamellar in μm scale), the longer they take to adsorb and reach a full coverage of the sensor surface. This can be explained by a slower diffusion onto the surface, and a longer-lasting reorganization and ordering once they are adsorbed. One has the possibility to explore the impact of various parameters on the vesicle kinetics (inclusion of new constituents, salts, etc.)

QCMD can also be used to detect phase transitions between various lipid phases in SLBs and SLVs (Cordoyiannis et al., 2021). For SLBs, the phase transitions signatures are weak and often difficult to probe, whereas in SLVs they can be nicely seen. After the SLV formation, temperature ramps can be set.
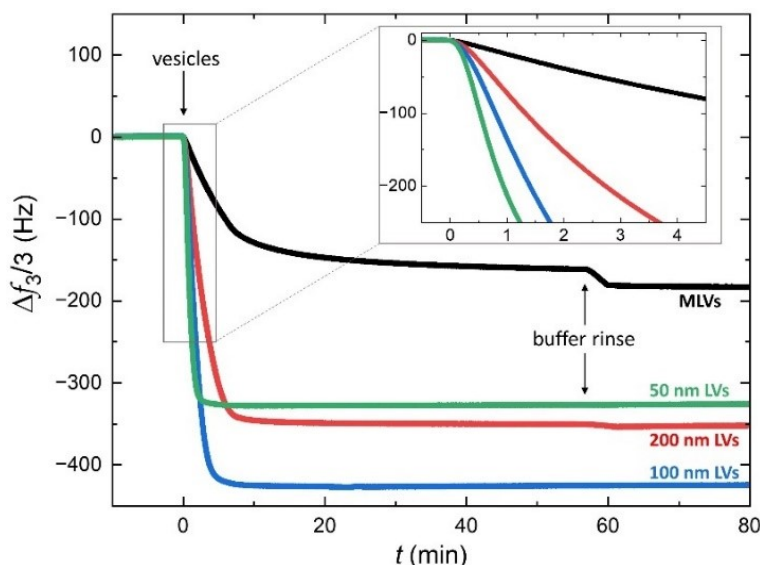
Figure 2: Kinetics of the adsorption of DPPC vesicles (0.5 mg/ml in Tris buffer) of three different sizes and their multilamellar representative, on Au sensor at 50μl/min and 20°C. The inset is a zoom in the first minutes of injection showing the slopes of frequency shifts.

During the heating or cooling runs, frequency and dissipation changes show irregularities at temperatures when a phase transition occurs. The temperature derivatives of $\Delta f_n/n$ and ΔD data make these irregularities appear as clear peaks. In **Figure 3**, an example of such lipid phase transition is presented. DPPC SLVs are heated from the low-temperature, ordered gel phase at 20 °C to the disordered liquid phase at 50 °C. Two anomalies highlight two phase transitions from gel-to-ripple (at 32.5 ± 0.5 °C) and from ripple-to-liquid phase (at 42.3 ± 0.2 °C). These two transitions are referred to as pretransition and main transition respectively. The intermediate ripple phase possesses ordered and disordered domains of few nm ranges. QCMD enables the observation of how an additional component (ex. cholesterol or some proteins) in the vesicle can affect its phase transitions between various phases and, thus, its stability (Cordoyiannis et al., 2021).
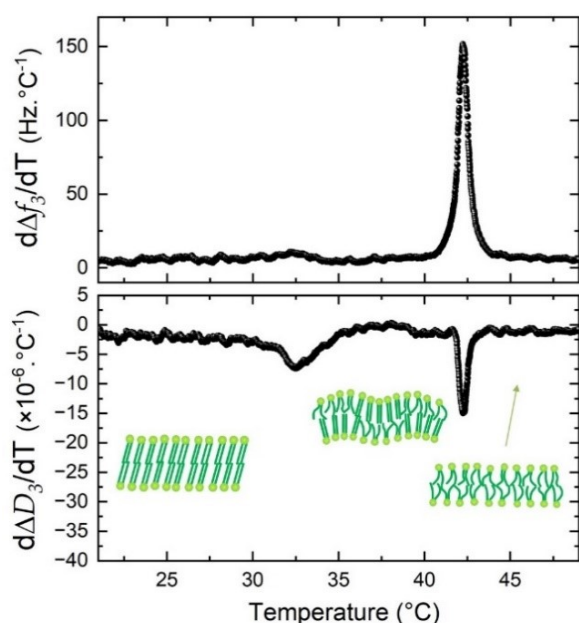

Figure 3: Frequency and dissipation temperature derivatives mark the phase transitions between three lipid phases (gel, ripple, liquid) in DPPC SLVs: (a) from the ordered gel phase to the ripple phase, and (b) from the ripple phase to the disordered liquid phase at high temperatures.

4. Conclusions

We have briefly presented the QCMD as a useful tool to study biomimetic membranes, such as SLVs and SLBs. Examples have been given about how we probe phase transitions between various lipid phases occurring upon changing temperature, as well as regarding the kinetics of lipid vesicle adsorption.

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

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Research

Estimation of Direct Interaction between Membrane Inclusions

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Abstract:
Within the statistical mechanical description or the phospholipid bilayer membrane, each monolayer is described as composed of patches containing very many inclusions that are characterized by intrinsic curvatures. A patch is subjected to local membrane curvature whereas summing up the free energies of the patches yields the free energy of the membrane. The origin of the single inclusion energy is the mismatch between the intrinsic curvature of the inclusion and the local curvature of the membrane, however, also direct interactions between inclusions contribute to the free energy. Here we upgrade the description by elaborating direct interactions between inclusions. We assume that the direct interactions are subject to van der Waals forces acting on the interfaces between the inclusion and its nearest neighbours. The expression for the interaction depends on the geometry of the inclusions, distance between them and Hamaker constant. The estimated direct interaction between inclusions of the size of small membrane rafts (20 nm) distanced for 0.2 nm with Hamaker constant 7×10^{-21}J is $W \approx 1800 \ kT$.

Keywords: Membrane free energy; Membrane curvature; Membrane shape; Shape of the phospholipid membrane; Membrane biophysics; Hamaker constant

1. Introduction

Theoretical description of biological membranes provides an important link between physical laws and features, observed experimentally in complex systems. Previous studies have shown, that the observable shape of the membrane-enclosed structures can be explained by minimization of the membrane free energy at relevant constraints (e.g. prescribed membrane area, enclosed volume, average mean curvature, average mean curvature deviator) (Kralj-Iglič et al., 2020 with included references). The derivation of the free energy is based on the mismatch of the curvature tensor of an infinitesimal membrane element and the curvature tensor of this element in its intrinsic state (Kralj-Iglič et al., 2020). For anisotropic constituents there may be also contribution of the orientation of the inclusion with respect to the principal axes system of the membrane (Kralj-Iglič et al., 2020). The fluid crystal mosaic model (Kralj-Iglič, 2012) emphasizes the effect of orientational ordering of membrane constituents which becomes important in highly anisotropically curved regions such as in the narrow necks (Kralj-Iglič et al., 2006). **Figure 1** shows that considering the orientational ordering of phospholipid molecules becomes noticable in the neck (Panes B) which may considerable impact the equilibrium free energy of the entire vesicle and therefore indicate the direction of spontaneous change of the shape (Kralj-Iglič et al., 2006). In the calculation of the results presented in **Figure 1** the direct interactions between membrane constituents were taken into account as estimated by the van der Waals interaction between phospholipid molecules. It was shown that the direct interactions acted synergistically with the curvature mismatch and that their contributions were of the same order of magnitude (kT) where k in the Boltzmann constant and T is the temperature (Kralj-Iglič et al., 2006).
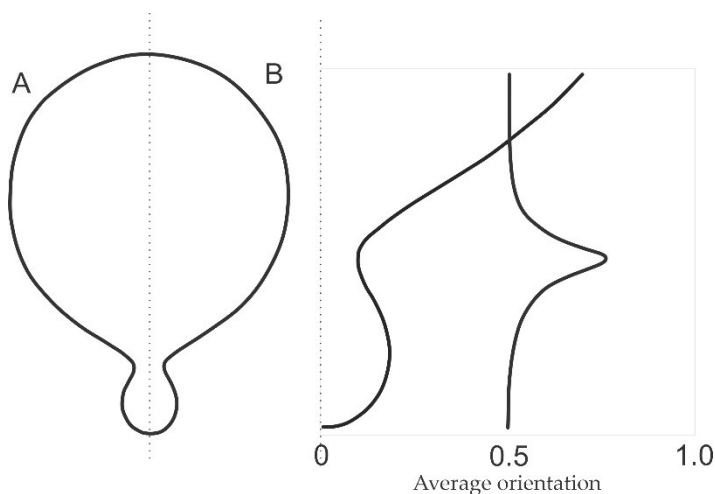

Figure 1. A: Equilibrium shape (shape of minimal free energy of the membrane) of a vesicle enclosed by a phospholipi bilayer membrane as calculated by taking into account orientational ordering of phospholipid molecules. B: The corresponding average orientation of the molecules in the neck (detail from Panel A). The model assumes two possible orientations (with minimal and maximal energy) and value 0.5 indicates that half of the constituents are in each of these two states; value 1 indicates that all of the constituents are in the energetically more favourable state. Adapted from (Kralj-Iglič et al., 2006).

However, in (Kralj-Iglič et al., 2006) the focus was on the orientational ordering and curvature mismatch of lipid molecules. The model should be further developed. Here it is taken into account that the membrane may be viewed as composed of inclusions (complexes of molecules). We focus on the estimation of the direct interactions between the membrane inclusions based on the van der Waals interaction. The derivation of the interaction between two walls, of them one with infinitely extending surface and the other with finite surface area is implemented to estimate the energy of direct interaction of the membrane inclusion with its nearest neighbors.

2.1. Interaction between a small entity and an infinitely extending wall

To calculate potential of the interaction between a small entity and an infinitely extending wall separated by a perpendicular distance D (**Figure 2A**), the entity is represented by a black dot and the wall is imagined to be composed of thin slices of thickness dx. Further, the slice is imagined to be composed of rings of the area $dS = 2\pi r \, dr$, their radii extending from 0 to $\propto$ (**Figure 2**).
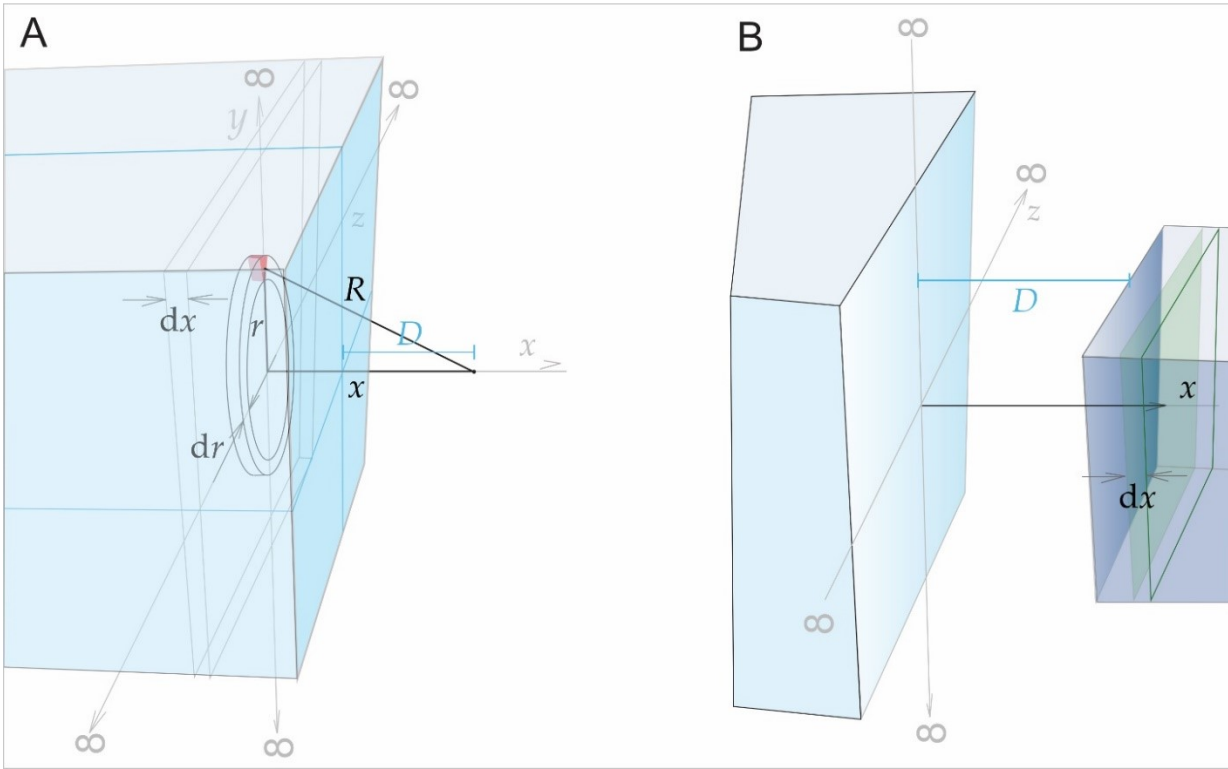

Figure 2. Illustration of the derivation of A: the van der Waals interaction between a wall and a small entity, B: the van der Waals interaction between an infinitely extending wall and a wall with finite surface and infinite length.

The entities within the ring are equally distanced from the chosen x. Following Israelaschvili (2011), it is taken that the potential V at point $(x,0,0)$ created by a small patch within the ring of the plate with radius r (**Figure 1**, red spot) containing dN entities is,

$$dV = - C_{vdW} \, R^{-6} \, dN , \qquad (1)$$

where (**Figure 1**)

$$R^2 = r^2 + x^2 \qquad (2)$$

and C_{vdW} is a constant. Considering Eqs. (1) and (2),

$$dV = - C_{vdW} \, (r^2 + x^2)^{-3} \, dN . \qquad (3)$$

To include the contributions of the entire wall, the potentials of all thin rings composing the plate are summed and then the contributions of all the slices are summed,

$$V(D) = -\int_x dx \int_r C_{vdW} \, (r^2 + x^2)^{-3} \, dN \qquad (4)$$

with

$$dN = n \, 2\pi r dr, \qquad (5)$$

and n the number density of the entities composing the wall. Integration is performed from $r = 0$ to $r = \propto$ and from $x = -\propto$ to D.

Inserting Eq.(5) into Eq.(4) yields

$$V(D) = - \int_x dx \int_r C_{vdW} (r^2 + x^2)^{-3} n\, 2\pi r\, dr \, . \tag{6}$$

We introduce a new variable

$$u = r^2 + x^2 \tag{7}$$

and the corresponding differential

$$du = 2r\, dr \tag{8}$$

with the boundaries

$$u(r = 0) = x^2 \tag{9}$$

and

$$u(r = \infty) = \infty \tag{10}$$

to obtain

$$V(D) = - \int_x dx\, (1/2)\, \pi\, n\, C_{vdW}\, x^{-4} \, . \tag{11}$$

Integration over x within the boundaries $x = -\infty$ to D gives the energy of the interaction between a small entity and a wall,

$$V(D) = - C_{vdW}\, n\, \pi\, D^{-3}/6 \, . \tag{12}$$

2.3. Interaction between a wall with a finite surface and a wall with an infinite surface

One of the walls extends infinitely in the y and z directions and the other is parallel to it, but of finite dimensions in the y and z directions, attaining the surface area S. It is imagined that the wall with the finite surface is composed of thin slices with thickness dx. The number of the entities composing a slice is

$$dN = n\, S\, dx \, , \tag{13}$$

where it is taken that the number density of the entities of the wall with the finite surface is the same as the number density of the entities of the wall with the infinite surface. Any point within the wall that is at the perpendicular distance D from the wall with infinite surface contributes the same to the energy of interaction. The contributions to the potential of the interaction are summed over the slices from $x = D$ to $x = \infty$,

$$V_{wall\text{-}wall}(D) = \int_x n\, V(x)\, S\, dx \, . \tag{14}$$

Insertion of Eq.(12) into Eq.(14) yields

$$V_{wall\text{-}wall}(D) = - \int_x (1/6)\, C_{vdW}\, n^2\, \pi\, S\, x^{-3}\, dx \quad . \tag{15}$$

The integration is performed from $x = \infty$ to $x = D$ to yield the energy of the interaction between a wall with finite surface area and a wall with infinite surface area (Israelaschvili, 2011),

$$V_{vdW}(D) = - C_{vdW}\, n^2\, \pi\, S\, D^{-2}/12 \, . \tag{16}$$

2.4. Interaction between two membrane inclusions

Within the model, membrane can be considered as composed of inclusions with given principal curvatures $C_1 = 1/R_1$ and $C_2 = 1/R_2$ (**Figure 3A**). Each inclusion shares interfaces with 4 nearest neighbors (**Figure 3B**). In the model, the direct interaction between inclusions is described by the van der Waals interaction between two surfaces. The surfaces of the walls are taken to be parallel (**Figure 2**). The surface areas of the interfaces are finite, however, as the van der Waals interaction falls off with 6th order of the distance between two entities, the smaller surface of the two interacting surfaces in the potential $V_{vdW}(D)$ for small D largely determines the potential, therefore Eq.(16) is applied. The potential of the interaction of the inclusion with its 4 nearest neighbors is

$$W = 4\, V_{wall\text{-}wall}(D) \quad . \tag{17}$$

Considering Eq. (16),

$$W = - C_{vdW} \, n^2 \, \pi \, S \, D^{-2}/3 \quad . \tag{18}$$

The product $C_{vdW} \, n^2 \, \pi^2$ is Hamaker constant A (Hamaker, 1938),

$$A = C_{vdW} \, n^2 \, \pi^2 \, . \tag{19}$$

Considering Eqs.(18) and (19), we get
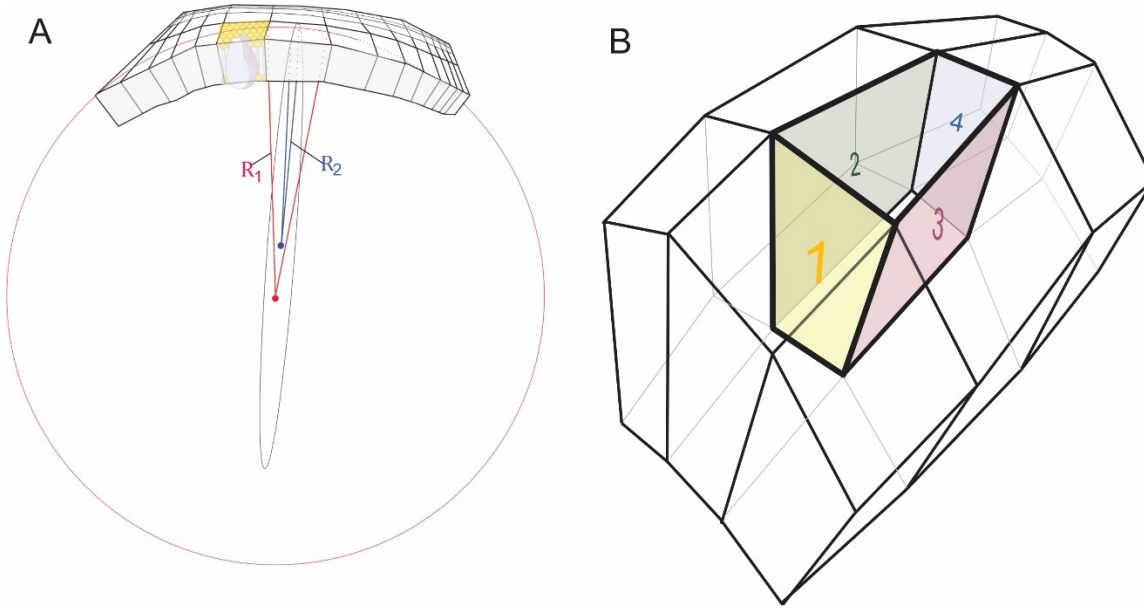
$$W = ASD^{-2}/3\pi \, . \tag{20}$$

Figure 3. A: Within the model, the membrane patch is composed of inclusions with certain principal curvatures $C_1 = 1/R_1$ and $C_2 = 1/R_2$. B: Interfaces between inclusions are subjected to inter-surface interactions. The inclusion is viewed as composed of molecules. Each inclusion interacts with its four nearest neighbours.

Hamaker constant for lipid bilayer composed of DPPC was estimated as $A = 6.9 \times 10^{-21}$J while for DPPE it was 7.5×10^{-21}J (Kienle et al., 2014). The size of the inclusion is estimated by the size of the lipid raft (20-200 nm) (Pile, 2008). The distance between the inclusions is estimated by the void between the phospholipid tails. Roughly, the C-C bond attains length below $d = 0.2$ nm (Ishigaki et al., 2018), so the distance D was estimated to be smaller than 0.2 nm.

The estimated direct interaction between inclusions in the lipid bilayer membrane is calculated by using Eq.(20) subject to the above data ($A = 7 \times 10^{-21}$J, $S = 400$ nm^2 (for lower limit of the rafts of the size 20 nm) and $D = 0.2$ nm),

$$W \approx 7500 \times 10^{-21} \text{J} \tag{21}$$

which is at $T = 300$ K

$$W \approx 1800 \, kT \quad . \tag{22}$$

The effect of the van der Waals interactions on the membrane shape was previously estimated by considering the phospholipid molecules as inclusions (Kralj-Iglič et al., 2006). It was assumed that the molecules were distributed in a quadratic lattice. The nearest tails of the neighbouring molecules were described as cylinders were taken into account. The estimated energy of the interaction was $W \approx 1 \, kT$ (Kralj-Iglič et al., 2006). Although already the estimation by phospholipid molecules showed that the direct interactions contributed noticeable to the orientational ordering (Kralj-Iglič et al., 2006), it follows from the above that describing the membrane as composed of inclusions with intrinsic principal curvatures gives the effect of the direct interactions that may be 3 orders of magnitude higher. These estimations suggest a possibility of the major contribution of the van der Waals forces within the hydrocarbon region of the membrane to the membrane free energy. This is in accordance with estimation of energy contributions of dipole-dipole interaction of the headgroups by

Garcia et al. (2019) who found the dipole-dipole energy of around 0.15 kJ mol⁻¹ while the experimentally determined total enthalpy change for a gel-to-liquid-crystalline phase transition of dimyristoyl phosphatidyl choline of 23–29 kJ mol⁻¹ suggesting that dipole–dipole interactions between phosphatidyl choline dipoles of the head group in the plane of the membrane are likely to play only minor role in the energetics of the gel-to-liquid crystal phase transition (Garcia et al., 2019).

The term "membrane inclusion" was previously used to describe large molecules embedded in the lipid bilayer membranes (Marcelja, 1976; Owicki & McConnell, 1979; Aranda-Espinoza et al., 1996). Here, the complexes of molecules that compose the membrane patch are taken as the inclusions. Such model was developed based on previous theoretical results considering statistical physical description of membrane (Kralj-Iglič et al., 1996; Kralj-Iglič et al., 1999) and of the electric double layer (Kralj-Iglič & Iglič, 1996).

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

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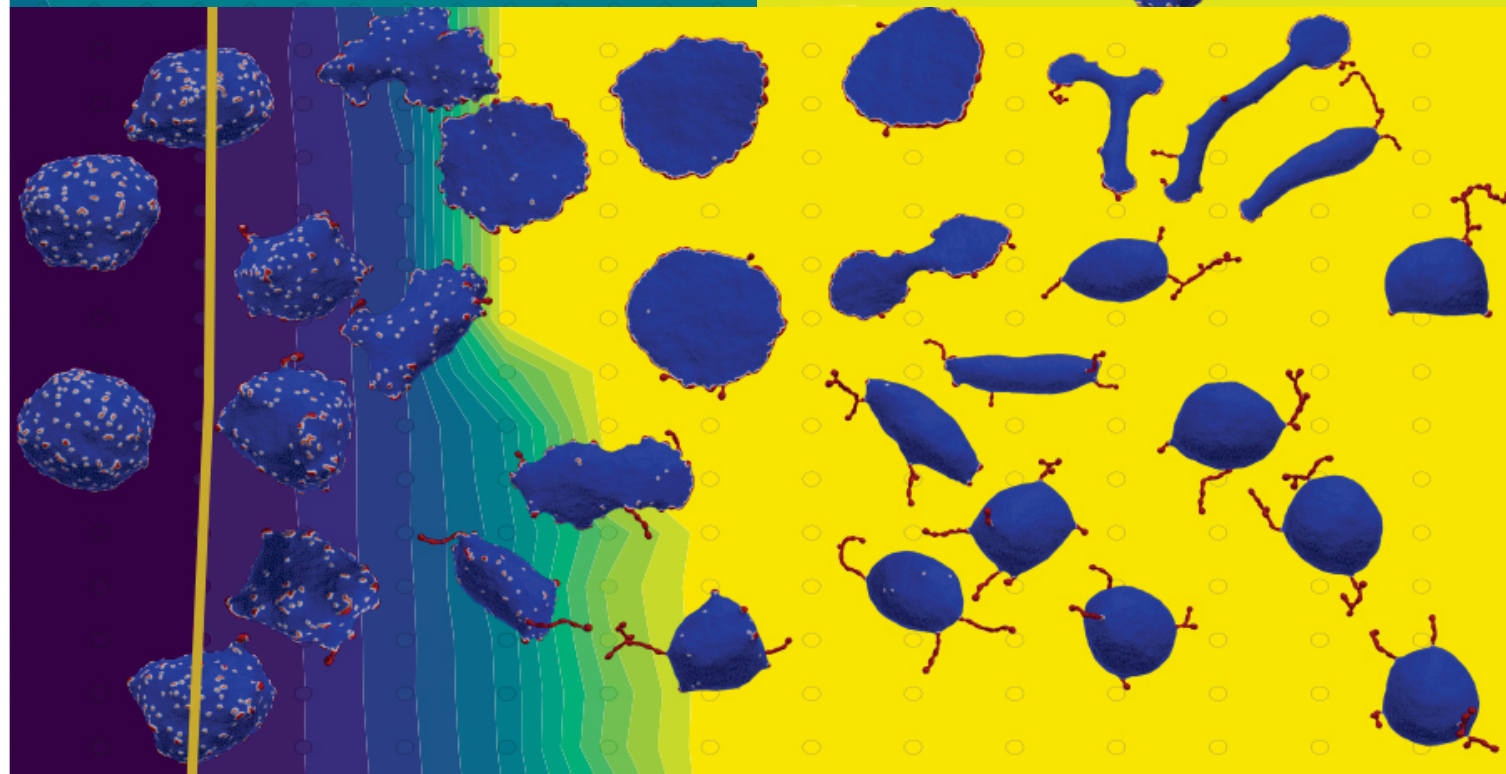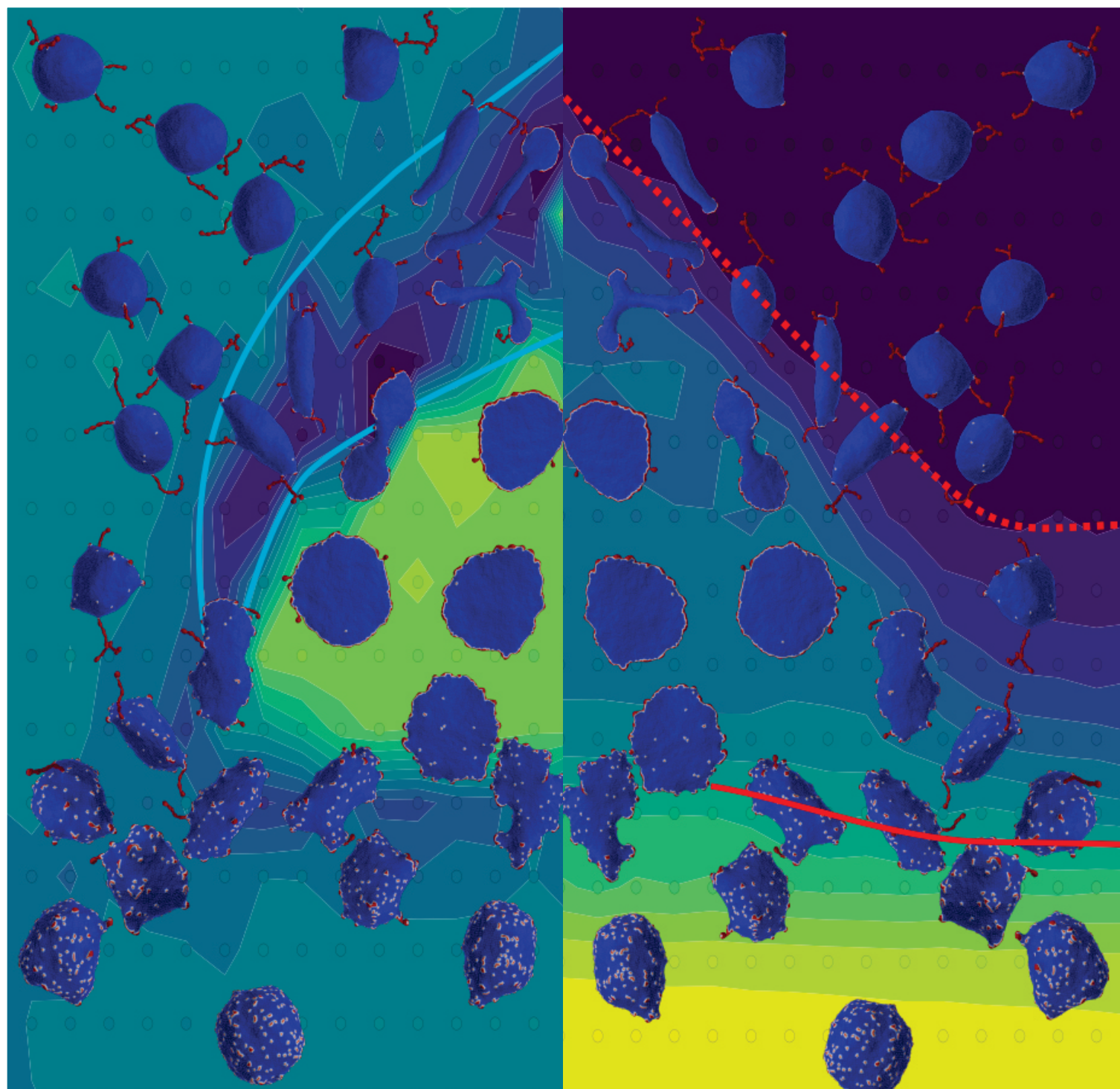
Research

Titanium Dioxide Substrates as Sensors for Detection of Platelets and Extracellular Particles from Blood Plasma

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Abstract:

Biosensors are pivotal in biomedical applications, particularly for disease detection, diagnosis, treatment, health management, and monitoring. Titanium dioxide (TiO_2) is a prominent material for biosensors due to its biocompatibility, corrosion resistance, and availability in various nanostructured forms. This study explores the interaction of platelets and extracellular vesicles (EVs) with different TiO_2 surface morphologies using flow cytometry (FCM) and scanning electron microscopy (SEM). Blood plasma samples were incubated with various TiO_2 surfaces to evaluate particle counts and characteristics. FCM results indicated a higher abundance of platelets compared to EVs, with significant fragmentation observed post-centrifugation. SEM analysis confirmed platelet activation and fragmentation, with the microflowered TiO_2 surface displaying fewer vesicles due to its rough topography. The findings suggest that while TiO_2 surface structuring minimally impacts particle counts, it influences platelet and EV interactions, highlighting the need for advanced detection techniques and further investigation into surface interactions.

1. Introduction

Biosensors play a crucial role in the biomedical field, contributing to disease detection, treatment, diagnosis, health management, and monitoring. Biosensors hold a significant position in biomedical field since they provide early stage diagnostics of diseases, targeted drug delivery to diseased cells and nanorobots which are basically miniaturized biomedical devices. Titanium dioxide (TiO_2) is the most widely used metal oxide nanomaterial for biosensors, due to its advantageous properties such as bio inertness and resistance to corrosion in body fluids, making it suitable for various biological applications. The advancement of biosensors using nanomaterials has significantly facilitated the assessment of abnormalities in body fluids like blood, saliva, urine, and serum. These biosensors are particularly effective in detecting biomarkers, such as cells or mutated DNA, associated with tumorous or pre-tumorous tissues (Turner, 2013). In the fabrication of biosensors, nanoparticles are promising candidates as they can enhance sensitivity by improving system conductivity and amplifying signals providing more active sites for biological entities because of their high surface to volume ratio (Solanki et al., 2011). TiO_2 is chosen for its nontoxicity, biocompatibility, low cost, and good conductivity. A distinguishing feature of TiO_2 from other metal oxide nanomaterials is its availability in various morphologies, such as nanoparticles, nanorods, nanotubes, and nanobelts (**Figure 1**). For constructing a biosensor, a crucial step is a biomarker for specific condition or disease to detect these species from biofluids. This involves a microsystem where a bioreceptor (comprising of nucleic acids, membranes, enzymes, antibodies, extracellular vesicles (EVs), etc.) is connected to a transducing element. This transducer (which can be optical, electrochemical, magnetic, piezoelectric, micromechanical, or thermometric) produces, intercepts, and converts signals after the analyte attaches to the biosensor (Mavrič et al., 2018; Mittal et al., 2017). Biosensors are efficient in differentiating specific analytes, even at lower concentrations, offering easier data collection and interpretation for various applications.
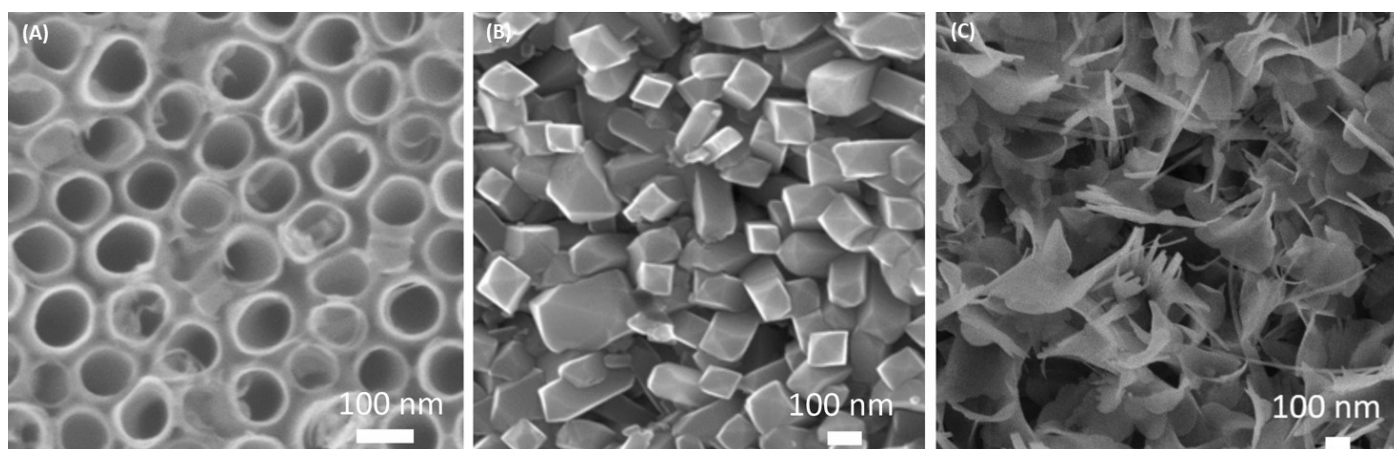
Figure 1. TiO_2 nano-structured surfaces a) nanotubes, b) nanocubes and c) nanoflakes.

Nanomaterials exhibit distinct physicochemical properties (such as solubility vapour, boiling point, pressure and reactivity) compared to their bulk counterparts, influenced by their high surface-to-volume ratio and quantum confinement effect (QCE). Their large surface energy, arising from more exposed atoms, makes nanomaterials particularly suitable for sensing technology. Since most structures, molecules and biological processes of human system occur at nanoscale ranges (eg. DNA has approximately 2 nm diameter) therefore nanomaterials are preferred in sensing technology. The absence of effective measures to control the coronavirus resulted in worldwide lockdowns, significantly impacting the global economy and leading to a standstill. Metal oxide nanostructures are used in various biosensor contexts, extending beyond the scope of the novel coronavirus disease (Covid-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). For this purpose, Vadlamani et al. (2020) developed an economical yet highly sensitive electrochemical sensor using cobalt-functionalized TiO_2 nanotubes (Co-TNTs) for the rapid detection of SARS-CoV-2. This sensor operates by sensing the spike protein, specifically the

receptor binding domain (RBD) of the virus. The synthesis of TNTs involved a conventional one-step anodization (Junkar et al., 2016; Kulkarni et al., 2015) setup, followed by cobalt functionalization using the incipient wetting method. The entire system was connected to a potentiostat for data collection. Remarkably, even at low concentrations ranging from 14 to 100 nanomolar (nM), the sensor successfully detected the S-RBD protein of SARS-CoV-2. Furthermore, the sensor exhibited a linear response in the detection of the viral protein at various concentrations. This underscores the effectiveness of the system in rapidly detecting SARS-CoV-2 S-RBD protein in approximately 30 seconds, making it suitable for point-of-care diagnostics using nasal secretions and saliva samples.

In the early stages of cancer detection using biosensors, the analysis of biomarkers in urine, blood, and body fluids is essential for oncology, aiding in cancer diagnosis and prognosis (Golubnitschaja and Flammer, 2007; Strimbu and Tavel, 2010). Cancer biomarkers are essentially proteins overexpressed in body fluids like blood or serum, originating from cancerous cells. Detecting these biomarkers during the initial phase of cancer is challenging due to their low concentrations (Wulfkuhle et al., 2003). For this purpose, extracellular vesicles (EVs) serve as ideal cancer biomarkers, offering a minimally invasive diagnostic method with added advantages for biosensors (Kralj-Iglič, 2015). EVs have diverse applications, including the diagnosis of bodily abnormalities and targeted drug delivery. TiO_2 nanoparticles have been investigated for a smart pH-responsive drug delivery system, where TiO_2 nanoparticles loaded with daunorubicin form a daunorubicin/ TiO_2 nanocomposite for the treatment of solid tumors and hematological malignancies (H. Zhang, Wang, Chen, & Wang, 2012). TiO_2's drug-eluting mechanism has also been applied in coronary stents (Junkar et al., 2016a) and orthopedic implants (Popat et al.,2007). Nanotubes, with their favorable transport pathways, enhanced substrate adhesion, and high surface area, present several advantages over different nanomaterial morphologies, making them well-suited for biosensing applications (Xiao et al., 2011). A sophisticated TiO_2 system, such as the TiO_2–NTs/CdS:Mn/CdTe sensor, has been investigated, with nanotubes acting as the base. This sensor is applicable for detecting MMP-2, whose overexpression is associated with various cancers and serves as the basis for biomarker-based expression techniques like ELISA, RIA, and IHC. The TiO_2-based system demonstrates definite electron transfer and inhibition of charge recombination, associated with a cascade effect of charge carriers. In this system, SiO2 coated with MMP-2 antibodies was employed for signal amplification, resulting in a low detection limit of 3.6 fg/mL for MMP-2 detection (Fan et al., 2014).

The utilization of TiO_2 in sensors for the detection of platelets and extracellular particles from blood plasma represents a promising avenue in biomedical research and diagnostics. In the context of blood plasma analysis, the detection of platelets and extracellular particles is crucial for understanding various physiological and pathological conditions. Platelets play a key role in hemostasis, wound healing, and immune response, while extracellular particles, such as extracellular vesicles (EVs) (Božič et al., 2022; Romolo et al., 2022), carry valuable information about cellular activities and can serve as biomarkers for diseases. Liquid biopsies (Martín-Gracia et al., 2020) represent a highly promising alternative to traditional tissue biopsies for the detection of cancer, monitoring tumor progression, and tracking tumor evolution (Castro-Giner et al., 2018). Recently, tumor-derived extracellular vesicles (EVs) have emerged as an alternative source of biomarkers in liquid biopsies. Despite initially being considered cellular waste, it is now understood that EVs play a significant role in intercellular communication and are involved in various normal and pathological processes, including cancer (Cordonnier et al., 2017). The cargoes carried by EVs depend on their parent cells, making them promising prognostic elements (Zhang et al., 2019). However, the clinical translation of EVs has been hindered by the use of complex and time-consuming traditional methods for isolation and analysis (Dong et al., 2019). Additionally, the high heterogeneity of EV isolates, containing a mix of EVs from different origins, sizes, and cargo contents, poses a challenge for their characterization (Li et al., 2019; Lim et al., 2020). In this context, there is a need for new analytical platforms that can conduct high-throughput analyses in an easy and sensitive manner without requiring extensive sample pre-treatment. Ideally, point-of-care (POC) biosensors would enable the sensitive, selective, and rapid detection of EVs while being user-friendly and cost-effective. Although significant efforts have been invested in developing novel biosensors for EV

analysis based on microfluidics, nanomaterials, or plasmonics, the majority of these platforms are still at the proof-of-concept stage and have not yet entered the market. Therefore, extensive research on TiO_2-based sensors for the detection of platelets and extracellular particles from blood plasma needs to be done, as it would offer a sensitive, specific, and biocompatible platform for understanding and monitoring various physiological and pathological conditions.

2. Material and Methods

2.1. Blood sampling

Blood was donated by an author (a female with no record of disease). 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 was centrifuged at 300g and 18°C for 10 minutes. Supernatant (taken cca 5 mm above the haematocrit boundary) was diluted with phosphate and citrate buffered saline in proportion 1 to 8 to obtain plasma samples.

2.2. Preparation of microflowered TiO_2 substrates

Titanium foils, which were 0.10 mm thick and had a purity exceeding 99.6%, were utilized to manufacture nanostructures. Pre-anodization, the foils underwent cleaning via successive ultrasonication in acetone, ethanol, and deionized water for 5 minutes each, followed by drying in a nitrogen stream. Electrolytes consisting of ultra-pure deionized water, sodium chloride (NaCl) and ethylene glycol were used. The anodization experiments were conducted at room temperature under 60 volts for 1 hour, employing an atmospheric-pressure plasma jet (APPJ) as the cathode. Our custom-built APPJ setup comprised a tubular capillary (acting as a discharge microreactor) crafted from an insulating material like glass or quartz, several electrodes (one grounded and the other supplying power to the discharge), an RF step-up transformer, and an RF (375 kHz) power source. The grounded electrode was structured as a ring surrounding the glass tube near the gas outlet, while the powered electrode (a slender, lengthy metallic tube) was situated within the glass tube. A gas supply mechanism (comprising bottles and mass flow controllers) regulated the desired flow rates (up to 1000 sccm for helium or argon) within the discharge zone.

2.3. Preparation of plasma treated TiO_2 substrates

The untreated Ti foil underwent oxygen plasma treatment within a plasma reactor. The plasma was generated using an inductively coupled RF generator running at 13.56 MHz and delivering around 800 W of power. Oxygen from a commercial source was introduced into the discharge chamber, maintaining a constant pressure of 50 Pa, which allowed for the highest level of gaseous molecule dissociation as indicated by catalytic probes. The samples, positioned on a glass holder, underwent treatment for 10 seconds.

2.4. Incubation of plasma

Plasma samples were incubated in Eppendorf tubes alone or with an added mica plate at room temperature on Orbital shaker for 1 hour.

2.5. Interaction of plasma with titanium dioxide plates

Plates (plate size: approx. 5×5mm) were inserted into eppendorf tubes (1.5mL, polypropylene), 460 µL of the sample was added and incubated at room temperature on a mixer (orbital shaker, 250-300/min) for 1h. 20 µL was taken for FCM analysis. Then, the samples were centrifuged for 20 min at 5000g and 4°C in the Centric 200R centrifuge with Lilliput rotor (Domel, Železniki, Slovenia). The supernatant (430 µL) and the pellet (30 µL) were pipetted off and the plate was prepared for scanning electron microscopy (SEM).

2.6. Flow cytometry of samples

The particle numbers in samples from plasma were estimated by flow cytometry using a MACSQuant Analyzer flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) and the related software. The following instrument settings were employed: CV settings: 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). Samples were mixed by pipetting before measurement, and 20,000 events per well were acquired. Data were analyzed using Aurora software or FlowJo software (BD Biosciences, Franklin Lakes, NJ, USA). The gates defining regions in the scatterplots pertaining to the populations of particles (P2*, and Pa*) were set in a preliminary study on the basis of experience with previously analyzed several blood samples (Božič et al., 2022). The P2* gate pertains mostly to platelets and the Pa* gate pertains to sub-cellular sized particles. The regions in the smaller scale settings were set by the shape of the event clouds.

2.7. Scanning Electron Microscopy

The samples were fixed with OsO_4 as adapted from [48]. Samples were imaged on titanium/mica plates. The plates were incubated in 39.3 mM double distilled water solution of OsO_4 for 2 h. Then they were washed 3 times with distilled water (10 min each), dehydrated in graded series of ethanol (30%, 50%, 70%, 80%, 90%) and absolute ethanol, each step 10 min. Absolute ethanol was replaced twice. Then they were washed in hexamethyldisilazane (mixed with absolute ethanol; 30% and 50%) and in absolute hexamethyldisilazane, each step 10 min. The samples were left to dry in air overnight. For examination under JSM-6500F Field Emission Scanning Electron Microscope (JEOL Ltd., Tokyo, Japan), the samples were sputtered with Au/Pd (PECS Gatan 682).

3. Results and Discussion

Table 1 shows flow cytometric count (Jeran et al., 2023) of P2* and Pa* particles in the samples. The P2* particles were considerably more numerous than the Pa* particles, in particular in the samples that underwent incubation and in the pellet after centrifugation. Comparing the incubated samples with the centrifuged ones it can be seen that centrifugation depleted the samples of P2* particles while some increase of the Pa* particles was observed in the pellet after centrifugation. This can be interpreted by destruction of platelets during centrifugation resulting in fragments that were then detected within the Pa* gate. However, the majority of EVs is expected to be smaller than the threshold limit of the FCM and were therefore not detected. It can be seen that the surface structuring of TiO_2 did not have a notable effect on the count of both types of particles, however, mica seems to have destructed platelets more effectively than TiO_2.

Table1. Flow cytometry count of P2* and Pa* particles in diluted plasma (PVRP) samples treated by incubation or centrifugation.

	Incubation		Supernatant after centrifugation		Pellet after centrifugation	
	P2* $(10^6/mL)$	Pa* $(10^6/mL)$	P2* $(10^6/mL)$	Pa* $(10^6/mL)$	P2* $(10^6/mL)$	Pa* $(10^6/mL)$
PRP	52.99	0.92	0.18	0.06	32.70	1.44
PRP+EV	54.96	1.05	0.23	0.55	31.79	1.52
PRP on Ti foil	53.71	0.96	0.23	0.05	34.24	1.56
PRP+EV on Ti foil	55.30	1.06	0.51	0.48	29.84	1.48
PRP on Flowers_TiO_2	52.91	1.04	0.35	0.07	33.50	1.47
PRP+EV on Flowers_TiO_2	57.63	1.28	0.25	0.53	34.45	1.57
PRP on P_TiO_2	52.99	0.91	0.28	0.06	30.43	1.43
PRP+EV on P_TiO_2	55.15	1.08	0.50	0.50	31.97	1.51

P2* corresponds mainly to platelets, in case of fragmentation of erythrocytes and leukocytes also larger microvesicles; the Pa* population contains EVs, lipoproteins, and other submicron particles.

Based on the results of the analysis of blind samples (without foil), there was no apparent binding of particles to any of the surfaces. In all samples, after centrifugation, we measured a total of more particles in the P2* regions than in the blind sample, except in the case of mica, where the result of this measurement was unreliable due to plate fragmentation and removal of part of the pellet with it. According to the results of flow cytometry (FCM) after incubation (with mixing), it appears that 5-30% additional EVs were formed in samples with microstructured Ti and mica, while untreated and nanostructured were not significantly different from the blind. After centrifugation, generally in the supernatants of the test samples (with Ti plates), there were more platelets (on average +70%) and fewer EVs (on average -7%) than in the blind sample. Differences between pellets were minimal, <10%, and trends in comparing PRP and PRP+EV were often inconsistent (reversed), making it very difficult to draw any conclusions. So, this time we rely entirely on SEM analysis; because we detect EVs less reliably with flow cytometry, it will be interesting to see if they have bound better to any surfaces. Several factors could contribute to the "poor" binding in this experiment - the high position of the foil in the microcentrifuge, higher dilution with buffer (impact on interactions).

Figure 2 shows samples containing platelets and EVs on untreated Ti foil (Ti foil) as imaged with SEM. It can be seen that the platelets (indicated by black arrows in Figure 2B and 2D) are highly activated exhibiting tubular protrusions. It can therefore be expected that the shed fragments and remnants of the cells contribute to the Pa* count by FCM. In Figures 2A and 2B numerous dendritic and extended platelets along with numerous vesicles can be observed whereas in case of Ti_PRP+EV only occasional vesicles were observed.
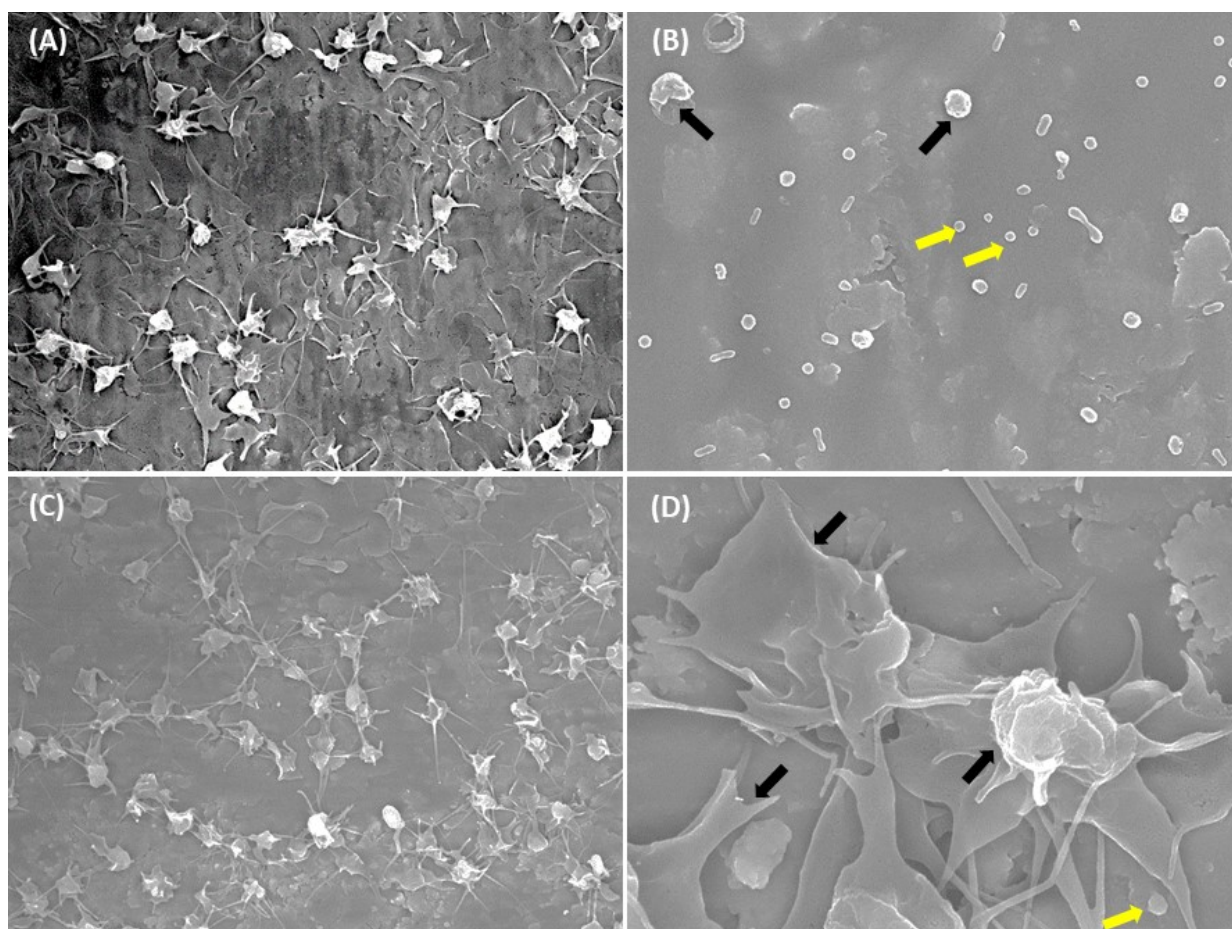

Figure 2. SEM of platelets and extracellular particles on A: untreated titanium foil (Ti foil), B: Ti foil+ PRP; C,D: Ti foil +PRP+EV. Yellow arrows indicate extracellular particles and black arrows indicate platelets at 3000 and 20000 magnifications respectively.

Figure 3 shows samples containing platelets and extracellular particles on microflowered Ti foil (Flowers_TiO$_2$) as imaged with SEM. It can be seen that only individual dendritic platelets existed (indicated by black arrows in Figure 3B and 3D). Furthermore, it can was difficult to observe vesicles on the surface. This could be due to the microstructuring of the surface due to roughened surface topography.
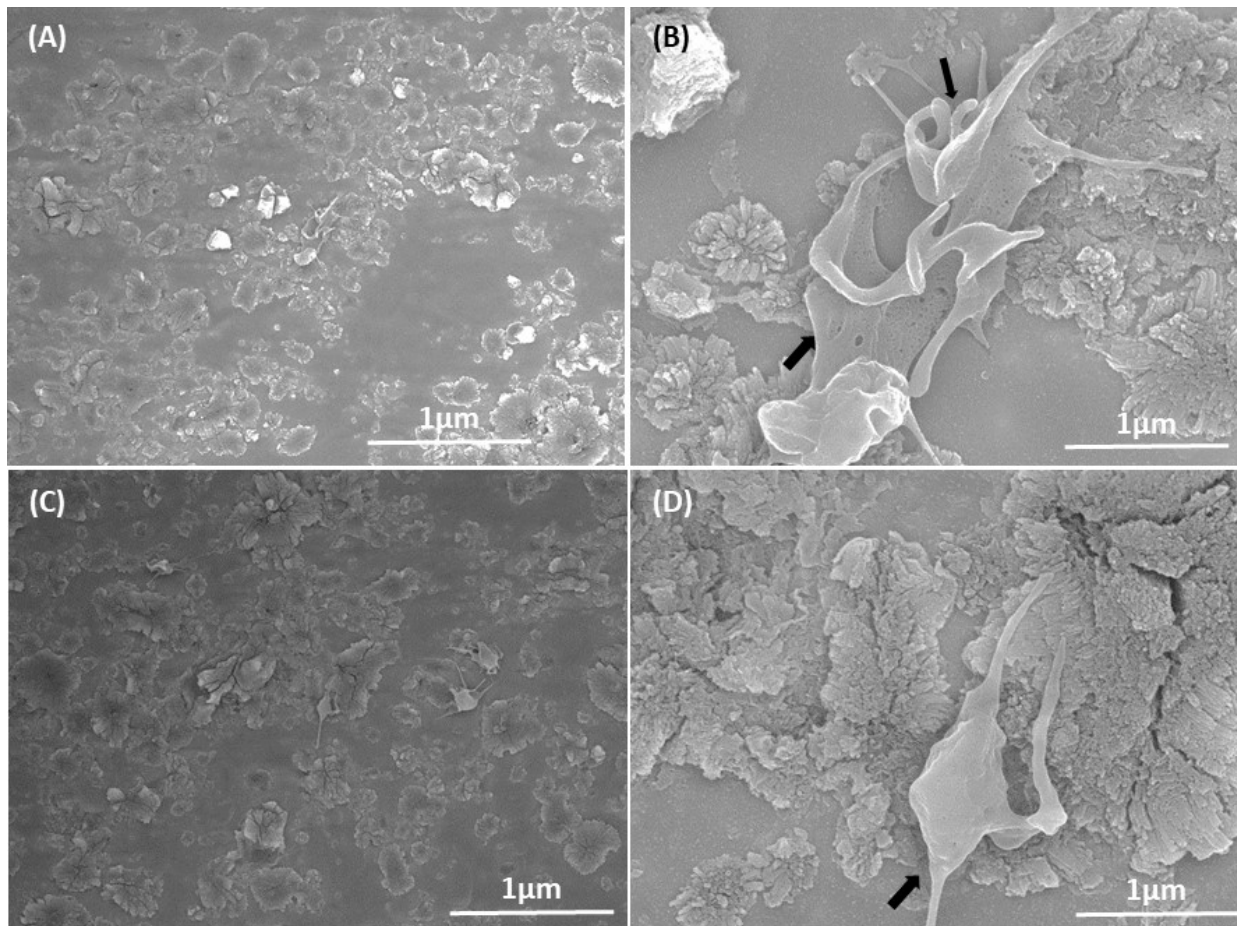
Figure 3. SEM of platelets and EVs on microflowered titanium surface (flowers_TiO$_2$): A, B: Flowers_TiO$_2$+ PRP; C,D: Flowers_TiO$_2$+PRP+EV. Yellow arrows indicate EVs and black arrows indicate platelets at 3000 and 20000 magnifications respectively.

Figures 4 and **5** show samples containing platelets and EVs on plasma treated titanium surface (P_TiO$_2$) as imaged with SEM. It can be seen that the platelets (indicated by black arrows in Figure 4B and 4D) are highly activated exhibiting tubular protrusions. In Figure 4A and 4B numerous dendritic and extended platelets along with occasional vesicles can be ob-served whereas in case of P_TiO$_2$_PRP+EV no dendritic platelets were observed.
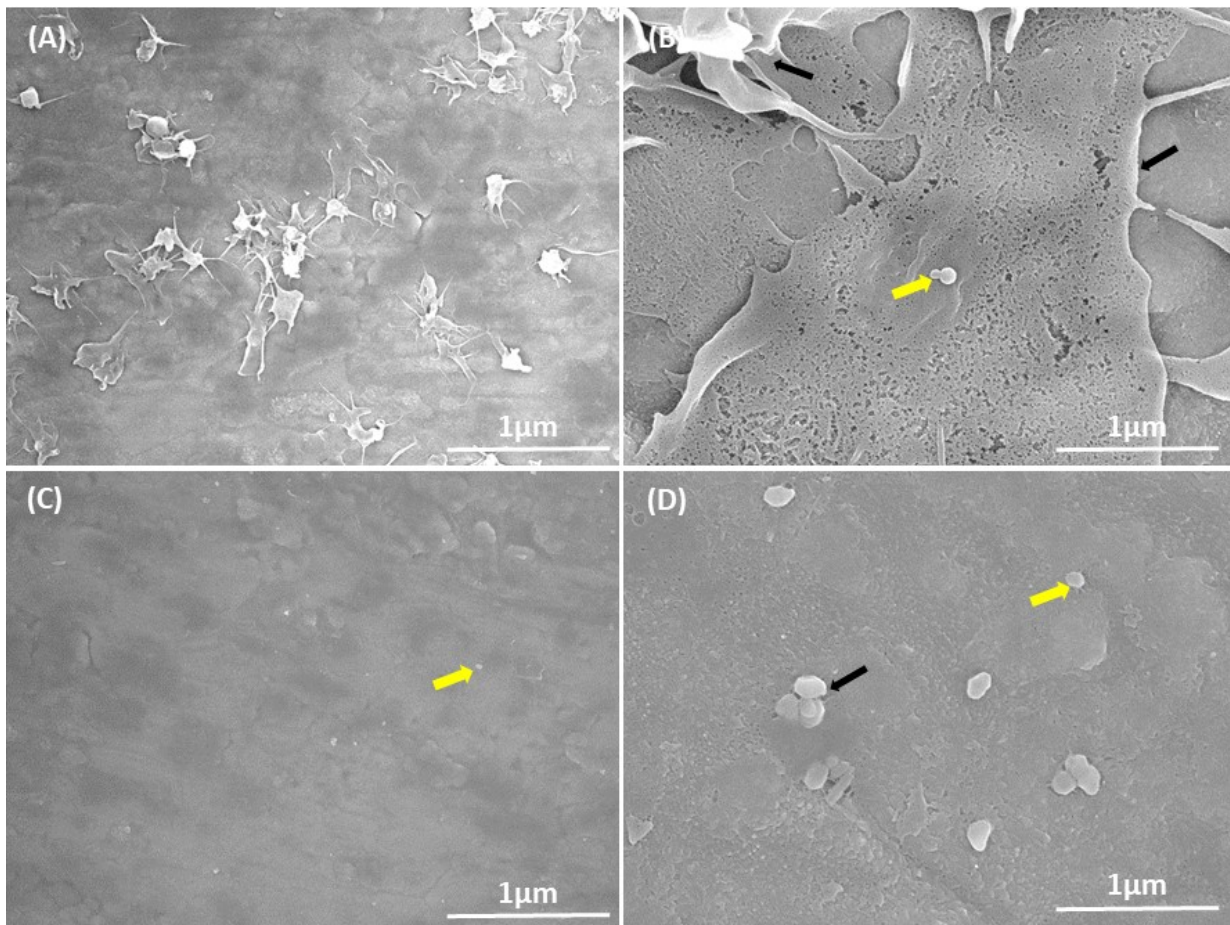
Figure 4. SEM of platelets and EVs on plasma treated titanium surface (P_TiO₂): A, B: P_TiO₂+ PRP; C,D: P_TiO₂+PRP+EV. Yellow arrows indicate extracellular particles and black arrows indicate platelets at 3000 and 20000 magnifications respectively.
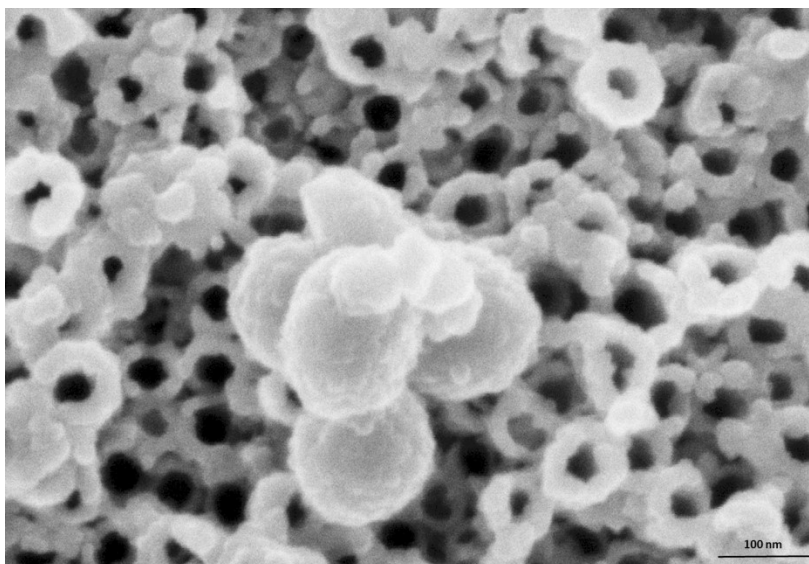

Figure 5. Scanning electron micrograph of EVs on TiO₂ nanotubes.

4. Conclusion

In present study we investigated the effect of different titanium surface treatments on the count and characteristics of platelets and EVs using FCM and SEM. FCM results revealed that P2* particles (predominantly platelets) were significantly more abundant than Pa* particles (primarily EVs) across all samples, particularly after incubation and in the pellet post-centrifugation. Centrifugation reduced P2* particles and slightly increased Pa* particles, indicating potential platelet fragmentation. SEM analysis corroborated these findings, showing high platelet activation and fragmentation across various Ti surfaces. However, the microflowered TiO_2 surface showed fewer vesicles, possibly due to its rougher topography. The study concluded that surface structuring of TiO_2 did not significantly affect particle counts, though mica effectively fragmented platelets. Overall, the SEM analysis provided more reliable detection of EVs, highlighting the need for further investigation into surface interactions and the limitations of FCM in this context.

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

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Review

Demand Aggregation and Joint Purchasing of Natural Gas in the European Union: Analysis of the AggregateEU Mechanism

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Abstract:
Following the energy crisis in 2022, EU adopted the REPowerEU Plan proposing several actions to mitigate its consequences. One of the proposed actions was the introduction of demand aggregation and joint purchasing of natural gas, known under the name AggregateEU. This paper describes the AggregateEU mechanism and analyses its framework and implementation from the perspective of economic efficiency. It compares the mechanism with the economic model of buyers' groups and monopsony, identifying potential benefits and costs of such a market structure. The paper shows that the original idea of joint purchasing was not fully implemented which reduces the overall efficiency of the AggregateEU mechanism.

Keywords: Demand aggregation, Joint purchasing, AggregateEU, REPowerEU, Natural gas, Monopsony

1. Introduction

1.1. Origins of the crisis

The outbreak of the Russian-Ukrainian war in 2022 had significant economic consequences across many sectors and the energy sector was no exception. Energy prices, in particular for natural gas, rose significantly over a short period of time and Europe was hit by an energy crisis of major proportions, affecting both businesses and households. This situation required a national response as well as a coordinated response from the European Union (later denoted as "EU") (Statista, 2023).

The year 2022 was marked by significant volatility in the global natural gas market. Several key factors converged to create a crisis-like situation, particularly impacting Europe but with worldwide repercussions (IEA, 2022).

The natural gas market crisis in 2022 was largely precipitated by geopolitical tensions and infrastructural limitations. The invasion of Ukraine by Russia in February significantly exacerbated an already fragile situation. This geopolitical event disrupted Russian gas supplies to Europe, which had been a major source of natural gas for the region (IEA, 2022). Prior to the conflict, Europe had been attempting to fill gas storages, but these efforts were hampered by Russia's strategic withholding of gas supplies. This resulted in soaring prices, with the Title Transfer Facility (TTF) benchmark in Europe peaking at over USD 90 per million British thermal units (MBtu) during the year (IEA, 2022).

Additionally, a lack of new gas project investments, weather-driven demand increases, and LNG outages tightened the global supply, further driving up prices (IEA, 2022).

1.2. Response to the crisis

In response to the immediate crisis and high prices, Europe and other regions intensified their discussions on energy policy and market reforms. This included efforts to better manage gas supplies and protect consumers from price volatility. European Union and its member states debated ways to decrease reliance on Russian gas, focusing on diversifying their energy sources (IEA, 2022).

Simultaneously, several major policy initiatives were launched globally to promote a shift towards cleaner energy. The United States introduced the Inflation Reduction Act, the European Union pushed forward with its Fit for 55 package, focusing on reducing greenhouse gas emissions by 55% by 2030, Australia enacted the Climate Change Bill to legally bind emission reduction targets, Japan unveiled the GX Green Transformation plan, focusing on accelerating its green energy transition (IEA, 2022).

The crisis showed the inadequacies of this long-term strategies that failed to address immediate and critical energy supply disruptions and reactive measures that could quickly mitigate the impact of such crises became essential (IEA, 2022).

In response, the European Union introduced RePowerEU Plan. It was specifically designed to decrease the EU's dependence on Russian natural gas swiftly and effectively. This approach prioritized rapid response over long-term planning alone. In essence, while long-term energy transition strategies are vital, they must be complemented by flexible, responsive measures that can address sudden disruptions and safeguard energy security in real-time (IEA, 2022).

The European Commission (later denoted as "Commission") proposed several measures to combat the energy crisis. In this paper, we present one of these measures – an instrument of demand aggregation and joint purchasing of natural gas, also known as the AggregateEU mechanism. It was introduced at the end of 2022 and implemented in the form of the first tender in the beginning of 2023. Although designed as a temporary instrument, its validity was extended and is thus still in force today. In the paper, we present the legal framework of the instrument and analyse it from the perspective of economic efficiency.

The paper is structured as follows. Section 2 provides a general overview of the EU response to the energy crisis and section 3 describes the AggregateEU mechanism into detail. Section 4 analyses the economic effects of demand aggregation and joint purchasing in general, while section 5 applies these findings to the AggregateEU mechanism with the purpose of establishing whether it is economically efficient.

2. REPowerEU Plan: A response to the energy crisis

Following the Russian invasion of Ukraine, on March 8, 2022, the Commission published a communication entitled REPowerEU: Joint European Action for more affordable, secure and sustainable energy. Its purpose was twofold. The first part of the communication was aimed at addressing the emergency of the energy crisis, particularly mitigating high energy prices. The goal of the second part was more strategic and argued for eliminating EU's dependence on Russian fossil fuels through gas supply diversification and renewable energy transition (*Communication 1*, 2022).

The Commission further expanded on these ideas in its next communication. On May 18, 2022, the REPowerEU Plan was published and highlighted, as main drivers of action, high energy prices, energy security concerns and high amounts paid to Russia for energy imports. The plan put forward a set of actions that can be divided into four segments: (i) energy saving, (ii) diversifying energy imports, (iii) substituting fossil fuels by accelerating EU's clean energy transition and (iv) smart investment (*Communication 2*, 2022).

Energy saving was proposed to be achieved through further increases in existing energy efficiency targets. Similarly, the REPowerEU Plan proposed to increase the target share of energy from renewable resources. The new binding target by 2030 is currently 42.5 %. Additionally, the Commission promised to enhance the regulatory framework to enhance solar, wind and heat pump technologies. Diversifying energy imports was proposed to be implemented through setting up of an EU Energy Platform, which was implemented as the AggregateEU mechanism. All these measures are complemented by smart investment. The Commission estimated that the implementation of REPowerEU Plan would require additional 210 billion Eur of investment until 2027. This is in addition to the substantial investments needed to implement the 2019 European Green Deal (*Communication 2*, 2022; European Commission, n. d.-*a*).

3. REPowerEU Plan: A response to the energy crisis

In the segment of diversifying energy imports, the REPowerEU Plan proposed the creation of an EU Energy Platform, which would introduce "demand aggregation and structuring" and a "joint purchasing mechanism" (*Communication 2*, 2022). Under the name of AggregateEU, this idea became one of the first achievements of the REPowerEU Plan to be implemented (Marin, 2023).

Upon a proposal from the Commission, the Council of the EU adopted the Council *Regulation (EU) 2022/2576* of 19 December 2022 enhancing solidarity through better coordination of gas purchases, reliable price benchmarks and exchanges of gas across borders (later denoted as "Regulation"). The Regulation covered, in section II of chapter II, demand aggregation and joint purchasing.

The process has essentially four phases, which are illustrated schematically in **Figure 1** (*Report*, 2023).
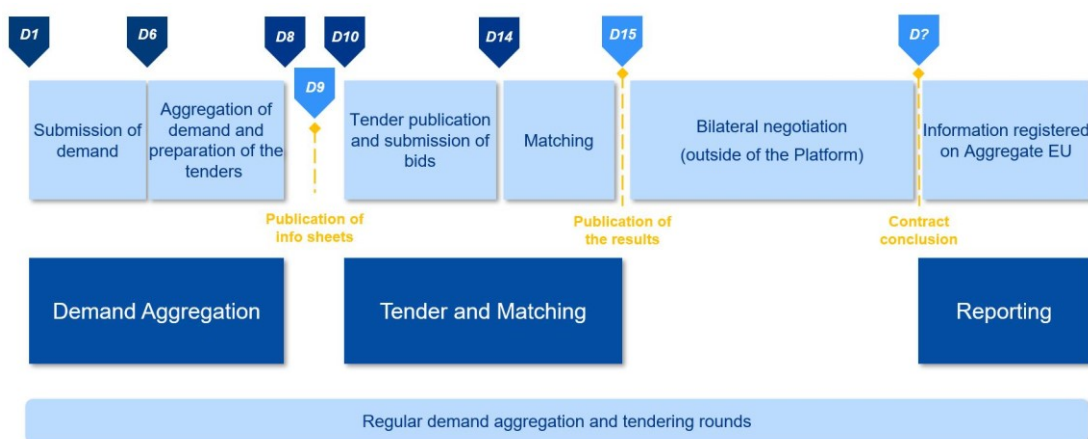

Figure 1. AggregateEU mechanism design (*Report*, 2023).

The first phase is demand aggregation. Participation is open to all natural gas undertakings and undertakings consuming gas established in the EU and Energy Community Contracting Parties that wish to purchase natural gas ("buyers"), regardless of the volume of natural gas requested. Russian undertakings are precluded from participating (Article 8 of the *Regulation*). The buyers can submit their demand through the IT system to a service provider that is authorised to organise demand aggregation and joint purchasing. All the submitted demand is aggregated by the service provider. The process is entirely voluntary and optional for undertakings. Pursuant to Article 10 of the *Regulation*, however, Member States have to require the natural gas undertakings and undertakings consuming gas to participate in demand aggregation with volumes equal to 15 % of the volumes necessary to meet the filling targets for underground gas storage facilities (*Proposal*, 2022; *Regulation*, 2022; European Commission, n. d.-*b*).

The second phase is tendering. In this phase, the gas suppliers ("sellers"), submit their bids. In each bid, the quantity that the seller is willing to sell and the price at which the seller is willing to sell gas must be indicated. Sellers must not be Russian undertakings (European Commission, n. d.-*b*).

The third phase is matching of sellers and buyers. The supply bids are ranked from the lowest to the highest price offered and then allocated to the given demand on a pro-rata basis, ensuring the lowest average price within the tender while guaranteeing equal treatment of all buyers. The pro-rata approach applies both in the case of excess supply and excess demand. After the matching is carried out, the quantities allocated are communicated to the sellers and buyers, together with general information to ensure contact between them (*Regulation*, 2022; European Commission, n. d.-*b*).

The fourth phase is contracting. As the matching of supply and demand is not binding, the sellers and buyers start negotiating outside the AggregateEU once contact between them is established. These negotiations can lead to the conclusion of a contract or not. The bid is thus only a starting point for negotiations and is not binding on the seller. Nevertheless, the sellers must act in good faith, as they can be excluded from the AggregateEU platform in case of manipulative behaviour. For the purpose of negotiation, in particular to achieve better prices and conditions, the *Regulation* allows buyers to form a gas purchasing consortium, within which they can coordinate prices, volumes, delivery times and delivery points (Article 11 of the *Regulation*). Competition law must, however, be fully complied with. In practice, the Commission has also allowed the two following forms of cooperation: the Agent model and the Central Buyer model, the difference being that an agent is a third party while a central buyer is one of the buyers that have submitted the demand (European Commission, n. d.-*b*).

Upon the adoption of the *Regulation*, the validity of the instrument was limited to one year and would thus expire on December 30, 2023 (Article 31 of the *Regulation*). In September 2023, the Commission gave its report on the functioning of the *Regulation* and found that the *Regulation* has "played an important role in contributing to stabilising the gas market and ensuring an adequate supply of gas to the EU" (*Report*, 2023). Upon the recommendation of the Commission, the Council extended the validity of the *Regulation* to December 31, 2024.

In its Report, the Commission also promised to consider whether some of the measures of the *Regulation* could be integrated in a more structured way (*Report*, 2023). This does not rule out a permanent mechanism as AggregateEU is still only temporary.

4. Economic effects of demand aggregation and joint purchasing

Cooperation between firms at the level of purchasing is considered in economic theory in two forms: (i) a buyer cartel and (ii) a buyers' (also: buying) group. A buyer cartel is an agreement between firms to restrict competition between them in any aspect of purchasing with the aim of reducing prices or otherwise influencing the supplier's behaviour. It is a coordinated approach to purchasing, but the companies do not integrate the purchasing process – each company does its own purchasing. On the other hand, firms participating in a buyers' group enter into a joint purchasing agreement (JPA) and integrate the purchasing function (Carstensen, 2010).

The distinction between the two forms is sometimes difficult but important because of the different competition law treatment. In the US, purchasing cartels constitute a per se violation of the Sherman Act, whereas buyers' groups (*i. e.* joint purchasing arrangements) are subject to the 'rule of reason' doctrine. In the EU, purchasing cartels are also prohibited, while the assessment for buyers' groups will depend in particular on market power (OECD, 2022). As demand aggregation and joint purchasing, as established by the AggregateEU mechanism, is a buyers' group, we focus only on this category.

By aggregating demand and joint purchasing, the firms in the buyers' group gain market power *vis-à-vis* the supplier. In the extreme, where all buyers in the market participate in a buyers' group, a monopsony is established – a state where there is only one buyer in the market. Buyers' groups organised for the purpose of gaining bargaining power can, as established in economic theory, be treated in the same way as a monopsonist (Chen, 2007). In the following subsections, potential benefits and costs of such a market state are explored.

4.1 Potential benefits

Groups of buyers with a joint purchasing agreement can lead to cost savings. As the supplier negotiates with the buyers' group as a single entity made up of a large number of companies, transaction costs associated with negotiating and contracting are reduced for both parties, since unnecessary duplication of tasks is eliminated (Björkroth, 2013).

Cost savings are also possible where the supplier enjoys economies of scale, meaning that its average cost declines as output increases (Besanko et al., 2017). In this case, the supplier will be able to increase the volume of production as a result of the order from a group of (a large number of) customers, thereby reducing its average costs (OECD, 2022). Where part of these cost savings is passed on to the buyers' group via a lower price (*e. g.* through volume discounts), the benefits will accrue to both parties.

Since a buyers' group has a high bargaining power *vis-à-vis* the supplier compared to individual buyers, it can negotiate certain advantages, in particular a reduction in the price charged by the supplier. This is also implied by the monopsony model in **Figure 2**, in which the monopsonist lowers the price from the socially optimal level w^* to the level w^m (Chen, 2007). Such a price reduction benefits the buyers' group, while the supplier is worse off, losing part of its revenue.
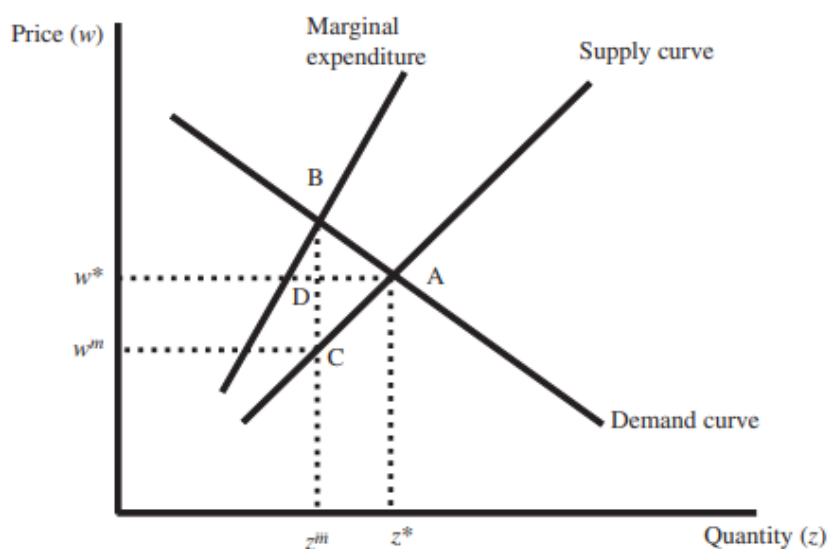

Figure 2. Monopsony model (Chen, 2007).

The question is whether the benefit achieved by the buyers' group on the upstream market in the form of a price reduction is passed on to consumers in the form of a reduction in

final prices on the downstream market. Although the literature is not entirely unanimous, the most common view is that the benefits of a price reduction can be passed on to consumers when there is a sufficient degree of competition in the downstream market (Colen et al., 2020; OECD, 2022). Particularly where there is a monopoly in the upstream market, the buyers' group exhibits a countervailing buyer power which enables it to lower prices and pass these savings (at least partly) to the consumers (Chen, 2007; Colen et al., 2020). established in economic theory, be treated in the same way as a monopsonist (Chen, 2007).

4.2 Potential costs and inefficiencies

If, based on the monopsony model in Annex 2, we could conclude that increased bargaining power allows the buyers' group to lower the price and thereby gain a benefit, the supplier incurs a loss. This is where the redistribution of wealth occurs. However, since the buyers' group gains less than the supplier loses, there is in part also an erosion of wealth. A so-called deadweight loss occurs, which leads to a reduction in economic efficiency and social welfare. The deadweight loss arises irrespective of whether the downstream market is perfectly competitive or not (Chen, 2007). In the monopsony model, the deadweight loss is represented by triangle ABC, while the redistribution of wealth from the supplier to the buyers' group occurs only in the w^*DCw^m part.

Another negative consequence of the high bargaining power of buyers' groups is the so-called waterbed effect. According to this effect, suppliers who reduce the price towards a strong buyer (*e. g.* a buyers' group) will compensate such reduction by increasing the price towards the remaining (smaller) buyers (Björkroth, 2013). Such buyers are put at a disadvantage because they are subject to two opposing forces – on the one hand, they will want to pass on higher prices from the upstream market to consumers through higher final prices in the downstream market, while on the other hand, competition with the strong buyer, who achieves lower prices in the upstream market, will force them to lower prices in the downstream market (Dobson & Inderst, 2007). If, due to these effects, they are forced to exit the market, the downstream market will become more concentrated, leading to higher prices and less consumer choice overall.

5. Is the AggregateEU mechanism under the Regulation economically efficient?

The AggregateEU mechanism introduced by the *Regulation* aggregates, in the first phase, the demands of the companies that decide to participate in the process. This phase is not (yet) joint purchasing, but it is nevertheless important because it creates a single demand. As the Commission notes in its proposal, this allows the EU to "use its collective purchasing power to negotiate better prices, reduce the risk of Member States [meaning, of course, undertakings established in Member States] outbidding each other on the already tight market and, in doing so, counter-productively driving up prices" (*Proposal*, 2022). While it is difficult to argue with these reasons, Barnes (2023) points out that such EU intervention is unnecessary, as the demand aggregation market is already effectively used in the so-called wholesale gas hubs.

The next phases are tendering and matching of buyers and sellers. Publicly available data on the tenders carried out so far show that the procedures have been relatively successful. The results are summarized in **Table 1**. For example, in the first round of short-term tenders, bids in the total amount of 18.7 bcm of gas fully met and exceeded the aggregate demand of 11.6 bcm. The recent first round of medium-term tenders was particularly successful, with bids reaching almost three times aggregate demand (European Commission, 2024). In total, more than 43 bcm of gas has been secured in five short-term tenders to meet European demand (European Commission, n. d.-*c*). For context: in 2022, EU gas consumption was 343.4 bcm (Statista, 2023), which means that the AggregateEU platform met 12.5% of Europe's annual gas demand in its first year of operation. Some caution is, however, necessary in interpreting these results since, firstly, some negotiations were perhaps unsuccessful in the next stage of the process, and secondly, the contracts can have a duration of more than 1 year, meaning that comparisons with annual data are not necessarily correct. Nevertheless, given that AggregateEU is a novelty in the European context, the results are not insignificant.

Table 1. AggregateEU tendering rounds results (*Report*, 2023; Luca, 2023; European Commission, 2024). Values are reported in billion cubic meters (bcm).

		Supply	Aggregated demand	Difference
Short-term tender rounds	First round (April 2023)	18.7	11.6	+ 7.1
	Second round (June 2023)	15.2	15.9	− 0.7
	Third round (September 2023)	18.1	16.5	+ 1.6
	Fourth round (November 2023)	9.1	10.1	− 1
Mid-term tender	First round (February 2024)	97.4	34	+ 63.4

The final phase is negotiating and concluding the contract. As Barnes (2023) notes, the AggregateEU mechanism does not even make use of the joint purchasing instrument, although this is supposed to be the core of the mechanism. Rather, it is simply a platform for matching supply and demand. It is precisely the lack of joint purchasing and the binding nature of the bids that is the source of the inefficiency of the AggregateEU mechanism. The supplier is not bound by its bid, which may lead to an increase in the gas price in the negotiations. This is, in fact, quite possible because the negotiations are conducted individually and there is therefore no real joint purchasing in which the buyers as a group would have more bargaining power. The Regulation does allow for the creation of a consortium of buyers, but it has not been used in practice (*Regulation*).

The ultimate test for AggregateEU, as Barnes puts it, is "if it leads to contracts being signed, and at lower prices than could be achieved if buyers bought gas via existing market mechanisms". Given that data on contracts concluded, volumes, durations and prices are not public, it is, however, impossible to ascertain whether the price was indeed, as predicted, lower (Barnes, 2023).

For the AggregateEU to be more efficient, the *Regulation* should integrate joint purchasing into the mechanism, since it is currently only a matching platform. In this way, benefits such as reduced transaction costs, (potential) economies of scale and, in particular, lower gas prices would be achieved. It is true that, as described, joint purchasing also has some inefficiencies, however the benefits seem to outweigh them. Redistribution of wealth from gas suppliers to buyers would mainly benefit the EU, as the EU mainly imports gas from third countries. Thus, the loss of wealth would mainly affect companies outside the EU. The waterbed effect remains problematic, but could be fully eliminated if all companies participated in the joint purchasing process. Given the openness of this system, any company could join if it saw advantages in the mechanism over purchasing gas individually.

6. Conclusion

The paper presented a comprehensive analysis of the AggregateEU, a mechanism aimed at diversifying natural gas imports by "demand aggregation and joint purchasing" of natural gas for the participating undertakings. The analysis, however, showed that the instrument as implemented does not fully follow the original idea. The first part – demand

aggregation – is functional and implemented via the service provider who aggregates the demand submitted by the buyers. The second part – joint purchasing – is, on the other hand, not implemented although declared at first to be of equal importance. After matching of supply and demand, the buyers and sellers negotiate individually for the conclusion of the contract, relativising the bargaining power of the buyers.

Although the success of AggregateEU cannot yet be precisely measured, the available data on natural gas quantities indicate that is has been successful. Nonetheless, economic implications of the so-called buyers' groups show that AggregateEU could be even more efficient if the joint purchasing part of the mechanism was fully implemented. This might be tricky due to certain competition law limitations, however it might be key in reducing energy prices which is, after all, essential for the competitiveness of EU economy.

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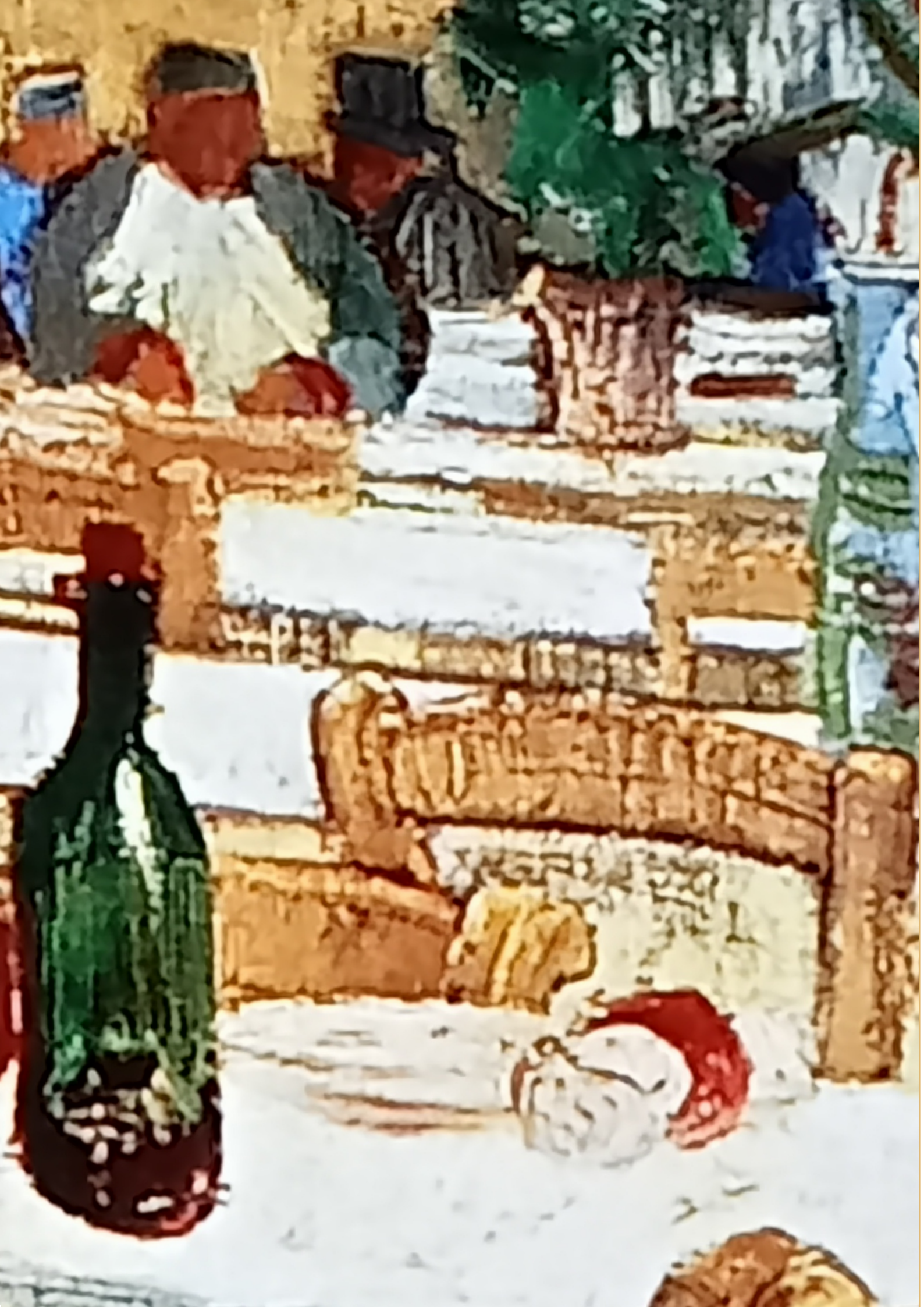
Review

Economic Analysis of the New Regulation of the European Parliament and Council on Artificial Intelligence

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Abstract:
This paper presents a comprehensive economic analysis of the new regulation on Artificial Intelligence (AI) proposed by the European Parliament and Council. The regulation aims to establish a harmonized framework for the development and deployment of AI across member states, ensuring safety, transparency, and ethical standards. We evaluate the potential economic impacts of this regulation, focusing on innovation, market competitiveness, and compliance costs for businesses. Additionally, the paper explores the concept of the "Brussels Effect," a phenomenon where European Union regulations influence global standards beyond the EU's borders. By exploring how this new AI regulation might shape international norms and practices, we assess its implications for global trade and the international AI landscape. We examine the ways in which the regulation could set benchmarks for AI governance worldwide, potentially affecting non-EU companies and markets. Through this dual examination, the paper provides insights into the economic opportunities and challenges posed by the EU's proactive regulatory approach to AI.

Keywords: The artificial intelligence act, The Brussels effect, Impact assessment, Global influence, Costs of new AI regulation

1.   **Introduction**

In this paper, the new Regulation of the European Parliament and of the Council laying down harmonised rules on Artificial Intelligence (AI) will be presented (the Artificial Intelligence Act) (Regulation of the European Parliament and of the Council laying down harmonised rules on Artificial Intelligence: *Law 1*), which represents an important step towards the legalization of this relatively new technology. The purpose of the regulation is to improve the functioning of the internal market by establishing a unified legal framework for the development, marketing, and use of artificial intelligence in accordance with the values of the Union. The decision-makers behind the Act emphasize that their goal is to establish a comprehensive legal framework for managing artificial intelligence. This framework aims to promote innovation, protect fundamental rights, and provide legal certainty for both developers and users. With the adoption of this regulation, significant changes will occur in the development and use of AI within the EU. This will impact numerous sectors and use cases, marking the first truly comprehensive legislation in the field of artificial intelligence.

2.   **Economic impacts of artificial intelligence regulation**

The development of modern advanced artificial intelligence technologies necessitates the establishment of a comprehensive regulatory framework to manage their deployment and integration across multiple sectors. This regulation is crucial as it addresses several impacts that arise from the integration of AI. This chapter explores these challenges and provides insights into the effects of regulatory measures needed in various sectors.

2.1. Impact on productivity and innovation and cost implication on businesses
AI regulatory frameworks should balance innovation with proper risk management. Research indicates that very well-calibrated regulations help offer a "safe space" for innovation that can boost productivity in all sectors (Comunale et al., 2024). High-quality regulations can lower uncertainty about standards and expectations, promoting investment in AI technologies that will lead to the much-desired productivity gains critical for economic growth and competitiveness on the international level (Comunale et al., 2024). While the potential for augmented productivity and innovation through AI is relatively high, the compliance-related costs at the outset are high for firms. The turn-around cost of acquiring new technologies, training, and compliance mechanisms that come with implementing regulations on AI is usually quite huge and a heavy burden for small- and medium-sized enterprises. However, these costs may be mitigated over time as standardized practices may lead to economies of scale and lower the price of compliance-related technologies and services (Comunale et al., 2024).

2.2. Labor market transformations
There are enormous labor market consequences, as well. Regulation involving ethics and safety considerations will determine which AI technologies can be developed and scaled in deployment and, by extension, which are developed and deployed at all: the cascading effects on the creation and displacement of jobs would be huge. For example, regulations to control any form of automation could be safeguards for jobs but could also slow the pace of productivity growth. Conversely, policies supporting AI adoption within safe and controlled environments can accelerate the displacement of routine jobs and potentially create new high-skilled positions in the governance, development, and monitoring of AI (Comunale et al., 2024).

2.3. Global market dynamics and long-term economic growth
In the long term, effects on economic growth due to the regulation of AI will be dependent on the balance reached in facilitating innovation and mitigating risks. Proper regulation may underpin sustained economic growth as it develops a conducive environment where safe and ethical development of artificial intelligence technologies can flourish (Gunst et al., 2021). It can ensure more trustworthy and integrated AI across the economy, allowing efficiency gains through a new capacity that lies beyond which the increase of growth is

driven by higher efficiency in new capabilities for different parts of GDP (Comunale et al., 2024).

In a word, the economic effects of AI regulation are profound and multifaceted. Compliance would carry an immediate cost, which over the longer term would be dwarfed by the benefits of establishing an enabling regulatory environment that is appropriate, safe, and fosters innovation. As AI technologies continue to advance, it would prove essential for countries to continually assess their economic impacts and risks alongside the evolving of the required regulation frameworks (Comunale et al., 2024).

3. The artificial intelligence act as part of the EU digital strategy

The European Parliament and Commission have adopted the AI Act as part of the EU Digital Strategy, in line with the European Commission's political priority to make Europe fit for the digital age. The AI regulation is a key step in preparing EU member states for the challenges posed by the rapid advancement of this technology. The Act is ambitiously designed to create a legislative framework on which further regulations of AI systems will be based, both at the member state level and in other EU regulatory bodies. The EU aims to lay the foundations for a truly comprehensive legal environment that will enable the further development of AI systems, striving to become a leading global player in this field (Mišič Jančar et al., 2024).

An important question regarding artificial intelligence, which must be addressed when attempting to regulate the field, is how to classify the various models of programs that fall under the term "artificial intelligence". There is no consensus on what AI encompasses, and it is evident that AI can be used in so many different ways and sectors that a uniform regulation without distinction cannot achieve the desired results (MacCarthy, 2023) Regulating this field is thus challenging, which is why the preparation for the adoption of a concrete regulation took several years and involved many different stakeholders (European Commission, 2024).

Therefore, the EU, in its impact assessment, examined various policy options with different levels of regulatory intervention, from the least intrusive to the most stringent regulation. Based on its methodology, the Commission assessed each regulatory intensity option concerning economic and social impacts. The most appropriate option was chosen based on risk identification. They opted for a horizontal legislative instrument based on a proportionate risk-based approach with additional codes of conduct for AI systems that do not pose significant risks. The AI Act thus creates a risk-based approach and introduces regulatory burdens only when an AI system is likely to pose a high risk to fundamental rights and safety. For AI that does not pose a significant risk according to the criteria set in the Regulation, only very limited obligations are prescribed (Mišič Jančar et al., 2024).

The framework established by the regulation precisely defines the spectrum from minimal to unacceptable risk and assigns different categories to AI based on the level of threat they may pose.

4. Cooperation with International stakeholders

In 2020, the European Commission published a *White Paper on Artificial Intelligence* (White Paper on Artificial Intelligence – A European approach to excellence and trust: *Law 2*) and initiated a consultation aimed at gathering opinions and views from the interested public on how AI regulation should proceed. Most participants agreed on the need for action due to legislative gaps in the field of artificial intelligence. However, they cautioned the Commission to avoid duplication, over-regulation, and conflicting obligations. A clear and precise definition of artificial intelligence and key terms was highlighted as crucial. It is important to emphasize that in regulating any industry, there are several issues that need to be approached carefully. Regulatory rules that are too lenient will not have the desired effect, while overly strict regulation will curb and reduce progress in developing innovative technological solutions, thereby limiting market competitiveness (Heller, 2023). The EU understands that increasing costs for businesses and end-users also creates obstacles for further and faster development. This is especially critical in AI regulation, as the EU does not want to fall behind other countries in AI development and instead aims

to create the best possible conditions to enhance competitiveness and the European industrial base in the field of artificial intelligence (Mišič Jančar et al., 2024).

5. The Brussels Effect

The rules of the European Union do not only impact within its borders but also have a significant influence beyond them. The concept known as "Brussels Effect" describes this process, where EU rules effectively extend beyond the Union's borders, often through market mechanisms (Bradford, 2020). This effect can manifest in two ways: the *de facto* Brussels Effect and the *de jure* Brussels Effect.

The *de facto* Brussels Effect occurs when companies voluntarily comply with EU regulations in non-EU countries, even if the local laws do not require it. Conversely, the *de jure* Brussels Effect arises when foreign jurisdictions adopt rules that are directly or indirectly influenced by EU legislation (Siegmann and Anderljung, 2022). This dynamic demonstrates the EU's expanding influence and the adoption of its standards and practices on a global scale.

The Brussels Effect results in the EU shaping and influencing the legislation of other countries that engage economically with the EU, as well as changing the practices and operating conditions of companies, especially large corporations, that operate in the EU market. Similar to the first-mover advantage in the market, there is also a comparable regulatory first-mover advantage. This term refers to the benefits gained by an entity that takes early action in forming regulatory policies or frameworks in a particular field. When a country or region becomes a pioneer in regulation, it gains an advantage in setting future rules and standards (Siegmann & Anderljung, 2022).

A notable example of this influence is the General Data Protection Regulation (GDPR), adopted in 2016. It prompted major technology companies to adjust their business practices to align with EU standards to continue operating in the EU market (Gunst et al., 2021).

If the AI Act aims to replicate the impact of the GDPR, the key factor is the EU's market power. This element is fulfilled due to the extensive and relatively affluent population and the single EU market, attracting AI providers who do not want to lose millions of users and will thus need to comply with the AI Act (Almada and Radu, 2024). Moreover, artificial intelligence is a new technology with little established regulatory practice, giving the EU an advantage in regulation and encouraging other countries to follow the EU's lead (Almada and Radu, 2024). However, market factors alone are not sufficient to predict the Brussels Effect at the level of the GDPR. It is also noted that the demand for AI programs is relatively inelastic; if it were elastic, regulation could shrink the EU AI market, and multi-nationals might be more willing to leave the single market due to increased costs. Since the AI market is generally inelastic, there is a predisposition for the Brussels Effect (Siegmann and Anderljung, 2022).

It can be concluded that both the *de facto* and *de jure* Brussels Effects are possible and likely due to the specific nature of the AI and EU markets and the relative lack of regulation in this field by other major markets. While the Brussels Effect may not be as pronounced as with the GDPR, it is still too early to draw definitive conclusions with certainty.

6. Costs of new regulation for individual companies

The EU's impact assessment of the AI Act (Impact Assessment Accompanying the Proposal for a Regulation of the European Parliament and of the Council Artificial Intelligence Act: *Law 3*) estimates that compliance with the regulatory requirements will cost approximately €6,000 to €7,000 for supplying an average high-risk AI system. AI users would also incur annual costs for the time spent ensuring human oversight, estimated at around €5,000 to €8,000 per year. Verification costs could add an additional €3,000 to €7,500 for suppliers of high-risk AI. Companies or public authorities developing or using any AI applications not classified as high-risk would have minimal information obligations but may choose to join others in adopting a code of conduct that follows the regulation, which could result in higher costs.

At the same time, there have been calls highlighting that the cost of AI regulation will not only be reflected in direct expenses but also in the price the economy will pay in terms of

lost opportunities. A study by the Center for Data Innovation indicated that the adoption of the AI regulation could cost the European economy €31 billion (Mueller, 2021). The study further suggests that the regulation is likely to trigger a "chilling effect" on AI investments in Europe, particularly in systems classified as high-risk. They also believe that the AI Act will lead to high opportunity costs for the European economy, causing the European AI market to develop much more slowly than it potentially could (Mueller, 2021).

However, the study is not without criticism. Several responses argue that the study cited incorrect and unfounded information about the expected costs of implementing AI regulation. The Centre for European Policy Studies, which also conducted an analysis on which the Center for Data Innovation based its figures, highlighted the incorrect referencing of their research in the study and stated that their numbers were used misleadingly and inaccurately, leading to the study's conclusions (Laurer, 2021).

7. Conclusion

The European Union's AI regulation represents a pioneering step towards creating a safe, ethical, and innovation-friendly digital environment. Rooted in the broader EU Digital Strategy, the regulation lays a solid foundation for managing AI technologies, ensuring alignment with the Union's fundamental values. By introducing a risk-based regulatory framework, the regulation skilfully balances the delicate equilibrium between promoting technological innovation and protecting fundamental rights and public safety, aiming for the development of ethical AI.

As discerned from this paper, there is a strong likelihood that the adoption of the AI regulation will have both *de facto* and *de jure* effects. EU regulation is likely to influence other countries that decide to regulate this field, as well as international economic actors who wish to remain in the EU single market. It is still too early to assert with certainty how the regulation will impact other actors.

With the new regulation imposing additional costs for AI development and classification, policymakers emphasize their intention to carefully balance these costs with the benefits accrued in other areas.

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Research

MOOCs and their contribution to non-formal learning in the realities of Ukrainian business education

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Abstract:

In today's rapidly changing educational landscape, the role of MOOCs (Massive Open Online Courses) has become increasingly significant, especially in the context of non-formal learning. This paper delves into the evolving landscape of Massive Open Online Courses (MOOCs), examining global and Ukrainian platforms. Two primary models of MOOCs are highlighted: cMOOCs, which emphasize connectivist principles like creation and autonomy, and xMOOCs, which follow traditional higher education methods with pre-recorded lectures and standardized assessments. The diverse MOOC landscape is highlighted, including major global platforms and Ukrainian initiatives, with a discussion on the comprehensive challenges and potential solutions to enhance the effectiveness of MOOCs.

Despite their promise of making education more accessible, flexible, and cost-effective, MOOCs grapple with significant obstacles. These include low completion rates, particularly in emerging economies, access disparity due to the digital divide, and a mismatch between course offerings and the intended audience. Engagement issues also persist, with limited involvement from educational professionals and high dropout rates among employed learners. Additionally, the perceived value of MOOC credentials remains questionable, and geographical and socioeconomic barriers further hinder their effectiveness. Addressing these challenges through continuous innovation and improved course design and delivery is essential for enhancing the impact and accessibility of MOOCs globally.

Keywords: Non-formal education, MOOCs, Business Education, Learning platform, Lifelong learning

1. *Understanding MOOCs*

1.1.   Characteristics and diverse models of MOOCs

According to the UNESCO Institute for Information Technologies in Education (Witthaus et.al., 2016), MOOCs are online courses designed for large-scale participation and open access via the internet. They typically include video lectures, readings, quizzes, and discussion forums, creating a comprehensive and interactive learning environment.

MOOCs have developed into two primary forms: cMOOCs and xMOOCs (Oxford reviews, 2024; Smith et al., 2013). cMOOCs, or "connectivist" MOOCs, encourage learners to actively contribute through blog posts, tweets, and other social media interactions. Connectivist MOOCs (cMOOCs) are based on the theory of connectivism (Siemens, 2004), emphasizing "creation," "creativity," "autonomy," and "social networked learning". These cMOOCs follow explicit principles of connectivism, autonomy, diversity, and openness. These contributions are collected and shared by course organizers via email or newsletters, fostering a sense of community and connection among participants. Learners in cMOOCs are encouraged to pursue their own learning goals and engage in networking within the wider online community.

On the other hand, xMOOCs mimic traditional classroom-based courses and use conventional higher education teaching methods. They offer pre-recorded video lectures and scalable assessments. Interaction in xMOOCs usually takes place within structured forums on a single platform, rather than through distributed content creation across the web. This approach emphasizes a more standardized and centralized learning experience, similar to that of a traditional educational environment.

Non-formal learning, as facilitated by MOOCs, takes place outside traditional educational settings, focusing on personal or professional development goals. The popularity of MOOCs stems from their accessibility, flexibility, and affordability, making education more inclusive and empowering learners globally. These courses enable individuals to gain new skills, advance their careers, and engage in lifelong learning.

1.2.   The impact of MOOCs on education

Over the past decade, MOOCs have revolutionized the educational sector. They offer global access to high-quality content from top institutions, driven by advancements in online learning platforms, the rising demand for lifelong learning, and the need for continuous skill development in a rapidly changing job market. Up to the research (Dhawal, 2021) In 2021, MOOCs attracted 220 million learners worldwide, not counting China. Providers introduced more than 3100 courses and 500 microcredentials. Additionally, 40 million new learners enrolled in at least one MOOC during the year.

The flexibility and accessibility of MOOCs dismantle traditional barriers such as geography, cost, and time, leading to millions of enrolments each year. As online learning continues to evolve, MOOCs are set to remain a transformative force in education, reshaping how we acquire new skills.

MOOCs are a game-changer, providing open access to education for anyone with an internet connection, regardless of location or background. They democratize education by offering high-quality learning resources on a global scale. MOOCs are scalable, accommodating thousands of learners simultaneously through sophisticated online platforms. They cover a diverse range of subjects, allowing learners to pursue topics of interest or professional relevance. Interactive elements like quizzes, assignments, and discussion forums enhance learner engagement and promote active learning. This makes MOOCs particularly suitable for busy professionals and working adults who need to balance their studies with other commitments. Overall, MOOCs are an effective and accessible means of enhancing skills and knowledge for learners worldwide.

2. The diverse MOOC landscape

2.1. World MOOCs platforms

The MOOC ecosystem is diverse and dynamic, providing numerous opportunities for online learning. Major platforms like Coursera, edX, and Udemy offer a wide variety of courses from leading institutions, catering to learners from various backgrounds and interests. These platforms have significantly expanded global access to education, offering flexible and affordable learning options.

Coursera has 148 million learners and more than 7,000 campuses, businesses, and governments that have come to Coursera to access world-class learning (Coursera, 2024). Collaborating with over 300 prominent universities and companies, it delivers flexible, affordable, job-relevant online learning to individuals and organizations globally. Our offerings encompass a diverse array of learning opportunities, spanning hands-on projects, courses, job-ready certificates, and degree programs.

The MOOC landscape is diverse and expansive (Pickard et al.,2024; Pratt, 2023) with platforms like FUN offering free academic and professional courses in multiple languages, including French and English. FutureLearn collaborates with over 260 institutions worldwide, providing pathways for learners to pursue micro-credentials, degrees, and professional development. Iversity, created by Springer Nature, offers MOOCs across academic and professional disciplines in multiple languages. OpenClassrooms focuses on professional training with over 500 free-access courses, Kadenze specializes in art and creative technology education, while MITx Online delivers courses developed by MIT faculty with interactive elements.

In the last decade, an increasing number of learning platforms have emerged, aiming to provide online courses to a global audience either for free or at affordable rates. Several of these platforms are operated by businesses. For instance, LinkedIn Learning features over 20,000 online courses taught by industry professionals.

2.2 Overview of Ukrainian MOOC platforms
In Ukraine, several MOOC platforms are making significant strides in transforming education and expanding access to learning opportunities. These platforms not only cater to traditional academic subjects but also play a crucial role in enhancing business education and vocational skills.

Prometheus Platform stands out as a pioneer in democratizing education by offering free online courses in collaboration with prestigious educational institutions and international organizations (Sharov et al, 2021). Starting 2014 it hosts over 400 courses across various disciplines, engaged 2 860 000 learners by 2024 (Prometheus, 2024). Its partnerships with international bodies like IREX Ukraine, British Council, and USAID underscore its commitment to global educational standards. Additionally, Prometheus and the U.S. Embassy have teamed up to forge a pioneering educational initiative, offering Ukrainians valuable access to the world's premier courses at no cost and in their native language. Over the next three years, Prometheus will provide 50 online courses from prestigious global universities in Ukrainian, delivering substantial educational value to learners. The program features courses from esteemed institutions including Harvard University, Massachusetts Institute of Technology, University of Michigan, Johns Hopkins University, Duke University, University of Illinois, and University of California.

The platform emphasizes blended learning models, integrating online resources with traditional classroom methods. This approach not only enhances educational outcomes but also supports lifelong learning initiatives essential for modern business environments.

EdEra focuses on innovation within Ukrainian education, developing online courses and interactive textbooks tailored to diverse educational needs. Beyond traditional subjects, EdEra addresses vocational training and corporate education, aligning closely with the evolving demands of the business sector. By collaborating with the Ministry of Education and Science of Ukraine and other local organizations, EdEra ensures the relevance and quality of its educational offerings.

It has enrolled over 2 million learners since its inception, with a course completion rate exceeding 70% (EdEra, 2022). Its interactive approach fosters active engagement among learners, enhancing their readiness for professional challenges in business and industry.

OUM has engaged participants in its civic education programs, fostering a robust understanding of democratic principles and civic responsibilities among Ukrainians (Sharov et.al., 2021). It focuses on civic education through non-formal online channels, promoting active citizenship and civic competencies. While its primary focus is not business education per se, the development of civic competencies is increasingly recognized as essential for effective business leadership and corporate governance.

Addressing the educational challenges in Ukraine requires innovative solutions. One clear answer lies in the development of national learning platforms that not only provide educational resources but also offer active consulting on business support issues. By leveraging these platforms, Ukraine can enhance its educational framework, making learning more accessible and practical for all.

The evolution of MOOC platforms in Ukraine intersects significantly with the realm of business education. These platforms not only facilitate access to foundational business knowledge but also support ongoing professional development and specialization. By forming partnerships with international organizations and incorporating cutting-edge educational technologies, Ukrainian MOOCs boost the global competitiveness of local businesses. Moreover, the emphasis on vocational and corporate education reflects a strategic alignment with industry needs. Businesses benefit from a skilled workforce equipped with up-to-date knowledge and practical skills, thereby driving innovation and economic growth.

3. Comprehensive challenges and issues in MOOCs

MOOCs represent the future of non-formal learning. They make education more accessible, flexible, and cost-effective, allowing learners to study at their own pace and balance their education with other commitments. MOOCs cater to diverse learning needs and styles, offering various course formats and subjects. They are often less expensive than traditional education, with many courses available for free. Additionally, MOOCs provide learners with opportunities to connect globally with peers and instructors, fostering valuable networking opportunities. By removing barriers to education, MOOCs enable learners to acquire new skills, explore interests, and stay current in their fields.

Massive Open Online Courses (MOOCs) have revolutionized access to education, making it possible for millions of learners around the globe to participate in a variety of courses. However, this innovation comes with its own set of challenges and limitations. Below, we provide a comprehensive overview of these challenges, rephrased and expanded upon to highlight the complexities facing MOOCs.

3.1. Low completion rates

The average completion rate for MOOCs is notably low (MOOC statistics, 2022), with only about 5.5% of learners finishing their courses. In emerging economies, the situation is even more dire, with completion rates falling below 1%. This suggests significant barriers to sustained engagement and successful course completion. But it is worth mentioning that some researchers (Hew et.al.,2020; Badali et al., 2022) argued that completion rates should not be singularly used to measure MOOC success, as many users participate without intending to finish the entire course, often concentrating instead on specific segments or topics.

3.2. Access disparity

In 2019, MOOCs enrolled 110 million students globally (Shah D, 2019). However, only 12% of these learners were from low-income countries, underscoring a significant digital divide. This disparity highlights unequal access to online education, often due to lack of internet connectivity and technological resources in these regions.

3.3. Mismatch with Target Audience

There is a notable disconnect between MOOCs and their intended audience. For instance, half of Coursera's users are corporate employees, indicating that these courses might not be reaching the broader public or fulfilling their potential to democratize education (Coursera, 2023). This misalignment suggests that MOOCs may not be effectively targeting their educational goals.

3.4. Engagement issues

Based on research of 276 MOOCs (Jordan, 2015) it was found that the typical MOOC attracts around 43,000 students, but only about 6.5% of these participants are educators. This lack of engagement from educational professionals could be a significant barrier to enhancing the quality and effectiveness of courses, as their involvement is crucial for curriculum improvement and student support.

3.5. Impact of Employment Status

A significant portion of MOOC learners, about 51%, are employed full-time (Moocs statistics, 2019) . This high employment rate among participants may hinder their ability to dedicate sufficient time to complete courses, affecting overall completion rates and the effectiveness of MOOCs as a tool for career advancement.

3.6. Limited Reach Beyond Students

In 2020, 75% of MOOC users were classified as non-traditional students. This demographic composition underscores the significant appeal of MOOCs beyond traditional educational settings, attracting individuals seeking flexible learning opportunities outside of conventional academic pathways. This trend reflects MOOCs' role in democratizing education by catering to diverse learner needs and preferences, thereby expanding access to knowledge and skills worldwide.

3.7. Questionable Credential Value

MOOCs offer certificates of completion, but their value is often questioned. About 80% of MOOC participants already hold a bachelor's degree or higher, which diminishes the perceived benefit of these certificates. This raises concerns about the actual utility and recognition of MOOC credentials in professional and academic settings.

3.8. Motivation and Completion Challenges

While Coursera reports that over 60% of its users enroll in MOOCs to enhance their career prospects, overall completion rates remain low (Baladi, 2022). This indicates a need for better motivational and support mechanisms to help learners stay engaged and complete their courses successfully.

3.9. Need for Quality Assurance

EdX has reported a 59% completion rate for students who sign up for a verified certificate. This figure highlights the necessity of improving course quality and implementing effective engagement strategies to ensure higher completion rates and learner satisfaction.

3.10. Educational Background of Learners

Among the 34% of MOOC learners who are over 30 years old, most already possess a bachelor's degree or higher (Mooc statistic, 2023). This demographic trend suggests that MOOCs may not be effectively reaching less-educated individuals who could benefit the most from accessible online education.

3.11. Geographical and Socioeconomic Barriers

Although 35% of Coursera's users come from developing countries, completion rates in these regions remain low (Witthaus, 2016). This highlights the challenges MOOCs face in engaging learners from diverse backgrounds and overcoming socioeconomic barriers that hinder access and completion.

3.12. User Satisfaction

MOOCs on EdX receive an average user rating of 75 out of 100 (Pickard et al., 2023). While this indicates general satisfaction, there is considerable room for improving course quality and the overall user experience to meet the diverse needs of learners.

3.13. Market Saturation and Competition

By the end of 2021, there were 19,400 MOOCs available worldwide (Shah, 2021) . This high level of market saturation creates a highly competitive environment, potentially diluting the impact of individual courses and platforms. This saturation can also make it challenging for new courses to attract attention and for existing ones to maintain their relevance.

However, these obstacles can be addressed through continuous innovation and improvements in course design, delivery, and engagement strategies. MOOCs play a valuable role in expanding global access to education, and with ongoing enhancements, their impact can be significantly increased.

3. Conclusions

MOOCs have demonstrated their ability to democratize education by breaking down traditional barriers and providing open access to high-quality content from leading institutions. Their scalability and diverse subject offerings make them particularly suitable for a wide range of learners, including busy professionals and working adults.

Despite their numerous benefits, MOOCs face several challenges, including low completion rates, access disparities, engagement issues, and limited impact on certain populations. Addressing these challenges requires continuous innovation and improvements in

course design, delivery, and engagement strategies. Enhancing digital literacy and ensuring access to necessary technologies are critical steps in bridging the digital divide and maximizing the impact of MOOCs.

The exploration of MOOCs (Massive Open Online Courses) within the context of Ukrainian business education reveals their substantial potential to revolutionize non-formal learning. These platforms offer significant advantages such as flexibility, accessibility, and cost-effectiveness, making them a powerful tool for personal and professional development. The integration of MOOCs into the educational framework can significantly enhance Ukraine's educational landscape, particularly through the development of national learning platforms that provide both educational resources and business consulting services.

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

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The Role of Music in the Ukrainian Stories of Nikolai Vasilyevich Gogol

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Abstract:
This article discusses the role of music in the stories of Nikolai Vasilyevich Gogol that take place in Ukraine. The musical examples used refer to a selection of stories that were published in the collections *Ukrajinske povesti (Ukrainian stories)* from 1943, translated into Slovene language by Franc Terseglav, and stories that were published under the title *Maloruske povesti* in the book *Izginulo pismo* translated by Urša Zabukovec in 2010. By citing and analyzing examples of music from the mentioned stories, the aim is to find out how music is involved in Gogol's prose, in which situations it is used and what it expresses. In addition, the second part of the article mentions examples of operas whose librettos are based on Gogol's Ukrainian stories. One of the composers who wrote such an opera, is Pyotr Ilyich Tchaikovsky, who was also of Ukrainian origin by his ancestors. He spent a lot of time in Ukraine and in his work, he used Ukrainian folk music as one of the sources of inspiration.

Keywords: Gogol; Ukraine; Tchaikovsky and Ukraine; Folk songs and dances

1. Introduction

1.1. *Gogol's origin and his first experiences with the literature*

According to Miha Javornik's study, published in the edition of a selection of Gogol's prose (Gogolj, 2010: 547-548), three cultures influenced the creativity of the most famous [1] Ukrainian classicist Nikolai Vasilyevich Gogol (1809-1952). He had Polish[2] roots on his father's side, he was born in Ukraine and initially wrote in Ukrainian. Then he moved to Russia, where he settled in St. Petersburg and began writing in Russian. Gogol inherited his literary talent from his father, who was engaged in writing, and his father inspired him to paint[3] as well. He was also influenced by German romantic literature, including Schiller and Goethe, which he enthusiastically read (Gogolj, 2010: 549-550). Gogol met Pushkin, who, according to Franc Terseglav (Gogol, 1943: 6), became even his best friend, and on his advice he began to read all the world's classics, such as Cervantes, Dante, Shakespeare and Dickens, and devote extraordinary care to a perfect expression in the Russian language. Pushkin was impressed by Gogol's first collection of Ukrainian stories, *Evenings on the Farm near Dikanka*, which made the author famous. (Gogolj, 1943: 6).

1.2. Influences of Ukrainian folk traditions

It is characteristic for Gogol that he collected folklore material, namely Ukrainian folk tales, songs and descriptions of folk customs and traditions. The collection of folk material was characteristic especially for the early period of his literary creation. He asked his mother for help to provide him folk tales and as detailed as possible descriptions of the customs and habits of Ukrainians (Gogolj, 2010: 9). Gogol was particullary interested in stories where evil forces[4] appear. Thus, the real, everyday life of the Ukrainian man mixes with the fantastic-demonic world.

1.3. *Gogol's Ukrainian stories*

Gogol set all his Ukrainian[5] stories in the environment he was most familiar with: his native village and its surroundings. He was born in the village of Vasilyevka in the Myrhorod Raion, Poltava Oblast, near the villages Velyki Sorochyntsi and Dikanka (Gogolj, 1943: 5). His Ukrainian stories contain three volumes of story collections, namely two volumes of *Evenings on the Farm near Dikanka* (from 1831 and 1832) and *Mirgorod* (1835). Both parts of

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[1] According to the online portal culture.ru (Culture Russia, Михаил Булгаков), Mikhail Bulgakov (1891-1940), whose favorite writer was Gogol (at least in his youth), was born in Ukraine, namely in Kiev, where he graduated in medicine. Since 1900, his family also owned a holiday house (dacha) in the village of Buča near Kiev. According to various sources (including Nestruck, 2008) and (Wikipedia, Mikhail Bulgakov)), Bulgakov was born to Russian parents and wrote his works in Russian. He moved to Moscow in 1921. In his life and work, music had been very important. According to an online article on culture.ru (https://www.culture.ru/persons/8263/mikhail-bulgakov), he listened the music at home as a child, as his parents were playing in their free time; they also occasionally went to concerts and opera.

[2] As Miha Javornik writes, Gogol did not accept his Polish origin with affection, which could be confirmed by his negative attitude towards Polish culture that can be observed, for example, in the stories *A Terrible Vengeance* and *Taras Bulba* (Gogolj, 2010: 548).

[3] His novella *The Portrait* is related to painting.

[4] As Franc Terseglav states in his description of Gogol's life and work, Gogol's mother was somewhat excessively religious, as she perceived the world as a threat from evil forces. In the second half of Gogol's life, the influence of "negative faith" became apparent in Gogol, which developed into a life crisis, leding to his death (Gogol, 1943: 5). Gogol spent the last twelve years of his life abroad, he traveled to Germany and France, and he was most impressed by Italy. In search of spirituality, he even made a pilgrimage to Palestine, and for the last four years of his life he lived in painful self-denial, visiting monasteries and searching for God, first in his native Ukraine and then in Russia (Gogolj, 1943: 7-10).

[5] Another name for "Ukrainian" is "Little Russian" stories. According to the »Fran« dictionary, "Maloruski, Malorus, Malorusinja" are ancient terms for "Ukrainian, Ukrainian" or "Ukrainian woman" (Fran). Little Russia is a geographical and historical term for Ukraine (Wikipedia, Little Russia). Miha Javornik (in Gogolj, 2010: 552), however, states that the term "Malorossiya or Little Russia" was used at the Russian court to refer to the land around the Dnieper where Ukrainians lived.

Evenings on the Farm near Dikanka consist of four stories each, which are connected in pairs: the story from the first part "has a structural and ideological thematic parallel in the second" (Gogolj, 2010: 555). The first part contains the stories *The Fair at Sorochyntsi*, *St. John's Eve*, *May Night, or the Drowned Maiden* and *The Lost Letter*. The second part consists of *Christmas Eve, Ivan Fyodorovich Shponka and His Aunt, A Terrible Vengeance* and *A Bewitched Place*. The *Mirgorod* collection also contains four stories (also structurally and thematically divided into two parts): *The Old World Landowners* and *Taras Bulba* and in the second part, *The Tale of How Ivan Ivanovich Quarreled with Ivan Nikiforovich* and *Viy*.

2. Music in Gogol's Ukrainian tales
2.1. Sounds in the nature: bird calls
In his stories, Gogol romantically describes the beauty of Ukrainian nature in addition to the everyday life of Ukrainians, their characters, customs and rituals. The sound image of the landscape and the calls of various birds are also important. For example, in the first paragraph of *The Fair at Sorochyntsi*, Gogol creates a hymn to the summer in his homeland. *"How intoxicating, how magnificent is a summer day in Little Russia! […] Everything might be dead; only above in the heavenly depths a lark is trilling and from the airy heights the silvery notes drop down upon adoring earth, and from time to time the cry of a gull or the ringing note of a quail sounds in the steppe."* (Gogol, 1999: 19). Even when describing the fantasy night scene in the story *Viy*, when the hero flies in the air with a witch on his back, sound impressions are important: *"Is he seeing it, or is he not? Is he awake or asleep? But what now? Wind or music: ringing, ringing, and whirling, and approaching, and piercing the soul with some unbearable trill…"* (Gogol, 1999: 92).
We find a description of the night in the tale *May Night, or the Drowned Maiden*: *"Do you know the Ukrainian night? Oh, you do not know the Ukrainian night! Look at it: the moon looks out from the centre of the sky; the immense dome of heaven stretches further, more inconceivably immense than ever; it glows and breathes; the earth is all bathed in a silvery light; and the exquisite air is refreshing and warm and full of voluptuousness, and an ocean of fragrance is stirring. Divine night! Enchanting night! […] The glorious clamour of the Ukrainian nightingale bursts upon the night and one fancies the moon itself is listening in mid-heaven… […]"* (Gogol, 1926: 86-87).

2.2. Folk dances
There are many references to singing and dancing on various occasions in the everyday life of Ukrainian people. Gogol likes to describe the joyful spirit of the Ukrainian people. When they are happy, they usually dance folk dances trepak or gopak (hopak). According to the Oxford Dictionary of Music, these are "Russian folk dances in 2/4 time" (Kennedy 1996: 746, 296). According to the translator's note to the story *The Lost Letter* (Gogolj 2010: 13) and as confirmed as well as by the Russian musician Elena Starceva-Somun (Starceva-Somun 2024), gopak is an Ukrainian national dance. Gogol also mentions other dances, such a Russian folk dance kazachok, which is also a fast dance in two-quarter time (Gogolj, 2010: 24). The officers in the regiment in the story *Ivan Fyodorovich Shponka and His Aunt* danced the mazurka, the Polish national dance (Gogolj, 2010: 75-76). In the story *A Terrible Vengeance*, where he mentions the revelry of the Poles in the tavern, they dance the Polish dance krakowjak (Gogolj, 1943: 49).

The main character in the story *Viy* likes to dance: **"***The philosopher Khoma Brut was of a merry disposition. He liked very much to lie about and smoke his pipe. When he drank, he was sure to hire musicians and dance the trepak.* **"** (Gogol, 1999: 89). When Khoma Brut once with his friends drank a lot, he suddenly jumped up and shouted: "*Musicians! We must have musicians!*" — *and, without waiting for the musicians, broke into a trepak in the cleared spot in the middle of the yard. He danced until it came time for the afternoon snack, when the household people, standing in a circle around him, as is usual in such cases, finally spat and went away, saying, " Look how long the man's been dancing! "* (Gogol, 1999: 106).

In the story *May Night, or the Drowned Maiden* we find a description of how a drunk farmer dances a gopak in the middle of the night: "*But that's not the way to dance the gopak. I feel that it won't come right somehow. What was that my crony was saying... ? Oh yes: hop, tra-la! hop, tra-la! hop, hop, hop!* " *So a middle-aged peasant, who had been drinking and was dancing down the*

street, talked to himself. "I swear, that's not the way to dance the gopak. Why should I tell a lie about it? I swear it's not right. Come: hop, tra-la! hop, tra-la! hop, hop, hop!" (Gogolj, 1999: 87).

2.3. Music in everyday life

In the story *A Terrible Vengeance*, Gogol cites a lullaby that a mother sings to her child: *"Lullay, lullay, lullay, Lullay, little son, lullay, Grow up, grow up wise, Win glory in the Cossacks' eyes And punish their enemies. "* (Gogol, 1999: 39).

Songs can even be sung by an "unclean force", for example, by the witch from the story *A Terrible Vengeance*. The poem is about the death and burial of her husband (Gogolj, 2010: 60-61).

In *Viy*, Gogol describes the life of seminarians who go home for vacation during the summer. On the way home, they "earned" food by singing in the choir in front of richer houses. Under their windows they *"begin a full-throated hymn".* (Gogol, 1999: 88). According to the website belcanto.ru (Keldysh), a hymn is a type of polyphonic song typical of Russia, Ukraine and Belarus in the 17th and 18th centuries. In the beginning, hymns had religious lyrics, but in the 18th century, the lyrics of songs could also be patriotic, military, or love. In the story *Viy*, the seminars moved an owner of one house and he ordered to his wife: *"Wife! what these students are singing must be something very intelligent; bring out some lard for them and whatever else we've got!"* (Gogol, 1999: 88).

2.4. Music in national customs and ceremonies

Similarly, Carolers[6] sang at houses and received gifts in the form of (festive) dishes, but not only at rich houses. Gogol mentions caroling with the singing of carols in the story *Christmas Eve*: *"Noisier and noisier sounded the songs and shouts in the streets. The crowds of jostling folk were increased by those coming from neighboring villages. The lads frolicked and horsed around freely. Often amidst the carols one could hear some merry song made up on the spot by some young Cossack. Then suddenly one of the crowd, instead of a carol, would roar a New Year's song at the top of his lungs: Humpling, mumpling! Give me a dumpling, A big ring of sausage, A bowl full of porridge! "* (Gogol, 1999: 20).

The Fair at Sorochyntsi ends happily with a wedding. All the guests, together with the bride and groom, old and young, among them eighty-year-old women, began to dance *"at one stroke of the bow of the fiddler, who had long twisted moustaches and wore a homespun jacket. Men whose sullen faces seemed to have known no gleam of a smile for years were tapping with their feet and wriggling their shoulders; everything was heaving, everything was dancing."* (Gogol, 1926: 54).

Also in *St. John's Eve* there is a passage that describes the celebration of the wedding: the customs, clothes, music and dances. *"[…] they baked a lot of cakes, sewed a lot of napkins and kerchiefs, rolled out a barrel of vodka; the young couple was seated on the table; the round loaf was cut; they struck up the bandore, cymbals, pipes and mandolins—and the fun began . . . Weddings in the old days were no comparison with ours. "* (Gogol, 1999: 6). […] *"how young women in tall headdresses, the upper part made all of gold brocade, with a small cutout behind and a golden kerchief peeking from it, with two little peaks of the finest black astrakhan, one pointing backward and the other forward, in blue jackets of the best silk with red flaps, stepped out imposingly one by one, arms akimbo, and rhythmically stamped away at the gopak"* (Gogol, 1999: 6).

The story *A Terrible Vengeance* begins with a description of a wedding, where musicians played drums, cymbals and violins, and one of the Cossacks danced the kazachok (Gogolj, 2010: 24).

2.5. Folk songs
2.5.1. Love song

In *The Fair at Sorochyntsi*, a girl in love takes a mirror in her hands and sings a happy, jumping Ukrainian folk song, which was her favorite: *"Little green periwinkle, Twine lower*

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[6]According to Margarita Kovyneva, singing carols is an old custom that is characteristic of the period from Christmas to Epiphany (in Orthodox countries, that is from January 7 to 19). According to Russian tradition, this time is called "svyatki" (святки). The carolers went from house to house singing carols, and people mainly gave them cakes and sweets.

to me! And you, black-browed dear one, Come nearer to me! Little green periwinkle, Twine lower to me! And you, black-browed dear one, Come nearer to me!" (Gogol, 1926: 52). (Зелевевький барвиночку, Стелисья низенько). During the girl's joyful singing, her father enters the room, and this scene made him dance with joy.

2.5.2. Folk instrument »bandura« (or »bandore«)

A typical Ukrainian folk instrument, which Gogol mentions in his work, is a type of lute called a »bandura«. Thus, for example, the tale *May Night, or the Drowned Maiden* begins with a serenade sung by the hero and accompanied on the bandura. "*Ringing song flowed like a river down the streets of the village. It was the hour when, weary from the cares and labours of the day, the lads and girls gather together in a ring in the glow of the clear evening to pour out their gaiety in strains never far removed from melancholy. […] It was already dusk, yet still the singing did not cease. Lyovko, a young Cossack, the son of the village Head, slipped away from the singers with a bandura in his hands. He was wearing an astrakhan cap. The Cossack walked down the street thrumming on the strings of his instrument and dancing to it.*" (Gogol, 1999: 78). When the hero approached the house where the girl lived, he sang a Ukrainian folk song, from which Gogol quotes the first stanza *"The sun is low, the evening's nigh, come out to me, my little heart!"* (Сонце низенько, вечер близенько , / Вийди до мене, моє серденко!). (Gogol, 1999: 79).

In the continuation of this story, a group of boys, accompanied by a bandura, sings a humorous song »*Laddies, have you heard the news now!*« ("Хлопцы, сличили ли вы?"), with which they want to tease the master (Gogol, 1999: 99-100).

There is another scene in the same story where the hero Levko sings a Ukrainian folk song accompanied by a bandura. It is a night scene by the lake near the home of a rusalka or an enchanted drowned woman. "*There was a sense of sweet stillness and space and freedom in Lyovko's heart. Tuning his bandura, he began playing it and singing: "Oh, thou moon, my darling moon! And thou, glowing clear sunrise! Oh, shine brightly o'er the cottage, Where my lovely maiden lies!*" (Gogol, 1999: 110).

2.6.1. Singing tradition in the town of Glukhov

In the conclusion of the story *A Terrible Vengeance*, Gogol describes the singing of a blind folk singer from Glukhov. Ukrainian folk singers and the town of Glukhov have historically been of great importance for all Ukrainian and Russian classical music: "*In the town of Glukhov people gathered around the old bandore player and listened for an hour as the blind man played his bandore. No bandore player had ever sung such wonderful songs or sung them so well. First he sang about the old hetmans, about Sagaidachny and Khmelnitsky. Times were different then: the Cossacks were in their glory; their steeds trampled down their enemies, and no one dared to mock them. The old man sang merry songs, too, and kept glancing around at the people as if he could see; and his fingers, with little bone picks attached to them, flew like flies over the strings, and it seemed the strings played of themselves; and the people around him, the old ones with their heads hanging, and the young ones looking up at the old man, dared not even whisper to one another*" (Gogol, 1999: 56-57).

According to a Russian musicologist Mikhail Kazinik (2021), Ukraine has always been famous as a country of singers. That is why Catherine the Great founded an Orthodox singing academy in Ukraine, namely in the city of Glukhov. The greatest Ukrainian composers, such as Maksim Berezovsky and Dimitry Bortnyansky, who were both born in Glukhov, graduated from this academy.

2.6.2. Dmitry Stepanovich Bortnyansky

Dmitry Stepanovich Bortnyansky (1751-1825) is classified in the Oxford Dictionary of Music as a Russian composer. As we learn from Wikipedia (Dimitrij Stepanovič Bortnjanski), at the age of eight he started singing in the choir of the court chapel in St. Petersburg. Between 1765 and 1768, the conductor of the court choir was the Italian composer Baldassare Galuppi, with whom he studied composition. According to the Oxford Dictionary, Bortnyansky followed Galuppi to Venice in 1768, where his own operas were staged in 1776, and two years later they were staged in Modena. He remained in Italy until 1779, when he returned to Russia and became the leader of the imperial church choir, for which

he wrote a large number of compositions. As we read further in the aforementioned dictionary, his ecclesiastical works were published in St. Petersburg in ten volumes published and edited by Tchaikovsky (Kennedy, 1996: 88). As Mihail Kazinik claims, Bortnyansky can be counted among the founders of the tradition of Russian classical music (Kazinik, 2022).

2.6.3. Maksim Sozontovich Berezovsky

There is no information about Maksim Sozontovich Berezovsky (c. 1745-1777) in the Oxford Music Dictionary, although according to Mihail Kazinik (2021 b) he was very important in the history of Ukrainian music. According to (Wikipedia, Maxim Berezovsky), together with Bortnyansky and Artemy Vedel, he belongs to the three most important Ukrainian composers of the 18th century. Just like Bortnyansky, Berezovsky was a member of the imperial singing chapel in St. Petersburg, where all the best talented musicians were sent, and he studied composition with the famous Italian composer Galuppi, and then went to Italy, namely to Bologna (Wikipedia, Maxim Berezovsky). Mihail Kazinik points out that Berezovsky became a honorary member of the Bologna Academy (Kazinik, 2020). Unfortunately, Berezovsky's career did not continue successfully after his return to the Russian Empire, because he did not achieve the same success and honor in his homeland as he did abroad. In the Russian Empire in that period, foreign artists were preferred, who also earned a lot in Russia, especially by writing operas. Berezovsky, as further describes Mihail Kazinik, has been forgotten for many years in history (Kazinik, 2020).

2.6.4. Gogol's Ukrainian stories as opera libretto

Gogol's stories have often served as a template for opera librettos written by famous Russian composers. Thus, Modest Mussorgsky is the author of the opera *The Fair at Sorochyntsi*, for which he himself wrote a libretto after a Gogol's story (1876-81). The opera remained unfinished at the composer's death, and different composers contributed their own version of the ending, including César Cui and Nikolai Cherepnin (Wikipedia, The Fair at Sorochyntsi). In this Mussorgsky's opera, the most famous passage is the »Gopak« from the third act, for which there exist many arrangements for different instruments and ensembles. Another famous example of a setting of Gogol's prose in an opera is P. I. Tchaikovsky's opera *Vakula the Smith* (1874), based on the libretto by Yakov Polonsky, adapted from the story *Christmas Eve*. Tchaikovsky reworked this opera in 1885 and changed the title to *Cherevichki (The Little Shoes)* (Wikipedija, Cherevichki).

3. Ukrainian melodies in the work of Pyotr Ilyich Tchaikovsky

3.1. Tchaikovsky's origins

Like Nikolai Gogol, Pyotr Ilyich Tchaikovsky also had several nationalities in his background. As we can find out on the kulturologia.ru website, the composer's great-grandfather was born in Ukraine and was a Cossack of the Myrhorod regiment who participated in the Poltava battle.[7] At that time, his surname was »Chaika«, and it was only during the lifetime of his son, the composer's grandfather, that it took on the form Tchaikovsky. On his mother's side, the composer had European roots: his great-grandmother, who was from an Austrian family, married a Frenchman, and their son emigrated to Russia, as states the aforementioned website.

3.2. Tchaikovsky's First piano Concerto

Mihail Kazinik (2021a) points out, Ukrainian music was very important for Tchaikovsky, as he drew from it as from one of the sources, and therefore there are many influences of Ukrainian folk music. As Kazinik further explains, Tchaikovsky spent the happiest periods of his life in Ukraine, in the village of Kamenka, where his sister Alexandra and her family

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[7] A battle in 1709 when the Russian army defeated the Swedish army (Wikipedia, Bitka pri Poltavi).

lived (this was the composer's family, as he did not create his own family). It was in Ukraine that the composer heard the melody performed by a blind »lirnyk« (that is a traveling Ukrainian singer who plays the lyre) and it became the main theme of his famous *First piano concerto*. He reworked this theme in such a way that it no longer sounded as tragic as the lirnyk performed it, but it became more bright. Tchaikovsky's *First piano concerto* was first called *Concerto on Ukrainian Themes.* He later changed the title because in the second movement he used a French melody that his brothers liked to sing (Kazinik, 2021a). Even the finale of this concert, written in the form of a rondo, is based on a Ukrainian theme, namely the so-called "vesnianka" - this is a song that celebrates the arrival of a spring. A spring and a new life return in cycles, such as the rondo form, where the chorus constantly returns in a circle (Kazinik, 2021b).

3.3. *Tchaikovsky's Symphony No. 2*

Tchaikovsky's Symphony No. 2 is connected with Ukraine and Ukrainian music as well. It is also called the »Little Russian« or "Ukrainian" symphony or the "Crane symphony" (Kazinik, 2022b). In its finale, the composer used the Ukrainian folk song 'Crane', which he orchestrated differently each time it appeares. According to Kazinik, this finale influenced Stravinsky and his *Firebird*. In the continuation of the last movement of the symphony, another theme appears, according to Kazinik, an "oriental" theme, reminiscent of Borodin and his *Prince Igor*. In this mouvement the connection between Russian and Ukrainian music can be felt (Kazinik, 2022b).

4.   Conclusion

Gogol in his Ukrainian stories often describes Ukrainian folk songs and dances. Music accompanies people's everyday life: for instance, a mother is singing for her child in a cradle; singing and dancing are present at weddings and other celebrations. Singing in many cases expresses joy, because Gogol like to describe a joyful character of Ukrainians, but it also expresses sadness or pain. In Gogol's literature, a real life mixes with fantasy and the demonic, and even fictional or folklore creatures can express themselves through music or singing.

Gogol's Ukrainian stories have been the basis for opera librettos several times, as in the case of famous Russian operas by Tchaikovsky, Mussorgsky and Rimsky-Korsakov. Tchaikovsky, who also had Ukrainian blood in his origins, in his compositions often used melodic themes of Ukrainian folk music.

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Reflection

An Entertaining Lesson for Paediatric Oncology Patients: Learning Natural Science through Play and Demonstrating Chemistry and Physics Experiments

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Abstract:

Experimental work plays an important role in learning the natural sciences (chemistry, physics and biology). It is also an important tool in various interdisciplinary fields. Many natural laws and phenomena of everyday life can be observed through simple chemical and physical experiments. With the help of a researcher from a scientific institution, we transferred the described concept from ordinary classrooms to a hospital environment, namely in the Clinical Department of Paediatric Haematology and Oncology of the University Children's Hospital in Ljubljana (Slovenia). A dynamic interaction was found that motivated the young patients to learn science in this way. Through their active participation in challenging diagnoses, they can immerse themselves for a moment in effects that literally enchant them. In addition to curiosity and creativity, cooperation and teamwork were also encouraged.

Keywords: Experimental work, Natural sciences, Paediatric haematology and oncology, Learning, University Children's Hospital, Motivation, Curiosity

1. Introduction

At the Division of Paediatrics – University Children's Hospital, University Medical Centre Ljubljana, the diagnosis and treatment of cancer as well as peripheral hematopoietic stem cell and bone marrow transplantation in children and adolescents up to the age of 18 or until the end of schooling is carried out. This institution is also performing sophisticated diagnostic and therapeutic procedures for children and adolescents with non-malignant blood diseases and blood clotting disorders, severe forms of congenital immunodeficiencies and some congenital metabolic disorders. Advanced forms of treatment, including immunotherapy such as CAR-T (*Engl.* Chimeric Antigen Receptor) are introduced and implemented. Annually 80-90 patients with newly diagnosed cancer are treated and an average of 10 transplantations are carried out (University Medical Centre Ljubljana, n. d.).

Treatment of paediatric cancer requires close multidisciplinary cooperation with various laboratory specialities and clinical subspecialties. Therefore, close cooperation among different subspecialities is essential and established, not only within the University Medical Centre Ljubljana (UMCL), but also with the University of Ljubljana, Faculty of Medicine and Institute of Oncology in Ljubljana. Radiotherapy of children is performed at the Institute of Oncology, where treatment is performed by radiotherapists dedicated to paediatric radiotherapy. When proton irradiation is preferred, it is performed at the Proton Center in Trento, Italy, with which a multi-year exemplary collaboration has been established (University Medical Centre Ljubljana, n. d.). In this case, all costs of treatment abroad and accompanying parents are covered by the public health insurance company and are not a burden on the family (University Medical Centre Ljubljana, n. d.).

The Department of Paediatric Oncology and Haematology is a member of the European Reference Network for Paediatrics Oncology (ERN PAEDCAN) (University Medical Centre Ljubljana, n. d.), International Organization of Paediatric Oncology (SIOP), European Society of Blood and Marrow Transplantation (EBMT) and Berlin-Frankfurt-Münster Group (BFM group). Within the framework of these professional organizations, it is involved in international academic research in the field of treating children with cancer. Active research participation in some of the European Union projects (EU projects) is carried out. Quality comprehensive treatment is key to the successful treatment and rehabilitation of children treated for cancer. Three psychologists, one dietician and one physiotherapist work in the department (University Medical Centre Ljubljana, n. d.). Tight and regular cooperation with the National Institute of Rehabilitation, Ljubljana is established (University Medical Centre Ljubljana, n. d.).

Children and adolescents treated at the Department of Paediatric Oncology and Haematology are also involved in the hospital kindergarten and school. During the stay of the child in the hospital, the education takes place in the hospital and is carried out by the teacher of Ledina Hospital school, Ljubljana. Whereas, when the child is at home, the teaching is carried out by the teachers of the school the child attended before the illness. Funds for individual teaching at home, are provided by the Ministry of Education based on a decision, issued by a special commission, to which parents address their inquiries (Žugman, 2021). The majority of children in Slovenia complete school regularly, despite a long-term illness (Kandus, 2023).

The hospital school departments within the Ledina Primary School (Ljubljana, Slovenia) have existed since 1958, and school work in the hospital began in Ljubljana in 1951 (Ledina hospital school, n. d.). Today, the entire hospital school consists of 23 different departments, in which 23 teachers teach. Around 2,700 primary school pupils attend the school every year, 600 of whom are secondary school students. They are taught individually by the hospital teachers in the general education subjects. The secondary school pupils and students attend the school to make their stay in hospital more varied and enjoyable and, above all, to make it easier for them to integrate into the life and work of their school when they return from hospital. Due to the treatment methods for certain diseases, some students come from home to attend school at the hospital; and for some, the hospital teachers come to their homes and teach at home, the latter mainly with the help of information technology (Ledina hospital school, n. d.).

Pupils and high school students who attend the hospital school over a longer period of time are also assessed (oral and written knowledge tests, seminar papers). Everyday school life in the hospital takes place in small groups and the learning process more often takes place on individual level. Frequently lessons take place in patients' rooms, at their bedsides. The pupils and students also enjoy many school activities – special cultural, scientific and sports days, creative activities, various workshops, from cooking to poetry, visits from famous singers, actors, athletes, poets and writers as well as excursions, etc. (Ledina hospital school, n. d.).

This paper will present the enrichment of the day in the hospital with experiments in chemistry and physics. By conducting a lecture and by demonstration of experiments, the cooperation with scientific research institution in the transfer of knowledge, with the method of interdisciplinary knowledge integration of scientific content was presented to the pupils.

2. Practical activity

The thorough practical part consisted of building the triangle of members of the system (**Figure 1**). The researcher of the scientific research institution led the discussion between the young patients and the staff of the department. Fundamental concepts from the classical educational process and their application in real life situations were incorporated through the presentation. In our case, the parents of the primary school pupils were also involved and have actively participated in the discussion. Doctors and nurses were involved in some experiments.
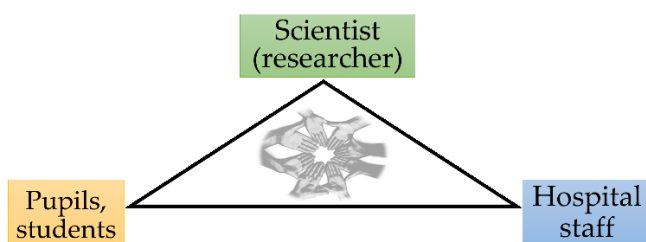

Figure 1. Triangle of cooperation in the implementation of the educational day in the hospital department.

2.1. Air and its components – physical experiments

The active and dynamic collaboration of all participants began with the topic of air. The discussion focused on the components of air and their isolation. A large proportion of the participants knew the composition of the gasses in the air. After the theoretical background, the important components of air were demonstrated in practice.

The young people were most enthusiastic about liquid nitrogen. Through simple experiments, we found out what the extremely low temperature of −196 °C means in practice. Some interesting experiments should be mentioned here. The participants had not expected that the balloon deflated in liquid nitrogen would return to its original state at room temperature (molecular dance in motion). When the balloon is at room temperature, the air inside moves quickly and takes up a lot of space. When the balloon is cooled by the liquid nitrogen, the air inside moves much more slowly as it loses heat to the liquid nitrogen. This causes the air inside to condense and the balloon can "deflate" and fit into the thermos flask. After the balloon has been pulled out, the air inside heats up again and expands the balloon back to its original size (Liquid nitrogen, n. d.). We also made a banana hammer. When a banana comes into contact with liquid nitrogen, the water in it quickly freezes and turns to ice, making such objects "rock hard". They can use it to hammer nails into a wooden base.

By interpreting the Leidenfrost effect on the skin, we have established that liquid nitrogen is extremely cold and that our body is extremely warm compared to liquid nitrogen. There is a big difference between body temperature and the temperature of liquid nitrogen. When liquid nitrogen comes into contact with our hand for the first time, part of the liquid

immediately turns into gas. The rest of the liquid lands on this layer of gas and rolls on without ever touching us. This effect only works if the hand is not exposed to the cold liquid for long. Prolonged exposure can cause the gas layer to escape, which can lead to frostbite or worse. Due to the working conditions described above, we must wear suitable protective equipment when experimenting with liquid nitrogen (Liquid nitrogen, n. d.). Special gloves are necessary, because classic laboratory gloves become very sensitive on contact with the gas and can cause skin ulcers. By demonstrating the effect of liquid nitrogen on classic laboratory gloves, we can also demonstrate the fragility of such materials.

Following on from the other experiments, we also investigated what happens when we try to seal a bottle containing a small amount of liquid nitrogen with a plastic stopper. When liquid nitrogen is trapped in a sealed bottle, the gases in the container begin to expand as it constantly boils and the pressure in the bottle increases. Approximately one ml of liquid nitrogen turns into 700 mL of nitrogen gas (Tretiakov, 2014). The force blows the cork away from the bottle opening. The alternative demonstration with the bottle, in which a hole is drilled through the cork and it can be observed how the nitrogen escapes through the hole, was also demonstrated. The effect is similar to the fountain – in our example, the nitrogen fountain.


Figure 2. Demonstration experiments with liquid nitrogen: (**a**) cold vapours of liquid nitrogen can already be felt with the hand near the container, (**b**) Leidenfrost effect of liquid nitrogen on the skin and (**c**) gas leakage through the capillary: nitrogen fountain (Photo: Alenka Klun with permission of the University Medical Centre Ljubljana).

2.2. Chemical reactions

By demonstrating chemical reactions, the visitors learned about chemical reactions in nature and deepened their knowledge of them. It was also considered when the chemical reaction takes place.

Fire is a chemical reaction in which a fuel and oxygen are converted into carbon dioxide and water. It is an exothermal reaction, *i. e.* a reaction in which heat is generated. The reason for this is that the chemical bonds in the oxygen molecule are relatively weak and the newly formed bonds are more stable – so there is a net production of energy (NewScientist, n. d.). The general combustion equation of fire is (**Eq. 1**):

Fuel + oxygen (O_2) → carbon dioxide (CO_2) + water (H_2O), (1)
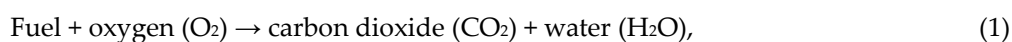
a theorem that was drummed into many of us by our teachers at school. However, the combustion reaction does not proceed directly from oxygen to carbon dioxide. Instead, a whole series of intermediate molecules are involved along the way. Sometimes incomplete combustion occurs, in which these intermediate molecules are formed in unusually large quantities (New Scientist, n. d.). This model was used to demonstrate the combustion reactions of everyday ingredients – absorbent cotton and dry bread. The visitors were then

able to observe the combustion of the aforementioned oxygen-enriched raw materials. The greatest wave of enthusiasm was triggered by the oxygen-enriched absorbent cotton (nitrocellulose), which caught fire very quickly. All of the absorbent cotton was converted into gaseous products, resulting in complete combustion. This was followed by a demonstration of the northern lights, a chemical reaction in which kitchen aluminium reacts with hydrochloric acid with the addition of copper(II) sulfate pentahydrate. The reaction produces hydrogen gas, which colours the flame green due to the copper ions present (Fleming, 2014).

Also we considered the production of light in nature and its application in chemical laboratories. In general, the type of luminescence is defined by the type of energy emitted. Bioluminescence often occurs in nature when an enzyme catalyses the oxidation of a substrate molecule (*e. g.* in fireflies; enzyme: luciferase, substrate molecule: luciferin) (Marinko et al., 2024). Light can be used for defense or attack, for communication, for mating or simply to illuminate the environment. Two phenomena of photoluminescence are known: fluorescence and phosphorescence. Fluorescence is a photoluminescent phenomenon that occurs under a constant supply of energy (usually in the form of radiation), while phosphorescence can persist even if the energy supply is interrupted (Jeran et al., 2020*a*; Jeran et al., 2020*b*). The fluorescence of various dyes was presented, including fluorescein, which occurs in fluorescent markers. Chemiluminescence is the generation of electromagnetic radiation in the form of light through a chemical reaction. In this type of luminescence, light generation of light was demonstrated in the reaction of forensic luminol and hydrogen peroxide. Due to the enthusiasm of those present, it was necessary to repeat some of the lighting effects (**Figure 3**).
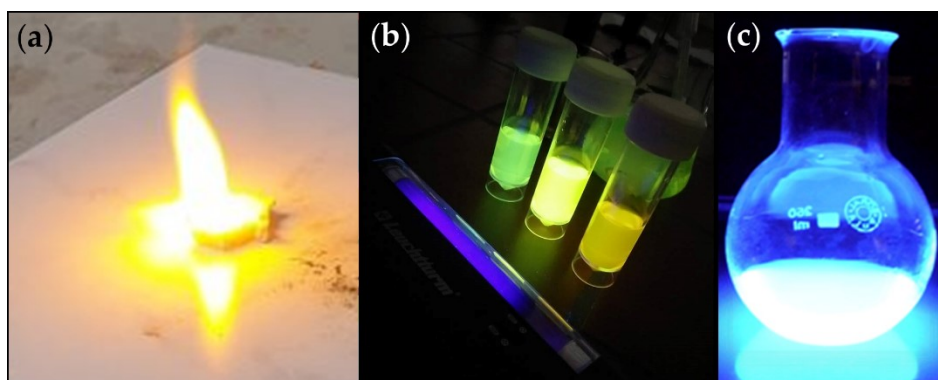

Figure 3. Demonstration of lighting effects: (**a**) ignition of bread, (**b**) fluorescence of fluoresceins and (**c**) chemiluminescence of luminol with diluted hydrogen peroxide solution (Photo: Marko Jeran).

There was also a colourful party with lollipops. When the lollipop dissolves in the sodium hydroxide solution containing manganese, at least five different colours can be distinguished, corresponding to the different oxidation states of the manganese. In a series of redox reactions, electrons are continuously released from the glucose to successive manganese compounds. At each step in this chain, a colour change becomes visible. Manganese is ideal for this experiment because it has more stable oxidation states than any other transition metal (from +2 to +7), each of which has a different colour (Prolongo and Pinto, 2018).

Beer was produced using detergent and an aqueous solution of potassium iodate (KIO_3) and a solution of sodium sulfite (Na_2SO_3) in sulphuric acid with the addition of ethanol. The contents of both solutions were simultaneously poured into a beaker in which "beer" was formed. We waited about 15 seconds for the "miracle", as the reaction took place with a slight delay. The participants thought this was a mistake. The yellowish-brown colour of the "beer" came from the precipitated iodine and the foam from the detergent.

At the end of the demonstration, we asked a little fighter, who was celebrating his 4ᵗʰ birthday that day, to stand in front of the experiment table. We all congratulated him and wished him well, but he was keen to experiment for himself. Using an aqueous solution of iron trichloride ($FeCl_3$) and a cotton bud, he made a drawing and wrote the text on the

filter paper (**Figure 4**). The invisible writing and drawing were made visible by spraying with potassium thiocyanate (KSCN). The experiment is also known as artificial blood (blood magic), in which a reaction takes place between the two reactants, resulting in the formation of dark red iron(III) thiocyanate ($[Fe(SCN)_3]$) (**Eq. 2**) (MEL Science, n. d.).
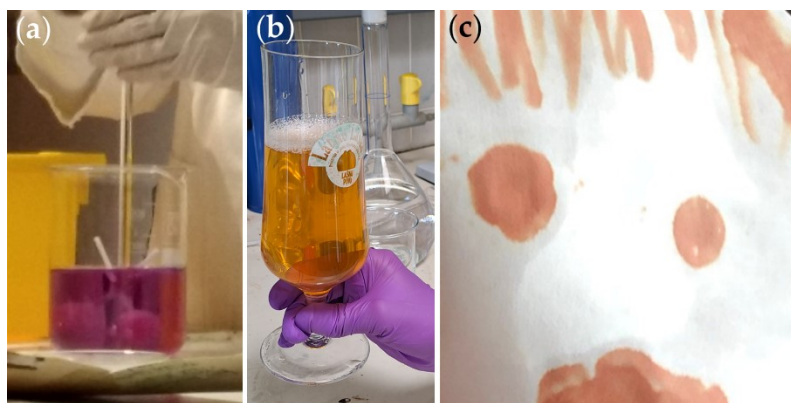
$$FeCl_3 + 3\ KSCN \rightarrow Fe(SCN)_3 + 3\ KCl \quad . \tag{2}$$

Figure 4. Demonstration of: (**a**) the course of a redox reaction between potassium permanganate and sucrose (lollipop) in a basic solution, (**b**) chemical beer and (**c**) an art work product after the reaction of iron trichloride and potassium thiocyanate (Photo: Marko Jeran).

3. Conclusions

Experimental work plays an important role in learning the natural sciences (chemistry, physics and biology). Many natural laws and phenomena of everyday life can be observed through simple chemical and physical experiments. From the interaction between all those involved, it can be concluded that this method of imparting knowledge has proved its worth in hospital work. In addition to this type of learning, we also organized entertainment for the young people, which they really need with such demanding diagnoses. For a moment, they can indulge in effects that literally enchant them. In addition to curiosity and creativity, we have also promoted interdisciplinary cooperation and teamwork.

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

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Reflection

Applications of Archetypal Art Therapy Techniques in School Counselling

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Abstract:
Archetypes are universal symbolic images present in the collective unconscious of every individual. In children, these archetypes can manifest through play, fantasies, dreams, and artistic projects. Art therapy provides children with the opportunity to explore these images and express their internal experiences and conflicts through creativity. For example, the archetype of the mother can be expressed in a child's drawing depicting their connection to a caring adult or feelings of loss and separation. In the article I explore how the archetypes of Shadow and Magician (wise person, wizard, healer) enables adolescents to become aware of their inner resources and find new strategies for responding to life's challenges.

Keywords: Archetypes, Shadow Archetype, Magician Archetype, School counselling, Pupils Mental Health, Psychological Healing

1. Introduction

The modern world is changing rapidly and dramatically. Children are not only witnesses to the challenges of the contemporary world but also participants in complex global processes such as pandemics, international conflicts, the high pace and speed of life, political and economic instability, and so on. All these processes inevitably impact the psychological climate of families and the well-being of adults, which, in turn, affects the mental health of children.

Wellbeing and raising awareness about mental health among children is a strategic goal of "Haileybury Astana," the first international school in Kazakhstan. We teach children that mental health is just as important as physical health and that it is crucial to understand their own mental health and how best to take care of it. As a school psychologist, I think that mental health is just as important as physical health and that it is crucial for children to understand their own mental health and how best to take care of it. Here I share my experience on how archetypal art therapy helps in individual and group counselling with children at school. This article explores how the archetypes of the Shadow and the Magician (wise person, wizard, healer) enable adolescents to become aware of their inner resources and discover new strategies for responding to life's challenges.

2. Diagnostics and psycho-correction effects of archetypal art-therapy in counselling pupils

The main issues for which children seek psychological consultations and meetings include problems related to low academic achievements and abilities, misbehaviour in class such as indiscipline and disobedience, communication and relationship issues with peers including conflicts, low mood, lack of interest and motivation to study, low participation and class engagement, self-image and self-assessment, self-confidence, eating disorders, and addiction to electronic devices. Pupils experience a high workload in high school, with a challenging curriculum and a large volume of material for independent study and homework. In addition, there are daily scheduled clubs, sports sections, and extra lessons with tutors. All of this often leaves insufficient time for leisure, hobbies, and socializing with peers, leading to emotional breakdowns, occasional bouts of aggression, or apathetic and depressive states. Consequently, these issues are symptoms of deeper psychological problems such as: feelings of insecurity and anxiety, feelings of guilt and obligations towards parents, perfectionism and excessive efforts to meet expectations Burnout and demotivation in senior school children

This article explores how the archetypes of the Shadow and the Magician (wise person, wizard, healer) enable adolescents to become aware of their inner resources and discover new strategies for responding to life's challenges.

This article explores how the archetypes of the Shadow and the Magician (wise person, wizard, healer) enable adolescents to become aware of their inner resources and discover new strategies for responding to life's challenges.

In these conditions, the presence of sufficient emotional support from adults, the existence of trusting relatitonships with adults, and the ability to seek and receive support from peers, parents, teachers and specialists become critically important.

Art therapy is an excellent method for working with children of different ages, serving both as a diagnostic tool and a corrective mechanism without intensive interventions from the psychologist. The creative process during activities such as drawing, sculpting, and sand play helps children express their feelings in a non-verbal, softer, and more natural way, allowing them to externalize and explore their internal conflicts. The creative process can have a calming effect, helping children relax and reduce their levels of stress and anxiety.

Archetypal art therapy has proven to be very effective psychological tool in art therapy for pupils because it closely aligns with fairy tale and mythical themes and images, allowing children to create their own stories using archetypal images and symbols (Abbenante & Wix, 2015).

Archetypal art therapy is based on Carl Gustav Jung's theory of 12 archetypes, which are recognized as universal images of the collective unconscious and manifest in myths, legends, dreams, and art. (Martin, 2018). According to Carl Jung (1977), an archetype is akin to a human instinct that regulates psychic life and controls human behaviour at a biological level. In other words, it is an internal regulator of psychic life that determines the quality and nature of mental processes. Thanks to archetypes, people react, experience, and act in a specific manner. An important characteristic of archetypes is their unconscious nature. The opportunity to become aware of inherent or innate patterns arises during exploration in art therapy at the archetypal level.

The possibilities of art therapy in working with children include the use of the following techniques according to Allan and Clark (1985).

- Depicting Archetypal Images: for example, using techniques like the Hero's Journey, Conversations with the Shadow, and Inner Healer (Sage).
- "Mask, I Know You": creating masks through techniques like drawing, sculpting, and papier-mâché.
- "Fairy Tale in the Sand": creating stories using figures and toys that include archetypal elements.
- Creating Mandalas: drawing mandalas with pencils, paints, using colouring books, and incorporating stones.
- "Stone Garden": Stone therapy, which allows for the recreation of scenes and stories through archetypal symbols.

Below are examples of how art therapy can be used through Shadow Archetype and Magician Archetype in individual counselling with schoolchildren.

3. Results and Discussion: Cases

Drawings of the children that were subjected to counseling are shown in **Figure 1**. Written consent has been obtained by the children and their parents to publish the images.

3.1 Case 1

Girl H, 8 years old. was referred for counselling by her parents. The problem under investigation: panic fear of entering school in the morning. Individual sessions using archetypal art therapy explored the figure of fear (Shadow Archetype). The girl painted her fear in the form of a dark frightening figure (**Figure 1A**), which she interpreted as a man who is very strong and does not let her into school, because she could not get the certificate of the best pupil in a term. Subsequently, during several meetings and the compilation of a story about the Shadow, as well as the physical accompaniment of the girl in the morning to the class, the problem of fear and rejection was resolved, also as a result of family therapy with parents.

3.2 Case 2

Girl S, 10 years old, was sent to psychological support by teachers as she refused to go to lunch to the school canteen. However, she was willing to eat if the food was brought to her to the classroom at recess. At an individual meeting, the girl described her condition at entering the dining room as weakness, powerlessness and fatigue. She was asked to draw this state of weakness and fatigue. She drew her head **(Figure 1 B1 and B2)** and composed a fairy tale about many small people appearing in her head who begin to march and make noise when she enters the dining room (Погосова, 2006). When compiling a story about what these people want, the girl composed a story that these little people are from another planet, and that they want to get out of her head and fly back to their planet. After a series of meetings with the use of figurative art therapy, the girl managed to cope with her problem.

3.3 Case 3

Figure 1C shows an image of a figure of fear of a girl who experienced sadness and grief over the loss of her grandmother.

3.4. Case 4

Figure 1D shows an image of the Shadow archetype called the Pest which periodically caused girl S abdominal pain.

In all cases, working in the technique of archetypal art therapy helped children to create stories reflecting their inner experiences and externalize emotions in the form of images and symbols, which means seeing their separation from these images and then, performing transformative actions with them that led to more adaptive behaviour.

Figure 1. Images drawn by A: Case 1 girl, B1 and B2: Case 2 girl, C: Case 3 girl and D: Case 4 girl.

One of the favorite archetypal techniques is the practice of "Inner Healer," which has proven itself well during a pandemic. This resource practice allows to turn to internal forces and consciously dispose of them in moments of difficult situations.

The materials applied for archetypal art-therapy are paper, liquid oil and ear cleaning sticks. Pupils are asked to draw random lines for half a minute, just scratch on a piece of paper with a cotton swab without any idea to depict something. Then the resulting picture is viewed in a mode resembling assessment of an X-ray image. In the random lines, the Inner Healer is sought to manifest himself, or – his message is manifested to suggest what is best for the subject at the given point and what will help him/her to heal.

Children with great pleasure come into contact with an Inner Healer, create and interpret images and symbols regarding him, and decipher his messages. Thus, contact is made with an internal unconscious resource, which acquires clear features and images children's own strength. The author of the drawing presented in **Figure 2** claimed that he would never have been able to arbitrarily draw the image by himself, but with the help of the technique of the Internal Healer, he was able to identify and prioritize his actual needs.

Figure 2. Illustration of the Inner Healer in the form of a smart and cheerful elephant, reminding of the importance of daily routine and hygiene and performing physical exercises.

4. Conclusions

Thus, the attractiveness of the psycho-correction method based on archetypical art therapy with children is based on the following provisions: lack of teaching and moralizing; safe space and psychological security in contact with the adult; imagery, fabulousness and metaphorical language; the presence of mystery and magic. The use of elements of archetypal art therapy helps to understand a child's inner world, explore their true experiences and resources, and integrate unconscious deep elements of the psyche.

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

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Research

Longevity Through Storytelling: The Silver Passport Case Study

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Abstract:
The Silver Passport Case Study investigates the preservation and dissemination of collective wisdom through storytelling, focusing on the active engagement of older adults as knowledge sharers. This initiative addresses the digital divide among older adults in Peru, fostering intergenerational connections through innovative methodologies for capturing and sharing personal histories. The study details the project's inception, implementation, and impact, underscoring its role in enhancing social inclusion and emotional well-being among senior participants. The primary results indicate notable improvements in digital literacy, social connectivity, and emotional health among the older adults. The project effectively bridged generational gaps and fostered mutual understanding between age groups. Future research should explore the long-term effects of digital storytelling on social inclusion and psychological health across different cultural contexts. This exploration is essential for developing strategies to address the challenges of aging populations and the digital divide.

Keywords: storytelling, digital inclusion, intergenerational connections, senior engagement, collective wisdom, emotional well-being

1.   Introduction

Digital literacy among s in Peru remains a significant challenge, despite a reported increase in internet usage to 25% by 2022 (INEI, 2023). This lack of digital skills limits their ability to share valuable life experiences and cultural heritage. The Silver Passport project seeks to bridge this gap by equipping older adults with the necessary tools and platforms to share their stories digitally. This initiative aims to preserve their wisdom and foster inter-generational connections, contributing to cultural preservation and community building (Tessler et al., 2021; Palma, 2024). Barbosa et al. (2019) confirm that digital communication enhances social connectedness among older adults, reducing isolation and loneliness.

Hülür & Macdonald (2020) state that "Digitalization has provided new ways to communicate and develop social ties, contributing to more positive social experiences and well-being in old age." The Silver Passport project adopts a user-centered design approach, essential for developing assistive technologies tailored to the needs of older adults. Wang et al. (2019) highlight the importance of involving older adults in the design process to uncover their unique perspectives, leading to more effective and accepted technologies. They emphasize that "technology can support independent living, but it must align with the real-world needs and preferences of older adults, emphasizing usability, privacy, and co-design participation" (Wang et al., 2019).

Under the mentorship of the International Center for Journalists, the Silver Passport initiative effectively mitigated the digital divide and promoted social inclusion among older adults by involving them in storytelling activities with younger generations.

The project's inception in the post-COVID era back in August 2021 highlighted the critical need to document the life stories and cultural heritage of older adults, who were disproportionately affected by the pandemic's social isolation. By employing innovative methodologies for digital storytelling, the Silver Passport initiative not only preserved the collective wisdom of older adults but also fostered intergenerational connections that contributed to the emotional well-being of all participants.

The focus group sessions and online survey revealed that both older adults and youth valued the opportunity to connect and share stories. The use of familiar technology, such as WhatsApp, facilitated these interactions and made the process accessible (Arbanas et al., 2024).

2.   Material and Methods

The case study method was selected for this project as it is a common approach for conducting qualitative research, allowing for an in-depth exploration of the Silver Passport initiative. Focus groups were utilized as the primary means of data collection, incorporating techniques such as interviewing and observation to gather rich, detailed insights from participants.

The findings suggest that storytelling can be a powerful tool for preserving cultural heritage and enhancing emotional well-being (Dimoulas, 2022). By providing a platform for older adults to share their stories, the Silver Passport initiative fosters a sense of belonging and purpose among older adults participants while bridging generational gaps (Tessler et al., 2021). This aligns with broader trends in cultural heritage management, where user engagement and participation are increasingly recognized as vital components (Palma, 2024).

In addition to focus groups, the study also employed online surveys to gather supplementary data on participants' digital habits and preferences. These mixed methods provided a comprehensive understanding of the project's impact and effectiveness. The integration of qualitative and quantitative data reinforced the conclusion that digital storytelling not only supports the preservation of cultural narratives but also significantly contributes to the social inclusion and emotional well-being of older adults.

2.1. Focus Groups

The focus groups were conducted in El Parque Infantil de Chiclayo, an open area in Chiclayo, Peru, to adhere to COVID-19 prevention measures. This setting was chosen to ensure a safe and conducive environment for interaction between participants.

The focus group sessions were conducted, each involving three older adults and three youth participants. The older adults, aged over 60 years, were considered part of a vulnerable group, particularly impacted by the pandemic's social isolation. The sessions were meticulously designed to facilitate intergenerational connections through structured activities, user testing questions, and technology training. Each session lasted for two hours and included the following activities:

1. Welcome and Introduction: Participants were introduced to the project, and consent was obtained for recording the sessions. This segment aimed to create a welcoming atmosphere and explain the project's objectives and significance.

2. User Testing Questions: Both older adults and youth were asked about their preferences for storytelling formats and platforms. This activity was designed to gather insights into their digital habits and preferences for sharing stories.

3. Technology Training: Participants were trained on using WhatsApp for recording and sharing stories. This practical session aimed to equip both older adults and youth with the necessary skills to utilize digital tools for storytelling.

4. Storytelling Sessions: Older adults shared their stories with the youth, who then reflected on what they heard. This reciprocal storytelling activity fostered a deeper understanding and connection between the generations.

2.2. Online Survey

An online survey was administered to 22 senior participants to gather additional insights into their digital habits and interests. This method was chosen to specifically target older adults who are relatively tech-savvy, ensuring that the respondents had a basic level of digital literacy necessary to complete an online survey. The survey included questions about their internet usage patterns, preferred storytelling formats, and favored platforms for sharing stories. The survey's design aimed to understand the older adults' engagement with digital tools, which is crucial for tailoring the Silver Passport initiative to better suit their needs and preferences. By identifying the most effective formats and platforms, the project could enhance its approach to preserving cultural heritage and fostering intergenerational connections through digital storytelling.

Figure 1. A: Participants of the Silver Passport initiative engage in a focus group session at El Parque Infantil de Chiclayo, Peru. B: A senior participant shares a personal story while a youth participant records the interaction on a smartphone during a focus group session of the Silver Passport initiative in Chiclayo, Peru.

3. Results
3.1. Focus Group Findings
Key findings from the focus groups, which included a total of three older adults and three youth participants, were as follows:
• Participation and Interest: Both older adults and youth showed a strong interest in sharing and hearing stories. Older adults were particularly interested in topics such as the economy, politics, family stories, health, local history, and personal experiences.

- Technology Use: All older adults used WhatsApp, with preferences for text and video formats. Youth participants also favored WhatsApp and expressed interest in podcast formats.
- Content Length: Older adults preferred longer, detailed stories (around 5 minutes), while youth favored shorter stories (1-3 minutes).
- Emotional Impact: Both older adults and youth reported positive feelings after participating, with older adults feeling heard and youth appreciating the opportunity to learn and help.

3.2. Online Survey Results

An online survey was conducted with 22 senior participants to obtain additional insights into their digital habits and interests:

- Internet Usage: Only a small percentage of older adults actively used the internet, highlighting the need for digital literacy support.
- Story Preferences: Older adults expressed a desire to share stories that conveyed personal experiences and cultural heritage.
- Platform Preferences: Older adults were more inclined to share stories via Facebook, while youth preferred platforms like TikTok for content consumption.

4. Discussion

The name "Silver Passport" symbolizes the empowerment of older adults to explore and share their rich life histories and cultural experiences, effectively giving them a 'passport' to connect with the digital world. Participants recounted a variety of stories, from traditional folklore to personal anecdotes, which not only preserved cultural narratives but also provided a sense of identity and continuity for the younger generation.

5. Conclusions

The Silver Passport project, launched in August 2021 post-COVID, aimed to preserve cultural heritage and enhance the emotional well-being of older adults through storytelling. This initiative was introduced to address the digital divide among older adults in Peru, equipping them with the necessary tools and platforms to share their life stories and cultural heritage digitally. The project targeted older adults aged 60 and above as active knowledge sharers, and younger individuals aged 18-30 as listeners and digital facilitators.

By fostering intergenerational connections, the Silver Passport demonstrated the power of storytelling in bridging generational gaps and promoting social inclusion. Integrating digital technology enriched the lives of older adults and provided the younger generation with valuable cultural insights. The positive outcomes observed in both target groups underscore the importance of preserving cultural narratives, contributing significantly to the emotional and social well-being of participants.

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

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Scientists as Bearers for the Interpretation and Teaching of the General Society: Broadening the Model from the Municipality to the National Region

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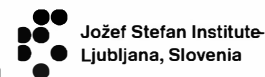
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OUTLINE

Due to the pace of life in modern society, the development of various disciplines, and not least, the progress of science, we have decided to organize monthly forum under the title *"Good Evening, Science"*. In discussions with various experts on everyday topics,, we will shed light on their regularities and look for answers to various questions in a relaxed atmosphere. The event is organized in cooperation with the Library Domžale, where our talks take place.

ACTIVITY

In the first year, we dealt with interesting and current topics, met extraordinary personalities who are active in both the academic and applied fields. At the end of the event, a popular article is always published in the local newspaper, informing citizens and conveying the basic message of the event. The event was well received by the residents of the region. After the main description of the topic and the introduction of the guests, the moderator (M. Jeran) leads a 90-minute discussion and enriches the event with various anecdotes. The event attracts many visitors every month who follow the discussion and ask various questions. Questions from the general public are very welcomed as they usually further support some scientific content in practice (**Figure 1**).

Events in a 1st year

- Urban planning
- Water and its influence on the quality of our lives
- Modern society and mental health care
- The role of women in modern society
- Stories from the past and the preservation of traditions – cultural heritage as the basis for our future
- The influence of music and dance on human brain function

Figure 1: Highlights of the last event *"The influence of music and dance on human brain function"*. It was particularly festive because, in addition to a neurologist, a cognitive scientist and a doctor turned singing teacher, we also had young dancers from the Ana Ballet School, Domžale and a young singer from the Voice Academy as guests. It turned out that even fundamental scientific topics can be combined with art. We discovered that music and dance have influence our brains. Studies have shown that listening to music reduces anxiety and pain, lowers blood pressure and improves sleep quality, mood, mental alertness and memory. If we want to keep our brains active as we age, listening to and playing music is an excellent tool. It provides a complete workout.

CONCLUSION

An important task of every scientist is to communicate the passion for their work and its importance to society. This includes communicating effectively with other professionals and people outside their own field, as well as with the general public. Science and technology are increasingly changing modern life. To make informed decisions about health, the environment and modern technologies, we need accurate, unbiased information. In this case, scientists are the most direct and constructive source of information. Through such activities, decision-makers in areas such as regulation, science policy and funding can be influenced, which can have a significant impact on scientific progress and also lead to advances in society.
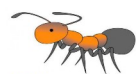
Insect Bioconversion Technology of Black Soldier Fly (Hermetia Illucens) Larvae in the Transition to a Sustainable Food System

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OUTLINE

One of the greatest global challenges of the 21st century is to meet the ever-increasing demand for food while limiting the negative impact of agriculture on the environment and nature. As the human population continues to grow, the demand for proteins of animal origin increases proportionally. If the trend of population growth continues, food production will have to almost double in the coming years. Climate change and the accumulation of organic waste also pose an increasing challenge, and the escalation of food production will only exacerbate the associated problems. One of the most attractive alternatives are insects, which have been part of the human diet since ancient times and can easily be included in the daily diet.

BLACK SOLDIER LARVAE

The black soldier fly originally comes from South America, from where it has spread all over the world. It occurs in various colours, from yellow and green to dark blue and black. Its head is small and narrower than its body, its eyes are compound, quite large and widely spaced. The entire life cycle of the black soldier fly lasts 40 to 45 days and consists of four stages: egg, larva, pupa and adult fly.
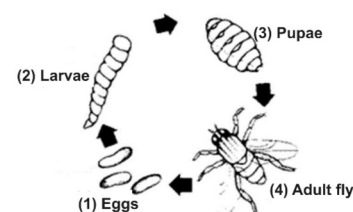
LIFE CYCLE

Figure 1: Life cycle of the black soldier fly. (**1**) The first phase is the egg phase. The female lays 500-900 white, oval-shaped eggs. This phase lasts 4 days, after which the larvae hatch from the eggs. (**2**) The next phase is the larval stage, which lasts 18 days. During this time the larva goes through 6 molts. During this time, the insect is already suitable for consumption. (**3**) The third phase is called the pupal stage. It lasts about 8 days and is divided into 4 sub-stages – ecdysis, cryptotheca pupa, phanerocephalic pupa and pharate adult fly. (**4**) The last phase is the adult fly stage, which lasts up to 2 weeks.

METHOD
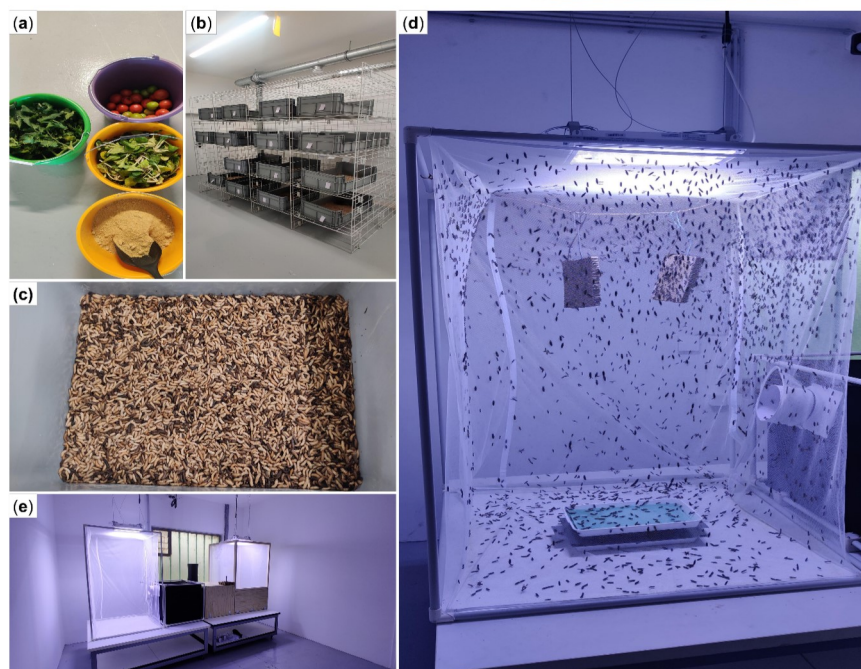
Larval rearing began with approx. 40,000 larvae. The larvae were sorted into 4 weight groups and placed in 8 small plastic trays containing about 1 kg of substrate. The substrate consisted of 35% chicken feed and 65% water, which should ensure optimal conditions for survival and growth given the sensitivity of their larvae in the early life stages. After 4 days, the larvae were transferred to larger dishes with more substrate. Additional substrate was applied at each feeding stage to ensure continued larval growth. Once the larvae had reached the desired size and colour, they were separated from the substrate using a sieve system and then frozen in liquid nitrogen for later analysis.

When the larvae had reached the later developmental stage, suitable containers were selected and the larvae were separated from the feeding substrate. Some larvae were then transferred to dark cages where they developed into adult flies. After hatching from the dark cages, the flies were released into specially lighted cages for mating, where mating and oviposition took place. Egg collection was made easier by the use of wire mesh to create oviposition chambers above the food containers. The hatched larvae were used for subsequent breeding cycles. This process was repeated depending on the procedure to ensure a uniform age of the organisms and to optimize the efficiency of breeding and reproduction.

Figure 3: (**a**) The substrates used, such as plant residues, tomatoes (fruits and leaves) and cornmeal. (**b**) Breeding larvae intended for inactivation in a specific area on wire racks. (**c**) A container filled with substrate and approximately 10-day-old larvae. (**d**) Breeding larvae intended for maintaining a colony and mating. In such a cage, the larvae mature into adult flies. (**e**) Preparation of cages in which the larvae mature into adult flies.

RESULTS

Table 1: Composition of the samples and their yields.

Sample	Age	Composition at inactivation	Substrate composition (%)	Feed Conversion (FCR)[1]	Yield (%)
1	14	FRASS + larvae FRASS Larvae	Corn meal (22.20) Water (55.60) Chicken feed (22.20)	6.53	15.31
2	16	FRASS + larvae FRASS Larvae	Tomato, fruit (34.32) Water (45.06) Chicken feed (11.63) Sugar beet (7.72) Tomato, leaves (1.28)	6.96	14.37
3	16	FRASS + larvae FRASS Larvae	Tomato, fruit (44.76) Tomato, leaves (12.11) Corn meal (23.98) Sugar beet (15.06) Water (4.10)	8.23	12.15
4	30	FRASS + larvae FRASS Larvae	Corn meal (46.51) Water (53.49)	14.14	7.07
5	22	FRASS + larvae FRASS Larvae	Corn meal (29.44) Sugar beet (11.23) Water (48.13) Tomato (5.35) Eggplant (5.85)	11.99	8.34
6	17	FRASS + larvae FRASS Larvae	Chicken meal (39.19) Water (60.81)	10.10	9.90

[1]Feed conversion ratio (FCR) is the conventional measure of livestock production efficiency: the weight of feed intake divided by the weight gained by the animal. Lower FCR values indicate higher efficiency. FCRs are typically 6.0–10.0 for beef, 2.7–5.0 for pigs, 1.7–2.0 for chicken, and 1.0–2.4 for farmed fish and shrimp.

CONCLUSION

The larvae in the samples grew at different rates. It was found that the larvae fed with cornmeal, water and chicken feed had the highest mass yield, but these larvae also consumed the greatest amount of the initial substrate. The larvae that gained the least weight were those fed only cornmeal and water, although they also consumed a considerable amount of substrate. The best relationship between substrate consumption and weight gain was observed in larvae fed tomatoes, water, chicken feed and sugar beet.

Practical Approach in the Chemistry of Natural Sources on the Example of Studying the Qualitative Effect of *Nicotiana tabacum* Plant Macerates on Baker's Yeast

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OUTLINE

The tobacco plant *Nicotiana tabacum* (**Figure 1**) is a perennial plant characterized by upright growth and glandular hairy plant parts. It belongs to the *Nicotiana* genus and the nightshade family (*Solanaceae*). It is a slightly branched plant that reaches a height of 1 to 2 meters. It is characterized by large green leaves that are oblong-lanceolate and pointed at the end. The flowers are stalked and white, pink or even red, 4 to 5 cm long and usually in broom-like inflorescences. All parts of the plant are sticky and covered with short hairs that secrete a yellow secretion. These are plant metabolites, alkaloids, the best known of which nicotine. In addition to it, tobacco leaves also contain other alkaloids such as nicotimine, anabasine, anatalline and nor-nicotine. In addition, organic acids such as caffeic acid and oxalic acid as well as terpenoids were found in the leaves. *Nicotiana tabacum* has antibacterial, antifungal and antimicrobial effects, acts on the nervous system, cardiovascular system, digestive tract, exocrine glands, has a hematologic effect and affects body weight (Kishore, 2014).

Figure 1: (**a**) Dried tobacco and (**b**) the structure of the biologically active substance nicotine contained in tobacco.

Nicotine (**Figure 1**) is present in all parts of the tobacco plant but especially in the leaves. It represents between 0.3 and 5 % of the dry weight of the plant. Its biosynthesis takes place in the roots, and it accumulates in tobacco leaves. It is a powerful neurotoxin and a component of numerous insect control preparations. In low concentrations, nicotine can be a stimulant, which unfortunately leads to excessive smoking. After smoking tobacco leaves, nicotine breaks down into pyridine, furfural, collidine, hydrocyanic acid, carbon monoxide and others. The toxic effects of tobacco smoke are due to these compounds (Kishore, 2014).

Figure 2: *Saccharomyces cerevisiae* on agar plate. The cells are spherical to egg-shaped and have a diameter of 5 to 10 μm.

In the research work, extracts were prepared from the dry tobacco leaves using three solvents of different polarity, which were then tested on the Baker's yeast (*Saccharomyces cerevisiae*). *Saccharomyces cerevisiae* (**Figure 2**) is a unicellular organism and belongs to the yeast genus. Since ancient times it has played an important role in wine production, baking and brewing. It is one of the best-studied eukaryotic organisms, which is why *Saccharomyces cerevisiae* is also referred to as a model organism in molecular and cell biology. An important characteristic of a model organism is rapid growth and reproduction, which in the case of yeast is called budding or sexual reproduction (Hartwell, 1974).
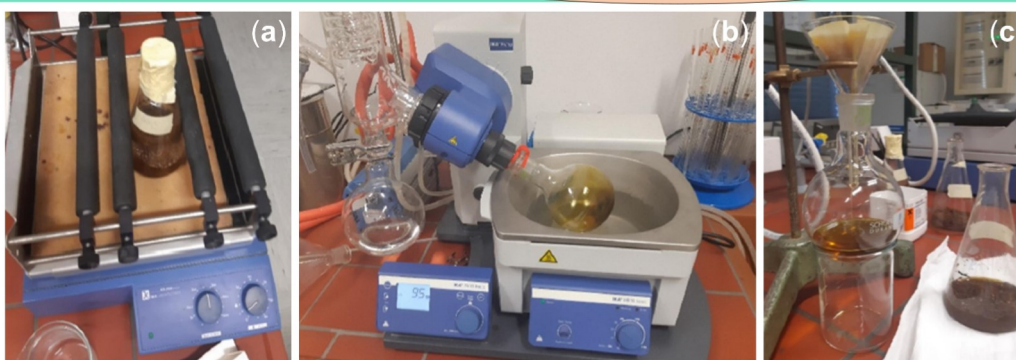
EXPERIMENTAL METHODS

Figure 3: Maceration process or extraction *"solid – liquid"*.
(**a**) Exposure of the tobacco to an organic solvent: shaking of the contents on a laboratory shaker, (**b**) separation of the liquid and solid phases after maceration and (**c**) evaporation of the solvent under reduced pressure a rotary evaporator.

Maceration
20.00 g of tobacco (Blue Camel, Reynolds Tobacco Company) was weighed into each of three Erlenmeyer flasks and poured with 250 mL of extraction solvent (ethanol, dichloromethane, or hexane) (Sigma-Aldrich). The Erlenmeyer flasks were closed airtight and placed on a laboratory shaker. Maceration on the mixing table was carried out for 4 days at 250 rpm. The contents were separated by filtration and the liquid was evaporated on a rotary evaporator (**Figure 3**).

Biological activity
Dry baker's yeast (Spar, Slovenia) suspended in brine (100 mg in 2 mL of 0.9% aqueous NaCl solution) was aseptically inoculated onto the prepared YGC agar culture medium (Sigma-Aldrich, 9576). By using an L-shaped field hockey-stick, we ensured an even distribution on the culture medium.

RESULTS

Three different solvents were used to macerate dry tobacco leaves, which differed in their dielectric constant (measure of polarity, ε): ethanol (ε = 22.4), dichloromethane (ε = 8.9) and hexane (ε = 1.9) (Jeran et al., 2021). The largest amount of the isolate was obtained by extraction with dichloromethane, slightly less with ethanol and the least with hexane (**Table 1**).

The results of biological activity on *Saccharomyces cerevisiae* showed that the zone of inhibition was formed only in culture media in which concentrated ethanol macerate was present. Since ethanol is the most polar of the three solvents used, this indicates that a wider range of compounds was extracted with it. The other two solvents were more selective extracting less polar components which did not show any noticeable activity.
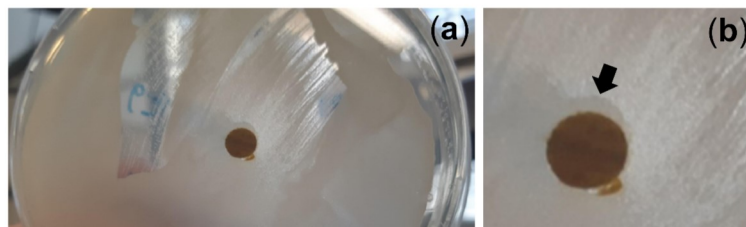
Table 1: Isolated masses of oils after maceration.

Maceration solvent	Weight of macerate (g) per 20 g tobacco
Ethanol	1.5
Dichloromethane	1.7
Hexane	1.2

For quick and effective identification of nicotine in prepared extracts, UV/Vis spectroscopy can be used in school laboratories to determine the absorbance of the desired solution. The method is based on the reaction of nicotine with potassium permanganate in the presence of sodium hydroxide, resulting in a water-soluble green product with an absorbance maximum at 610 nm (Al-Tamrah, 1999). Looking at the curve of the macerates, it is clearly seen that the peaks of the characteristic signal for nicotine are of different sizes, which indicates different nicotine contents. The method described method is simple and easy to understand, which is why it has been included in secondary school lessons (and practical experimental work).

Figure 4: The solid diffusion method is a simple method for determining the enhancement of a model organism against a target drug (*e.g.* fungicide). There is an area around the application site where yeast has not grown – this is called the inhibition zone. The diameter of the inhibition zone is measured with a ruler. The agent with the largest inhibition zone is the most effective in killing this type of model organism. In this case, we can observe the formation of the largest inhibition zone of *Saccharomyces cerevisiae* with ethanol macerate.

CONCLUSION

The results of the experimental work show that a highly polar solvent dissolve many components of the plant material, which together contribute to the biological activity. Stereoisomerism must also be considered, especially of the main active ingredient nicotine. Nature knows how to synthesize one form of stereoisomers, and it often turns out that one form is harmful, while the other can be beneficial.

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Peptide designing for Triple-Negative Breast Cancer treatment using Bioinformatics tools

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ABSTRACT

Binding of the ligand to the NOTCH receptor causes the cleavage of this receptor and ultimately the separation of the internal part of the NOTCH receptor, which forms the NICD-MAML1-CSL complex in connection with the MAML1 and CSL proteins. This complex acts as a transcription factor, and the transcription of genes affected by it is one of the factors that cause triple-negative breast cancer. This signaling pathway is active in triple negative breast cancer stem cells and plays a role in resistance to treatment and metastasis. Targeting the NOTCH signaling pathway by inhibiting the NICD-MAML1-CSL complex can play a role in the treatment of triple negative breast cancer.

We designed two antagonist peptides for CSL protein called Antagonist 1, and Antagonist 2 for blocking NICD-MAML1-CSL complex that is involved in mechanisms leading to TNBC, by mutating the native CSL protein, and using Molecular Dynamics simulations.

METHODS

We first chose a sequence of the CSL protein to mutate, and after that, we mutated the sequence by the OSPREY program, which gave us two antagonist peptides. Then, the antagonist peptides were rationally mutated by using the Nanome program. We simulated the two antagonist peptides using the molecular dynamics algorithms, by GROMACS 2022.1. Analysis were conducted by GROMACS 2022.1, and g-MMPBSA programs.

CONCLUSION

The overall RMSD diagram of all three complexes shows that these complexes have stabilized.

Examining the results of MM-PBSA analysis shows that the binding free energy without considering the polar solvation energy in antagonist 1 complex is more negative than antagonist 2 complex and the native complex, which indicates the effect of mutations in antagonist peptides 1 and 2. The ligand and receptor binding have become stronger in both complexes. Also, the different mutation in the C chain causes the binding of antagonist 1 with the receptor to be stronger than that of antagonist 2.

RESULTS

a)

b)
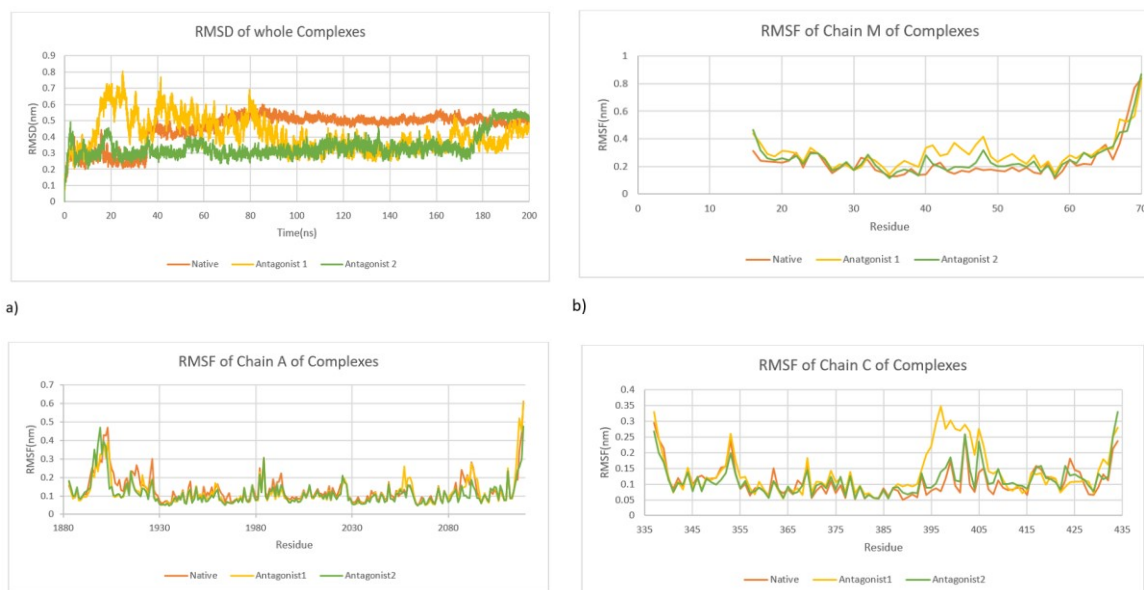
Complex / Energy	Native	Antagonist 1	Antagonist 2
Polar Solvation Energy	1451.833 +/- 170.732 kJ/mol	1526.802 +/- 173.236 kJ/mol	1520.521 +/- 478.938 kJ/mol
SASA	-69.563 +/- 4.996 kJ/mol	-72.487 +/- 3.253 kJ/mol	-69.951 +/- 20.263 kJ/mol
Binding Energy	845.701 +/- 102.405 kJ/mol	783.224 +/- 104.308 kJ/mol	823.375 +/- 359.531 kJ/mol

MM-PBSA analysis of all three complexes

Figure 1: