




# SOCRATIC LECTURES



10TH INTERNATIONAL SYMPOSIUM, LJUBLJANA, 9. DECEMBER 2023  
PEER REVIEWED PROCEEDINGS, PART II  
EDITED BY:  
VERONIKA KRALJ-IGLIČ,  
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## Socratic Lectures

### 10th International Symposium, Ljubljana, December 9, 2023

Peer Reviewed Proceedings, Part II

Edited by Veronika Kralj-Iglič, Yelena Istileulova and Anna Romolo

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Program of the Symposium Socratic Lectures, December 9, 2023, 10:30 – 14:00 (Ljubljana time)

10.30 Welcome to participants (Veronika Kralj-Iglič, University of Ljubljana) <https://uni-lj-si.zoom.us/j/98919724250>

10.35 -11.15 Plenary lecture: Bernd Giebel, Institute for Transfusion Medicine University Hospital Essen, Germany: Clinical Potential of MSC-EVs and Translational Challenges

## Scientific sections

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

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

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

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

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

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

### Section 2 : Human medicine orgnaized by Larisa Melia

#### Second group:

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11.50 - 12.10 **Khelaia A:** Metabolic endotoxemia and male infertility

12.10 - 12.25 **Sulukhia R, Melia L, Davidova N, Pkhaladze L:** Pregnancy complications in patients with endometriosis

12.25 - 12.45 **Mantskava M:** Blood rheology properties in patients with acute lymphoblastic leukemia

12.45 - 13.00 **Khatuna K:** Sebaceous nevus in children: clinical-dermoscopic differences

13.00 - 13.15 **Jabua M, Gognadze T:** Jprner's syndrome caused by ultrasound guided supraclavicular nerve block (Aversi Clinic)

13.15 - 13.30 **Vojkovič R:** Epidemiology of periprosthetic proximal femur fractures



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### Section 3: Veterinary medicine

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- 11.30 - 11.50 Mamukelashvili N, Kalandia E,** Georgian Mountain Dog – Kartuli Nagazi
- 11.50 - 12.10 Mamatsashvili G, Kereselidze M, Beruashvili M, Mikadze K:** Justification of the ways of landscape-epizootological-ecological monitoring in order to determine the risk of a possible or expected outbreak of anthrax disease and to minimize the risk
- 12.10 - 12.30 Nebieridze S, Kereselidze M, Beruashvili M, Zibzibadze M:** Food safety problems in Georgia
- 12.30 - 12.50 Arko M et al.:** Extracellular particles from bovine and equine milk
- 12.50 - 13.10 Chkhikvishvili M, Milashvili N, Omarashvili N:** Benefits of flank spay in cats – compared to midline spay in cats - compared to midline spay
- 13.10 - 13.30 Plavšič Z, Bee-Wellness:** Apitherapy and the pursuit of vitality

### Section 4: Veterinary medicine

**Second group:** organized by **Vladimira Erjavec**

- 11.30 – 11.50 Šimundić M:** Urinary tract infection in dog and cat
- 11.50 - 12.10 Dučić N:** Feline tooth resorption - Experiences from the clinic of veterinary dentistry at University of Sarajevo
- 12.10 - 12.30 Vejzović A:** Exotic animals vascular system characteristics and its application in clinical practice
- 12.30 - 12.50 Beletić A:** Glycosylation research in bovines-the significance and recent updates
- 12.50 - 13.10 Vasić Vilić J:** Apitherapy - our experience
- 13.10 - 13.30 Erjavec V, Lukanc B:** Retrospective review of 27 cases of congenital portosystemic shunt in dogs from 2015 to 2023

### Section 5: Physiotherapy organized by **Renata Vauhnik**

- 11.30 - 11.45 Ošlak A,** Effects of therapeutic approaches in treating varus malalignment of the knee joint
- 11.45 - 12.00 Vrbinc NK,** The Impact of sleep deprivation on physical activity
- 12.00 - 12.15 Urbančič Š,** Physiotherapy approach for treating lateral epicondylalgia
- 12.15 -12.30 Štuhec M,** Physiotherapy approach for treating De Quervain tenosynovitis during and after pregnancy
- 12.30 - 12.45 Močilar M,** Telerehabilitation to improve balance and mobility in patients post- stroke
- 12.45 - 13.00 Battalian T,** Respiratory management of Acute Respiratory Distress Syndrome (ARDS) in the ICU from early diagnosis: narrative review
- 13.00 - 13.15 Hemery K,** Adherence of musculoskeletal patients to home exercise programmes
- 13.15 - 13.30 Vauhnik R, Zuil Escobar JC, Martinez Cepa C,** Functional approach to musculoskeletal injuries in physiotherapy: A COIL project in Physiotherapy



**Section 6: Prosthetics** organized by **Monika Jenko, Drago Dolinar, Boštjan Kocjančič**

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- 12.15 - 12.30 **Merčun A**, Trabecular tital hip arthroplasty - glimpse of past
- 12.30 - 12.45 **Hojker M**, Implant failure in metal-on-metal hip endoprosthesis
- 12.45 - 13.00 **Kocjančič B**, Oxidized zirconium in hip prosthesis
- 13.00 - 13.15 **Dolinar D**, Study of surface and biointerface phenomena of implant materials and biosystems
- 13.15 - 13.30 **Jenko M**, The mechanisms of premature fracture in modular-neck stems made of CoCrMo/Ti6Al4V and Ti6Al4V/Ti6Al4V Alloy

**Section 7: Green transition** organized by **Tjaša Griessler Bulc, Sylwester Rzoska**

- 11.30 - 11.50 **Rzoska S**, When physics meets life: high pressure processing for food and pharmaceuticals
- 11.50 - 12.10 **Zaskavska K, Salun M**, Strategies for resilience in a dynamic world. From VUCA to BANI
- 12.10 - 12.30 **Lavtižar V**, Circular Economy: Designing a new, greener world
- 12.30 - 12.50 **Cepec E, Griessler Bulc T, Istenič D**, Uncovering algae biomass potentials: from wastewater to biostimulants
- 12.50 - 13.10 **Klemenčič L, Istenič D, Griessler Bulc T**, Challenges of the efficient algae harvesting
- 13.10 - 13.30 **Mežnar E, Štuhec A, Istenič D, Godič Torkar K**, Occurrence of antimicrobial resistance in bacteria of faecal origin at different stages of treatment in two municipal waste water treatment plants

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- 11.30 - 11.50 **Uridia R, Kereselidze M, Barbakadze N, Karkashadze N, Tserodze N**, Aflatoxin-inhibiting Adsorptive Nanocomposites Based on Natural Raw Material - Lignin
- 11.50 - 12.10 **Nenadović M, Kržišnik K, Trebše P, Bavcon Kralj M**, Effect of time, pH, alcohol and sugar content on nicotine release from pouches available on Slovene market
- 12.10 - 12.30 **Kaporov A, Zore A, Maniecki T, Trebše P**, Synthesis and application of Co-MOF compounds based on 2-methylimidazole and 2,5-dihydroxyterephthalic acid
- 12.30 - 12.50 **Roganović A, Istileulova Y**, Lithium-ion battery project: ESTEAM perspectives
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- 11.45 - 12.00 **Schlosser G**, Mass spectrometry of samples with extracellular particles



- 12.00 - 12.15 **Michelini S**, Quantification of growth and inflammatory factors in platelet- and EV-rich plasma (PVRP): an update
- 12.15 - 12.30 **Starzonek S**, Following erythrocyte sedimentation by optical methods
- 12.30 - 12.45 **Penič S**, Sensor for sedimentation of erythrocytes
- 12.45 - 13.00 **Berry M**, Validation of Interferometric Light Microscopy for assessment of extracellular vesicles in plasma: Preparing the path for future clinical practices
- 13.00 - 13.15 **Paliska N**, Operating sirens - The highest notes in Mozart's vocal works
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- 11.50 - 11.10 **Robledo G**, Microfluidics for extracellular vesicles
- 12.10 - 12.30 **Schabussova I**, Bacterial extracellular vesicles
- 12.30 - 12.50 **Cillo F**, Extracellular vesicles – virus interplay
- 12.50 - 13.10 **Vaino S**, In vitro analysis of farm extracellular vesicles
- 13.10 - 13.30 **Kwang-Pyo K**, Mass spectrometry – based extracellular vesicles analyses

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- 11.40 - 11.50 **Viltušnik B**, Industry 4.0 and sustainable development
- 11.50 - 12.00 **Oberleitner A**, Controlled modification of biopolymers and their versatile applications
- 12.00 - 12.10 **Bolka S, Nardin B, Pešl T, Rozman T**, The effect of size and surface treatment of nucleating agents on PA6 morphology studied by Flash DSC
- 12.10 - 12.20 **Verbič A, Golja B, Likozar B, Novak U**, Unveiling PFAS-free solutions for Hydrophobic and Oleophobic textile coatings
- 12.20 - 12.30 **Hriberšek M, Kulovec S**, Design Optimization and Fatigue Evaluation of Wood Composite Gears
- 12.30 - 12.40 **Bolka S, Nardin B, Pešl T, Rozman T**, The Use of Different Fractions of Waste Thermosets for Thermoplastic composites
- 12.40 - 12.45 **Kusič D**, Sustainable use of polymers using the AMT2P e-platform in the field of robotics, 3D printing and injection molding
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- 12.50 - 12.55 **Kusič D**, Optimisation of Production by 3DP
- 12.55 - 13.00 **Kusič D, Kusič D**, Presentation of the AIS measuring system for polymer injection control



**13.00 - 13.10 Sever Škapin A, Mušič B, Weathering effect of the cellulose acetate microplastic from discarded cigarette butts**

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**12.10 - 12.30 Mesarec L, Orientational ordering of curved membrane proteins attached to curved membrane surfaces**

**12.30 - 12.50 Benčina M, Stainless steel in biomedical applications**

**12.50 - 13.10 Rawat N, Nanostructured TiO<sub>2</sub> for selective biological response**

**13.10 - 13.30 Birk L, Use of gaseous plasma for dental applications**

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**13.00 - 13.10 Chikh N, Effect of a lightning strike on an airplane**

**13.10 - 13.20 Pinilla Doblaz E, The Doppler effect**

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**12.30 - 13.00 Krajnc T, Goričan G, Čokor E, Bucik D, Kralj S, Particle description of nature**

**13.00 - 13.10 Savujec M, Caloric effects in liquid crystals**

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- 11.45 -12.00 **Nurseitov A**, Innovative Approaches to Language Learning through the Lens of Use Artificial Intelligence
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- 12.15 - 12.30 **Dybach I**, Features of the formation of cross- cultural communication skills in it business
- 12.30-12.45 **Vlasenko T, Nemashkalo K, Shapovalova I**, Digital transformations: trends and perspectives worldwide
- 12.45-13.00 **Salun M, Zaslavska K**, Strategies for resilience in a dynamic world: from VUCA to BANI
- 13.00 - 13.15 **Haborets O, Kushkovyi A**, OSINT Technologies: Applications and Challenges in the Digital Age
- 13.15 -13.30 **Lunhol O, Torhalo P**, Artificial Intelligence in Law Enforcement: current state and development prospects

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- 11.30 - 11.50 **Kublashvili B, Maglakelidze T**, Transformative trends: A paradigm shift to student-oriented learning in higher education
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- 13.10 - 13.30 **Istileulova Y**, Innovations, arts and artificial intelligence: Innovative teaching and learning through STEAM approach (SMS method)

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- 12.00 - 12.15 **Drab M**, How to shape cells using membrane inclusions
- 12.15 - 12.30 **Turk J**, How to model small length scale deformations of injection-molded parts
- 12.30 - 12.45 **Jan M**, How to think about the nature of Descartes' ideas
- 12.45 - 13.00 **Sojicka AA**, How to think about Universal patterns of global population change: from doomsday day growth to the reversed criticality
- 13.00 - 13.15 **Kostanjevec J**, How to say the right thing? Remarks on expresivity and thinkability in recent debates on category theory
- 13.15 - 13.30 **Marin A**, How to hide: Camouflage from ultraviolet to infrared





**13.35-13.55 Honorary lecture for all participants**

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

**13.55-14.00 Cultural program**

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

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



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## Editorial

Proceedings of the **10th Socratic Lectures** is divided into two parts as we obtained 47 contributions that were after review accepted for publication; the Part I is composed of 30 papers and the Part II is composed of 17 papers and 10 posters that were presented at the Symposium. This volume presents Part II. The 10<sup>th</sup> Socratic lectures consisted of three events: an Organ recital of Roberta Schmid from Naples, Italy at the Church of Assumption, Tromostovje on Friday, 8.12. 2023, the Scientific symposium on Saturday, 9.12.2023, online and a social - cultural event on Monday, 11.12.2023 the accompanying social event was held at the Academy of Music, Palace Kazina. The event included classical and contemporary music performed by musicians and by scientists. We have embedded Socratic lectures into the so-called Z-STEAM project (STEAM means Science, Technology, Engineering, Arts and Mathematics). Welcome to the future activities within Z-STEAM project. We welcome contributions that support life-friendly methods and collaboration in all fields of knowledge and creation. Contact [kraljiglic@gmail.com](mailto:kraljiglic@gmail.com) or [annaromolo@gmail.com](mailto:annaromolo@gmail.com)

Veronika Kralj-Iglič, Yelena Istileulova and Anna Romolo



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# Myalgic Encephalomyelitis/Chronic Fatigue Syndrome – Etiology, Pathophysiology, Diagnosis and Treatment

Zupanc Timon<sup>1,\*</sup>, Milovanović Branislav<sup>1</sup>

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## Abstract:

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex condition characterized by a broad spectrum of overlapping symptoms and manifesting in multiple systems of the body. Patients experience symptoms such as profound fatigue, post-exertional malaise (PEM), unrefreshing sleep, and cognitive impairments. It affects millions worldwide, yet much is still unknown about its etiology and pathophysiology. The condition's onset is frequently linked to infectious triggers, including viral infections, suggesting a dysregulated immune response as a central component. Diagnosing ME/CFS poses significant challenges due to many unspecific symptoms that overlap with various other conditions. Current treatment strategies focus primarily on symptomatic relief and lifestyle modifications to manage disease impact. The COVID-19 pandemic has further spotlighted ME/CFS, drawing parallels between long COVID and ME/CFS symptomatology and underscoring the urgent need for comprehensive research.

**Keywords:** Myalgic Encephalomyelitis, Chronic Fatigue Syndrome, Infectious trigger, Post-Exertional Malaise, COVID-19, Post-COVID syndrome



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## 1. Introduction

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex and disabling, multi-systemic chronic disease affecting millions of people around the world. Despite its increasing prevalence and debilitating nature, it is still largely unknown to both the public and many medical professionals. Understanding of its etiology and pathophysiology is limited, diagnostic criteria inconsistent, and diagnostic and treatment guidelines inadequate and outdated (Committee on the Diagnostic Criteria for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome, 2015). The disease was initially defined only as a “persistent or relapsing, debilitating fatigue of new onset and unknown origin” (Holmes et al., 1988). In recent years it has been established that it is a disease characterized by a broad spectrum of overlapping symptoms manifesting in multiple systems of the body. These symptoms are often widespread, and shared by many other conditions, making ME/CFS difficult to diagnose. Marked variability in presentation, severity, course, and duration of disease in patients as well as lack of a definitive diagnostic test or biomarker, has resulted in controversy and lack of consensus around its diagnosis, with over a dozen different clinical definitions being used. The inconsistency in diagnostic criteria, along with lack of interest and funding, made it difficult to conduct conclusive research and studies that would help us better understand this disease (Committee on the Diagnostic Criteria for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome, 2015; Bested and Marshall, 2015).

## 2. Etiology and pathophysiology

The onset of disease appears to be precipitated by both genetic and environmental factors (Bested and Marshall, 2015). Very often there is a triggering event present at the start of the illness, usually a prolonged infection. Infectious triggers connected to ME/CFS include: Epstein-Barr virus (EBV), Cytomegalovirus, Varicella-Zoster virus, *Borrelia burgdorferi* (Lyme disease), *Coxiella burnetii* (Q fever), parvovirus B19, Coxsackie B virus, *Chlamydia pneumoniae*, mycoplasma, and SARS-CoV-2. Patients may also experience reactivation of previously latent infections (eg. Herpes viruses) (Frémont et al., 2009). Several studies found high titers of specific EBV antibodies in ME/CFS patients as well as the presence of a specific IgG that was not detected in healthy individuals with previous EBV infection (Loebel et al., 2017). The onset of ME/CFS could be explained as an EBV infection-triggered immune system dysregulation. One of the most consistent findings in ME/CFS patients is poor cytotoxic activity of natural killer (NK) cells (Bateman et al., 2021). NK cells are the body’s first line of defense and play a crucial role in surveillance against tumor cells and infections, preventing latent viruses from reactivating. A study of cytokine networks has found significant alterations in the cytokine profiles of ME/CFS patients, which showed differences in the relationships between the cytokines when compared to healthy controls (Broderick et al., 2010). A common hypothesis states that an abnormal antiviral immune response to the original state of microbial inflammation triggers a state of low-grade systemic inflammation. There is decreased ability to fight active or latent infections due to decreased NK cell function as well as chronic immune activation even in the absence of microbial infection, characterized by consistently elevated levels of proinflammatory cytokines and oxidative stress. This constant proinflammatory state of the body may bring about changes in different systems of the body, explaining the wide range of symptoms of ME/CFS. Another mechanism heavily contributing to the severe lack of energy seen in patients is the impairment of aerobic respiration. Mitochondria are a major producer of reactive oxygen species (ROS) in the cell which also makes them extremely susceptible to damage by oxidative stress. Increased levels of proinflammatory cytokines and increased oxidative stress damage mitochondrial DNA and membrane and interfere with the process of oxidative phosphorylation, resulting in decreased ATP production by mitochondria (Bested and Marshall, 2015).

## 3. Symptoms and manifestations

ME/CFS manifests itself in multiple systems of the body. Patients usually exhibit a cluster of signs and symptoms. Some develop all symptoms shortly after the onset, while others develop additional symptoms over time (Committee on the Diagnostic Criteria for Myalgic



Encephalomyelitis/Chronic Fatigue Syndrome, 2015). Fatigue is a broad and common symptom that presents in many different diseases. However, the fatigue experienced by ME/CFS patients is more profound, intense, debilitating and longer-lasting than in other diseases. It is also not a consequence of ongoing exertion and is not alleviated by rest (Bateman et al., 2021). Post-exertional malaise (PEM) refers to the worsening or exacerbation of disease symptoms following physical or mental exertion that was normally tolerated before disease onset. It is the hallmark symptom of ME/CFS, distinct from symptoms of other chronic diseases (Committee on the Diagnostic Criteria for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome, 2015). PEM differs from normal exercise intolerance in that it has a delayed onset, an abnormally prolonged recovery period. It usually sets in 12-48 hours after activity and may last anywhere from a few days to many weeks (Jason et al., 2021). Most patients with ME/CFS experience sleep dysfunction. The most common sleep-related symptom that patients report is unrefreshing or non-restorative sleep, often describing it as “feeling as tired upon waking as before going to bed” (Bateman et al., 2021). Patients also suffer from symptoms of neurocognitive dysfunction. When asked to summarize their problems, they usually characterize them as so-called “brain fog” (Bested and Marshall, 2015). This includes symptoms such as confusion, absent-mindedness, difficulty concentrating, disorientation, inability to multitask, and problems with short-term memory (FDA, 2013). In ME/CFS both the sympathetic and parasympathetic parts of the autonomic nervous system (ANS) and the equilibrium between them may be disturbed, manifesting in dysautonomia (Van Cauwenbergh et al., 2014). The most prevalent manifestation of dysautonomia, present in up to 97% of patients is orthostatic intolerance (OI) (Committee on the Diagnostic Criteria for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome, 2015). OI is a group of clinical syndromes in which symptoms worsen upon assuming and maintaining a standing position and are alleviated by lying down. This includes symptoms such as fatigue, lightheadedness, dizziness, syncope, nausea, headaches, heart palpitations, and visual disturbances. Specific subtypes of OI found in ME/CFS are: postural orthostatic tachycardia syndrome (POTS), neurally mediated hypotension (NMH) and delayed postural hypotension. Other common symptoms include: chronic pain (fibromyalgia), gastrointestinal symptoms, food and chemical hypersensitivities, and psychiatric symptoms (Bested and Marshall, 2015).

#### 4. Prognosis

Prognosis in ME/CFS is generally considered to be fairly poor. Full recovery is rare with only 5% of patients returning to pre-morbid levels of functioning (Cairns and Hotopf, 2005). About 40% report substantial improvement in their condition, allowing them to resume daily activities, but still functioning at a lower level than pre-disease (Cairns and Hotopf, 2005). Degree of functional impairment ranges from mild to severe. Around 25% of patients are mildly impaired, capable of participating in daily activities at a reduced level. About 50% suffer from a moderate form of disease, are severely limited in their daily activities, and usually incapable of work. Severe form of disease is present in 25% of patients, who spend their days mostly bedbound, requiring assistance to perform basic activities (Committee on the Diagnostic Criteria for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome, 2015).

#### 5. Diagnosis

The foundation for suspected ME/CFS is a presentation of new-onset fatigue accompanied by a substantial reduction in functional activity that persists for 6 months, along with additional symptoms. A diagnosis of ME/CFS is made if diagnostic criteria are met following an appropriate history, physical examination, and diagnostic testing. Table 1 contains the data on, and comparisons between four of the most used ME/CFS diagnostic criteria in the 21st century (Committee on the Diagnostic Criteria for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome, 2015; Fukuda et al., 1994; Carruthers et al., 2003; National Institute for Health and Care Excellence, 2021).



**Table 1.** Comparison between different diagnostic criteria for ME/CFS.

Criteria	1994 Fukunda	2003 CCC	2015 IOM	2021 NICE
New onset	Required	Required	Required	Required
Functional impairment	50% decrease	Substantial	Substantial	Significant
Minimal duration	6 months	6 months	6 months	3 months
Fatigue	Required	Required	Required	Required
Motor-sensory disturbances		2 symptoms from either	CP or OI	Required
Cognitive problems (CP)	4 symptoms required from any	Required	Required	Required
Pain		Required	Required	Required
Sleep disturbances		Required	Required	Required
PEM	5 categories	Required	Required	Required
Flu-like symptoms		1 symptom from these 3 categories		
Infection susceptibility				
Food sensitivities				
Gastrointestinal problems		1 symptom from these 3 categories		
Genitourinary problems				
Orthostatic intolerance (OI)			CP or OI	
Respiratory problems		1 symptom from these		
Cardiovascular problems		4 categories		
Temperature intolerance				
Thermostatic instability				

**Table 2.** Differential diagnoses of ME/CFS.

<b>Neurological disorders:</b> multiple sclerosis, vitamin B12 deficiency, spinal stenosis, craniocervical instability	<b>Gastrointestinal disorders:</b> celiac disease, food allergy/intolerances, inflammatory bowel disease, small intestinal bacterial overgrowth	<b>Infectious diseases:</b> HIV, tick borne diseases, hepatitis B/C, giardia, West Nile virus, Q-fever, Epstein-Barr virus, parvovirus 19
<b>Endocrine disorders:</b> primary adrenal insufficiency, hyper-cortisolism, hyper- or hypo-thyroidism	<b>Rheumatological disorders:</b> systemic lupus erythematosus, rheumatoid arthritis, polymyositis, polymyalgia rheumatica	<b>Cardiovascular disorders:</b> cardiomyopathy, coronary disease, valve disease, arrhythmias
<b>Sleep disorders:</b> sleep apnea, narcolepsy	<b>Psychiatric disorders:</b> anxiety, depression, bipolar disorder	<b>Hematological disorders:</b> anemia (iron deficiency, other forms), iron overload
<b>Other:</b> Gulf War illness, cancer, adverse medication effects, severe obesity, COPD		

**Table 3.** Comorbidities of ME/CFS.

<b>Neurological disorders:</b> sensory hypersensitivities, migraine headaches, peripheral neuro-pathy, small fiber neuropathy	<b>Gastrointestinal disorders:</b> food allergy/intolerances, gut motility issues, celiac disease, irritable bowel syndrome, small intestinal bacterial overgrowth	<b>Immunological disorders:</b> new or worsened allergies, mast cell activation syndrome, multiple chemical sensitivities, immunodeficiency
<b>Endocrine disorders:</b> hypothyroidism, HPA axis dysregulation, metabolic syndrome	<b>Rheumatological disorders:</b> fibromyalgia, Ehlers-Danlos syndrome, temporomandibular joint dysfunction, Sic casyndrome (dry eyes/mouth)	<b>Autonomic dysfunction:</b> postural orthostatic tachycardia syndrome (POTS), neurally mediated hypotension (NMH), orthostatic hypotension
<b>Sleep disorders:</b> sleep apnea, restless leg syndrome	<b>Psychiatric disorders:</b> secondary anxiety, secondary depression	<b>Gynecological disorders:</b> endometriosis, premenstrual syndrome
<b>Other:</b> nutritional deficiencies, vitamin B12 and D deficiencies, obesity		

ME/CFS is often a diagnosis of exclusion. Patients present with a wide array of symptoms, many of which are nonspecific and can be commonly observed in other chronic or acute conditions. When making a diagnosis of ME/CFS these diseases need to be excluded as the primary cause of the patient’s problems. Many diseases can also co-exist with ME/CFS and precipitate or potentiate some of its symptoms (Bested and Marshall, 2015). Diagnosis of these comorbid conditions does not exclude diagnosis of ME/CFS but can help guide





treatment and improve the patient's health, function, and quality of life. **Tables 2** and **Table 3** detail many of the potential differential diagnoses and comorbidities of ME/CFS as outlined in the 2020 US ME/CFS Clinician Coalition Recommendations (US ME/CFS Clinician Coalition, 2020).

## 6. Treatment

Currently, there is no treatment that would entirely cure ME/CFS. The disease can be managed and controlled through symptomatic treatment, non-pharmacological therapy, nutritional supplementation as well as alternative approaches (ME/CFS treatment recommendations, US ME/CFS Clinician Coalition, 2021). The goals of ME/CFS treatment are the improvement of current symptoms, functioning, and quality of life, prevention of symptom worsening, and helping patients understand and cope with the impact of the disease (International Association of CFS/ME, 2014). A drug that would interfere with the pathophysiological processes of ME/CFS has not been identified and approved for use in ME/CFS. In 2021 the first official recommendations for pharmacological therapy of ME/CFS, which focus on symptomatic treatment were outlined and released by the US ME/CFS Clinician Coalition (ME/CFS treatment recommendations, US ME/CFS Clinician Coalition, 2021). Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently prescribed to manage muscle pain and headaches. Tricyclic antidepressants are usually given to improve sleep quality. Fludrocortisone and fluid expansion therapy have proven to be effective in managing orthostatic intolerance. One of the most effective non-pharmacological methods for the management of disease is to teach patients about physical and cognitive “pacing”. Pacing is an individualized approach centered around the adaptation of the patient’s lifestyle based on their functional capabilities by balancing rest and activity, with the goal of conserving energy and avoiding flare-ups of post-exertional malaise. Once the patient establishes a baseline level of functioning through pacing, gradual increases in activity can be applied. The use of alternative approaches such as meditation, massage therapy, acupuncture, and chiropractic treatments is not well documented in literature, but may improve the general well-being of patients (International Association of CFS/ME, 2014).

## 7. COVID-19 and future

Over the last two years, the COVID-19 pandemic has resulted in 530 million cases and 6 million deaths worldwide (Worldometer, 2021). While the devastating acute effects of COVID-19 have been well documented, recently the research community’s interest has turned towards the chronic sequelae of the disease (Wong and Weitzer, 2021). Around 87% of patients affected by COVID-19 continue to experience at least one symptom two months after disease onset and up to 30% of people continue to experience at least one symptom, six months after onset (Carfi et al., 2020). This chronic phase of COVID-19 has been given names such as “long COVID” or “post-COVID syndrome. The most frequently reported symptoms six months after onset were fatigue, post-exertional malaise, and cognitive dysfunction (“brain fog”) (Wong and Weitzer, 2021). Considering the characteristic pattern of symptoms along with the post-viral onset, parallels can be drawn to ME/CFS and suggest that post-COVID syndrome could be a very similar disease.

## 8. Conclusion

In conclusion, ME/CFS represents a growing global issue for patients, healthcare professionals and economies. The advent of the COVID-19 pandemic has not only amplified the global burden of chronic, post-viral syndromes but also highlighted the similarities between long COVID and ME/CFS and the need for new research into the etiology, pathophysiology, diagnosis, and treatment strategies of these diseases. Embracing a multidisciplinary approach to research and patient care will be key in finding a path towards identifying disease triggers, understanding its mechanism refining diagnostic criteria and exploring effective treatments.



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# Respiratory management of Acute Respiratory Distress Syndrome (ARDS) in the Intensive Care Unit from Early Diagnosis: Narrative Review

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## Abstract:

This study aims to determine the optimal ventilatory technique for different levels of acute respiratory distress syndrome (ARDS) severity to reduce the need for endotracheal intubation (ETI) in the ICU. Secondary objectives include identifying predictors of failure of non-invasive ventilation (NIV), assessing the role of physiotherapists in the ICU across countries, and identifying areas for further investigation. A review of articles from 2013 to 2023 involving adults with ARDS in the ICU was conducted. The PEDro scale assessed article quality, and exclusion criteria included specific aetiologies of acute respiratory failure, extreme obesity, and pregnancy, as well as case series and case study designs. Data were extracted from PUBMED, EBSCO, and ScienceDirect, and a validated questionnaire was administered to physiotherapists in Chile, Spain, and France. Eight studies (number of patients n = 883) were included, and a questionnaire was completed by 22 physiotherapists (6 from France, 6 from Spain, 10 from Chile). Findings highlighted the management of high flow nasal cannula (HFNC) and NIV in different patient populations. Helmet NIV had an 18.2% failure rate and better outcomes than facemask interface. NIV non-response correlated with increased mortality rates. Cox analysis identified predictors of NIV failure. Caution is advised in NIV use, considering predictors of failure, and HFNC may be beneficial in mild ARDS. The majority of included studies had small sample sizes, limiting generalizability. The questionnaire results were constrained by a small sample size and lack of meta-analysis.

**Keywords:** Acute respiratory distress syndrome, high flow nasal cannula, non-invasive ventilation, endotracheal intubation, predictors of failure, physiotherapy.



## 1. Introduction

ARDS is a syndrome characterized by acute respiratory failure due to increased permeability of the alveolar-capillary membrane, leading to non-cardiogenic pulmonary oedema, hypoxemia, and respiratory system compliance impairment. (Thompson et al., 2017; Grasselli et al., 2023). The pathophysiology of ARDS is complex and involves various inflammatory mediators and cellular mechanisms.

The initial insult to the lungs can be caused by pulmonary or extrapulmonary factors. Pulmonary ARDS is often caused by direct injury to the lungs, such as pneumonia, inhalation of toxic gases or fumes, aspiration of gastric contents, non – protective ventilation (i.e., Ventilator induced Lung Injury) or drowning (Saguil and Fargo, 2020). In these cases, the inflammatory response is initiated within the lungs, leading to damage to the alveolar-capillary membrane and resulting in pulmonary oedema and hypoxemia.

Extrapulmonary ARDS, on the other hand, is caused by systemic inflammation due to a variety of factors such as sepsis, trauma, pancreatitis, or transfusion-related acute lung injury (Kassirian et al., 2020).

One of the primary treatments for ARDS is invasive mechanical ventilation (Fan et al., 2017; Tasaka et al., 2022) which involves the use of a ventilator to provide mechanical support to the lungs. The use of invasive ventilation in ARDS has been a topic of extensive research and debate in recent years, with the goal of optimizing patient outcomes and minimizing complications.

On the other hand, non-invasive therapies such as NIV and HFNC have been used more extensively in the treatment of ARDS. NIV is a respiratory support technique that aims to deliver positive pressure to the lungs without the need for an endotracheal tube. It is typically delivered through a mask, nasal prongs or a helmet and the positive pressure helps to improve oxygenation and reduce carbon dioxide retention (Peter et al., 2006; Thompson et al., 2017).

There is already strong level of evidence for NIV effectiveness in case of exacerbation of Chronic Obstructive Pulmonary Disease (COPD) (Rochweg et al., 2017) and for cardiogenic oedema (Peter et al., 2006).

HFNC is a type of respiratory support that delivers heated and humidified air or oxygen to the patient through a nasal cannula at a flow rate of up to 60 liters per minute (Tasaka et al., 2022). The high flow rates and humidification make it a comfortable and well-tolerated alternative to conventional oxygen therapy or NIV. The use of HFNC is based on the principle that high flow rates and humidification can improve gas exchange, reduce work of breathing, and Positive End Expiratory Pressure (PEEP) to prevent atelectasis. HFNC can be used in a variety of clinical settings, including acute respiratory failure, post-extubation support, pre-oxygenation, and bronchiolitis in infants (Frat et al., 2015; Tasaka et al., 2022).

It is known that invasive ventilation through endotracheal intubation can lead to complications such as barotrauma, ventilator-associated pneumonia, and hemodynamic instability (Saguil and Fargo, 2020; Coleman and Aldrich, 2021).

In the realm of professions to treat it, ARDS requires many of them. It is important to note that medical and nursing care are usually the ones described when considering the approach.

Physical therapy is a cornerstone in the re-education of patients, starting in the ICU. Little is known about the implication of physiotherapists in interventions regarding patients with ARDS in the ICU. Even less is known about the differences that could exist in this same level of implication according to different countries.

Therefore, this narrative review proposes to determine - according to the grade of severity of the pathology - which ventilatory technique should be adopted from the early diagnostics, to avoid as much as possible ETI as a primary objective. Additionally, the best non-invasive ventilation support interface discernment will be the secondary objective.

To start understanding physiotherapist's role in the ICU according to the country will be the primary objective of the questionnaire that has been produced during this study.



## 2. Methods

For the narrative review, the search was done from October 2022 until December 2022 in the following databases: PubMed, EBSCO, Science Direct. The identification process retrieved a total of 296 articles (13 from PubMed, 135 from EBSCO and 148 from Science Direct). The software Mendeley was used as a reference tool. The following data in addition to the reasons of exclusion in the assessment of eligibility are represented in **Figure 1**.

The updated preferred reporting items for systematic reviews and meta-analyses (PRISMA) has been used to report the following study.

The eligibility criteria encompass individuals aged 18 or older diagnosed with acute respiratory distress syndrome (ARDS), with consideration given to articles published between 2013 and 2023. Inclusion requires adherence to a PEDro scale score of 22 or 23 greater than 5/10 for randomized controlled trials and a focus on the intensive care unit (ICU) population. Conversely, exclusion criteria encompass individuals with other pathologies leading to acute respiratory failure, hypercapnic conditions such as Chronic Obstructive Pulmonary Disease (COPD), clinically extremely obese patients (BMI > 35 kg/m<sup>2</sup>), or those who are pregnant. Additionally, case series and case studies are excluded from consideration. The 2012 Berlin classification being the updated version of the AECC (American European Consensus Conference) for the diagnosis of ARDS is used by each article.

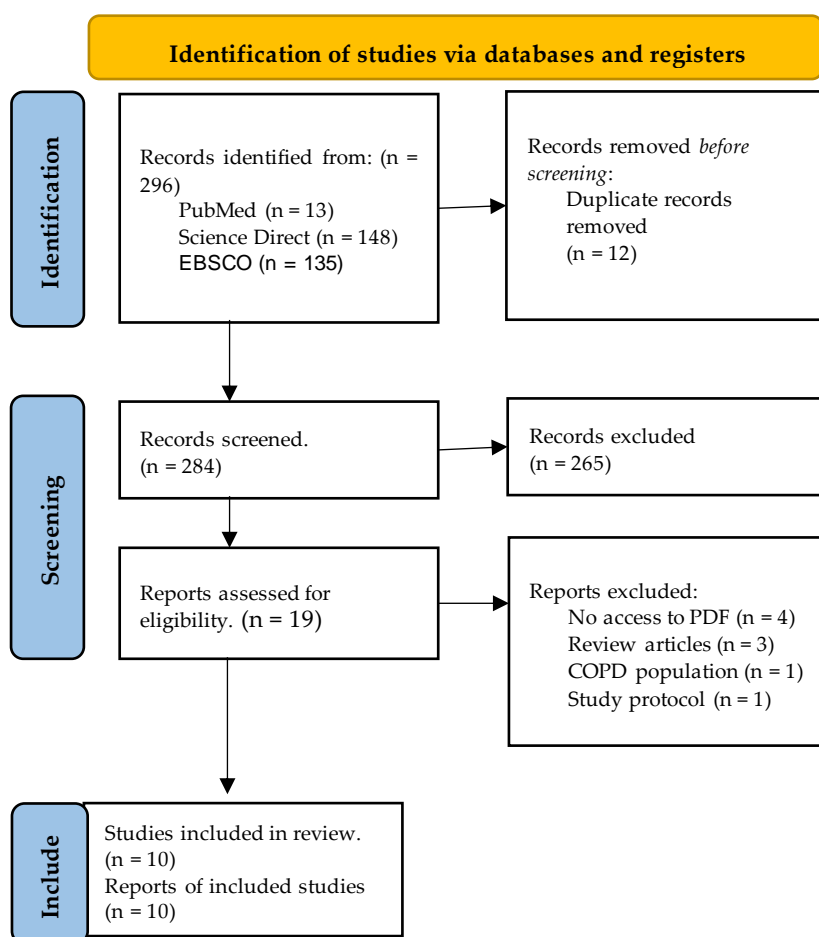


Figure 1. PRISMA Flow chart.



A questionnaire on the topic of the role of physiotherapists in ICU wards on the management of ARDS and the decision-making process has been conducted. This questionnaire has been answered by physiotherapists who work or used to work in ICUs from France, Spain, and Chile. The primary objective of the later was to determine whether the role of the professional was drastically different between countries, secondarily to have a state of the current practice, and lastly to determine if the COVID 19 pandemic changed the approach.

The answers got collected from October 2022 to February 2023. The confection of the questionnaire is based on the current literature and guidelines about the management of ARDS (Arcelo et al., 1998; Peter et al., 2006; Ranieri et al., 2012; Slutsky and Ranieri, 2013; Fan et al., 2017; Munshi et al., 2017; Rezoagli et al., 2017; Thompson et al., 2017; Dries, 2019; Saguil and Fargo, 2020; Grasselli et al., 2020; Grieco et al., 2020; Coleman and Aldrich, 2021; Tasaka et al., 2022) and has been validated in October 2022 by a panel of one experimented teacher in cardiorespiratory physiotherapy of the CEU San Pablo, and the two head of intensive physiotherapy of the Clínica Dávila Vespucio de Santiago de Chile. It has been created with the platform *Google Forms*, in three languages (Spanish, French and English). Chilean and Spanish answers were collected on two distinct versions.

The questionnaire (see **Appendix 1**) is divided in four sections for a total of 30 questions: Section 1: Personal information – 7 questions; Section 2: Diagnosis of the pathology – 4 questions; Section 3: Management of the pathology – 13 questions; Section 4: Physiotherapist involvement – 6 questions.

Overall, the questionnaire got a total of 22 answers (n = 10 from Chile, n = 6 from France, n = 6 from Spain).

### 3. Results

Based on the questionnaire, interpretations can be drawn. While 100% of French physiotherapists found the Berlin classification relevant, 33.3% of Spanish physiotherapists and 40% of Chilean physiotherapists did not. 60% of patients in Chile received between 3 – 5 sessions of physiotherapy per day, but nor Spanish neither French patient exceeded 3 sessions. Respectively 50% of physiotherapists in Spain and Chile did not include the exclusion of cardiogenic oedema as a diagnosis criterion, while only 26.7 of physiotherapists in France did not. 60% of Chilean physiotherapists believed that COVID 19 pandemics changed the diagnostic method while only 33.3% of Spanish and 16.7% of French physiotherapists did not. All the participants used protective factors when setting up mechanical ventilation. However, only 50% of Spanish physiotherapists considered the driving pressure, while 100% of Chilean and French professionals did. Additionally, only 83,3% of French physiotherapists but 100% of Spanish and Chilean physiotherapists considered tidal volume between 6 – 8 ml/kg as a protective factor. From Chilean practice, 70% considered they were completely in charge of HFNC (5/5 points), and 60% considered themselves completely in charge on NIV (5/5 points). Both of those parameters are dropping to 33% for French physiotherapists, and 16% for Spanish ones. 50% of French physiotherapists considered their role in the daily gestation of ventilation parameters as a 3/5, when the majority (70%) of Chilean professionals graded it between 3 and 4/5, and 50% of Spanish graded it 0/5. 100% of Spanish physiotherapists graded 0/5 on their implication in ETI manoeuvre, 66% graded 0/5 and 33 graded 1/5 in France, 40% graded 2/5 and 30% graded 3/5 in Chile. 66% of Spanish physiotherapists graded between 0 and 1/5 of involvement in the decision-making process for evolution between treatment strategies, 70% in Chile graded between 2 and 3/5, 33% in France graded 4/5, and 33% as a 2/5. Lastly, 100% of Chilean physiotherapists but only 33% of French and 66% of Spanish physiotherapists considered that their role in those points were more important after the pandemic.

This review is including 7 observational studies ((Frat et al., 2015; Messika et al., 2015; Sehgal et al., 2015; Chawla et al., 2016; Bellani et al., 2017; Menzella et al., 2021; Yaroshetskiy et al., 2022) and one randomized control trial (Patel et al., 2016). They are classified in three categories according to the technique of their investigation. The first



category regards the use of HFNC as a treatment method and includes two articles (Frat et al., 2015; Messika et al., 2015). The second one regards NIV and includes 4 articles (Sehgal et al., 2015; Patel et al., 2016; Bellani et al., 2017; Menzella et al., 2021). Lastly, the third category regards predictors of NIV failure and includes 2 articles (Chawla et al., 2016; Yaroshetskiy et al., 2022). We decided to separate group 2 and 3 since group 2's results are enhancing the focus on the facts and group 3's results are focused on drawing conclusions about the failure predictors of NIV. Firstly, we will present the results of the articles, in order to analyse those in a critical way.

3.1. Group 1: Publications on treatment with High Flow Nasal Canula (HFNC)

The summary of the characteristics along with the study design, the intervention, and a brief view of the results of the publications in Group 1 can be found in **Table 1**.

3.1.1. Article characteristics

Two studies evaluated the effect of HFNC on ARDS patients. Both publications dated from 2015. The population of interest in each of the article is based on the Berlin classification from 2012 and is scoping patients with approved diagnosis of ARDS, that are above 18 years old and are admitted to the ICU. However, Messika et al. (2015) are not taking into consideration in their diagnosis the parameter that requires a PEEP of 5 cm/H<sub>2</sub>O on NIV since the treated population would be with HFNC. The sample size of the studies was 23 (Frat et al., 2015) and 45 (Messika et al., 2015).

**Table 1:** The summary of the characteristics, the study design, the intervention and a brief view of the results of the Publications on treatment with High Flow Nasal Canula (HFNC) (Group 1).

	Population characteristics	Study design	Intervention	Results
Frat et al. (2015)	<ul style="list-style-type: none"> <li>- n = 23 within a year</li> <li>- ARDS patients from Berlin classification</li> <li>- Male gender 78%</li> <li>- Median age 61 years</li> <li>- SAPS II 36 points</li> <li>- Pneumonia 64%</li> <li>- Severity (mild 34%, moderate 61%, severe 4%)</li> </ul>	Prospective observational	2 hours session of HFNC, then 1 hour session of NIV for a total of 16 hours of HFNC and 8 hours of NIV per day	Failure rate of 8/23 subjects (35%) requiring ETI No ETI for intolerance reasons Increase of PaO <sub>2</sub> in comparison standard oxygen Increase of PaO <sub>2</sub> /FiO <sub>2</sub> ratio only in NIV Decrease of RR and HR HFNC better tolerated than NIV, although second session of NIV was better than the first
Messika et al. (2015)	<ul style="list-style-type: none"> <li>- n = 45 within a year</li> <li>- ARDS from Berlin classification without considering the NIV criterion.</li> <li>- Male gender 49%</li> <li>- Median age 57.9 years</li> <li>- SAPS II 36 points</li> <li>- Pneumonia 80%</li> <li>- Severity (mild 29%, moderate 38%, severe 33%)</li> </ul>	Prospective observational single centre	Use of HFNC for first line treatment of ARDS when need >9L/min of oxygen to maintain SpO <sub>2</sub> > 92%	Failure of 18/45 subjects (40%) requiring secondary ETI Success of 26 subjects only with HFNC and one in combination with NIV Additional organ failure increases HFNC failure rate

3.1.2. Methodology

Each study is based on a prospective observational approach, with a (STrengthening the Reporting of OBServational studies in Epidemiology) score of 19 out of 22. On the STROBE score, the lack of study's funding sources and assessment of the generalizability of the study findings, are the items downgrading them to 19 points (**Table A2.1** in **Appendix 2**).





### 3.1.3. Population characteristics

It is quite similar on the following points: median age (61 years (Frat et al., 2015) vs 57.9 years (Messika et al., 2015)) and the Simplified Acute Physiology Score II of 36 in both cases. However, the aetiology is different since population in Messika et al. (2015) were treating cases from pneumonia in 80% of the patients, and Frat et al. (2015) were treating 64% of pneumonia-induced ARDS. The severity of the pathology in the patient group from Messika and al. (2015) is notably homogenous, when the other population is focused to 61% of patients with moderate ARDS.

### 3.1.4. Results of the therapy

Messika et al. (2015) are describing the use of HFNC as first line treatment in 29% of the subjects admitted for acute respiratory failure. Additionally, HFNC was used at all stages of the pathology, from first line treatment to pre-ETI, post-extubation or palliative care. The study of Frat et al. (2015) that is combining HFNC and NIV, points out that PaO<sub>2</sub> increased in 20 out of 28 patients included in the study for acute respiratory failure, and that it was even higher when in combination with NIV in comparison with standard oxygen therapy. On the other side, only NIV caused a decrease in the PaO<sub>2</sub>/FiO<sub>2</sub> ratio. Moreover, remaining stable throughout the entire HFNC/NIV sessions, respiratory rate and heart rate significantly decreased after the initiation of the therapy.

### 3.1.5. Tolerance of the therapy

HFNC was better tolerated than NIV, according to a lower score on the visual analogue scale of 16 mm versus 61 mm (P=0.004). However, comfort was better on the second session of NIV at 49 mm on the scale (Frat et al., 2015).

### 3.1.6. Failure rate

In the study of Messika et al. (2015) is about 40% of the patients, and the main reasons for ETI were worsening of hypoxemia in 72%, onset of hemodynamic failure with 22% and onset of neurological failure with 6%. The subjects included in the failure of HFNC group had significantly higher SAPS II scores in comparison to the success group. The failure rate on the combined treatment in the study of Frat et al. (2015) is 35% for ARDS patients and 36% for acute respiratory failure, and the reasons for ETI are worsening of distress in 70% patients, shock for 20% of them, and respiratory arrest for the last 10%

### 3.1.7. Predictors of failure

It is associated to lower PaO<sub>2</sub>/FiO<sub>2</sub> ratio after initiation of HFNC (115.3 vs 145.3 mmHg, P=0.26), SAPS II scores (46 vs 29), and hemodynamic failure in the univariate analysis, but only the SAPS II score in the multivariate analysis according to Messika et al. (2015). In the case of Frat et al. (2015) failure was associated to a breathing frequency of >30 breaths/min at 1 hour after initiation of the first HFNC session with a sensitivity of 94.1% and a specificity of 87.5%.

### 3.1.8. Mortality rate

According to Messika et al. (2015) mortality rate was 50% in patients in whom the treatment failed (p = 0.001 for the failed treatment) and 4% in patients in whom the treatment was successful. Therefore, meaning that mortality rate was much lower in successful patients. Frat et al. (2015) are reporting a mortality rate of 20% for the population in which the treatment failed, but it was not statistically significantly relevant (p = 0.12). Consequently, it does not precisely represent the percentage of deceased patients caused by the failure of the non-invasive treatment.

## 3.2. Group 2: Publications on treatment with non-invasive ventilation

The summary of the characteristics along with the study design, the intervention, and a brief view of the results of the publications in Group 2 can be found in **Table 2**.



**Table 2.** The summary of the characteristics, the study design, the intervention and a brief view of the results of the Publications on treatment with non-invasive ventilation (NIV)(Group 2).

Population characteristics	Study design	Intervention	Results
Bellani et al. (2017) <ul style="list-style-type: none"> <li>- n = 436 for 4 weeks</li> <li>- ARDS patients from Berlin classification aged &gt; 16 years</li> <li>- Male gender 58.9%</li> <li>- Median age 68 years</li> <li>- non-pulmonary SOFA 3 points</li> <li>- No etiology</li> <li>- Severity (mild 27.3%, moderate 53.2%, severe 19.5%)</li> </ul>	Prospective observational multicentre international cohort	NIV with any type of interface or ventilatory mode for at least 1 or 2 days to be enrolled as NIV patients	Failure rate of 131/436 subjects (37.5%) requiring ETL. Greater severity of ARDS associated with an increase of recognition of ARDS and worsening in outcomes including LOS and ICU mortality but not hospital mortality. Use of NIV didn't vary according to the severity category of ARDS. NIV patients had lower PEEP and higher respiratory rate in comparison to IMV. NIV failure in case of higher SOFA, lower PaO <sub>2</sub> /FiO <sub>2</sub> ratio and % of increase of PaCO <sub>2</sub> within first 2 treatment days.
Sehgal et al. (2015) <ul style="list-style-type: none"> <li>- n = 41 for one year</li> <li>- ARDS from AECC classification, &gt;18 years</li> <li>- Exclusion of severe ARDS patients</li> <li>- 65.7% women</li> <li>- Median age 30.9 +/- 11.4 years</li> <li>- APACHE II 18 points</li> <li>- Malaria 26.8%, Typhus 19.5%</li> <li>- Severity (mild 56.1%, moderate 43.9%, severe 0%)</li> </ul>	Prospective observational	NIV using ICU ventilators. First 24 hours, off only for oral intakes, then depending on patient, increase of off periods.	Failure of 23/41 subjects (56%) requiring ETL. NIV failure group had significantly higher APACHE II. Duration of ventilation higher in IMV. Significant decrease of respiratory rate in both groups. Predictors of NIV failure: no improvement of PaCO <sub>2</sub> /FiO <sub>2</sub> within 1 hour and higher baseline APACHE II.
Menzella et al. (2021) <ul style="list-style-type: none"> <li>- n = 79 over a month</li> <li>- COVID 19-ARDS patients</li> <li>- Male gender 56(71%)</li> <li>- Median age 66.5 +/- 11.4 years</li> <li>- SOFA 4.3 points</li> <li>- Charlson comorbidity index 3.4</li> <li>- Severity (mild 0%, moderate 100%, severe 0%)</li> </ul>	Retrospective observational	Use of NIV on patients with a PaO <sub>2</sub> /FiO <sub>2</sub> ratio >100 and <200 mmHg	Failure of 41/79 subjects (51.9%) requiring ETI in 21(26.6%) and death in 20(25.3%). 18/20 deceased were not eligible for ETI. Failure predictors according to a multivariate COX regression model: SOFA score. No higher mortality rate for failed NIV patients.
Patel et al. (2016) <ul style="list-style-type: none"> <li>- n = 83 for 3 years</li> <li>- ARDS with mask NIV&gt;8 hours, &gt;18yo, berlin criteria</li> <li>- Male gender 54% face mask, 55% helmet</li> <li>- Median age 60.9 years face mask, 58 helmet</li> <li>- APACHE II 26 points face mask, 25 points helmet.</li> <li>- Pneumonia 36%</li> <li>- Severity (60% PaO<sub>2</sub>/FiO<sub>2</sub>&lt; 200 mm Hg)</li> </ul>	Single centre randomized clinical trial.	After 8 hours of face-mask NIV Control (39/83): continue with face mask on a single limb ventilator. Intervention (44/83): switch to helmet interface with double limb ventilator.	ETI rate of 61.5% in face mask group, 18.2% in helmet group. PEEP of 8.0 (median) in intervention group VS 5.1 in control. Reduction of tachypnoea from face mask to helmet interface. Helmet group had more ventilator-free days (28 vs 12.5) and less ICU LOS (4.7 vs 7.8 days). Hospital and 90-days mortality significantly lower in helmet group. Independent association of APACHE II with 90-days death rate (still lower for helmet group)

### 3.2.1. Articles characteristics

The second part of the review consists of four articles, one randomized control trial (Patel et al., 2016) and three observational studies (Sehgal et al., 2015; Bellani et al., 2017; Menzella et al., 2021). The publication dates are ranging from 2015 to 2021 and they are all investigating the effect of NIV on ARDS patients.



### 3.2.2. Methodology

Two studies are using the Berlin classification as a diagnosis method (Bellani et al. (2017) and Patel et al. (2016), Menzella et al. (2021) are not specifying the diagnosis criteria but are mentioning the treatment of moderate to severe cases. In the case of Sehgal et al. (2015) they are using the now changed classification of the American European Consensus Conference (AECC) (Villar and Kacmarek, 2012) as a diagnostic criterion. Sehgal et al. (2015) are reporting cases of ARDS from tropical diseases such as malaria in 26.8% and typhus in 19.5% of the patients, only 9.7% was pneumonia related. The sample sizes are 436 patients in 4 weeks in Bellani et al. (2017), 41 patients in one year in Sehgal et al. (2015), 79 patients in one month in Menzella et al. (2021), and 83 patients in 3 years in Patel et al. (2016).

### 3.2.3. Patient's characteristics

Bellani et al. (2017) describe their population as being older, with a lower non-pulmonary SOFA score when comparing to intubated population. Additionally, they report that the NIV population is more prone to suffer from comorbidities such as chronic renal failure, congestive heart failure, and chronic obstructive pulmonary disease compared to intubated patients. Finally, they didn't find any significant difference about immunocompromised patients between both groups but was still an important number of subjects in each. Patel et al. (2016) are comparing their control and intervention group at baseline, and the findings are related to what Bellani et al. (2017) are stating. It was found that more than half of the overall non-invasive population are immunocompromised by virtue of cancer or transplant, and about one third had an immunocompromised pneumonia.

### 3.2.4. NIV failure

Regarding the outcomes, focusing on the failure of the treatment method, Sehgal et al. (2015) are reporting a rate of 56% of failure, 51.9% of failure for Menzella et al. (2021) with 26.6% requiring ETI, and 25.3% being deceased, 61.5% for face-mask group (control) and most importantly only 18.2% for helmet group (intervention) according to Patel et al. (2016). Lastly, Bellani et al. (2017) are reporting a rate of 37.5% of failure with a bigger sample size than any other of the studies. APACHE II has a high median value in the study of Patel et al. (2016) when looking closer to the same value in the study of Sehgal et al. (2015) (18 vs 25-26 points). Additionally, population in Sehgal et al. (2015) has a median age of 30.9 in comparison to the three other population ranging from 60.9 to 68 years.

### 3.2.5. Ventilation and patient's parameters

When comparing NIV with invasive ventilation, NIV had significantly lower levels of PEEP, and higher respiratory rates than the second one (Bellani et al., 2017). Moreover, measured tidal volumes and minute ventilation were greater in population treated by NIV. However, the latter and the former were not affected by ARDS severity in patients treated with NIV compared to patients treated with invasive ventilation. Patel et al. (2016) are reporting that patients from their control group had a median sustained PEEP level of 5.1 cm H<sub>2</sub>O (face mask group) and that their intervention group was at a level of 8.0 cmH<sub>2</sub>O (helmet group) ( $p = 0.006$  for both values). PEEP titration in the case of face-mask interface was a challenge because patients did not tolerate it well and because of excessive air-leak. Lastly, tachypnoea significantly decreased when patients got randomly assigned from the facemask to the helmet interface (from 27.7 breaths/min to 24.5 breaths/min) ( $p < 0.001$ ). It was pointed out in the study of Bellani et al. (2017) that in their consequent study population, NIV was used almost as frequently in the three severity groups of ARDS, 14.3% for mild, 17.3% for moderate and 13.2% for severe cases of the pathology.

### 3.2.6. Reasons for ETI

When coming to the decision to intubate, refractory hypoxemia and tachypnoea caused by the face mask are reported as being the main reasons (Patel et al., 2016, Sehgal et al., 2015). In one case, neurologic failure was described as altered sensorium



and in one case the Richmond Agitation Sedation Scale (RASS) dropped below 8 points. However, when comparing those results with the intervention group of the randomized control trial, the first reason for ETI is neurologic failure and not respiratory failure. This can easily be correlated to the failure rate from the intervention group with the helmet interface. Lastly, Bellani et al. (2017) are reporting that PaO<sub>2</sub>/FiO<sub>2</sub> ratio was increasing more rapidly in patients treated with invasive mechanical ventilation in comparison to NIV.

### 3.2.7. Secondary outcomes

Regarding the hospital length of stay Bellani et al. (2017) are reporting worsening of this outcome along with ICU mortality according to the severity of the ARDS, although it was also correlated with a higher clinical recognition. According to Patel et al. (2016) the helmet group spent only 4.7 days in the ICU compared to the 7.8 days for the facemask groups, and helmet group had more ventilator free days than the facemask group (28 vs 12.5 days). However, statistically, hospital length of stay was not different. Menzella et al. (2021) reported a longer duration of NIV for successful subjects compared to failed ones and deceased ones (8.7 vs 2.9 vs 6.3 days respectively). In the case of Sehgal et al. (2015) the total ventilation duration was recorded (non-invasive and invasive), with a result of 2.75 days for NIV success, and 5.2 days for failure. Additionally, only regarding the NIV ventilation, the duration was 2.75 days in success patients and 3 hours for failure subjects. However, there was no significant difference in the ICU stay between each group.

### 3.2.8. Mortality rates

According to Menzella et al. (2021) mortality rate was not significantly different between both groups of NIV (failure and success) (43% vs 36%, p=0.61). However, Sehgal et al. (2015) reported that out of the 23 subjects who failed NIV, 19 got deceased (82.6% of NIV failure death), and out of the 18 patients of success, none got deceased. The total percentage of death of the study reached 46.3%. Patel et al. (2016) are reporting that hospital and 90-days mortality was significantly lower in the helmet group compared to the facemask group (48.7% vs 27.3% for hospital, 56.4% vs 34.1% for 90-days). Additionally they found that APACHE II was independently associated to 90 days death with a confidence interval of 95%. Bellani et al. (2017) are reporting relevant data on the mortality rates. Firstly, between NIV and invasive ventilation, there was no significant difference in ICU and hospital mortality. However, in the case of failed NIV patients, the rates are reaching 42.7% of ICU mortality compared to the 10.6% for the success subjects (p<0.001). After performing a multivariate COX regression model adjusting covariates significantly associated with outcomes; NIV use was independently associated with increased ICU but not hospital mortality with a 95% confidence interval. Another multivariate COX regression analysis was performed on baseline characteristic of NIV group, that showed that chronic heart failure, hematologic or neoplastic disease, chronic liver failure, age, ARDS severity, percentage decrease of PaO<sub>2</sub>/FiO<sub>2</sub> ratio between days 1 and 2, total respiratory rate and non-pulmonary SOFA score were each independently associated to in-hospital death.

### 3.2.9. Predictors of failure for NIV

Sehgal et al. (2015) performed a univariate logistic regression analysis and found out that failure of improvement in the PaO<sub>2</sub>/FiO<sub>2</sub> ratio within an hour of treatment, and a higher baseline APACHE II scores, were associated with failure of NIV. It should be taken into consideration that this analysis is only univariate. Menzella et al. (2021) conducted a univariate and multivariate COX regression model on those parameters associated with NIV failure, and out the two analyses, only SOFA admission score was significantly correlated with failure, leaving PaO<sub>2</sub>/FiO<sub>2</sub> ratio out. This can show that COVID ARDS might behave in a different manner. Bellani et al. (2017) conducted a multivariate COX regression analysis that revealed that higher non pulmonary SOFA score, lower PaO<sub>2</sub>/FiO<sub>2</sub> ratio, and the percentage increase of PaCO<sub>2</sub> over the first days of treatment were independently associated to NIV failure within 28 days. The



sample size of this article gives a higher scientific weight, and on the contrary, the small sample size from Sehgal et al. (2015) is dragging its conclusions to a lower level.

### 3.3 Group 3: Publications on predictors of NIV failure

The summary of the characteristics along with the study design, the intervention and a brief view of the results of the publications in Group 3 can be found in **Table 3**.

#### 3.3.1. Articles characteristics

The analysis of two articles was done in the third part of this study. Yaroshetskiy et al. (2022) gathered a population of 80 patients within 6 months of study, and Chawla et al. (2016) gathered a population of 96 patients within 3 years of study.

All the patients were treated with NIV, through a full-face mask. Chawla et al. (2016) are using the Berlin classification as a diagnosis method for the patients, knowing that the aetiology of the ARDS is coming from pneumonia in 55.3% of the patients. However, Yaroshetskiy et al. (2022) are evaluating patients with COVID 19-ARDS complying with at least one of the following criteria: fatigue, excessive visible WOB assessed by the Patrick scale (Patrick et al., 1996) (4-5 points), SpO<sub>2</sub> < 92%. Precise ARDS classification diagnosis was not reported and only moderate to severe patients were included (**Table 3**).

**Table 3.** The summary of the characteristics along with the study design, the intervention, and a brief view of the results of the publications in Group 3

	Population characteristics	Study design	Intervention	Results
<b>Yaroshetskiy et al. (2022)</b>	<ul style="list-style-type: none"> <li>- n = 102 within 6 months, 80 with NIV</li> <li>- COVID 19 ARDS with at least one of the following criteria: fatigue, excessive visible WOB assessed by Patrick scale(Patrick et al., 1996) (4-5 points), SpO<sub>2</sub>&lt;92%</li> <li>- Male gender overall 54(56.3%)</li> <li>- Median age overall group 71.5</li> <li>- SOFA overall 4 points</li> <li>- Severity only from moderate to severe ARDS</li> </ul>	Prospective observational clinical	Use of oxygen therapy <15 L/min or CPAP outside ICU as screening, 2 hours NIV trial as entrance test. Then, if tolerated, NIV, if not, ETI	Failure rate of 57/80 subjects (71.3%) requiring ETI After 48 hours of NIV, if PaO <sub>2</sub> /FiO <sub>2</sub> < 112 mmHg, ROX < 5.02, P <sub>ET</sub> CO <sub>2</sub> <19.5mmHg and Patrick score >= 2 then failure can be predicted. Respiratory rate can also be considered. NIV failure higher in older and/or more frailer patients, longer COVID duration without NIV
<b>Chawla et al. (2016)</b>	<ul style="list-style-type: none"> <li>- n = 170 within three years, 96 with NIV</li> <li>- ARDS from Berlin classification &gt; 18 years</li> <li>- Male gender overall 61.8%</li> <li>- Median age overall 47.54 years</li> <li>- SOFA overall 8 points</li> <li>- APACHE II overall 17.42 points</li> <li>- Pneumonia 55.3%</li> <li>- Severity overall (mild 34.7%, moderate 41.8%, severe 23.5%)</li> </ul>	Prospective observational	NIV through a non-vented full-face mask with and ICU ventilator using a dedicated NIV mode	Failure of 42/96 subjects (43.8%) requiring ETI Failure rate higher in severe (83.3%) and moderate (73%) Low PaO <sub>2</sub> /FiO <sub>2</sub> , septic shock and severity of ARDS as factors associated to failure. Higher mortality in failure of NIV patients. Longer LOS in NIV patients ICU mortality: 30.2% for NIV, 45.9% for IMV

#### 3.3.2. Methodology

Yaroshetskiy et al. (2022) is an observational prospective clinical study with a score of 20 out of 22 points on the STROBE scale. Chawla et al. (2016) is a prospective observational study with a STROBE score of 21 out of 22. Study's funding source and an assessment of the generalizability of the study findings is lacking for Yaroshetskiy et al. (2022) and only the assessment of the generalizability of the study findings is lacking for Chawla et al. (2016).



### 3.3.3. Failure and mortality rates

According to Yaroshetskiy et al. (2022), out of 80 patients, 57 (71.3%) failed the treatment. Among those patients, they were all intubated and 3 were put under extracorporeal membrane oxygenation (ECMO). The other ones were not eligible for ECMO because of exclusion criteria (mostly >65 years old). The mortality rate was 100% for this group. According to Chawla et al. (2016) out of 96 patients, 42 (43.8%) failed NIV by requiring invasive mechanical ventilation. Depending on the severity of the ARDS, the failure rate was higher. In moderate ARDS the failure rate was 27/37 (73%) and in severe ARDS it was 5/6 (83.3%) as compared to mild ARDS with 10/53 (18.9%) ( $p=0.000$ ). Among the patients who failed NIV, 69% (29/42 patients) of them died, with 7/10 (70%) for mild, 18/27 (66.7%) for moderate and 4/5 (80%) for severe ARDS.

### 3.3.4. NIV failure predictors

In the study of Chawla et al. (2016) the univariate analysis showed that admission APACHE II and SOFA scores along with low admission  $\text{PaO}_2/\text{FiO}_2$  ratio, presence of severe sepsis or septic shock or multiorgan dysfunction, presence of confusion, absence of  $\text{H}_1\text{N}_1$  influenza A infection, and severity of ARDS were the 9 factors for prediction of NIV failure. In the multivariate analysis however, only low admission  $\text{PaO}_2/\text{FiO}_2$  ratio ( $p = 0.049$ ), presence of septic shock ( $p = 0.001$ ) and severity of ARDS ( $p = 0.007$ ) were validated. According to a ROC analysis, Yaroshetskiy et al. (2022) have stated that after 48 hours of treatment the following parameters can be described as predictors of failure of NIV: 1) ROX index  $<5.02$  ( $p < 0.001$ ), 2)  $\text{PaO}_2/\text{FiO}_2 < 112$  mmHg ( $p < 0.001$ ), 3)  $\text{P}_{\text{ET}}\text{CO}_2 < 19.5$  mmHg, 4) Patrick scale  $\geq 2$ . Lastly, they concluded that the probability of failure was higher in older/frailer patients and in patients with a longer duration of COVID 19 without NIV.

## 4. Discussion

The HFNC is a relatively new technique that has been incorporated into ICUs for good reasons. However, the findings of this review about the use of the latter as an effective treatment option for patients with ARDS are fragile. There are lots of limitations and precautions to consider when interpreting results from those articles. Starting by the study design of both, which is observational, and the fact that they don't comply with the entirety of the items in the STROBE score. This is additional to the population sample size, that even though growing in comparison to previous case series or case studies, stays low.

Moreover, the HFNC technique presents a limitation when referring to ARDS. It is thought to generate low levels of PEEP, depending on the volume applied (between 35 and 60 L/min) and most importantly depending on if the patient stays mouth closed. This lack of precise knowledge about the PEEP level, confronts itself with the essence of diagnosis method of ARDS. The Berlin classification is including one crucial parameter that makes it the evolution of the AECC, the minimum PEEP level of 5  $\text{cmH}_2\text{O}$ . In this case, HFNC cannot provide such information and then block ARDS proper identification by health professionals. A post hoc analysis from two prospective studies by Coudroy et al. (2018) comes in line to defend this position and try to revise the Berlin classification on this very precise point of minimum level of PEEP. However, even though debates are forming, in this precise time, minimum level of PEEP is required to consider a diagnosis of ARDS.

On the level of failure rate, it is clearly stated that the use of HFNC is related to less failure of the treatment with 35% (Frat et al., 2015) and 40% (Messika et al., 2015), in comparison to studies on the use of NIV that reports failure rate in between 37.5% and 61.5% in the case of full-face interfaces (Sehgal et al., 2015; Patel et al., 2016; Bellani et al., 2017; Menzella et al., 2021). However, one of the greatest concerns in any non-invasive ventilatory support, is not the failure rate itself, but the hospital/ICU mortality for the subjects that are part of the failure group. This is shown by Bellani et al. (2017) when taking as a study population, the largest ARDS database available nowadays. They reported with a ROX regression analysis that NIV was associated with mortality, and the use of HFNC on this matter remains unknown. If considering the



results of the two articles of this review, Messika et al. (2015) are reporting 50% of deceased patients from the failure group, but this result is drawn from a sample of 18 subjects in the failure of the treatment, which is really low. Frat et al. (2015) are not reporting such data. Lack of power of both studies on such matters prevents any conclusions from being properly deduced.

Messika et al. (2015) concluded that HFNC was not improving the PaO<sub>2</sub>/FiO<sub>2</sub> ratio itself but only the FiO<sub>2</sub>, which can prove that levels of PEEP provided by HFNC is not sufficient to improve the overall gas exchange. Frat et al. (2015) with their approach of combination of both techniques with NIV, is then using the beneficial effect of oxygenation improvement from the NIV.

In the combined use of HFNC and NIV, it has been found that tolerance levels were significantly better in the case of HFNC rather than NIV, and a previous study by Antonelli et al. (2007) depicted an ETI rate due to NIV intolerance of 25% (Antonelli et al., 2007) in patients with ARDS. However, when considering that patients have to keep their mouth closed to benefit from the effectiveness of HFNC, another concern can be drawn on whether the tolerance level assessment was made respecting this criterion. To continue the positive sense of combination of HFNC and NIV, Frat et al. (2015) have reported that no ETI due to interface intolerance was conducted, knowing that the interface used in this study is a full-face mask. Patel et al. (2016) have proven the efficacy of helmet interface in comparison to full-face mask on multiple level, such as failure rate and mortality. This could be a good opening for next research, evaluating the efficacy and failure rate of combined HFNC and Helmet NIV alternatively. It is advanced that HFNC have an effect on decreasing the respiratory rate (Frat et al., 2015). However, it is important to keep in mind which are the predictors of failure of NIV (Table 3) since this study is using both HFNC and NIV. As a matter of fact, respiratory rate is never going further in the predictors than a univariable analysis. By relying on multivariable analysis, then HFNC doesn't have any substantial/statistical effect on avoiding failure of NIV. When conducting their multivariable analysis on the predictors of mortality Bellani et al. (2017) found that respiratory rate was one of them. Thus, statistically, HFNC has an effect on reducing mortality, but not on reducing failure rate. This conclusion can be turned into a positive sense, since as mentioned before, one of the greatest concerns from the analysis of the entire articles is focused on the mortality rate of the failure group.

Messika et al. (2015) are observing the use of HFNC at any stage of ARDS, 33% as severe, 38% as moderate and 29% as mild. However, SAPS II scores are relatively low (median of 36 for all subjects, 29 for success and 46 for failure) and the majority of patients did not present with an additional organ failure. The use of HFNC can be done at any stage if the ETI timing is respected. The need for further research on the exact population of application is needed to establish a clear view.

The questionnaire conducted in this study gives multiple information on the management of ARDS in the ICU. HFNC is designed as the major first line treatment for mild ARDS for physiotherapists in Spain (along with standard oxygen therapy), France and Chile, and the first with NIV in the case of moderate ARDS but only for physiotherapists in Spain. Limitations are multiple to this questionnaire, such as lack of power due to sample size, or lack of statistical analysis of the results. However, the conclusion of this survey is that in nowadays practice, HFNC is used broadly as the first line therapy in mild ARDS.

NIV in its turn is an older technique that has been used for a long time in acute respiratory failure from COPD or cardiogenic oedema. Healthcare professionals in the ICUs are highly trained for the technique. However, in the case of ARDS, the studies of this review are creating a brand-new perspective.

Results from the studies are reporting many deductions, but one crucial point here is to point out which outcome should be the main one to be analysed. Failure rate of the therapy is the first one that would seem logical, in the sense that if the patient doesn't fail the therapy, then the outcomes are positive, which seems to be the case. (Bellani et al., 2017) is the largest study population sample of all the articles and has a failure rate of only 37.5% out of the 436 subjects enrolled. Sehgal et al. (2015) reported a failure rate of 56%, but this study presents a couple of limitations that deserves to be pointed



out. The study sample is clearly different from all of the other studies, by its small sample size of 41 patients, by its aetiology of ARDS coming in most of the cases from tropical diseases (extra-pulmonary ARDS) and leaving pneumonia for less than 10% of the patients, and most importantly, including in the research patients that are staying under NIV for extremely short period of time, hence the median timing of ventilation of 3 hours for patients who failed the treatment. A limitation of this study is also the exclusion of severe ARDS patients. Failure rates in other studies are around 50% for NIV, which can be positively interpreted by saying that in case of both extra-pulmonary and pulmonary ARDS, NIV is effective in half of the patients.

The results of Yaroshetskiy et al. (2022) are conflicting to interpret since they report a failure rate of 73%. In this case it is important to note that they only included moderate to severe ARDS population and that it has been shown in the predictors of failure that lower  $\text{PaO}_2/\text{FiO}_2$  at baseline was highly associated with NIV failure (Chawla et al., 2016; Bellani et al., 2017). This leads towards the fact that the use of NIV on more severe population leads to higher failure rates. However, Bellani et al. (2017) stated that the use of NIV was not different in practice between the three severity groups, against the current state of evidence on the subject. This raises questions on whether this “non-adequate” use of the therapy is deliberate or if lack of precision and sharpness of knowledge could be at the origin. As a result of the questionnaire, it is clear that Spanish physiotherapists are not in complete osmosis with the current knowledge since none of the participants considered ETI as a first line treatment for moderate ARDS and shows that Chilean professionals are more prone to take a scientifically based decision, with 40% of them considering ETI as first line treatment, and 60% for NIV as first line. On the contrary, most participants of the questionnaire apart from one Spanish subject (HFNC), and 2 French ones (depends on the patient) did not consider other possibilities than invasive ventilation in the case of severe ARDS, which clearly seem to be the best option when evoking failure rates and improvement of parameters.

One result that points out from the studies is the comparison in the randomized control trial of Patel et al. (2016) of facemask and helmet NIV. The failure rate in the facemask group is 61.5% when in the helmet group drastically drops to 18.2%. This result is conclusive on the net effect and difference of the two interfaces when regarding the failure rate.

Following a logical interpretation of the results, the focus of the question in NIV should not only be on the failure rate but also on the mortality rates (ICU, Hospital, 90-days). This concern has been raised in previous articles (Antonelli et al., 2001; Rana et al., 2006) and the results of this review are going in this sense. Bellani et al. (2017) reported 42.7% of mortality among the subjects who failed NIV which stands as the smallest and most optimistic value. Sehgal et al. (2015) reported 82.6% of mortality, Yaroshetskiy et al. (2022) 100%, Chawla et al. (2016) 69%, and Patel et al. (2016) 48.7% vs 27.3% for hospital, 56.4% vs 34.1% for 90-days, respectively in the case of facemask and helmet. Chawla et al. (2016) enhance this statistics by giving details depending on the severity of the pathology, and moderate and severe results are above 70% of mortality. Bellani et al. (2017) used multivariate COX analysis linking NIV and ICU mortality indicating that NIV in the case of moderate and severe cases seem to lead towards high mortality rates. This link could be explained by the fact that patients under NIV are according to two studies of this review older, frailer, and with a greater number of comorbidities. Although these parameters are defending the treatment technique, it is important to be aware of the risks of delayed ETI, and the consequences it can have. By showing that NIV was used for any severity of the pathology, Bellani et al. (2017) are unrevealing some points that could include persistence of NIV against ETI criteria, thus leading to the late ETI. Lastly, the same study is showing that mortality in patients with a  $\text{PaO}_2/\text{FiO}_2$  of less than 150 mmHg was 36.2% for NIV and 24.7 with IMV. This opens a new window of research in the field and calls for answers on what is the current state of practice on the ETI level. Being able to pick the right population when implementing any non-invasive technology could reduce failure rate, but mostly could allow mortality in this category to decrease.





In the case of NIV, outcomes are clearly differing when using a full-face mask in comparison to the helmet interface. In the ETI reasons, Patel et al. (2016) and Sehgal et al. (2015) are pointing out respiratory failure as the first reason for ETI in the case of full-face mask NIV. As an example, this includes refractory hypoxemia or tachypnoea. As a correlation, tachypnoea can be associated to worsening of the respiratory failure, but can also be coming from psychological reasons, and most importantly stress itself (Gerritsen and Band, 2018). Frat et al. (2015) clearly showed that full-face mask comfort for the patient is not optimal, and the grades given to it on the visual analogue scale are going in this way. This would be the first conclusion drawn from the randomized control trial, that helmet is more comfortable and less aggressive for the patient, thus can have an effect on tachypnoea. Moreover, the randomized control trial shows that helmet ventilation is providing higher levels of PEEP by 3 cm H<sub>2</sub>O more than the mask. This allows the tachypnoea to be reduced as well and to correct even better the PaO<sub>2</sub>/FiO<sub>2</sub> ratio level. Bellani et al. (2017) found that provided levels of PEEP were more important in invasive mechanical ventilation rather than NIV, but if the study had been done on helmet interfaces, the outcome would have changed, and possibly reaching the same levels. This outline one limitation of NIV through face-mask, since they are not able to provide high levels of PEEP, the management of severe and even moderate ARDS patients will be far from optimal, and ETI would be recommendable. However, in the case of using helmet interfaces, the titration of PEEP is not limited by air leak and patient discomfort, which would allow successful management of this more severe population of ARDS.

Continuing on the beneficial effect of helmet NIV, Patel et al. (2016) are showing that ICU length of stay (4.7 vs 7.8 days) and ventilator-free days (28 vs 12.5 days) are significantly and effectively reduced in the helmet group, and even though total hospital length of stay is not significantly different, being out of the ICU means avoidance of ICU related complications such as ICU – acquired weakness, or ventilatory related complications such as V-ILI or P-ILI or nosocomial infections.

Predictors of NIV failure are then crucial. They are bringing the answer to how and when to stop the non-invasive technique to switch the patient to ETI and avoid the delay of the latter. Almost each article is reporting its set of predictors but Sehgal et al. (2015) as an example are only treating data into a univariate COX regression model and not in the multivariate model. Thus, those data are not significant enough to be considered relevant. However, 4 studies are reporting multivariate COX analysis, and their conclusions are the following. Menzella et al. (2021) only achieved to bring the SOFA score at this position, and not have the low PaO<sub>2</sub>/FiO<sub>2</sub> ratio. This can be interpreted that it is one of the differences between ARDS and COVID-ARDS since the general systemic state will apparently weight more than the severity of the ARDS alone. Bellani et al. (2017) are reporting higher non-pulmonary SOFA score, lower PaO<sub>2</sub>/FiO<sub>2</sub> ratio, percentage of increase of PaCO<sub>2</sub> over the first days as failure predictors within 28 days. SOFA score is then cited in two of the articles. Chawla et al. (2016) are then using low admission PaO<sub>2</sub>/FiO<sub>2</sub> ratio, presence of septic shock and the severity of ARDS as predictors in their multivariate analysis.

Lastly, Yaroshetskiy et al. (2022) are the most precise by stating that if after 48 hours of ventilation, 1) ROX index < 5.02, 2) PaO<sub>2</sub>/FiO<sub>2</sub> < 112 mmHg, 3) P<sub>ET</sub>CO<sub>2</sub> < 19.5 mmHg (using a capnometer) and 4) Patrick score ≥ 2, then failure can be predicted.

Therefore, predictors can be uncovered before applying the therapy thanks to the SOFA score, lower PaO<sub>2</sub>/FiO<sub>2</sub> ratio, presence of septic shock, severity of ARDS and can be effectively assessed after 48 hours of ventilation with the protocol established by Yaroshetskiy et al. (2022).

These findings are limited by the lack of precise information on the correlation between SOFA score, PaO<sub>2</sub>/FiO<sub>2</sub> and severity of ARDS and failure prediction. Further investigation should be performed in order to determine the optimal limitation values when predicting failure. **Figure 2** is depicting the knowledge drawn from this review. The questionnaire is limited by lack of power due to sample size. Moreover, additional statistical data interpretation would allow the aforementioned to gain power. The latter is limited knowing that it serves only as informative data. The objective of this questionnaire is to have a global idea of the actual involvement and management of



the pathology of and by physiotherapists; therefore, conclusions can't be properly drawn. They can lead to further research on the topic to provide areas of involvement of the profession.

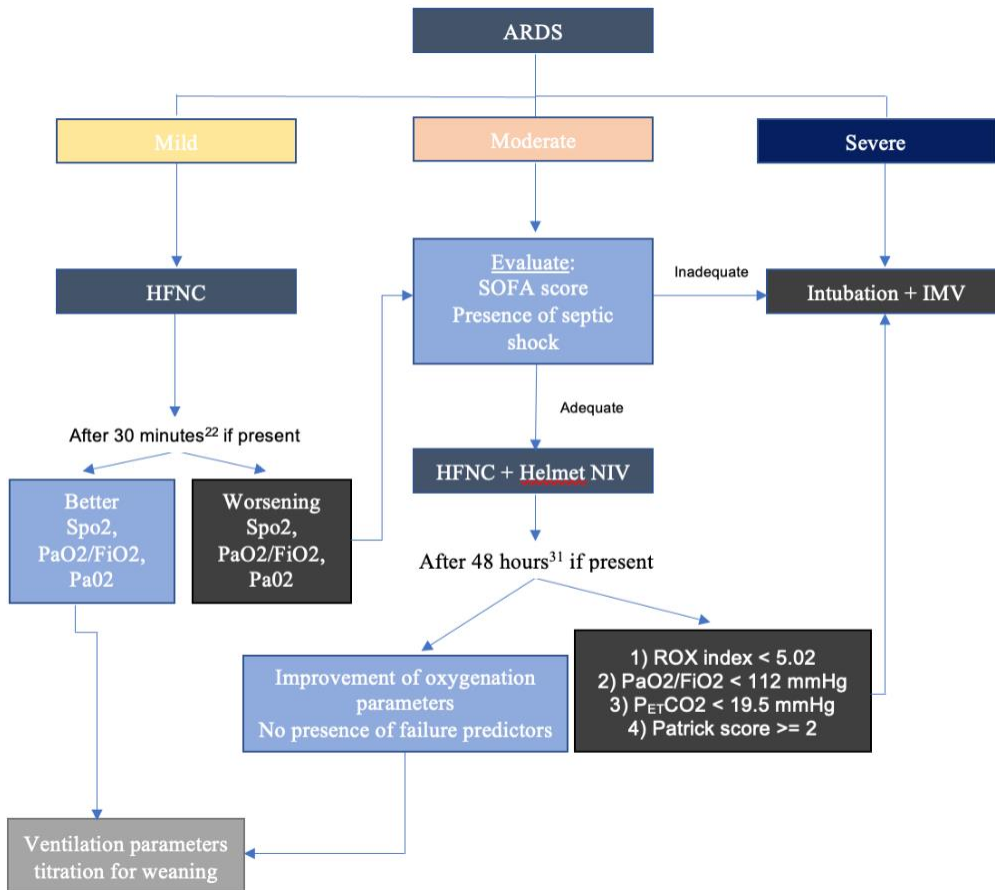


Figure 2. ARDS early ventilatory management scheme

### 5. Conclusions

To conclude, the effective use of High-Flow Nasal Cannula (HFNC) in conjunction with Non-Invasive Ventilation (NIV) is recommended for treating mild to moderate Acute Respiratory Distress Syndrome (ARDS), but caution is advised, and HFNC should be avoided in cases of severe ARDS. NIV application in moderate and severe ARDS requires careful consideration, with a thorough assessment of predictors of failure both before and during therapy. Notably, NIV shows an association with a higher failure rate in more severe ARDS cases. Survey responses indicate widespread utilization of HFNC as a primary treatment for mild ARDS.

Helmet NIV demonstrates superior outcomes in terms of mortality rates, failure rates, secondary outcomes, and patient comfort when compared to full-face masks. The use of helmet NIV is associated with enhanced comfort for patients compared to full-face masks.

Comparatively, physiotherapists in Chile exhibit greater involvement in the daily care of ICU patients with ARDS than their counterparts in France and Spain. Some responses from French and Spanish professionals deviate from current guidelines on ARDS management and are identified as areas for improvement.

Ongoing research is needed to investigate the potential combination of helmet NIV with HFNC in reducing mortality rates. Further exploration is required to enhance the precision of NIV predictors. Additionally, there is a need for research focusing on the use of helmet NIV in the moderate to severe ARDS population.



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

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6. **Appendixes**

6.1. *Appendix 1*

# Management of Acute Respiratory Distress syndrome in the ICU

Dear ones,

In the process of my 4th year's final year project in physiotherapy in the CEU San Pablo in Madrid, I am realizing a bibliographic review about the management of Acute Respiratory Distress Syndrome (ARDS) in the ICU from early diagnosis.

As a French student, studying in Spain and doing an international exchange in Chile, I would like to gather information about the management of the pathology according to the country, and put it in perspective as a discussion.

From a personal experience, ARDS is a pathology that is not perfectly understood yet and treated empirically, without a proper protocol. Furthermore, the role of respiratory physiotherapy is primordial in the prognostic of each individual patient and is not clearly established in each country.

Based upon those, I am kindly asking you to share your experience through this questionnaire that is completely anonymous and will last about 5 minutes.

I am available for any question or doubt at the following email: [b.theo@usp.ceu.es](mailto:b.theo@usp.ceu.es)

Thank you for your participation,

Théo Battalian

\*mandatory

Section 1: Personal information

1. Are you or have you been physiotherapist in Intensive care units (ICU)? \*

If the answer is negative, you don't need to continue answering.

*One answer only.*

Yes

No



2. How old are you? \*

*One answer only.*

- 18 - 25 years old
- 26 - 35 years old
- 36 - 50 years old
- 51 - 65 years old
- 66 and above

3. What was/is your country and city of practice? \*

\_\_\_\_\_

4. Which type of ICU's do/did you work in? \*

*Multiple answers possible.*

- Neonatal
- Pediatric
- Cardiac
- Severe burns
- Polyvalent
- Post-surgical
- Other: \_\_\_\_\_

5. Which type of physiotherapy treatment are/were you applying? \*

*Multiple answers possible.*

- Motor
- Respiratory
- Neurologic
- Other: \_\_\_\_\_



6. How many sessions of physiotherapy per 24 hours for each patient? \*

*One answer only.*

- Less than 3
- Between 3 and 5
- More than 5
- Other: \_\_\_\_\_

7. What is/was your experience in the ICU? \*

*One answer only.*

- Less than 1 year
- 2 - 5 years
- 5 - 10 years
- 10 - 20 years
- 20 + years

Section 2: Diagnosis of the pathology.

8. Are you aware of the Berlin classification of ARDS from 2011? \*

*One answer only.*

- Yes
- No



9. In your personal experience, which criteria are considered in the diagnosis of ARDS? \*

*Multiple answers possible.*

- Chest imaging
- Oxygenation level (PaFi)
- Origin of the oedema (non - cardiogenic oedema)
- Timing (> 1 week of symptomatology)
- Other: \_\_\_\_\_

10. Do you personally believe in this diagnostic method? \*

*One answer only.*

- Yes
- No

11. Did the pandemic change the diagnostic technic of ARDS? \*

*One answer only.*

- Yes
- No

Section 3: Management of the pathology.





12. In the case of mild ARDS (PaFi 200 – 300 mmHg) which type of ventilatory treatment do you usually use as a **first line of treatment**?

*One answer only.*

- High Flow Nasal Canula (HFNC)
- Non - invasive ventilation (NIV)
- Oxygen therapy
- Invasive ventilation through endotracheal intubationNone
- Other: \_\_\_\_\_
- 

13. In the case of moderate ARDS (PaFi 100 – 200 mmHg) which type of ventilatory treatment do you usually use as a **first line of treatment**?

*One answer only.*

- High flow nasal canula (HFNC)
- Non - invasive ventilation (NIV)
- Oxygen therapy
- Invasive ventilation through endotracheal intubationNone
- Other: \_\_\_\_\_
-



14. In the case of severe ARDS (PaFi < 100 mmHg) which type of ventilatory treatment do you usually use as a **first line of treatment**?

*One answer only.*

- High flow nasal canula (HFNC)
- Non - invasive ventilation (NIV)
- Oxygen therapy
- Invasive ventilation through endotracheal intubation
- Other: \_\_\_\_\_
- None

15. How do you measure the evolution after 1 hour of any ventilatory therapy or change ventilatory parameters?

*One answer only.*

- Clinical evaluation (monitor evaluation, signs, and symptoms)
- Gasometrical analysis
- Both
- Other: \_\_\_\_\_



16. Which are the intubation criteria in your ICU? \*

One answer per line.

	Not at all important	Low importance	Neutral	Important	Very important
PaFi < 150 mmHg	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Glasgow coma scale < 8 points	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Shock with unstable hemodynamic	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Hyperlactatemia	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

17. Any other intubation criteria you would consider?

\_\_\_\_\_

18. Do you use protective factors with mechanical ventilation? \*

One answer only.

- Yes  
 No

19. If yes, which ones?

Multiple answers possible.

- Control of PEEP  
 Tidal volume according to the ml/kg (ideal weight)  
 Driving pressure.  
 Neuromuscular blocking agents  
 Prone ventilation  
 Other: \_\_\_\_\_



20. Which PEEP level would you implement in patients with **moderate** ARDS in invasive ventilation?

*One answer only.*

- 5 - 10 cmH<sub>2</sub>O
- 10 - 15 cmH<sub>2</sub>O
- 15 - 20 cmH<sub>2</sub>O

21. Which level of PEEP would you implement in a patient with **severe** ARDS in invasive ventilation?

*One answer only.*

- 5 - 10 cmH<sub>2</sub>O
- 10 - 15 cmH<sub>2</sub>O
- 15 - 20 cmH<sub>2</sub>O

22. In which type of patients neuromuscular blocking agents are usually prescribed in your ICU?

*One answer only.*

- Mild ARDS
- Moderate ARDS
- Severe ARDS
- Other: \_\_\_\_\_



23. In which case would you pronate a patient? \*

*One answer only.*

- PaFi < 150 mmHg with FiO<sub>2</sub> 0.8
- Generalized Lung fibrosis
- Desaturation SpO<sub>2</sub> < 90%
- Severe hypoxemia
- Other: \_\_\_\_\_

24. In which case would you evaluate the patient for weaning from the invasive ventilation? \*

*One answer only.*

- PaFi > 200 mmHg; PEEP < 8 cmH<sub>2</sub>O, FiO<sub>2</sub> < 0.5
- PaFi > 150 mmHg; PEEP < 10 cmH<sub>2</sub>O; FiO<sub>2</sub> < 0.6
- PaFi > 150 mmHg; PEEP < 8 cmH<sub>2</sub>O; FiO<sub>2</sub> < 0.6
- Other: \_\_\_\_\_

#### Section 4: Physiotherapist involvement



25. In the following table, cross the level of implication you have as a physiotherapist in each treatment method.

0 = no involvement; 5 = completely in charge

One answer per line.

	0	1	2	3	4	5
High flow nasal canula	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Non - invasive ventilation	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Oxygen therapy	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Invasive ventilation	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Endotracheal intubation decision taking	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Endotracheal intubation maneuver	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Management of ventilatory parameters	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Weaning decision taking	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Pronation	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Decision between each treatment evolution	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

26. Did you notice a difference of involvement before and after the pandemic? \*

One answer only.

- Yes
- No



27. If yes, in the following table, cross the level of implication you had as a physiotherapist in each treatment method **before** the pandemic.  
 0 = no involvement; 5 = completely in charge

*One answer per line.*

	0	1	2	3	4	5
High flow nasal canula	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Non - invasive ventilation	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Oxygen therapy	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Invasive ventilation	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Endotracheal intubation decision taking	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Endotracheal intubation maneuver	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Management of ventilatory parameters	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Weaning decision taking	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Pronation	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Decision between each treatment evolution	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

28. Do you consider inter - disciplinary approach important in the management of a pathology in the ICU?

*One answer only.*

- Yes
- No



29. Do you generally feel satisfied with the physiotherapist role in the ICU and the decision taking?

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30. Would you like to add any comment to this study?

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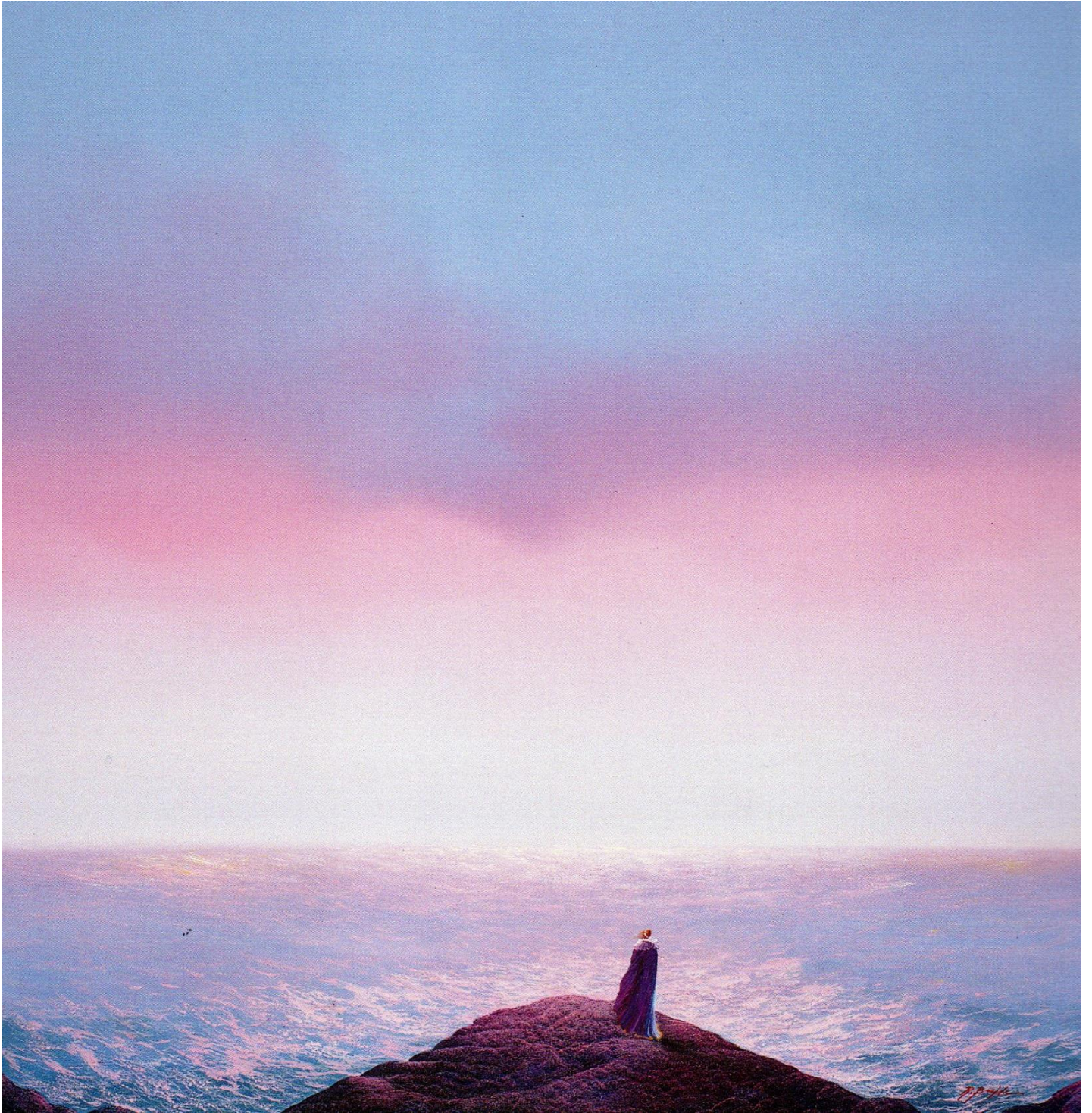




6.2. Appendix 2

**Table A2.1.** Scoring of articles according to STROBE statement and PEDro scale

	STROBE score (Vandenbroucke et al., 2007; Von Elm et al., 2008)	PEDro scale (Moseley et al., 2009; Macedo et al., 2010)	Missing information/items
Frat et al. (2015)	19 out of 22	x	- Study's funding sources - Assessment of the generalizability of the study findings.
Messika et al. (2015)	19 out of 22	x	- Study's funding source - Sample size calculation - Assessment of the generalizability of the study findings.
Menzella et al. (2021)	20 out of 22	x	- Study's funding source - Assessment of the generalizability of the study findings.
Bellani et al. (2017)	22 out of 22	x	x
Sehgal et al. (2015)	16 out of 22	x	- Study's funding - Potential sources of bias - Assessment of the generalizability of the study findings
Patel et al. (2016)	x	8 out of 10	- Blinding of therapists - Blinding of assessors for mortality outcome - Treatment or "intention to treat" for mortality outcome
Yaroshetskiy et al. (2022)	20 out of 22	x	- Study's funding source - Assessment of the generalizability of the study findings.
Chawla et al. (2016)	21 out of 22	x	- Assessment of the generalizability of the study findings





# Retrospective Review of 27 Cases of Congenital Portosystemic Shunt in Dogs from 2015 to 2023

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## Abstract:

The study aims to conduct a retrospective review of 27 cases of a congenital portosystemic shunt in dogs from 2015 to 2023, providing insights into classification, clinical manifestations, diagnostics, anaesthesia, surgery, and perioperative care. Initiation of medical therapy at least 14 days before surgery aims to alleviate clinical signs and mitigate anaesthesia and surgery risks, emphasising a low-protein diet and lactulose to trap ammonium ions in the intestinal lumen and was strictly followed in all patients. Changes in drug uptake, metabolism, and excretion should be considered when choosing an anaesthesia protocol. All dogs, except one with absent portal vein, underwent cellophane placement, facilitating gradual shunt closure. All dogs recovered uneventfully from surgery without evidence of portal hypertension and showed clinical improvement after that.

**Keywords:** Portosystemic shunts, dogs, cellophane attenuation, perioperative preparation



## 1. Introduction

A portosystemic shunt (PSS) in dogs is an abnormal connection between the portal vascular system and the systemic circulation (Buttler et al., 1990; Watson and Hertage, 1998; Mullins, 2019a). This condition diverts blood from the abdominal organs, bypassing the liver, which filters and detoxifies the blood before it returns to the heart (Sleight and Thomford, 1970; Tieman Mankin, 2015; Bertolini, 2019).

PSS are classified into acquired and congenital. Congenital PSS (CPSS) result from abnormalities in the development of the portal venous system or foetal vessels and are divided into two categories: extrahepatic and intrahepatic (Bertolini, 2019; Mullins, 2019a). CPSS predominantly enter the prehepatic caudal vena cava (Mathews and Gofton, 1988) at the epiploic foramen, termed portocaval shunts (Mullins, 2019b). Portophrenic shunts entering the left hepatic vein or caudal vena cava at the diaphragm level exhibit milder symptoms than other shunts (Berent and Tobias, 2018). Portoazygos shunts, typically involving the left gastric vein, occasionally the right gastric vein, transverse the diaphragm through the oesophageal hiatus and enter the azygos vein in the thorax (Harari et al., 1990). Acquired PSS are commonly caused by portal hypertension resulting from liver cirrhosis or liver failure (Boothe et al., 1996; Tieman Mankin, 2015; Berent and Tobias, 2018).

Breeds at an increased risk of extrahepatic CPSS are Cairn and Yorkshire Terriers, Maltese, Tibetan spaniels, miniature Schnauzers, Shih Tzu, Poodles (Tobias and Rohrbach; 2003), Pugs and Dachshunds (Wolschrijn et al., 2000; Tobias, 2003).

The clinical manifestations of CPSS lack specificity and may fluctuate over time, impacting the central nervous system, gastrointestinal system, and urinary tract (Konstantinidis et al., 2023). General signs in dogs include poor weight gain and stunted growth (Tobias, 2012). Hepatic encephalopathy and seizures may also be seen in PSS with the signs of hepatic encephalopathy, which are ataxia, depression, changes in behaviour, circling, disorientation, head pressing, blindness, seizures, and coma. In about 30% of dogs, vomiting, diarrhoea, anorexia, and salivation occur (Howe and Boothe, 2002; Konstantinidis et al., 2023). Clinical signs of the urinary system are associated with ammonium biuratur crystals and urate calculi and include dysuria, hematuria, pollakiuria, stranguria and decreased urine specific gravity (Howe and Boothe, 2002; Broome et al., 2004; Tobias, 2012; Caporali et al., 2015). In dogs, PSS can decrease the levels of various blood parameters such as total protein (TP), albumin, blood urea nitrogen (BUN), total cholesterol, and glucose. Elevated ammonia levels and serum bile acid are notable and serve as reliable indicators for diagnosing PSS in dogs (Ruland et al., 2010). Leucocytosis may be evident (Winkler et al., 2003), and mild to moderate microcytic normochromic nonregenerative anaemia is often observed alongside (Tobias, 2012; Tieman Mankin, 2015).

Surgical intervention is widely acknowledged as the preferred treatment for PSS, given that medical treatment alone cannot address the underlying vascular anomaly. The primary advantage of medical therapy lies in its ability to minimise ammonia absorption from the gastrointestinal tract, aiming to prevent hepatic encephalopathy and seizures. The increased ammonia concentration in plasma could also be attributed to bacterial degradation of amino acids in the gastrointestinal tract (Self, 2016). Before undergoing anaesthesia, dogs with PSS should undergo medical stabilisation (Dugdale et al., 2020).

Dogs with PSS are often young, and to avoid the risk of hypoglycemia, food should only be withheld four to six hours prior to anaesthesia (Self, 2016). Dogs with PSS require minimal premedication with opioids, induction with propofol or alfaxalone, and anaesthesia should be maintained with isoflurane or sevoflurane (Self, 2016; Dugdale et al., 2020). Low doses of alpha-2-adrenoceptor agonists can also be used for premedication, and opioids should be continued during surgery (Self, 2016). Nonsteroidal anti-inflammatory drugs should be avoided due to coagulopathies and hypoproteinaemia, and caution is advised also with paracetamol application because of hepatic insufficiency (Dugdale et al., 2020). Notably, due to their small body size and reduced metabolic heat production by the small liver, dogs with PSS are particularly prone to hypothermia (Self, 2016). Intravenous administration of Hartmann's solution is necessary during both the surgical procedure and the recovery period. Continuous monitoring of blood glucose levels and body temperature



is imperative during recovery, with particular attention to observing seizures. Seizures can be effectively managed by addressing metabolic disturbances such as hypoglycemia and elevated ammonia levels. Additionally, pharmaceutical options, including levetiracetam, phenobarbitone, propofol, or low doses of dexmedetomidine or medetomidine, are viable choices for seizure control (Dugdale et al., 2020). Post-surgery, ensuring adequate analgesia is essential, and opioids represent a recommended approach for managing pain (Self, 2016).

## 2. Materials and methods

Patient data regarding CPSS were collected from medical records at the Veterinary Faculty in Ljubljana, Slovenia, covering the period from 2015 to 2023. Out of the 27 dogs included in the study, detailed medical history and laboratory results were available for 23 dogs, while CT scans were available for 25. However, due to the retrospective nature of the study, complete data retrieval was not possible for all 27 dogs (Erjavec et al., 2024). Additionally, over one-third of the dogs were referred from other clinics and were under the care of their primary veterinarian after surgery; thus, complete data for these dogs were also not available. Since this study involved the review of existing medical records and did not involve any new interventions or procedures on animals, formal ethical approval was not required.

All dogs underwent surgical treatment, and among the 27 surgically treated patients, two (a Golden Retriever and a mixed breed dog) were found to have an intrahepatic PSS. The diagnosis was established based on clinical signs, laboratory findings (bile acids, blood ammonia, complete blood count (CBC) with differential, urea, creatinine, albumins, TP, potassium, sodium and chloride, glucose, ALT (alanine aminotransferase), urinalysis and confirmed with computed tomography (CT).

At least 14-day preparation regimen was implemented with the following oral therapies: lactulose (Portalak 667 mg/ml, Belupo, Koprivnica, Croatia) at a dose of 0.5 ml/kg, metronidazole 10 mg/kg/12h (Metrobactin, Dechra LelyPharma BV, Lelystad, Nederland), gastroprotective drugs - esomeprazole (Nexium, Grünenthal GmbH, Aachen, Germany), hepatoprotective (Epato 1500 plus, DRN s.r.l., Italy,  $\alpha$  IT000187AL, marketing@drnsrl.it) and ursodeoxycholic acid (Ursofalk 250 mg, Dr. Falk Pharma GmbH, Freiburg, Germany).

Additionally, a low protein diet (Hill's k/d) was administered, and on the day before surgery subcutaneous vitamin K (Konaktion MM, Phytomenadione 10 mg, Cheplapharm, Arzneimittel GmbH, Ziegelhof, Greifswald, Germany) at a dose of 5.0 mg/kg was given first day and continued 2 days after surgery at a dose of 2.5 mg/kg. The dogs that already had epileptic seizures received antiepileptic drugs phenobarbitone 1–4 mg/kg/12h (Epiphen 30mg, Vetoquinol, Buckingham, UK) and/or levetiracetam 20 mg/kg/8h (Keppra 100 mg/ml, UCB Pharma SA, Brussels, Belgium). Hospitalisation began a day before the scheduled surgery to facilitate preparation and blood examination (CBC with differential, urea, creatinine, albumins, TP, potassium, sodium and chloride, glucose, ALT, PT (prothrombin time), APTT (activated partial thromboplastin time). On the morning of the surgery, the dogs received their complete prescribed therapy, including lactulose, metronidazole, vitamin K, intravenously pantoprazole (Nolpaza, Krka, Novo mesto, Slovenia) and dogs with seizures antiepileptic drugs. All medicine except vitamin K was continued at least 2 weeks after surgery, i.e., to the first check-up, and then gradually discontinued according to the clinical signs and laboratory results.

Induction of anaesthesia was performed with analgetic fentanyl (Fentanil Torrex 50 µg/ml, Chiesi Pharmaceuticals GmbH, Wien, Austria), 2 µg/kg or remifentanyl (Remifentanil-hameln 1 mg, Siegfried Hameln GmbH, Hameln, Germany) 2 µg/kg and propofol (Propoven 10 mg/ml, Fresenius Kabi Austria GmbH, Graz, Austria) slowly to effect, 2–3mg/kg intravenously. Anaesthesia was maintained with isoflurane in oxygen, and constant rate infusion (CRI) of fentanyl (2 µg/kg/h) or remifentanil (5 µg/kg/h) was administered during surgery and continued as CRI two to three days after surgery. During anaesthesia lactated ringer solution (Hartmannova raztopina Braun, B. Braun Melsungen AG, Melsungen, Germany) 5 ml/kg/h was given, blood glucose levels were monitored at 30-minute intervals and monitoring of arterial blood pressure was assessed using a



Doppler flow detector (Model 811-BL, Parks Medical Electronic), end-tidal CO<sub>2</sub> concentration, oxygen saturation measured with a lingual SpO<sub>2</sub> probe, body temperature with an oesophageal thermometer, and electrocardiogram (ECG) was continuous.

In all animals with PSS, a ventral medial celiotomy was performed, PSS was identified, and a cellophane band was applied in all dogs except for one dog where the portal vein was absent. Before use, 1.2 cm wide strips of cellophane were prepared and sterilised. After taking them from the sterilisation pouch, cellophane strips were immersed in saline, and then one strip was folded longitudinally into 3 layers. Initially, the tissue around the shunt was carefully dissected with right-angled dissecting forceps, and subsequently, the cellophane strip was carefully passed around the shunt. Three to four titanium clips were used in alternating directions to fixate both ends of the cellophane, and the remaining parts of the cellophane were cut with scissors. Shunts were partially or not at all attenuated at the end of surgery. The abdomen was closed routinely in all dogs. Dogs were typically hospitalised for up to 3 days following surgery after which they underwent check-ups at 7-14 days post-surgery, followed by monthly appointments for 3-4 months thereafter. Further follow-up appointments were scheduled as needed based on the presence of clinical signs.

### 3. Results

The inclusion criteria for selecting cases encompassed a thorough review of medical records pertaining to 27 dogs, operated by the same surgeon (VE), comprising 18 females and 9 males, representing various breeds such as mixed breed (6), Yorkshire Terrier (5 dogs), Pug (2), Maltese (2), Shih Tzu (2), Jack Russell Terrier (2), Miniature Schnauzer (2), and one each of Bearded Collie, Chihuahua, Cavalier King Charles Spaniel, West Highland White Terrier, Golden Retriever, and Pomeranian. The age range of the dogs on the day of the operation was 4 to 100 months, with twelve out of 27 dogs being younger than 12 months. Dogs were presented with various clinical signs, 19/23 dogs were apathic, 14/23 dogs were vomiting, neurological signs such as seizures, disorientation, ataxia, restlessness, blindness, and head pressing were found in 13/23 dogs, 7/23 had polydipsia/polyuria, 6/24 had diarrhoea, and 2/23 were salivating.

On the day of surgery or a few days before blood analysis was performed, serum bile acids were elevated in all dogs (22/22), ALT was elevated in 13/25 dogs, urea was low in 17/25 dogs, PT was prolonged in 8/24, and APTT was prolonged in 15/24 dogs. Nephroliths or uroliths were found in 13/26 dogs. Ammonia was elevated in 8/8 dogs. Leucocytosis was found in 16/24 dogs. Eleven dogs from 25 (11/25) were hypoproteinaemic and hypoalbuminemic, 6/25 were anaemic with low red blood cells and low haematocrit. Before the operation, two dogs needed a transfusion. During anaesthesia, 9 of 25 dogs needed a bolus of 20% glucose (Glukoza Braun 200 mg/ml, B. Braun Melsungen AG, Melsungen, Germany) 1-2 ml/kg intravenously. Twenty out of 25 dogs required Voluvene for maintenance of blood pressure or due to hypoproteinemia (Voluven 60 mg/ml, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) administered either through CRI at 1-2 ml/kg/h or as a bolus (1-2 ml/kg) in case of observed hypotension. This paper does not present results from potassium, sodium, chloride, venous blood gas analysis, and urinalysis.

Based on CT angiography images of 22 dogs with extrahepatic shunts, the distribution included right gastrocaval shunts in 7 dogs (32%), each of spleno- and portocaval shunts in 3 dogs (14%), splenoazygos and left gastroazygos shunts in 2 dogs each (10%), and singular occurrences of gastrophrenic, left gastric, left gastrocaval, gastroduodeno caval, and right gastrocaval with a caudal loop shunt in one dog.

On the day after surgery, dogs required intensive care, and vital signs, including body temperature, pulse rate, respiration, urine production, capillary refill time, mucous membrane colour, arterial blood pressure, and blood glucose, were recorded hourly and monitored for neurological signs. Monitoring urine production and/or blood pressure was also undertaken to adjust postoperative fluid therapy. Fluid therapy using lactated Ringer's solution was given at 3-5 ml/kg/h. The dogs received intensive medical attention



until they regained the ability to move and independently consume a regular diet. Analgesia was maintained as described before, with the addition of methadone if necessary. Throughout the recovery phase, no indicators of portal hypertension, such as hypovolemic shock, progressive hypothermia, and severe abdominal pain (Tobias, 2012), were observed. We do not have uniform results after surgery because the dogs underwent check-ups at different times and with various veterinarians who did not monitor the same blood parameters. However, we found that bile acids were elevated in 12/16 dogs, ALT was elevated in 6/20 dogs, urea was low in 2/13 dogs, TP was low in 2/18 dogs, and albumins were low in 3/21 dogs (Erjavec et al., 2024).

#### 4. Discussion

The investigation of PSS in dogs provides valuable insights into the pathophysiology, clinical presentation, diagnosis and outcomes following surgical intervention. The clinical signs that raised suspicion of portosystemic shunt (PSS) in dogs in our study were primarily neurological and gastrointestinal, with a notable prevalence of apathy among the affected dogs. Notably, salivation, a more common clinical sign in cats than in dogs (Howe and Boothe, 2002), was also reflected in our study. We primarily confirmed the suspicion with results indicating elevated bile acids, ammonia levels and urinalysis, while the diagnosis was subsequently confirmed through CT imaging. Preoperative management is crucial in minimising ammonia absorption from the gastrointestinal tract, preventing hepatic encephalopathy and seizures in dogs, and enhancing postoperative recovery. When ammonia was measured, it was increased in all dogs. Although ammonia is a valuable parameter, we did not measure it in all dogs due to the complex process of sample preparation and transport to an external laboratory.

While coagulation times are frequently prolonged in affected dogs, they generally do not have significant clinical implications and spontaneous bleeding is uncommon (Kummeling et al., 2006; Kelley et al., 2013) which was consistent with our study where coagulation times were prolonged, but no issues with bleeding during surgery occurred, which we attribute to the administration of vitamin K. Following PSS occlusion, gastrointestinal ulceration and gastritis may persist years after surgery in majority of dogs, prompting consideration on proton pump inhibitors, especially for those receiving NSAIDs post PSS surgery (Weisse et al., 2014; Dugdale et al., 2020).

In cases of hypoalbuminemia, caution should be exercised regarding the infusion of large volumes of crystalloids. Instead, synthetic colloids or plasma infusions may be considered to maintain osmotic pressure (Self, 2016), which was carefully considered in our dogs, where the majority of dogs additionally received Voluven. The slow elimination of certain drugs may contribute to the prolonged recovery phase, potentially leading to hypothermia and hypoglycemia (Self, 2016; Dugdale et al., 2020), so maintaining body temperature is crucial, and warming measures were implemented from the premedication phase through full recovery.

Cellophane banding emerges as a straightforward surgical procedure for the progressive attenuation of PSS in dogs (Youmans and Hunt, 1998). This method involves applying a cellophane band around the shunt to reduce blood flow. While the benefits of surgically attenuating shunts are widely acknowledged, rapid closure or ligation of the shunt may not yield optimal results for many dogs, as it can lead to the development of portal hypertension and cardiovascular compromise. Because the occlusion of the PSS with cellophane progresses slowly through inflammatory reaction, the shunt closes gradually, while the portal vein can uptake larger volumes of blood (Holt, 1994; Youmans and Hunt, 1998; Frankel et al., 2006). Since this process occurs concurrently, no portal hypertension occurred. Using inexpensive and readily available cellophane allows progressive attenuation in the weeks following cellophane placement. Slow attenuation of CPS may also allow more time for the cardiovascular and central nervous systems to adapt to changing hepatic metabolism (Youmans and Hunt, 1998).

The incorporation of preoperative imaging can reduce both surgical duration and the extent of dissection required for evaluating the shunt (Or et al., 2016). Attenuation location for different shunt types vary; portocaval shunts are attenuated at the level of epiploic



foramen, portophrenic at the abdominal surface of the diaphragm and newer studies suggest attenuation of portoazygos shunts within the thorax (Mullins, 2019b), while older literature suggested attenuation at the diaphragm level (Or et al., 2016), where azygos shunts were also attenuated in our study. The following considerations guided us during the surgeries: A healthy canine circulatory system should exhibit no large vessels entering the caudal vena cava between the phrenicoabdominal vein and the porta hepatis (Berent and Tobias, 2018). Moreover, it is common to observe turbulent blood flow in the caudal vena cava where the shunting vessel enters (Mullins, 2019a).

After the surgery, laboratory parameters such as ALT, urea, TP, albumines, and bile acids improved in most dogs, alongside clinical signs. Subsequently, medical therapy was discontinued. Based on clinical signs, lactulose treatment should be sustained for a minimum of 4 weeks, coupled with a protein-restricted diet until liver function normalises, indicated by normal albumin levels. Serum bile acids may remain abnormal for over a year following shunt ligation (Tobias, 2012). However, if abnormalities persist nine months post-surgery, the likelihood of normalisation decreases. In such cases, additional laboratory values, including albumin, urea, and liver enzymes, should be utilised to monitor the patient's condition. If blood values fail to normalise six months post-surgery, a reevaluation is advised to explore potential issues such as incomplete shunt occlusion, multiple acquired shunts, or other liver diseases (Tobias, 2012). However, the primary goal of surgery is for the animal to be free of clinical signs and not require therapy. It is important to note that while laboratory findings often improve after surgery, they may still frequently remain abnormal, as observed in our study.

## 5. Conclusion

The absence of significant complications observed during the surgical procedure can be attributed to administering appropriate therapy to the dogs for a sufficient duration before the operation. This therapeutic approach was consistently maintained throughout the recovery period. Intensive patient monitoring was crucial in the initial 12-24 hours post-surgery. Furthermore, a thorough observation was kept for any neurological signs, ensuring prompt initiation of assistance if such signs manifested. No signs of portal hypertension were detected in any of the cases post-surgery.

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

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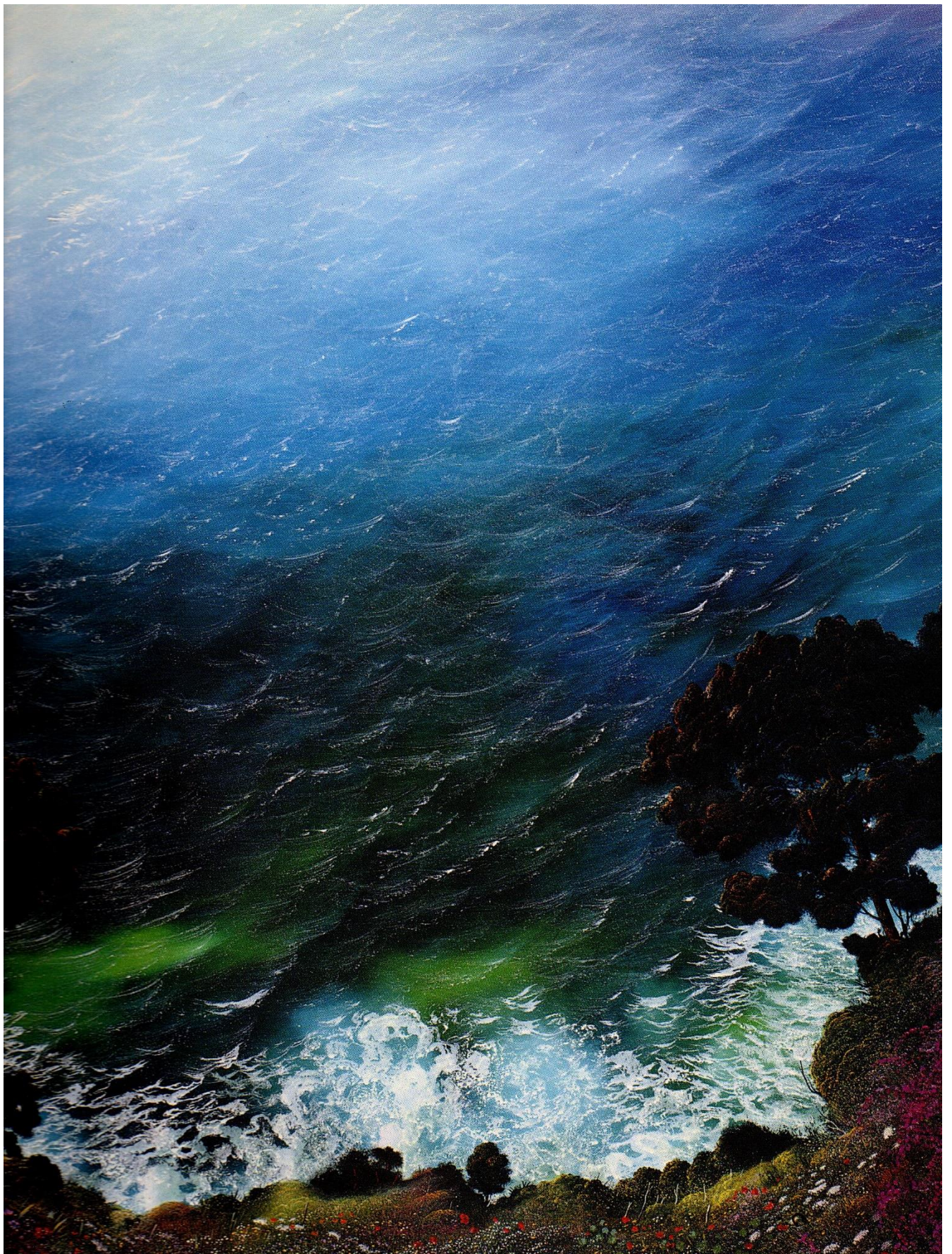




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# Data of 27 Cases of Congenital Portosystemic Shunt in Dogs from 2015 to 2023

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## Abstract:

Congenital portosystemic shunts (PSS) present a significant challenge in veterinary medicine, requiring comprehensive analysis for effective diagnosis and management. This repository offers an extensive overview of 27 cases of congenital PSS in dogs, spanning the period from 2015 to 2023. The data are shown in **Table 1**.

**Keywords:** Portosystemic shunts, dogs, clinical signs, types of PSS, laboratory results

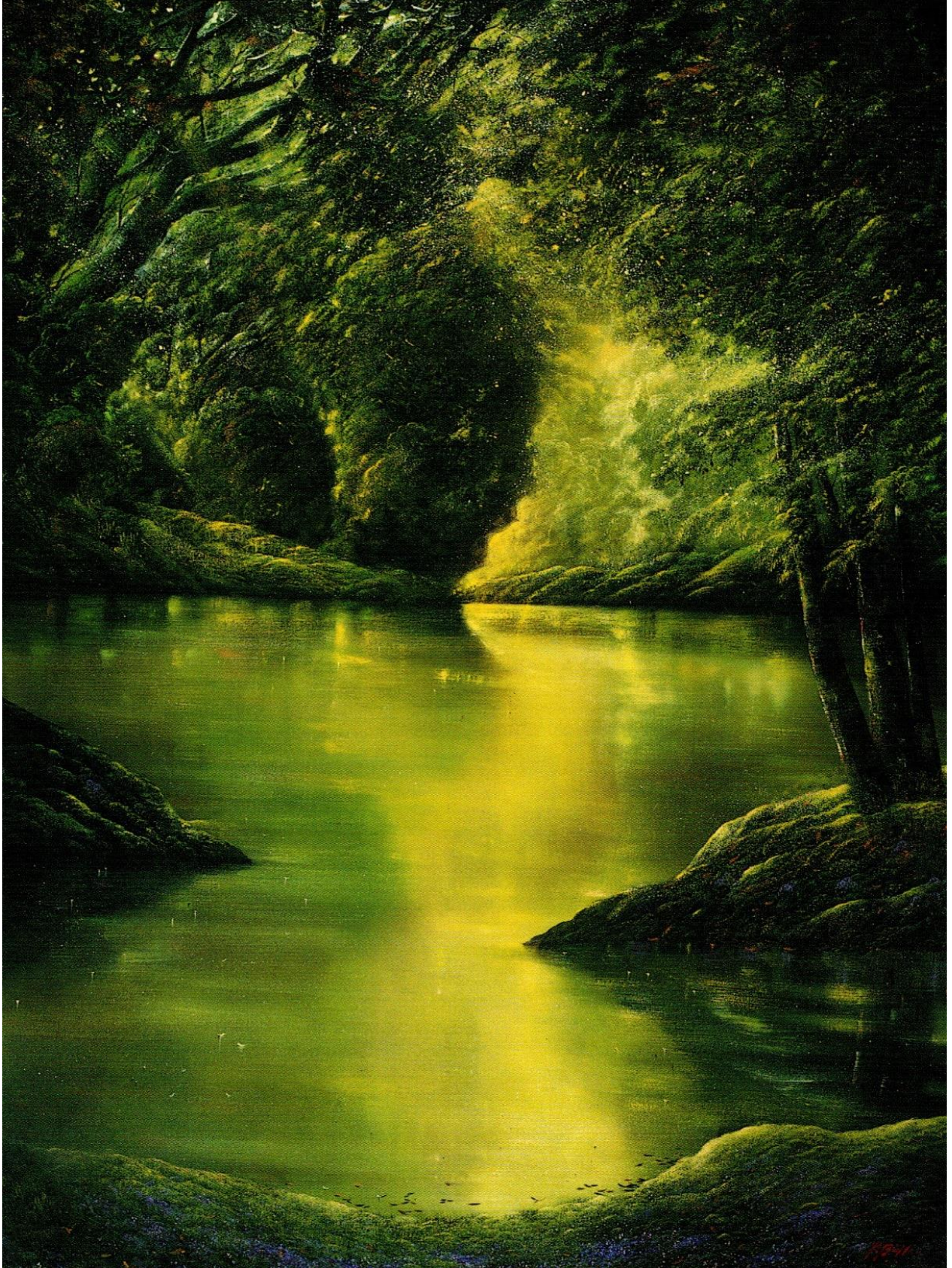
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**Table 1.** Data on dogs with Congenital Portosystemic Shunt from 2015 to 2023

No	Breed	Age Months	Weight kg	Gender	CT results	Epilepsy	Neurologic Signs	Apatic	Diarrhoea	Vomiting	PU/PD	Nephrolithiasis	Salivation	Anemia	RBC, HCT	↑bile acids Before s.	↑bile acids post surg.	↑ALT before	↑ALT post surg.	↑WBC before s.	↑BUN before s.	↓BUN post surg.	Total protein before s.	Total protein post surg.	ALB before s.	ALB post surg.	↑PT	↑APTT	Glucos	Voluven	Blood transfusion		
1	Pug	5	3.9	♀	spleno-caval, microhepatia,	Yes	Yes	Yes	NO	NO	NO	NO	NO	Yes	Yes	NO-5 mts	/	NO	NO-5 mts	Yes	NO	NO-5 mts	NO	NO-5 mts	Yes	NO-5 mts	NO	NO	NO	NO	Yes	Yes	NO
2	X	55	4.1	♀	L gastric, microhepatia, renal cyst	NO	Yes	Yes	NO	Yes	NO	Yes	NO	NO	NO	Yes	/	Yes	NO	NO	Yes	NO	NO	NO	NO	NO	NO	NO	NO	NO	Yes	Yes	NO
3	Pug	10	4.7	♂	R gastro-caval type, R gastric- L gastric prehepatic CVC, micro	NO	Yes	Yes	NO	Yes	NO	Yes	NO	Yes	Yes	Yes-1,5 mts; NO-1Y	/	NO	NO	Yes	Yes	NO	NO	NO-1,5 mts	Yes	Yes-1,5mt	NO	NO	Yes	Yes	Yes	Yes	
4	X	4	6.4	♀	azygos continuation of caudal vena cava, ectopic spleens	NO	NO	Yes	NO	NO	NO	NO	NO	NO	NO	Yes	Yes-7mts	/	NO	Yes-7 mts	Yes	Yes	NO	NO	NO-7 mts	NO	NO-7 mts	Yes	Yes	NO	Yes	NO	
5	X	20	10.8	♀	intrahepatic PSS, microhepatia	NO	Yes	Yes	NO	NO	NO	Yes	Yes	NO	NO	Yes	Yes-9mts	/	Yes	Yes-9 mts	Yes	Yes	Yes-3mts	Yes	NO	Yes	Yes-3 mts	Yes	NO	NO	NO	NO	
6	Shih-Tzu	42	3.8	♀	L gastric to caval shunt	NO	NO	NO	Yes	Yes	NO	NO	NO	NO	NO	Yes	NO-10 days	/	Yes	NO	Yes	NO	/	NO	/	NO	/	NO	NO	Yes	Yes	NO	
7	X	6	4.8	♀	R gastro-caval type, microhepatia	NO	NO	Yes	NO	Yes	NO	NO	NO	NO	NO	Yes	NO-3 mts, Yes-	Yes	NO	NO-3 mts	Yes	NO	NO-10 da	Yes	NO-1 mts	Yes	NO-1 mts	Yes	Yes	NO	Yes	NO	
8	Maltese	12	2.25	♂	gastroduodenal/R gastric caval, microhepatia, urinary/renal	NO	/	Yes	Yes	Yes	NO	Yes	NO	NO	NO	Yes	Yes-9mts	/	Yes	NO-1 and 9 mts	Yes	Yes	NO-9 mts	Yes	NO-1 mts	Yes	NO-1 mts	NO	Yes	Yes	Yes	NO	
9	CKCS	30	4.8	♀	R gastric type, microhepatia	NO	Yes	Yes	NO	Yes	NO	NO	NO	NO	NO	Yes	/	/	Yes	Yes-4 mts	NO	Yes	NO-4 mts	NO	/	NO	NO-4 mts	NO	Yes	Yes	Yes	NO	
10	Yorkshire	64	2.7	♂	gastrosplenic azygos, microhepatia	NO	Yes	Yes	NO	Yes	NO	Yes	NO	NO	NO	Yes	/	/	Yes	Yes-2 weeks	NO	NO	/	NO	/	NO	NO-2 wee	NO	Yes	Yes	Yes	NO	
11	CHD	7	/	♂	gastro-caval, microhepatia	/	Yes	/	/	/	/	Yes	/	/	/	Yes	/	/	Yes	/	/	Yes	/	Yes	/	Yes	/	/	/	/	/	/	
12	Yorkshire	20	1.8	♀	porto-left gastric azygos type, microhepatia	NO	Yes	Yes	NO	NO	Yes	NO	NO	NO	NO	Yes	/	/	Yes	NO	NO	Yes	NO	/	NO	/	NO	NO	NO	Yes	Yes	NO	
13	Pomeranian	35	1.45	♀	no portal vein, porto-caval, microhepatia	/	/	/	/	/	/	Yes	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	
14	Jack Russe	33	8.9	♀	gastro-splenic-azygos	NO	NO	Yes	Yes	NO	NO	Yes	NO	NO	NO	Yes	/	/	Yes	NO-2 mts	NO	NO	NO-2 mts	NO	NO-14 days	NO	NO-2 mts	NO	NO	NO	NO	Yes	NO
15	Shih-Tzu	15	8.2	♀	gastro-duodenal-caval, nephrolithiasis bilateral	NO	NO	NO	NO	NO	Yes	Yes	NO	NO	NO	Yes	Yes-2,5 mts	/	NO	NO-2,5 mts	Yes	Yes	NO-2,5 mt	NO	NO-2,5 mts	NO	NO-2,5 mt	NO	NO	NO	NO	NO	
16	BC	41	20.3	♀	L gastric to caval shunt, microhepatia, bilateral nephromegaly	NO	NO	Yes	NO	Yes	NO	NO	NO	NO	NO	Yes	/	/	Yes	YES-1 mts, NO-2 m	Yes	Yes	/	Yes	NO-2 mts	Yes	NO-2 mts	NO	NO	NO	NO	NO	
17	X	63	4.4	♀	R gastro-caval type, microhepatia, lack of formation of gastri	NO	NO	Yes	NO	Yes	NO	Yes	NO	NO	NO	Yes	Yes-1 mts	/	Yes	NO-1 mts	NO	Yes	/	NO	NO-1 mts	Yes	NO-1 mts	Yes	NO	NO	NO	NO	
18	Maltese	100	8.5	♀	spleno-gastro-phrenic type, microhepatia	NO	Yes	Yes	NO	NO	Yes	NO	NO	NO	NO	Yes	Yes-12 mts	/	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	Yes	NO	
19	WHWT	4	3.3	♀	spleno-caval, microhepatia,	NO	NO	Yes	Yes	Yes	NO	Yes	NO	NO	NO	/	NO-4 and 8mts	Yes	Yes	NO	Yes	Yes	NO	NO	NO-1 mts	NO	NO	NO	NO	NO	NO	NO	
20	Yorkshire	4	1.7	♀	/	NO	Yes	NO	Yes	NO	NO	NO	NO	NO	NO	Yes	/	Yes	NO	NO-10 days	Yes	NO	/	Yes	Yes-10 days	Yes	Yes-10 da	Yes	Yes	NO	Yes	NO	
21	MS	23	7	♀	double-loop right gastric-caval single	Yes	/	/	Yes	/	/	/	/	NO	/	/	/	/	NO	/	NO	Yes	/	NO	/	NO	/	NO	NO	NO	NO	NO	
22	Yorkshire	5	1.9	♀	R gastro-caval type, microhepatia	Yes	Yes	NO	NO	NO	NO	NO	NO	NO	NO	Yes	Yes-4 mts	Yes	NO	NO-14 days	Yes	Yes	/	Yes	NO-4 mts	NO	NO	Yes	NO	Yes	NO	Yes	
23	Jack Russe	7	4.9	♀	spleno-caval, microhepatia, portal vein hypoplasia	NO	NO	Yes	NO	Yes	Yes	NO	NO	NO	NO	Yes	Yes-1 and 3mts	Yes	Yes	NO-1 and 3 mts	Yes	Yes	/	Yes	NO-3 mts	NO	NO-3 mts	NO	NO	NO	Yes	NO	
24	MS	9	6.6	♀	/	/	/	/	/	/	/	Yes	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	Yes	
25	X	6	7.22	♀	porto-caval	NO	NO	Yes	NO	Yes	NO	NO	NO	Yes	/	Yes-5 and 6 mts	Yes	NO	NO-1 mts	Yes	NO	/	Yes	Yes-1 mts	Yes	NO-1 mts	Yes	Yes	NO	Yes	NO	Yes	
26	Yorkshire	9	2	♀	porto-caval	NO	Yes	Yes	NO	Yes	Yes	Yes	NO	Yes	Yes	Yes-4 mts	Yes	Yes	/	Yes	Yes	/	Yes	NO-2 mts	Yes	NO-mts	Yes	Yes	NO	Yes	NO	Yes	
27	GR	6	12.6	♀	intrahepatic, central, microhepatia	NO	NO	Yes	NO	Yes	NO	NO	NO	Yes	Yes	Yes-3 mts	/	NO	Yes-4,5 mts	Yes	Yes	Yes-3 mts	Yes	NO-3 mts	Yes	NO-3 mts	NO	Yes	/	/	/	/	
						9/24	13/23	19/23	6/24	14/23	7/23	13/26	2/23	6/25	22/22	12/16	8/8	13/25	6/20	16/24	17/25	2/13	11/25	2/18	11/25	3/21	8/24	15/24	9/25	20/25	2/25		

X: mixed breed, CKCS: Cavalier King Charles Spaniel, CHD: Chihuahua, WHWT: West highland white terrier, BC: Bearded collie, MS: Miniature Schnautzer, GR: Golden Retriever, L: left, R: right





# Exotic Animals' Vascular System Characteristics and its Application in Clinical Practice

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## Abstract:

Veterinary practice commonly involves caring for domestic and companion animal. Since the trend of owning exotic pets is rapidly increasing, veterinarians often treat exotic patients as well. As a result, veterinarians are facing increased demands for specialized skills and knowledge. This article aims to provide a concise review of the anatomy of the vascular system in exotic animals and its relevance in clinical practice. Snakes, lizards and chelonians have 3-chambered heart with 2 atria and one partially divided ventricle whereas crocodiles have 4-chambered heart. It is important to consider these characteristics during the anesthesia in reptiles since blood shunting affects changes in blood pressure, oxygen level and other anesthetic parameters. In birds, the cardiovascular system is crucial in enduring the exhausting physical activities such as swimming, flying or diving and running. Birds have renal portal system which consists of cranial and caudal renal portal vein. The recommendation of avoiding drug application in the hindlimbs of birds is present for a long time. This is valid not only for birds, but for reptiles, amphibians and most fish. The ventricle of amphibians is trabeculated, which minimizes blood shunting through various anatomical and physiological features. During biphasic systole, the left and right side of the ventricle contract separately, directing blood in different parts of aortic arches. Due to the complexity of the exotic animals, thorough education added in veterinary curricula is needed.

**Keywords:** amphibians, birds, cardiovascular, clinical, reptiles



## Introduction

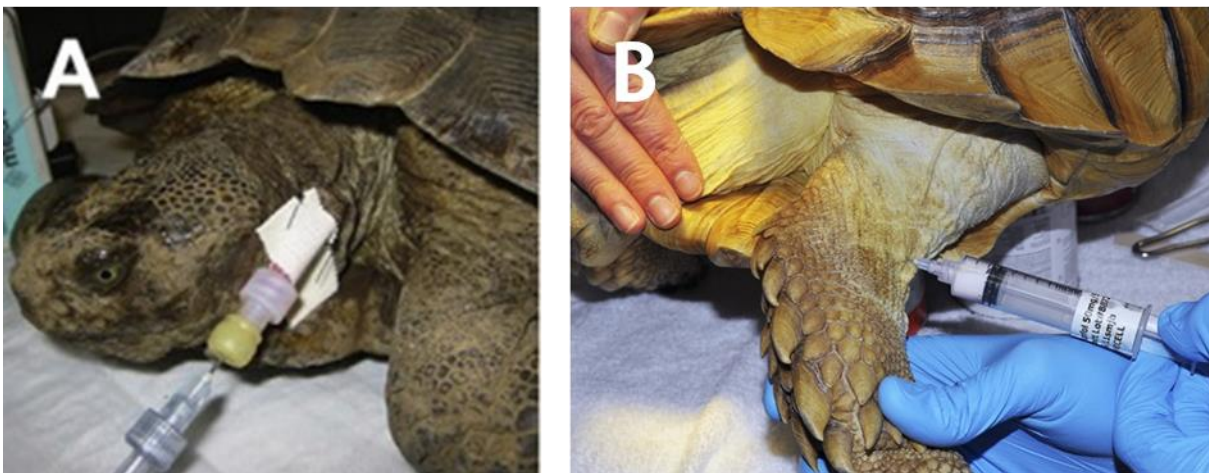
Common veterinary practice deeply relies on working with domestic and companion animals. Even though they are perceived as the most frequent patients, trend of owning exotic pets is rapidly increasing. Statistics show that the number of pet birds in Europe is around 52 million with 29 million small mammals, 10 million aquatic animals and around 9 million reptiles (Ostović et al., 2022). In the USA, the exotic animal trade market has seen significant growth in recent years. Between 2000 and 2006, United States imported around 1.5 billion wild-life animals from which 92 % was acquainted as pets (Smith et al., 2009). These numbers increase steadily every year. Thus, skills and knowledge required from veterinarians increase and become challenging (Espinosa García-San Román et al., 2023). Contrary to this, education about exotic pets and practical skills are not included in most of the veterinary schools or they are included insufficiently (Rosenthal, 2006). Institutions are often reluctant towards introducing the courses about exotic animals, not realizing that exotic pets as patients are more common than before and students should have some basic knowledge after the graduation. Recommendation of American College of Zoological Medicine regarding veterinary curricula implies that all students should be able to take history and perform physical examination of exotic patients. They also suggest that students acquire specific knowledge of physiology, methods of restrain, sample collection, and understanding of clinical data (Stoskopf et al., 2001). Many specificities characterize exotic animals in comparison with usual companion animals. However, the studies regarding cardiovascular system of exotic animals are scarce. Existing studies primarily focus on laboratory animals due to their widespread use in biomedical research. Although American mink (*Mustela vison*) is not a typical laboratory animal, its cardiovascular system has been extensively studied. Several studies (Hadžiomerović et al., 2016; Mrvić et al., 2017; Mrvić et al., 2021) investigated mink's vascular system including kidneys, liver and lungs. They described branching of hepatic, renal and pulmonary veins and arteries comparing it to the dog as a similar carnivore model. In other studies, Ninomiya et al. (2008) and Mazensky et al. (2012) explored vascular features of rabbit eyes using vascular corrosion casting and branching of renal veins and arteries. This article aims to condense specific anatomy of exotic animal's vascular system and to refer on its use in clinical practice. It further intends to help veterinary practitioners in process of clinical examination of these animals and to highlight what to be aware of regarding their anatomy and physiology.

## Reptiles

Reptiles belong to the large class of *Reptilia* consisting of several orders. Snakes and lizards (order - *Squamata*), chelonians and crocodiles make the majority of this class where squamates are the most species-rich order with approximately 7000 species (Jensen et al., 2014). Their cardiovascular anatomy greatly varies. Snakes, lizards and chelonians have 3-chambered heart with 2 atria and one partially divided ventricle whereas crocodiles have 4-chambered heart. All species have 2 aortic arches, right and left with 4 systemic veins including 2 cranial caval veins and caudal caval vein (Sladky and Mans, 2012; Jensen et al., 2014). These anatomical features cause mixing of arterial and venous blood. Even though considered pathophysiological in mammals and birds, blood shunting is proven to have several roles in reptile organism unlike previous hypothesis that treats shunting as evolutive remnant without physiological significance or even detrimental. It enables stabilization of oxygen level during breaks in respiration. Furthermore, right to left shunt can partially help heating process by increasing the amount of blood in systemic circulation. Left to right shunt, where the blood level in lungs is reduced, happens during the diving apnea of aquatic reptiles (Hicks, 2002; Mosley, 2005). It is important to consider these characteristics during the anesthesia in reptiles since blood shunting affects changes in blood pressure, oxygen level and other anesthetic parameters. The shunting can also delay or hasten the excretion of the inhalation anesthetics. Another vascular system characteristic specific to reptiles is renal portal system. Cranial and caudal portal veins collect blood from the tail, pelvis, limb and caudal part of the spine and intestines forming the plexus around the kidneys. Blood flow is controlled by autonomic nervous system and valves which direct blood through or around the kidneys (Sladky and Mans, 2012). Renal portal system could possibly affect the pharmacodynamics and pharmacokinetics of drugs



administered in the caudal part of the reptile's body. Some authors suggest that drugs administered in the caudal part could be nephrotoxic or that the excretion time of the drug could be decrease, consequently affecting the effective dose and treatment of the animal. Contrary to this, some studies oppose these statements due to the lack of scientific evidence. However, current recommendations advise against the administration of nephrotoxic drugs and those excreted primarily by kidneys in the caudal part of reptiles (Mosley, 2005; O'Malley, 2017). During the clinical examination of patient, it is significant to know the sites for blood sample collection as well as drug administration sites. In lizards, ventral tail vein, ventral abdominal vein, cranial vena cava, brachial vein and jugular vein are commonly used while in chelonians, for intravenous route and blood sample collection, jugular, dorsal tail vein and brachial vein are used (**Figure 1**).



**Figure 1.** A: Venipuncture sites in turtle – jugular vein (Courtesy of the University of California, Davis). B: Anesthetic administration in brachial plexus of turtle (Courtesy of Kurt K. Sladky).

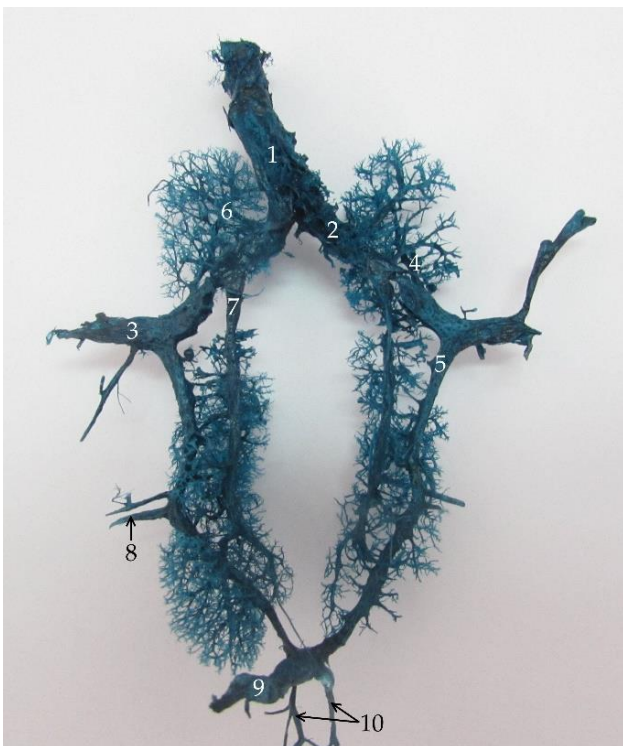
Intravenous route for drug administration in snake is jugular, palatine, and ventral tail vein. Important note for the examination of exotic animals is to restrain animal properly but in most cases, sedation or general anesthesia is necessary (Coutant et al., 2018).

### Birds

Avian heart is located in the median plane of cranioventral part of coelomic cavity surrounded by the lobes of liver. It consists of two atria and two ventricles. Mass of the avian heart in regard to body mass surpasses the mammal heart (Strunk and Wilson, 2003). The avian heart is well-adapted due to the high demands for oxygen. The cardiovascular system is crucial in enduring the exhausting physical activities in different birds such as swimming, flying or diving and running (Dzialowski and Crossley, 2022). Even though heart of mammals and birds share substantial amount of similarities, during the evolution, birds developed many physiological characteristics which reflect on the gross anatomy. For example, heart size of goose increases before the period of migration (Bishop et al., 1995). Peripheral resistance is lower in pulmonary blood vessels, therefore the right ventricle wall is thinner compared to left ventricle (Dzialowski and Crossley, 2022). Generally lower peripheral resistance in birds compared to mammals demands higher arterial pressure for the high cardiac output. Heart rate in birds is variable and depends on the size of the bird, physical activity and different physiological needs (King and Lelland, 1984). With powerful heart muscle birds achieve bigger heart stroke volume, large cardiac output and are able to increase the heart rate over 1000 beats per minute (Dzialowski and Crossley, 2022). Birds major systemic blood vessels consist of aorta which originates from right aortic arch and three caval veins including right and left cranial vena cava and caudal vena cava. Aorta gives branches to the two brachiocephalic trunks for the vascularization of cranial part of the body and continues as descending aorta. Right cranial vena cava and caudal vena cava terminate in right atrium separately from left cranial vena cava which enters to sinus venosus (Strunk and Wilson, 2003). Amongst other

characteristics in birds, vascular system of kidney is to be highlighted. Birds have renal portal system which consists of cranial and caudal renal portal vein. These veins drain blood from common iliac, external iliac, internal iliac, caudal mesenteric and ischiatic veins along with the internal vertebral venous sinus (**Figure 2**).

Arterial supply is done by renal arteries which enter each lobe separately (Burgos-Rodriguez, 2010). Kidneys of the bird itself have specific structure, embedded in renal fossa of the sacrum and divided into three parts, cranial, middle and caudal lobe. Birds have two types of nephrons, mammalian-like and reptile-like. Mammalian type is of more complex structure with longer proximal and distal tubules while reptilian type of nephron is missing Henley loop and the tubules are numerous but shorter (Burgos-Rodriguez, 2010; Hadžiomerović et al., 2021). Detailed description of kidneys and renal portal system encompasses the objectives of the study but it is assessed briefly to emphasize a complexity of these features in birds. This has clinical significance, since the recommendation of avoiding drug application in the hindlimbs of birds is present for a long time. Due to the lack of extensive studies that would distinguish which drugs are safe for application, most of the practitioners still avoid it. Many existing studies have opposite results and claims, therefore it is most appropriate to administer drugs in the cranial part of animal. This is valid not only for birds, but for reptiles, amphibians and most fish (Coutant et al., 2018). For the intravenous route in birds and blood sample collection in birds, mostly there are three blood vessels available including ulnar superficial vein, also known as basilic or wing vein, medial metatarsal vein and jugular vein. Important to note is that right jugular vein is often bigger than the left, hence it is more accessible (Coutant et al., 2018).



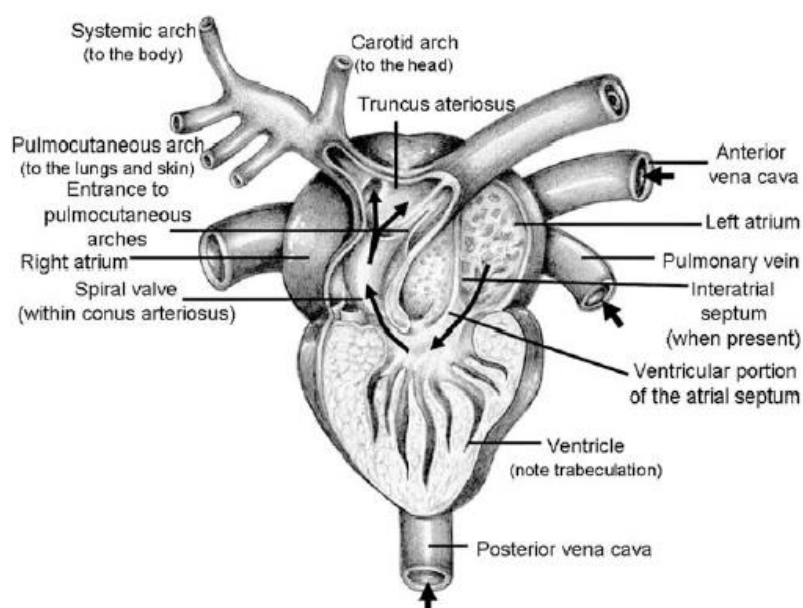
**Figure 2.** Venous vascularization of fowl's kidney and blood vessels of renal portal system (corrosion technique). 1. *V. cava caudalis*; 2. *V. iliaca communis*; 3. *V. iliaca externa*; 4. *V. portalis renalis cranialis*; 5. *V. portalis renalis caudalis*; 6. *V. renalis cranialis*; 7. *V. renalis caudalis*; 8. *V. ischiadica*; 9. *V. mesenterica caudalis*; 10. *V. iliaca interna*

### Amphibians

Among three types of animals reviewed in this article, species from the group of amphibians represent the rarest patients for veterinarians. Earlier studied mostly by

biologists, in the last few decades veterinarians are more involved in working with amphibians. Amphibians are divided into three groups: *Anura*, *Caudata* and *Caecilians*. Frogs and salamanders are the representatives of first two groups as the commonly studied species (Forzán et al., 2017). The three-chambered heart of amphibians is generally similar among species, but some differences can be noted. In anurans and salamanders, the right atrium is usually larger than the left, except in a few species of anurans, such as African Clawed frog (*Xenopus laevis*). The interatrial septum is fenestrated in salamanders, allowing for the mixing of blood, while it is complete in anurans. Blood from both atria enters the undivided ventricle (Sharma, 1961; Heinz-Taheny, 2009). Trabeculated ventricle in amphibians and different anatomical and physiological features minimize the blood shunting. During biphasic systole, the left and right side of ventricle contract separately forcing the blood in different parts of aortic arches. Separation of the blood is enabled by spiral valve in the conus arteriosus. Three separate trunks exit the ventricle, systemic arch, carotid and pulmocutaneous arch (**Figure 3**).

In the first phase of heart contraction, deoxygenated blood from the right side of the ventricle enters the pulmocutaneous arch which gives pulmonary and cutaneous arterial vessels carrying the blood for gas exchange in the lungs and skin of amphibians. In the second phase, blood is sent to the systemic and carotid arches for oxygenation of the cranial and caudal parts of the animal. Therefore, very small portion of blood from the right side of ventricle goes to the left side. The blood from the left side is directed mainly to the systemic circulation avoiding right side (Heinz-Taheny, 2009). Along with systemic and pulmocutaneous circulation, amphibians also have renal portal system. According to O'Malley (2005), since the blood from the hindlimbs does not bypass the kidneys, drug administration in the caudal part of the body may cause changes in drug distribution. Additionally, ventral abdominal vein, often used in clinics passes through the liver as a part of the hepatic portal system. This could affect pharmacokinetics of drug administered in this vein and its branches. Hence, it is suggested to avoid the application of drugs primarily metabolized in the liver in the caudal part of the body (Heinz-Taheny, 2009). Furthermore, taking in considerations amphibians are poikilothermic animals, clinicians should keep the patients in their optimal temperature zones due to the fact physiology, behavior and therapy are influenced by temperature (Whitaker et al., 1999; Wright, 2001). Venipuncture sites differ between the species. In the frogs, cardiac puncture, ventral abdominal vein, femoral vein and lingual vein are used. Ventral tail vein is most commonly used in salamanders. Amount of blood sample should not exceed 1% of body weight in healthy animal nor 0.5 % in diseased animal (Heinz-Taheny, 2009).



**Figure 3.** Anatomy of the anuran heart. Large block arrows indicate blood flow into the heart. Thin black arrows indicate flow of oxygenated blood (Courtesy of Frank Taheny).



### Conclusion

The number of exotic pets is increasing over the past few years. Aside from usual work with companion animals, veterinarians often treat exotic patients as well. This demands certain level of knowledge about specific anatomy and physiology of these animals. The article provides a brief insight in vascular system of reptiles, birds and amphibians with an emphasis on important clinical considerations regarding these characteristics. However, due to the complexity of the exotic animals, thorough education is needed.

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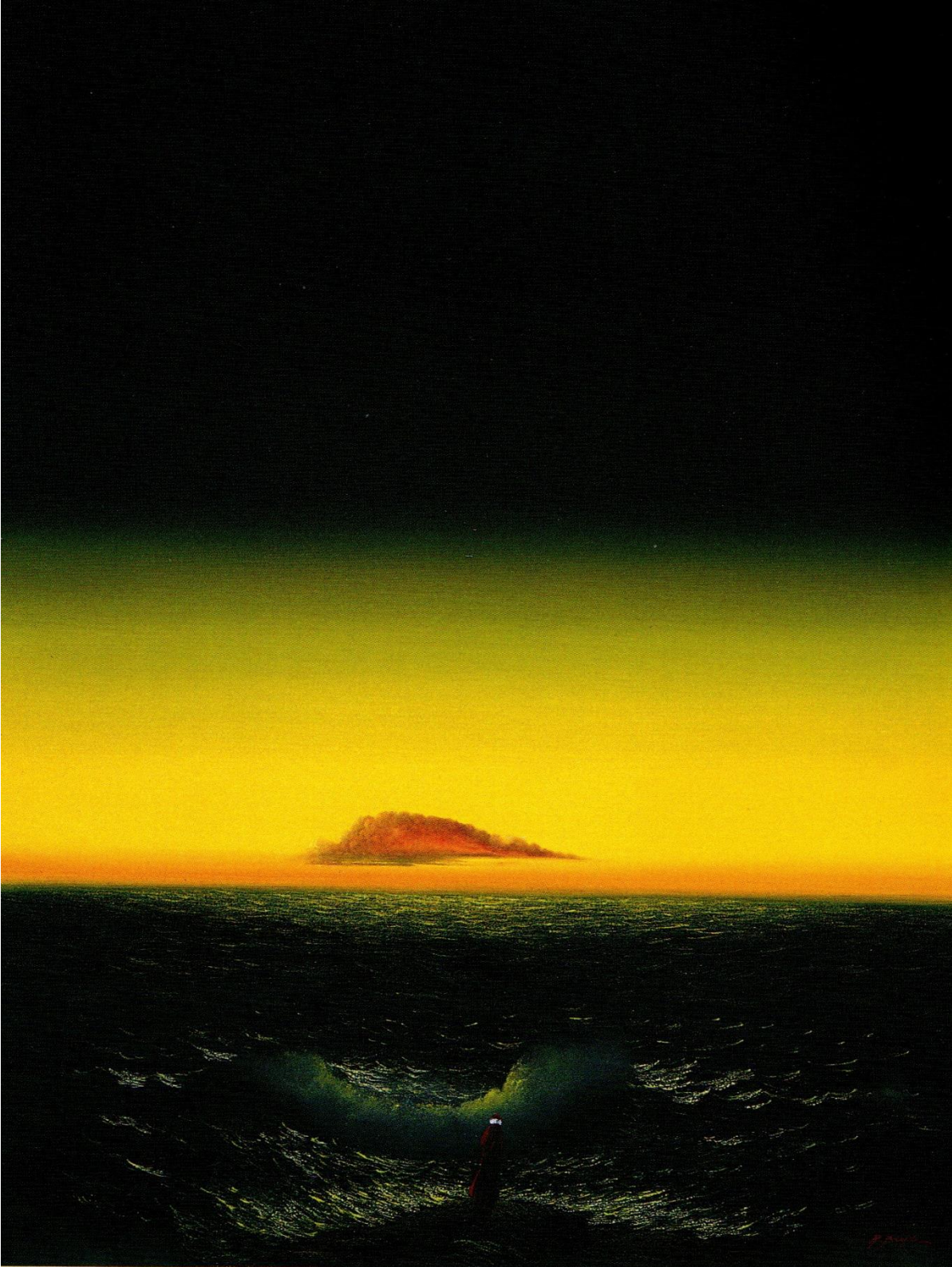
**Conflicts of Interest:** The authors declare no conflict of interest.

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# Milestones in Research of Small Cellular Particles. Membrane Modeling and Theoretical Description in Connection with *In vitro* and *Ex vivo* processes

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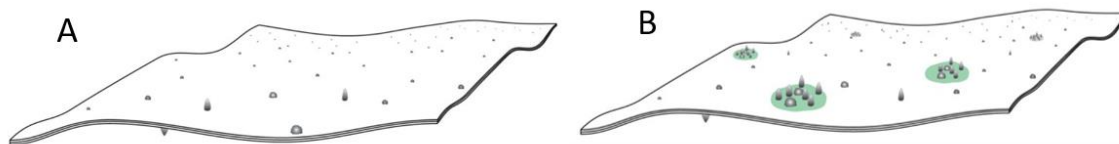
## Abstract:

Sub-micron sized small cellular particles (SCPs) (microparticles, microvesicles, exosomes, extracellular vesicles, extracellular particles) are recently gaining attention due to their potential role in all fields involving living organisms (e.g. medicine, food science, agriculture, ecology). Due to their small size and dynamic nature, their properties and biological roles are not yet understood up to a level to be widely used and remain potentially important. Employing the knowledge from different fields is highly warranted to progress towards better understanding of physiological and pathophysiological processes based on SCPs. This contribution outlines four milestones in SCP research: M1: Model of the biological membrane, M2: Membrane budding and vesiculation, M3: Theoretical description of the membrane shape and M4: Transferrin release from the reticulocyte membrane in the form of membrane – enclosed SCPs. Also we outline the increasing interest of the scientific society for SCPs reflected in highly cited papers and present a synthesis of the theoretical and experimental view on membrane-enclosed SCPs within the fluid crystal mosaic model of the membrane.

**Keywords:** Extracellular vesicles; Extracellular particles; Extracellular vesicles; Small cellular particles; Microvesicles; Exosomes

## 1. Milestone 1: Model of biological membrane

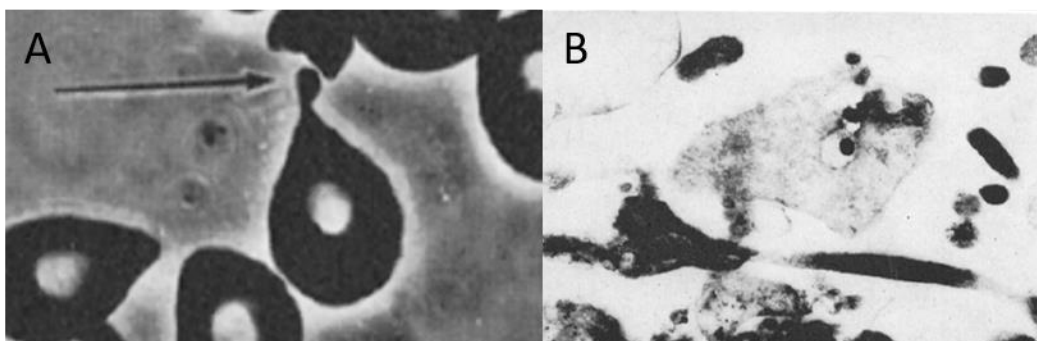
In 1972, a paper was published by Singer and Nicolson (1972) following thorough study of biological membrane thermodynamics and proposing the Fluid mosaic model. According to this model, the membrane is composed of a lipid bilayer with embedded proteins that are integral to the membrane (**Figure 1A**). The proteins are arranged with the ionic and highly polar groups protruding from the membrane into the aqueous phase, and the nonpolar groups largely buried in the hydrophobic interior of the membrane. The proteins are more or less free to move laterally over the membrane analogously to a two-dimensional solution of integral proteins (or lipoproteins) in bilayer solvent. In the next 50 years the agreement of this model with experimental results established it as a base for the description of membrane features. However, already in the paper (Singer and Nicolson, 1972), it was suggested that direct interactions between the embedded entities may take place and may have also consequences for biological features involving the membrane. Lateral segregation of the protein molecules was observed in connection with the pinocytosis of the membrane – endovesiculation - which refers to the uptake of substances by a cell. This concept was later further developed and reported in Simons and Ikonen (1997) and Brown and London (1998). Simons and Ikonen (1997) presented a model based on the dynamic clustering of sphingolipids and cholesterol to form rafts (**Figure 1B**) that move laterally within the fluid bilayer and function as platforms for the attachment of proteins. Furthermore, lateral segregation into rafts was connected with membrane curvature within the entities called calveolae. It was suggested that spingolipid-cholesterol rafts may be an essential feature of all organellar membranes involved in biosynthetic and endocytic traffic. Brown and London (1998) further up-graded the model by considering order within the particular regions in the lipid bilayer.



**Figure 1.** Illustration of the fluid mosaic model of the membrane in its original form (A) and by considering formation of membrane rafts (B). Adapted from Kralj-Iglič (2012).

## 2. Milestone 2: Membrane budding and vesiculation

Erythrocytes are convenient for the study of the properties of the cell membrane. Mature cells do not have nucleus nor cytoskeleton. Their shape is largely determined by the properties of the membrane (lipid bilayer with underlying membrane skeleton). The process of microvesiculation (**Figure 2A**) and the pinched-off vesicles (**Figure 2B**) were imaged and described (**Figure 3**) by Weed and Reed (1966).



**Figure 2.** A: Budding of the erythrocyte. B: Electron micrograph of “fragments”. Panel B has magnification 14000 X. Adapted from Weed and Reed (1966).



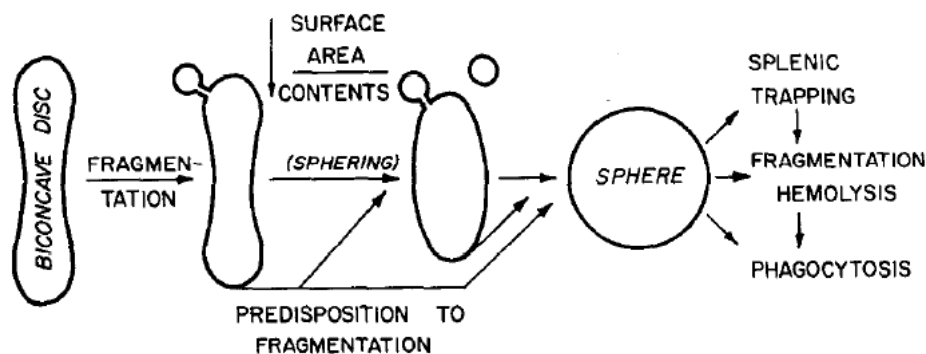


Figure 3. Scheme of erythrocyte membrane fragmentation as presented by Weed and Reed (1966).

In 1967, SCPs were identified as fragments shed from platelets (Wolf, 1967). Already then, their **potential** biological role was indicated (Wolf, 1967). Later it was observed that blood contains a mixture of young and old erythrocytes which differ in the size, density and surface charge (Lutz et al., 1977). It was suggested that microvesiculation is responsible for the loss of the erythrocyte membrane and therefore increase of the volume to area ratio is reflected in senescent rounding of erythrocytes (Lutz et al., 1977). In the following decades, erythrocyte shape change, hemolysis and vesiculation were thoroughly studied experimentally (Nelson et al., 1983, Isomaa, 1979, Isomaa et al., 1987, Hagerstrand et al., 1992). It was found that the erythrocyte membrane may bud inward or outward and if this process continues, it terminates in vesiculation (Isomaa et al., 1987, Hagerstrand et al., 1992). Procedures were developed to isolate and image microvesicles (Isomaa et al., 1987, Hagerstrand et al., 1992). It was found that the shape of the buds and of SCPs may vary depending on the added compounds that intercalate into the membrane (Figure 4).

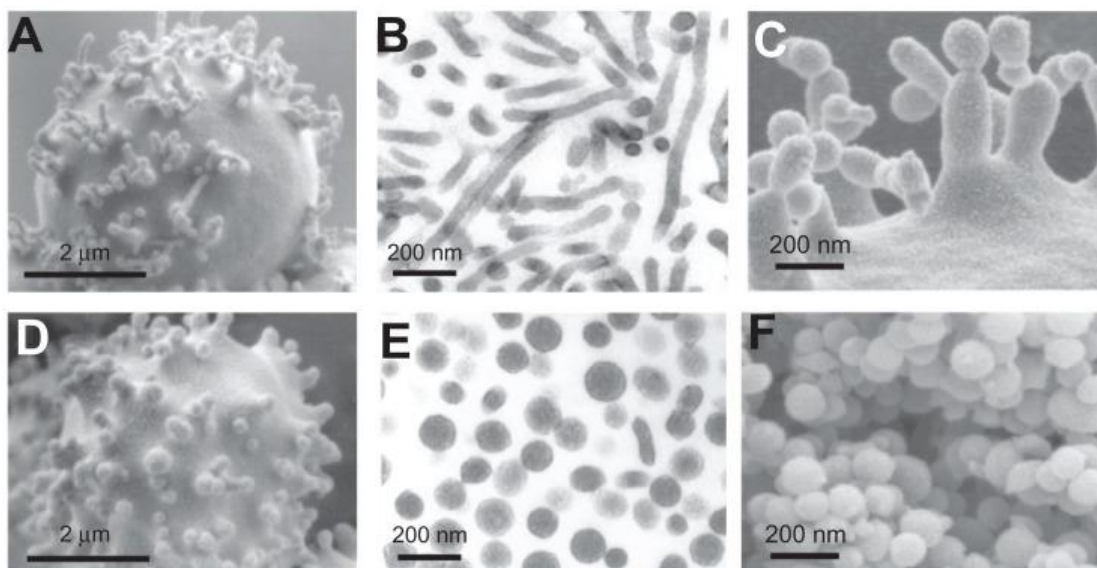
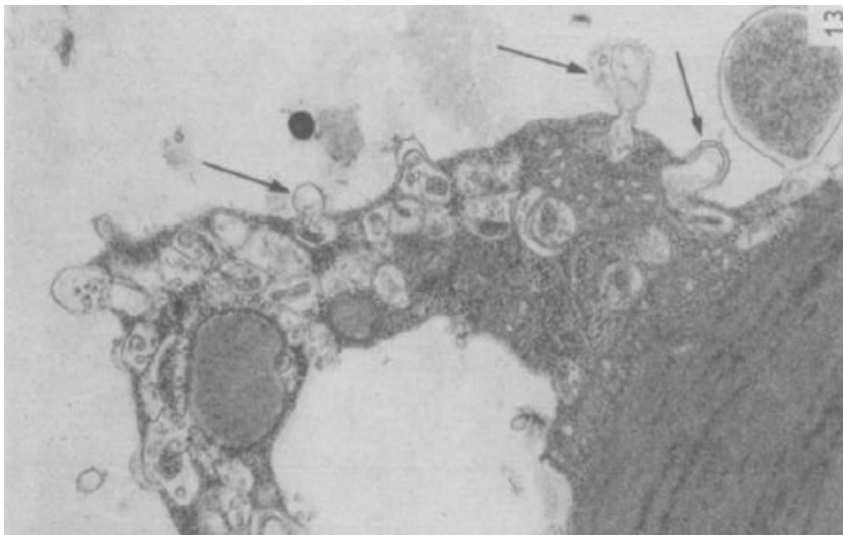


Figure 4. Budding of erythrocytes (panels A, C and D) and isolated microvesicles (panels B, E and F). The process was induced by adding amphiphilic molecules into the erythrocyte suspension (A,B: dodecyl maltoside; D,E,F: dodecyl zwittergent). A, C, D and F: scanning electron micrographs; B and E: transmission electron micrographs. Adapted from (Schara et al., 2009).

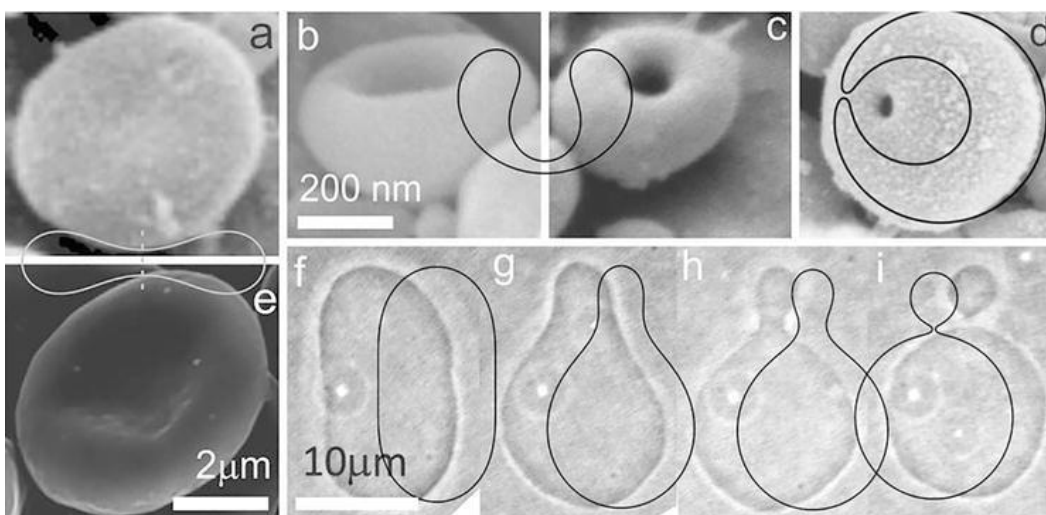
Budding and vesiculation of nano to micro – sized particles were detected and observed in different biological systems. Figure 5 shows budding and vesiculation in a microalgae *Ochromonas danica*. Assessment and visualization of SCPs in different samples led to the conclusion that cells of all types are prone to shed their fragments in the surrounding solution (Yanez-Mo et al., 2015). Being freed from the mother cell, they can travel with surrounding liquid, reach nearby or distant cells and interact with them.



**Figure 5.** SCPs emerging from the surface of the cell membrane of a 9-day-old *Ochromonas danica* cell; see arrows. Adapted from (Aaronson et al., 1971).

### 3. Milestone 3: Theoretical description of the bilayer membrane shape

Erythrocyte shape was described theoretically (Canham, 1970) by considering the membrane as a thin, locally slightly curved elastic shell. The contour of the rotationally symmetric shape was approximated by Cassini ovals and the bending energy of the shell was minimized at chosen geometrical constraints by determining the parameters of the ansatz (Canham, 1970). Good agreement was obtained between calculated shapes and shapes observed under the optical microscope (Canham, 1970). Theoretically advanced expression for the membrane free energy including the assumption that the membrane has spontaneous curvature was presented by Helfrich (1973). The description was improved by stating the variational problem for minimization of the free energy by a set of Euler-Lagrange differential equations (Deuling and Helfrich, 1976). This enabled a rigorous solution of the set of differential equations for the axisymmetric shapes. The calculated shapes shown in **Figure 6** (Kralj-Iglič et al., 2022) were obtained by using this method.



**Figure 6.** Observed and calculated membrane shapes corresponding to a sequence leading to formation of internal vesicle (endovesiculation) in SCPs found in isolates from blood and to a sequence leading to formation of external vesicle (exovesiculation) in a giant phospholipid vesicle. The theoretical shapes were taken as axisymmetric with respect to the vertical axis and were calculated by solving the variational problem presented as a system of the Euler-Lagrange differential equations. Note the relevance of the model over 100 fold size difference. From Kralj-Iglič et al., (2022).



Bilayer couple principle considering that the two membrane layers are in close contact but can slide over each other (Sheetz and Singer, 1974) was implemented to explain intercalation of compounds into a particular lipid layer (Helfrich, 1974, Evans, 1974) thereby changing average mean curvature of the membrane. The models were theoretically elaborated to yield the equilibrium membrane shape by minimization of the membrane free energy (reviewed by Seifert, 1997). Membrane budding and vesiculation were described by an increase (in the case of exovesiculation) or a decrease (in the case of endovesiculation) of the average mean curvature of the membrane. The pinching off of the bud was preceded by the thinning of the neck connecting the bud and the mother membrane. When the bud is pinching off, the opening in the membrane is very small and can be sealed with little energy change by rearranging the adjacent molecules.

In considering the models of the membrane it was taken into account that besides lateral redistribution and segregation the anisotropic membrane constituents may undergo orientational ordering with respect to the axis perpendicular to the membrane. This introduced curvature deviator as an important parameter (Fischer, 1992; Kralj-Iglič et al., 1999). Changing of the constraints and/or parameters (e.g. relative volume (expressing volume to area ratio  $v$ , average mean curvature  $\langle h \rangle$ , average curvature deviator  $\langle d \rangle$ ) yields different shapes of membrane-enclosed entities without internal structure. A  $(v, \langle h \rangle, \langle d \rangle)$  phase diagram featuring some shapes corresponding to the minimal free energy is presented in Figure 7. Regions are defined within limiting shapes composed of spheres, cylinders, tori and flat regions. Shapes composed of spheres lie in the  $\langle d \rangle = 0$  region. Red, blue and green lines indicate transformations of shapes due to changes in  $v$ ,  $\langle h \rangle$  and  $\langle d \rangle$ . Endo and exo vesiculation (Figure 6) are presented by the blue and red – marked sequences, respectively.

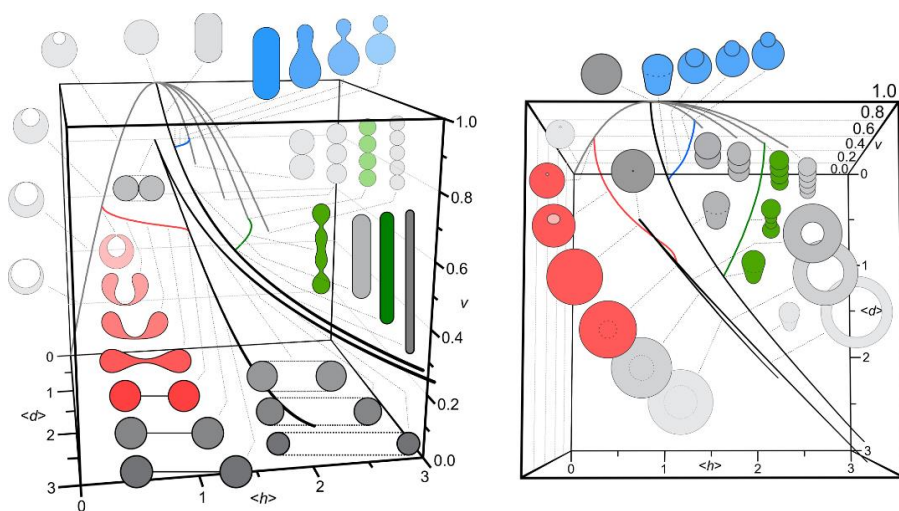


Figure 7. A  $(v, \langle h \rangle, \langle d \rangle)$  phase diagram of shapes calculated by minimization of the membrane free energy. Two aspects (side view (left) and top view (right)) are shown. From (Kralj-Iglič et al., 2020).

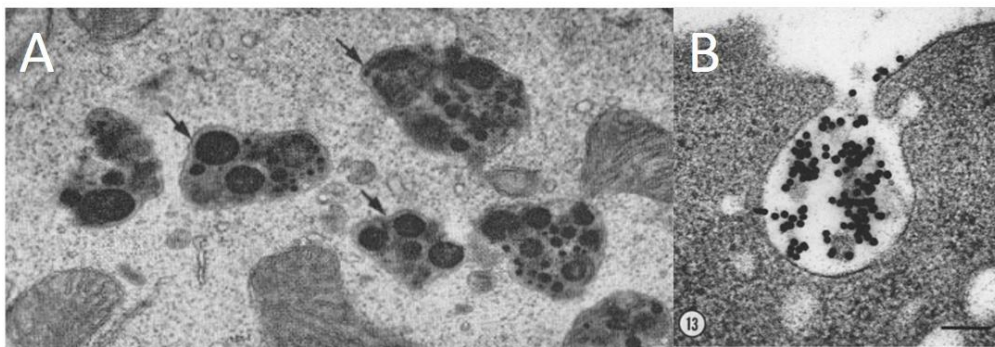
Taking into consideration orientational ordering of anisotropic membrane constituents indicated stability of thin (below cca 100 nm) tubular vesicles (Kralj-Iglič et al., 2002) and required revision of the model of the membrane (Kralj-Iglič, 2012). As these effects were similar to the ones observed in liquid crystals, the up-graded model which becomes relevant in nano-sized anisotropic membrane regions was called the Fluid crystal mosaic model (Figure 8).



Figure 8. Illustration of the fluid crystal mosaic model of the membrane. Adapted from Kralj-Iglič (2012).

#### Milestone 4: Transferrin release from the reticulocyte membrane in the form of membrane – enclosed SCPs.

While internal structures filled with SCPs were observed already in 1971 (Coons and Axtell, 1971) (**Figure 8A**), the importance of this feature was revealed in the study of transferrin release from maturing reticulocytes (Harding et al., 1983; Pan and Johnstone, 1983). It was suggested and supported by measurements and observations that transferrin was released in the form of small vesicles that are formed in invaginated compartments of reticulocytes (**Figure 8B**). These results were considered as a base for the general mechanism of formation of »exosomes« - vesicles that form within internal compartments of the cell and are eventually released into the surrounding solution.



**Figure 8.** A: Cross-section of an excretory tube cell of the mesostigmatid mite *Macrocheles muscaedomesticae* showing a group of lysosomes (arrows). Each is bounded by a single membrane and contains many round bodies of varying degrees of size and electron density. X 20,000. Adapted from (Coons and Axtell, 1971). B: Release of SCPs labelled with gold (black dots) from the invagination of an unfixed reticulocyte. The cell was incubated for 30 min with AuTf, subjected to a 20-min chase with unconjugated transferrin, and then quick-frozen without prior fixation and freeze-substituted. Bar, 200 rim. X 61,000. Adapted from (Harding, 1983).

#### Notorious SCPs

Although the existence of SCPs has been acknowledged, their roles and underlying mechanisms are not yet completely understood and are a subject of extensive study. As SCPs were discovered in different samples by scientists from different fields, they were called different names. International Society for Extracellular Vesicles (ISEV) is coordinating effort to nominate SCPs and impose quality requirements on experimental procedures. For his purpose ISEV has issued several »position papers«. Some of these works have become highly cited. We have browsed Google Scholar database for papers using keywords »extracellular vesicles«, »microparticles«, »microvesicles« and »exosomes« and outlined papers cited more than 2000 times in **Table 1**. It can be seen that the majority of highly cited papers are reviews, however the one listed with the highest number of citations in **Table 1** is the ISEV position paper (Thery et al., 2018). Recently, the requirements were updated in a new publication MISEV2023 (Welsh et al., 2024) with contribution of over 1000 ISEV members. The goal of this paper is to provide »snapshot of available approaches and their advantages and limitations for production, separation and characterisation of EVs from multiple sources, including cell culture, body fluids and solid tissues«. As SCP-based mechanisms are very basic and relevant in all living systems, new SCP research areas are emerging. As the biological roles of SCPs are acknowledged, their better understanding is highly warranted. However, further efforts are needed to accomplish this goal and achieve breakthrough of SCP-based methods in everyday practice. Model approach and theoretical work is therefore an important element in SCP research. For comparison, **Table 2** shows a list of highly cited papers on membrane models and theoretical descriptions and also some milestone papers referred to in this work.



**Table 1.** Highly cited papers on cellular fragments found in first 20 pages of Google Scholar database by keywords »extracellular vesicles«, »microparticles«, »microvesicles« and »exosomes« subject to more than 2000 citations.

Paper	N	Expression browsed	Journal	Type of the paper
They et al., 2018	8043	Extracellular vesicles	J Extracellular Vesicles	ISEV Position paper
Raposo and Stoorvogel, 2013	8027	Exosomes/microvesicles	J Cell Biol	Review
Van Niel et al., 2018	5864	Extracellular vesicles	Nature Rev Mol Cel Biol	Review
They et al., 2006	5821	Exosomes	Curr Prot Cell Biol	Research
Colombo et al., 2014	5639	Exosomes/Extracellular Vesicles	Ann Rev Cell Develop Biol	Review
They et al., 2002	5624	Exosomes	Nature Reviews Immunology	Review
Kalluri and LeBleu, 2020	5503	Exosomes	Science	Review
Skog et al., 2008	5481	Microvesicles	Nature Cell Biology	Review
Yanez Mo et al., 2015	4887	Extracellular vesicles	J Extracellular Vesicles	Review
Johnstone et al., 1987	3217	Vesicles	J Biol Chem	Research
Tkach and They, 2016	3001	Extracellular vesicles	Cell	Review
El Andaloussi et al., 2013	2985	Extracellular vesicles	Nature Reviews Drug Discovery	Research
Mathivanan et al., 2010	2728	Exosomes	J Proteomics	Review
Lotvall et al., 2014	2633	Extracellular vesicles	J Extracellular Vesicles	ISEV Position paper
Costa Silva et al., 2015	2548	Exosomes	Nature Cell Biology	Research
Mulcahy et al., 2014	2468	Extracellular vesicles	J Extracell Vesicles	Review
Pisitkun et al., 2004	2438	Exosomes	Proc Nat Acad Sci	Research
Gyorgy et al., 2011	2305	Extracellular vesicles	Cell Mol Life Sci	Review
Witwer et al., 2013	2251	Extracellular vesicles	J Extracellular Vesicles	ISEV position paper
Al Nedawi et al., 2008	2275	Microvesicles	Nature Cell Biology	
Doyle and Wang, 2019	2212	Extracellular vesicles	MDPI Cells	Review
Vlassov et al., 2012	2192	Exosomes	Biochim Biophys Acta	Review
Pan and Johnstone, 1983	2166	Vesicles	Cell	Research
Cocucci et al., 2009	2141	Exosomes		
Robbins and Morelli, 2014	2121	Extracellular vesicles	Nature Reviews Immunology	Review

N: number of citations on March 19, 2024.

**Table 2.** Selected milestone papers.

Paper	N	The scope	Journal	Type of the paper
Singer and Nicolson, 1972	13186	Membrane model	Science	Research
Simons and Ikonen, 1997	12015	Membrane model	Nature	Research
Helfrich, 1973	7186	Membrane shape	Z Naturforschung	Theory
Brown and London, 1998	3908	Membrane model	Ann Rev Cell Develop Biol	Research
Seifert U, 1997	2015	Membrane shape	Advances in Physics	Review
Canham, 1970	2005	Red blood cell shape	J Theor Biol	Theory
Wolf, 1967	1999	Platelet dust	Br J Haematol	Research
Harding et al., 1983	1988	Vesicles and tubules	J Cell Biol	Research
Sheetz and Singer, 1974	1940	Membrane model	Proc Nat Acad Sci	Research
Weed and Reed, 1966	268	Cell fragments	Am J Med	Research

N: number of citations on March 19, 2024.



### Conclusions

Biological roles of SCPs inspire great effort of the scientific community to fulfill the potential effects and uses of SCPs in health improvement of living organisms. As SCPs are tiny and dynamic entities, their harvesting, assessment and manipulation presents a challenge. Modeling and theoretical description are powerful tools which should be included in consideration of SCPs.

### Acknowledgement

Due to increasing interest in SCPs and large number of published works we are aware that some important works may not have been included in the survey.

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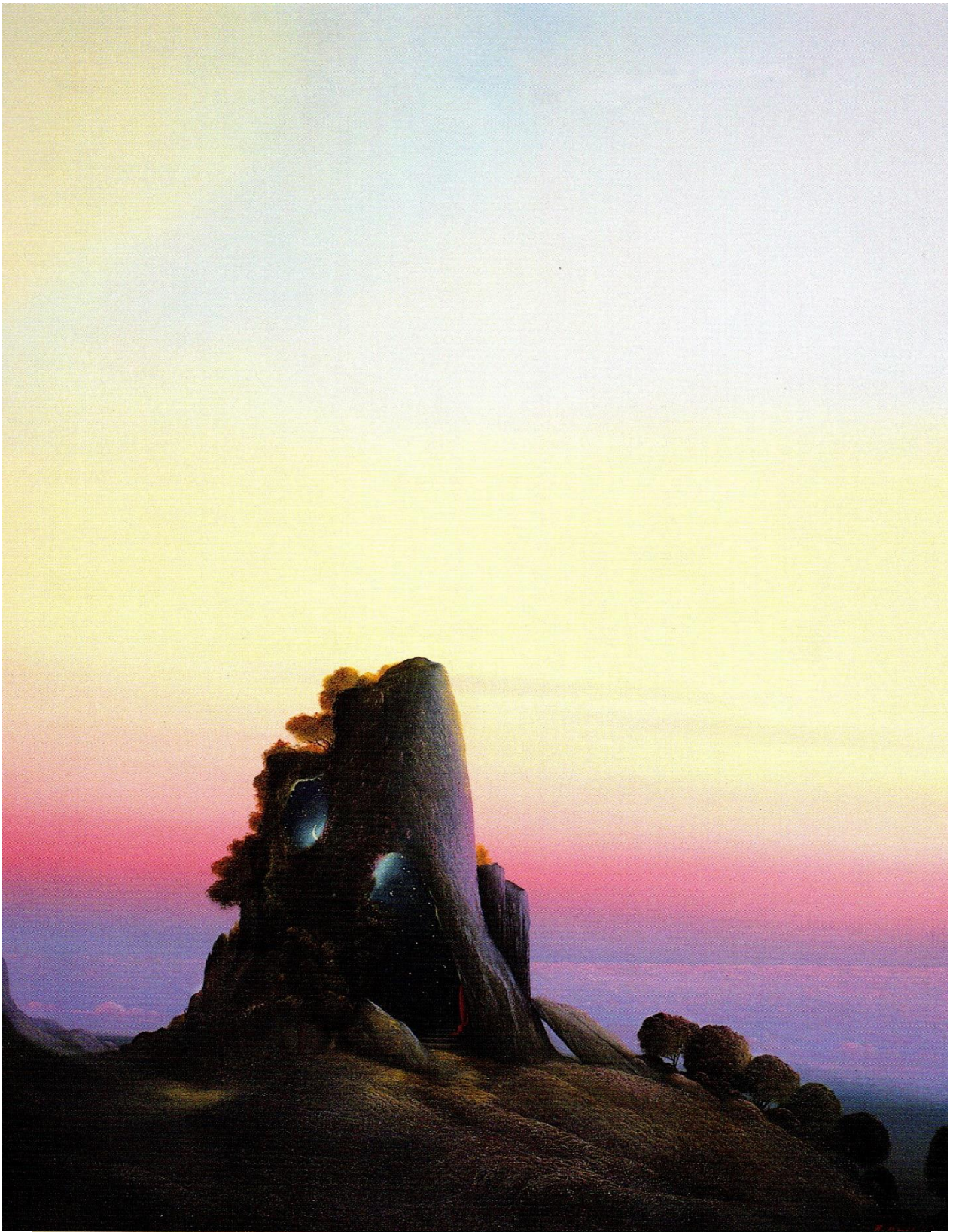


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# Effect of Time, pH, Alcohol and Sugar Content on Nicotine Re-release from Pouches Available on Slovene Market

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## Abstract:

Tobacco-free nicotine pouches, first introduced in Sweden, are made as a less hazardous product for cigarette consumers. Instead of tobacco leaves, they consist of nicotine-containing powder and other ingredients such as water, salts, natural aromatic oils and others, that will boost the flavor and effect and make it last longer. This research aimed to investigate the extraction efficiency of nicotine from pouches of different brands. The effects of alcohol and sugars present in saliva at different pHs and consumption times were investigated as well. We optimized a High performance liquid chromatography with diode-array detection method (HPLC-DAD) for nicotine determination and its quantification. We used reversed-phase chromatography (RP-HPLC), and a mixture of sodium hydrogen carbonate and acetonitrile as a mobile phase (85:15, v/v). Different brands of nicotine pouches were used. Nicotine extraction rate showed positive linear dependence on time. The percentage of extraction was measured at different intervals, up to two hours, without getting a plateau. Nicotine extraction lowers with the increasing volume of saliva. The obtained results have shown that the increasing ethanol concentration in saliva (from 5-40 %) leads to a higher extraction rate, coming up to 85 %. In the case of sugar addition, the results were variable. Experiments with *White fox* pouches have shown that extraction of nicotine decreases with a higher concentration. On contrary, *Siberia* pouches do not exhibit that correlation. When it comes to pH, we used pH range that usually varies in saliva and the results have shown no significant differences.

**Keywords:** Nicotine, Pouches, HPLC-DAD, Extraction

## 1. Introduction

As people's awareness regarding the hazard of cigarettes grows, many companies have come up with alternative/potentially less harmful products to replace their use. One of these products, nicotine pouches, also known as tobacco-free snus, hailing from Sweden. These pouches are weighing up to 1g, do not contain tobacco, but have nicotine powder, sweeteners, flavorings, salts, and plant-based fibers. They are placed between the lip (usually the upper lip) and the gums, and the holding time can be up to an hour, although it is usually 10-15 minutes. The long-term health impact of nicotine pouches is still unknown. Nicotine pouches contain nicotine which is harmful to young people in any form. Due to the absence of tobacco leaf, the Food and Drug Administration does not classify oral nicotine pouches as a smokeless tobacco product (Robichaud et al., 2020). Side effects of use can include irritation of the gums, sore mouth, hiccups, nausea, nicotine addiction and more. Only 10-20 % of nicotine reaches the systemic circulation. This means that only 1-2 mg of nicotine is absorbed into the blood from a one-gram pouch containing around 16 mg of nicotine (Miller-Holt et al., 2022). Nicotine is absorbed quite rapidly from Swedish snus. Studies on Swedish snus users have shown that the plasma steady-state levels of nicotine and its main metabolite, cotinine, are like Swedish snus users and cigarette smokers (Scherer et al., 2022, Miller-Holt et al., 2022). Various brands of tobacco-free snus are available on the Slovenian market, and for the purposes of this research, we chose only some of them and compared the results obtained. We investigated how much nicotine is released from the bag into the saliva, trying to simulate people's daily habits. As you can eat and drink prior to using snus, in addition to varying the pH and the time of use, we investigated the effect of alcohol and sweeteners on nicotine release, wanting to simulate people's everyday life this way (Bishop et al., 2022).



**Photo 1.** Nicotine pouches; *White fox* and *Siberia*.

## 2. Materials and Methods

### 2.1. Materials

Different brands of nicotine pouches were used: *White fox*, *Siberia* (GN Tobacco, Sweden) (**Photo 1**), *Velo* (R.J. Reynolds Vapor Company, USA), and *Thunder* (V2 Tobacco, Denmark), but the first two expressed better extraction rate, so we continued our research on them. We used three types of *White fox* pouches: The original (blue) and Double-mint (green), both with 12 mg of nicotine per pouch (0,75 grams), and Full-charge (red) with 16 mg of nicotine per pouch (1 gram). *Siberia* pouches were *Regular* (big) with 16.4 mg of nicotine per pouch and *Slim*, (small) with 10.66 mg of nicotine per pouch. To stay in accordance with scientific ethics, the results will be presented by the alternative names previously stated in brackets (blue, green, red, big and small). In Slovenia, nicotine pouches are available in every tobacco shop and gas stations, so we obtained them from one of these.



The artificial saliva solution was prepared by dissolving 0.69 g of Sodium phosphate monobasic monohydrate ( $\text{NaH}_2\text{PO}_4$ ), 0.4 g of sodium chloride ( $\text{NaCl}$ ), 0.795 g of calcium chloride ( $\text{CaCl}_2$ ), 0.4 g potassium chloride ( $\text{KCl}$ ), 0.005 g sodium sulfide ( $\text{Na}_2\text{S}$ ) and 1 g of urea, in distilled water in final volume of 1 liter.

## 2.2. Methods

For examining dependence of:

- volume of saliva, we used vials filled with 2, 4, 6, 8 and 10 milliliters of saliva
- pH of saliva, we used 1 M HCl and 1 M of NaCl for establishing wanted pH (5, 6, 7 and 8)
- alcohol concentration, we used different percentage (5, 10, 15, 20, 30 and 40 %) of ethanol mixed with saliva
- sugar (saccharose) concentration, we used 5, 10 and 15 % of sugar dissolved in saliva; for each of these experiments, we put 2 ml of prepared solution in the vials (except for investigating the impact of volume of saliva, where we put 2, 4, 6, 8 and 10 ml), placed one pouch inside, and left it in water bath on 37 degrees.

After 15 minutes, 1 ml from each vial was taken and placed in the vials for further detection on HPLC-DAD. For measuring time dependence, we used big vial filled with 10 ml of saliva. Then we placed pouch in vial and put it in the water bath on 37 degrees. We had 2 repeats per each type of nicotine pouches. 1 ml of saliva was taken after 5, 10, 15, 30, 45, 60, 90 and 120 minutes and placed into the vials for HPLC-DAD. Experiments are done in duplicate (for volume, time, and alcohol), or in triplicate (for pH and sugar dependence). Calibration curve was constructed for nicotine measurement from six standard solutions specifically: 50, 100, 150, 200, 250 and 500 mg/ml. The standard solutions were prepared by serial dilution of proper amount from stock standard solutions with saliva.

Chromatography Conditions: we used HPLC equipped with a diode array detector (DAD) for nicotine detection. C18 Agilent HP-5MS (19091S-433) Capillary Column (30M x 0.25mm x 0.25 $\mu$ m) was used. Mobile phase was consisting of acetonitrile (ACN) and sodium hydrogen carbonate buffer (pH 9.2, 2 M) (15:85, v/v), at a flow rate of 1,2 ml/min, running time 15 min and a UV detection achieved at 259 nm. For buffer preparation, we used 20 ml of sodium carbonate solution (2,2 g/100 ml, 0.2M), and 230 ml of sodium bicarbonate (1.68 g/100 ml, 0.2M). Filled to 1 liter with distilled water (Bansal et al., 2018).

## 3. Results

The results are shown in the **Tables 1-6**. We measured areas under the signals in chromatograms and calculated the percentage of nicotine extraction based on the known concentration of nicotine in pouches and in solutions diluted from the stock.

**Table 1.** Nicotine extraction rate (%) depending on the volume of saliva (White fox - green).

volume [ml]	green		
	Area	mg of nicotine	%
2	23935.35	5.08	42.36
4	16611.5	3.53	29.40
6	9343.6	1.98	16.54
8	6892.6	1.46	12.20
10	6027.65	1.28	10.67



**Table 2.** Nicotine extraction rate (%) depending on the alcohol percentage found in saliva (White fox - blue, red)

Alcohol %	blue			red		
	average	mg of nicotine	%	average	mg of nicotine	%
5	1037.2	0.22	18.35	20419.45	4.34	27.10
10	1347.2	0.29	23.84	19915.6	4.23	26.43
15	1373.4	1.29	24.30	21842.25	4.64	28.99
20	1641.45	0.35	29.04	53908.7	11.45	71.55
30	1853.05	0.39	32.79	52031.2	11.05	69.06
40	2104.7	0.45	37.25	56161.45	11.93	74.54

**Table 3.** Nicotine extraction rate (%) depending on the sugar percentage found in saliva White fox - (blue, red)

Sugar %	blue			red		
	average	mg of nicotine	%	average	mg of nicotine	%
0	4073.4	0.87	72.09	2891.55	0.61	51.17
5	2928.93	0.62	51.83	2800.6	0.59	49.56
10	2675.8	0.57	47.35	2554.63	0.54	45.21
15	2194.33	0.47	38.83	2311.37	0.49	40.90

**Table 4.** Nicotine extraction rate (%) depending on the sugar percentage found in saliva (Siberia - small, big)

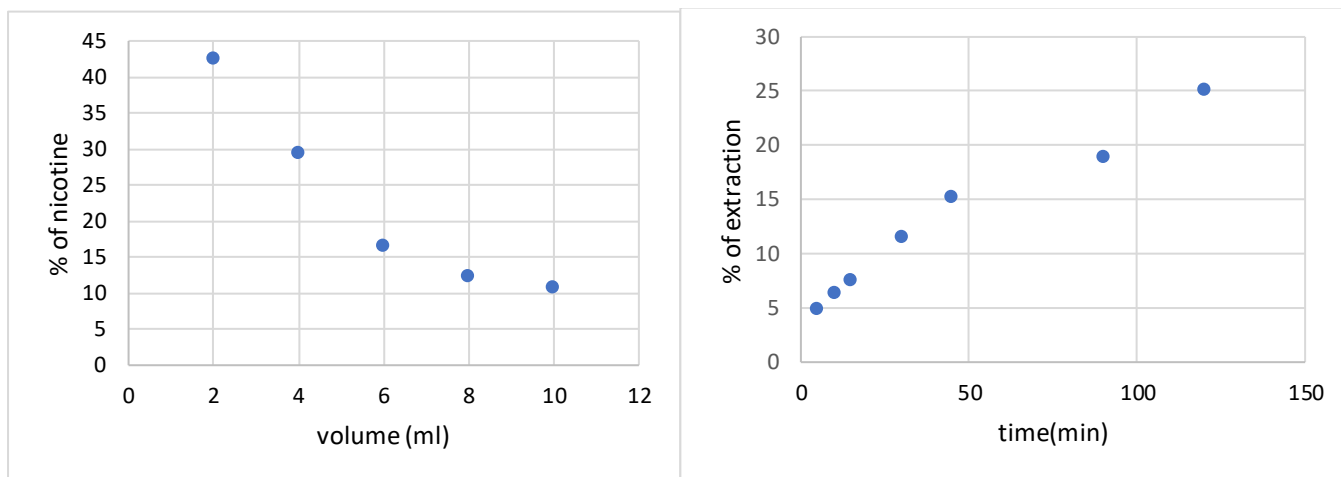
Sugar %	small			big		
	average	mg of nicotine	%	average	mg of nicotine	%
0	1658.40	0.35	33.04	2088.4	0.44	27.04
5	1462.73	0.31	29.14	1163.43	0.25	15.06
10	1070.83	0.23	21.33	1362.83	0.29	17.65
15	1443.93	0.31	28.76	1717.8	0.36	22.24

**Table 5.** Nicotine extraction rate (%) depending on the pH of the saliva (White fox - green, Siberia - small)

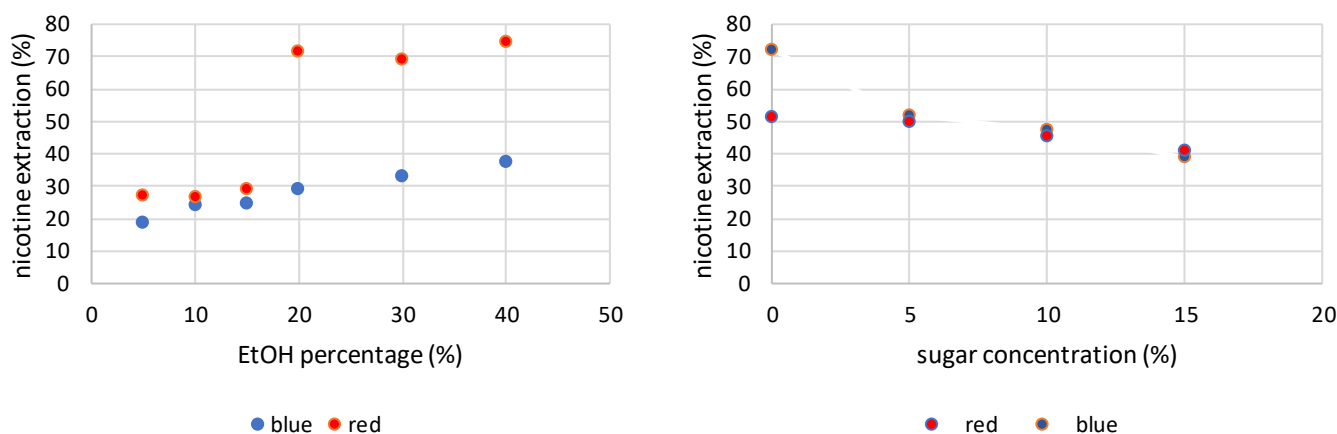
pH	green			small		
	average	mg of nicotine	%	average	mg of nicotine	%
5	20703.47	4.40	36.64	19902.80	4.23	39.65
6	23041.60	4.89	40.78	19617.23	4.17	39.08
7	21986.43	4.67	38.91	20003.13	4.25	39.85
8	19621.23	4.17	34.72	15342.93	3.26	30.56

**Table 6.** Nicotine extraction rate (%) depending on the time pouches spent in saliva (White fox - green, Siberia - small, big)

time [min]	green			small			big		
	average	mg of nicotine	%	average	mg of nicotine	%	average	mg of nicotine	%
5	2797	0.59	4.95	943.75	0.20	1.88	2521.95	0.54	3.27
10	3597.5	0.76	6.37	1886.85	0.40	3.76	4650.25	0.99	6.02
15	4308.15	0.91	7.62	2241	0.48	4.64	4565.45	0.97	5.91
30	6565.05	1.39	11.62	2747.55	0.58	5.47	4925.1	1.05	6.38
45	8649.35	1.84	15.31	4326.95	0.92	8.62	6223.2	1.32	8.06
90	10683.05	2.27	18.91	7564	1.61	15.07	12780.35	2.71	16.55
120	14238.75	3.02	25.20	7791.35	1.65	15.52	14246.85	3.03	18.45



**Figure 1.** Nicotine extraction rate (%) depending on the volume of saliva (left) and time pouches spent in the saliva (right); White fox - green pouches.



**Figure 2.** Nicotine extraction rate (%) depending on the percentage of alcohol (left) or sugar (right) found in saliva (White fox – blue, red pouches).

#### 4. Discussion

**Time dependence:** With all pouches, nicotine extraction rate showed positive linear dependence with time. The percentage of extraction was measured at different intervals, up to two hours, without getting a plateau. At the beginning, we have a linear increase of the extraction % with the time, but after 45 minutes, the increase is slower, but it continues to rise. Maximum of extraction was achieved after 120 minutes, and it was 25 % with the green pouches. Nicotine extraction lowered with the increasing volume of saliva. For 2 ml of saliva (which was the minimal volume enough to cover pouches completely), we found an extraction rate 42 % in some brands of pouches. **Ethanol dependence:** The obtained results have shown that the increasing ethanol concentration in saliva (from 5-40% EtOH) leads to a higher extraction rate, coming up to 85% with some pouches. We had a second peak following the first one, possibly as a product of nicotine degradation, or the difference occurred because of the protonated groups. **Sugar dependence:** In the case of sugar addition, the results were variable. Experiments with red and blue pouches have shown that extraction of nicotine decreases with a higher concentration (5, 10 and 15 %) of sugar. On



the contrary, big and small pouches do not exhibit that correlation, potentially because of the difference in pouch material or ingredients in them. We observed that the sacks are less tight and nicotine could be distributed unevenly. When it comes to pH, we used a pH range that usually varies in saliva (from 5 to 8), and the results have shown no significant differences. In some cases, we had very big areas measured under the peaks, so we had to dilute them 10 times to make it calculable.

## 5. Conclusion

We standardized the existing HPLC-DAD method for nicotine extraction and used it for nicotine quantification in saliva under specific conditions of extraction. As shown in the results, some of the substances, such as alcohol or sugar, can modulate nicotine extraction rate from pouches. Consumers could potentially adjust nicotine consumption by using products that contain these substances.

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

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# New Approaches for Testing the (Geno)Toxic Activity of Nano-particles *In Vitro*

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## Abstract:

The safety of nanomaterials, whether they are made of natural or artificial substances, represents a significant challenge because nanotechnology, as a young and up-and-coming field, is developing very quickly, while nanotoxicology and nanoecotoxicology are falling behind. Since the production, use, and consequently, the exposure of people to nanomaterials is increasing significantly, the acquisition of data on potential acute and chronic toxicity plays a crucial role. It is known that nanomaterials due to their high surface-to-volume ratio, high reactivity, and unique physical, chemical, and biological properties exhibit a greater risk of toxicity than the corresponding bulk material and that is why a comprehensive assessment of the toxicity of nanoparticles should always be done prior to their use.

We develop 3D cell models as a new *in vitro* methodological approach for nanoparticle (geno)toxicity assessment to better understand the impact nanomaterials have on environmental and human health. Currently, as a part of our ongoing study, core-shell iron nanoparticles are being examined, where the core consists of FeO, and the shell is made of Fe<sub>3</sub>O<sub>4</sub>. So far, *in vitro* cyto- and genotoxicity were assessed in the human hepatocellular carcinoma cell line HepG2, using the ATP assay and the comet assay, respectively, but due to ongoing genotoxicity testing and reservations about data publishing, the results will not be presented in this scientific contribution.

**Keywords:** 3D cell models; 2D cell models; Cytotoxicity; DNA damage; Genotoxicity; Iron-based nanoparticles



## 1. Introduction

Nanotechnology is a technological intersection with the nanoscale which straightforwardly links the macroscopic world of our perceptions with the nanoscopic world of individual biomolecules (Contera, 2019). It represents one of the most promising technologies of the 21st century (Bayda et al., 2019) strongly intertwined with our everyday life and society (He et al., 2019).

The prefix “nano” is of Greek origin meaning “dwarf” or something very small and depicts one thousand millionth of a meter ( $10^{-9}$  m) (Bayda et al., 2019) hence by the word nanomaterials we describe materials with one or more components that have at least one dimension in the range of 1 to 100 nm (Borm et al., 2006; T. Singh et al., 2017).

In the last decade, the production and use of nanomaterials have grown tremendously, and as a result, so has human exposure to these materials (Borm et al., 2006; Zhu et al., 2019). Since human exposure to nanoparticles is inevitable (Yang W, et al., 2021) much attention has been drawn to nanoparticle toxicology (Yang Y, et al., 2017) – especially to the potential acute and chronic adverse effects that nanoparticles may cause on humans (W. Yang et al., 2021) – mostly because nanomaterials due to their high surface-to-volume ratio, high reactivity, and unique physical, chemical, and biological properties (Awashra and Młynarz, 2023; Yang W, et al., 2021) exhibit a greater risk of toxicity than the corresponding bulk material (Hoet et al., 2004).

The study of nanoparticle adverse effects and toxicity is referred to as nanotoxicology (Elsaesser and Howard, 2012) and even though exposure to nanoparticles is increasing, information on their toxicological properties remains inadequate (Gornati et al., 2009). For a comprehensive assessment of the toxicity of nanoparticles, structure, and corresponding physicochemical properties need to be fully characterized because only then can the observed toxic effects be attributed to specific properties of nanoparticles in order to establish specific nanoparticle structure-activity/ toxicity functional relationships ( Yang W, et al., 2021). Furthermore, to better understand the mechanisms of nanoparticle toxicity studies at the cellular and sub-cellular levels need to be done (Awashra and Młynarz, 2023).

## 2. *In vitro* Cell Models

### 2.1. Two-dimensional (2D) cell models

Genetic toxicity testing is an essential part of drug and material safety assessment since DNA damage can lead to genetic changes, including mutations, chromosome damage, and genomic instability that can lead to cancer development (David, 2020; Maynard et al., 2011). Current EU legislation for chemical and material safety assessment demands testing of chemical/material on two types of *in vitro* tests: (i) an Ames test (a bacterial test) and (ii) one of two mammalian cell tests – micronucleus test or chromosomal aberration test – followed by an *in vivo* animal model study (Cimino, 2006; Corvi et al., 2013). For an adequate evaluation of genotoxicity, the evaluation of three parameters is required: gene mutations, structural changes, and numerical changes in chromosomes (R et al., 2013).

In *in vitro* toxicology, the golden standard for studying absorption, distribution, metabolism, excretion, and toxicity of compounds is considered to be human primary hepatocytes, which, compared to permanent liver cell lines, better reflect the properties and phenotype of hepatocytes *in vivo*, but in practice, due to the limited availability of human liver samples, they are replaced by cell lines such as HepG2, HepaRG, Huh7, SK-Hep-1, and others. In addition, primary liver cells are difficult to maintain under *in vitro* conditions. Not only can they be grown for a short period, but they also quickly lose their cuboidal morphology and liver-specific functions during cultivation. The high price and differences between donors due to polymorphisms are also a problem (Arzumanian et al., 2021; Klingmüller et al., 2006; Sefried et al., 2018; Shulman and Nahmias, 2012; Štampar et al., 2020; Zeilinger et al., 2016).

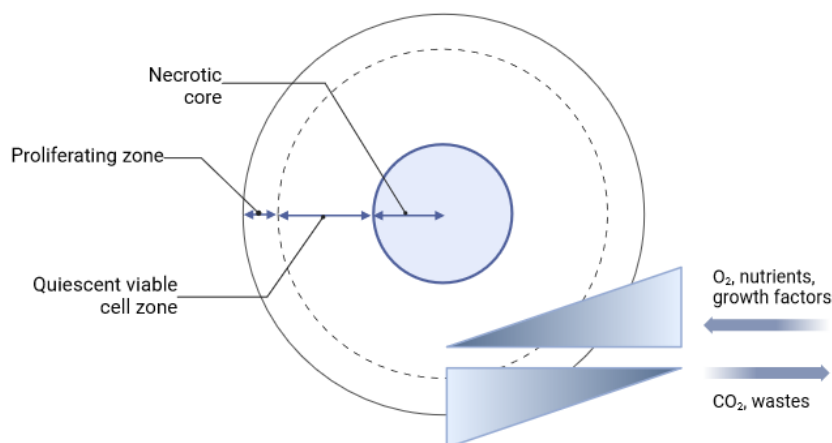
Both human primary hepatocytes and human hepatic cell lines are normally cultured as a monolayer of cells (2D cell model) for genotoxicity testing (Laohathai and Bhamarapravati, 1985; Shulman and Nahmias, 2012) with one main difference: primary human hepatocytes cannot be cultured indefinitely as they stop dividing under *in vitro* conditions (Shulman and Nahmias, 2012). In this specific respect using human hepatic cell lines represents a good alternative for genotoxicity studies. However, one must consider that hepatic cell

lines are immortalized, cancer cells, which means that despite retaining a certain degree of the properties of primary hepatocytes, they also show similarities to tumor cells (Arzumani et al., 2021).

While both primary hepatocytes and human hepatic cell lines as model systems have their advantages and disadvantages, both are usually used in 2D culture. 2D cell models have certain limitations for genotoxicity testing. They are indeed associated with simple and low-cost maintenance and performance of functional tests, yet they do not mimic the natural structures of tissues (Kapałczyńska et al., 2016) – 2D models lack the cell-cell and cell-extracellular matrix (ECM) signaling (Breslin and O’Driscoll, 2013; Kapałczyńska et al., 2016), which in turn leads to reduced cell differentiation and modified signaling of metabolic pathways (Aucamp et al., 2017). Furthermore, they lose the diversity of phenotype (Richter et al., 2021; von der MARK et al., 1977), and have unlimited access to the ingredients of the medium such as oxygen, nutrients, metabolites, and signal molecules (Kapałczyńska et al., 2016), forcing them into a polarization that does not reflect physiological conditions (Fontoura et al., 2020). Furthermore, the shortcomings of 2D cell models include the non-robustness of the models (Xiao et al., 2022) and misleading results (Saji et al., 2019) due to which according to ECHA (European Chemical Agency) additional *in vivo* studies need to be done (“ECHA - European Chemical Agency,” n.d.; R et al., 2013). For these purposes, the European REACH regulation promotes the 3R strategy (Replacement, Reduction, Refinement), to replace and reduce the use of animals in *in vivo* studies, while at the same time refining test systems, obtaining more relevant results for humans (Törnqvist et al., 2014). As a result of this and the many disadvantages of 2D models, a lot of attention is being paid to the development of new alternative models that will reflect the *in vivo* conditions more accurately (Ipek et al., 2023) – and one of these are 3D cell models.

## 2.2. Three dimensional (3D) cell models

In toxicology, 3D cell models also known as spheroids are a powerful tool for studying the genotoxic effects of chemicals/materials because they better mimic *in vivo* conditions (Ipek et al., 2023; Wang et al., 2021). Cells inside the spheroid comprise different cell layers (**Figure 1**) (Edmondson et al., 2014). The external layer is composed of cells displaying high proliferation rates, towards the middle resting cells can be observed, and non-dividing (necrotic) cells can be found in the core of the spheroid (Alvarez-Pérez et al., 2005; Edmondson et al., 2014; Nath and Devi, 2016). The high proliferation rate of cells in the spheroid's external layer can be explained by their easier access to oxygen and nutrients (Tredan et al., 2007). In contrast, cells within spheroids remain in a necrotic state due to the absence of oxygen (hypoxia) and nutrients (Minchinton and Tannock, 2006; Tredan et al., 2007).



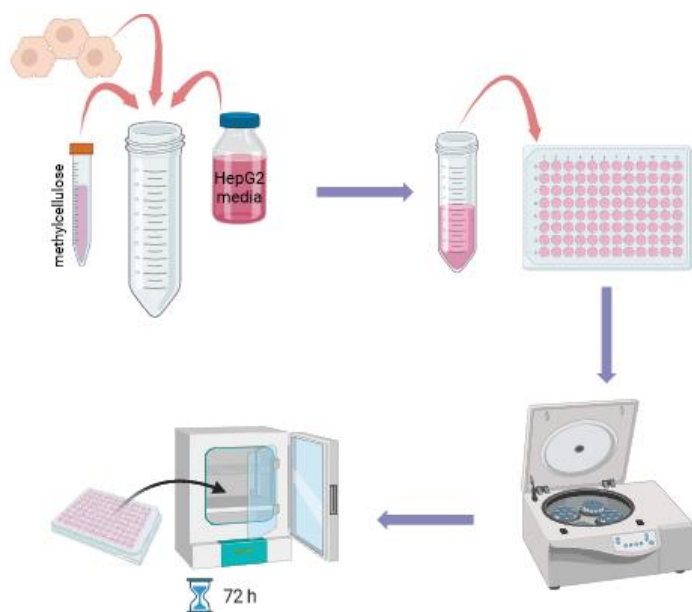
**Figure 1.** Structure of a spheroid (Edmondson et al., 2014; Nath and Devi, 2016). Created with BioRender.com

Apart from the 2D cell model, cells in the 3D model form better cell-to-cell connections and produce a matrix that promotes tissue-specific cell binding, direct cell-to-cell interactions, and cell-to-extracellular matrix interactions (Langhans, 2018). They also preserve their natural morphology (Costa et al., 2016) and maintain high viability for several weeks (Štampar et al., 2021). Gene and protein expression levels in spheroids better resemble levels found in cells *in vivo* (Costa et al., 2016; Langhans, 2018; Ravi et al., 2015). Therefore, by using 3D cell models, we can reduce the differences between *in vitro* and *in vivo* studies, decreasing the likelihood of needing to use animal models (Costa et al., 2016; Langhans, 2018; Ravi et al., 2015).

Despite all the advantages of 3D models, they still have certain drawbacks, including more demanding and expensive cell maintenance in culture (Costa et al., 2016; Langhans, 2018; Ravi et al., 2015), difficult replication of experiments, and more demanding interpretation of data (Kapałczyńska et al., 2016).

### 2.3. Spheroid formation

We are developing human hepatocellular carcinoma (HepG2) 3D cell models as a new methodological approach for nanoparticle (geno)toxicity assessment. In general, several approaches and materials can be used for culturing cells in 3D for instance, different hydrogel substrates, e.g., beads, injectable gels, moldable gels, and macroporous structures (Białkowska et al., 2020), or/and different methods such as the forced floating method (Štampar et al., 2019), pellet culture method, liquid overlay method, hanging drop method, etc. (Ryu et al., 2019). Within our department, HepG2 spheroids are routinely formed using the forced floating method (**Figure 2**), which will be described further on.



**Figure 2.** A simplified scheme of spheroid preparation with the forced floating method (Štampar et al., 2019). Created with BioRender.com

When preparing spheroids from HepG2 cells using the forced floating method, 96-well U-bottom low attachment microtiter plates are needed. The cell suspension is mixed with an appropriate volume of cold HepG2 medium (4 °C) – HepG2 medium is composed of MEME medium (MEME-10370-047) containing NEAA supplemented with 10% FBS, all from Gibco (Praisley®, Scotland, UK) and 2.2 g/L NaHCO<sub>3</sub>, 2 mM L-glutamine, 100 IU/mL penicillin/streptomycin and 1 mM sodium pyruvate from Sigma-Aldrich (St. Louis, MO, USA) – and 4 % methylcellulose in a way that cell density equals 15.000 cells/mL. Then 200 µL of the mixture is pipetted into each well and plates are centrifugated for 90 minutes at 28 °C and 900 g. Due to the centrifugal force, the cells formed clusters (aggregates), which



after 72 hours of incubation (37 °C, 5 % CO<sub>2</sub>) matured into spheroids suitable for further research (Štampar et al., 2019).

### 3. Methods for assessment of *In vitro* cell models

#### 3.1. ATP Luminescent Cell Viability Assay

The ATP Luminescent Cell Viability Assay is a method for determining the number of viable cells in culture based on the quantification of the ATP present. The measurement of ATP using firefly luciferase is the most frequently applied method for estimating the number of viable cells (Riss et al., 2016) – ATP serves as an indicator of metabolically active cells because when cells lose membrane integrity, they lose the ability to synthesize ATP and endogenous ATPases rapidly deplete any remaining ATP from the cytoplasm (Riss et al., 2016). The ATP assay proved sensitive and user-friendly cell viability assay. It was reported that ATP assay is less prone to artifacts than other viability assay methods (Riss et al., 2016). Another advantage of this assay is that an incubation step with a population of viable cells is not prerequisite to convert a substrate into a colored compound (as in MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assays), which also eliminates a plate handling step because cells do not need to be returned to the incubator to generate a signal (Riss et al., 2016).

##### 3.1.1. Luminescent cell viability assay protocol

For the performance of the cell viability assay, we prepared the spheroids as described in the section 2.3 and exposed them to graded nanoparticle concentrations prepared as described in the section 3.3. After 24-hour or 96-hour exposure, we transferred 5 spheroids for each concentration from the U-bottom microtiter plate to the white opaque walled microtiter plate and added 50 µL of the reagent (CellTiter-Glo®, Promega, Madison, Wisconsin, USA) which contains a detergent to lyse the cells, ATPase inhibitors to stabilize the ATP that is released from the lysed cells, luciferin as a substrate, and the stable form of luciferase to catalyze the reaction that generates photons of light (Riss et al., 2016). Then we resuspended the mixture of reagent and cells and incubated it for 10 minutes at room temperature before measuring the luminescence signal.

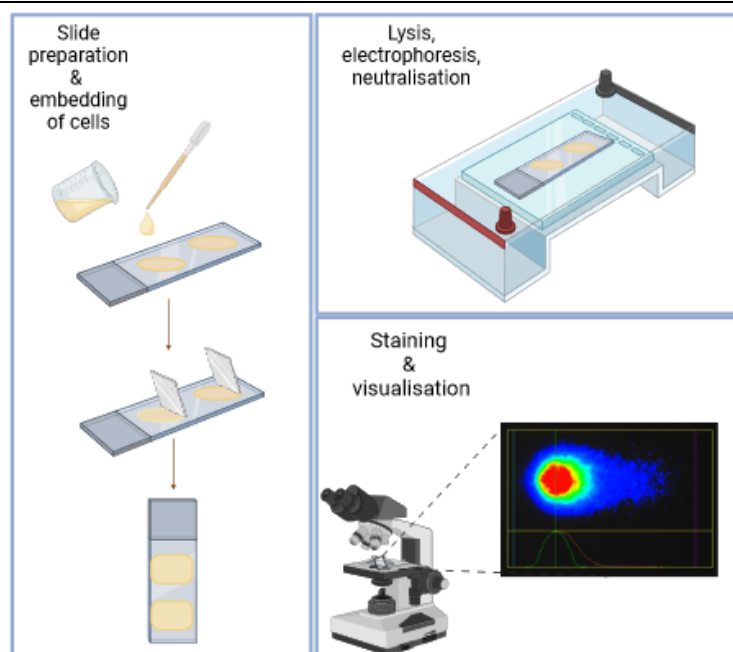
#### 3.2. Comet Assay

The comet assay, or Single-Cell Gel Electrophoresis (SCGE), is an extremely sensitive and fast quantitative *in vitro* method that detects DNA damage at the level of a single cell (Nickson and Parsons, 2014). The method enables the detection of single- and double-strand DNA breaks, alkali-labile sites, DNA-DNA, and DNA-protein cross-linking (Tice et al., 2000). DNA damage can be either endogenous or exogenous. Most of the endogenous DNA damage arises from the chemically active DNA engaging in hydrolytic and oxidative reactions with water and reactive oxygen species (ROS), that are naturally present within cells while exogenous DNA damage occurs when environmental, chemical, and physical agents such as UV and ionizing radiation, alkylating agents, and crosslinking agents damage the DNA (Chatterjee and Walker, 2017).

Nowadays the method represents one of the standard methods for DNA damage evaluation with applications in genotoxicity testing, human biomonitoring, molecular epidemiology, eco/genotoxicology, and basic research on DNA damage and repair (Collins, 2004; Cordelli et al., 2021).

In the SCGE method, the cells are embedded in an agarose gel, and lysed so that only the nuclei (DNA) remain in the gel. The gel is then exposed to an electric field in the electrophoresis and because the damaged DNA migrates at a different rate than non-damaged DNA using specific dyes we can observe structures, resembling a comet. The undamaged DNA in the comet structure is referred to as the “head” while the trailing damaged DNA band is referred to as the “tail”. The percentage of DNA in the tail is directly proportional to the percentage of DNA damage that has occurred in a particular cell (Nickson and Parsons, 2014; Vandghanooni and Eskandani, 2011).

A simplified procedure scheme is shown in **Figure 3**.



**Figure 3.** A simplified procedure scheme of the comet assay (Nickson and Parsons, 2014; Vandghanooni and Eskandani, 2011). Created with BioRender.com

### 3.2.1. Comet Assay Protocol

After the treatment (section 3.3), a suspension of viable single cells was obtained by the combination of enzymatic digestion and mechanical degradation. For each tested concentration we harvested 5 spheroids and transferred them to a 1,5 mL Eppendorf tube. Then we centrifuged them for 4 minutes at 1000 Rotations Per Minute (RPM) and discarded the media. We washed them with 1 mL 1x Phosphate Buffered Saline (PBS) and repeated the centrifugation step. After that, we discarded the PBS and added 50-100  $\mu$ L of an enzymatic mixture (10x diluted collagenase (50 mg/ml) solution with serum-free medium (MEME-10370-047, Gibco, Praisley®, Scotland, UK) and TrypLE (Gibco; 12604-013, Waltham, Massachusetts, USA) in the ratio of 1:2) into the Eppendorf Safe-Lock Microtube (Eppendorf, Hamburg, Germany) with spheroids. We incubated the spheroids for 8-10 minutes at 37 °C and 5 % CO<sub>2</sub>. After the incubation, we resuspended the spheroids and added 500  $\mu$ L of HepG2 growth media with supplements to deactivate the collagenase type I and centrifuged them for 4 minutes at 1000 rpm. We discarded the media and then the comet assay was conducted according to Singh et al. (1988) with minor modifications by Štampar et al. (2019).

### 3.3. Nanoparticle sample preparation

So far, our work has focused on (geno)toxicity assessment of core-shell iron nanoparticles – the core consists of FeO while the shell is made of Fe<sub>3</sub>O<sub>4</sub>. First, we evaluated the cytotoxicity of graded concentrations of nanoparticles using the ATP Luminescent Cell Viability Assay. Nanoparticles were dispersed into cell media with the highest concentration of 80  $\mu$ g/cm<sup>2</sup> and the lowest concentration of 0,2  $\mu$ g/cm<sup>2</sup> for 24-hour exposure while the highest concentration for 96-hour exposure was 40  $\mu$ g/cm<sup>2</sup> and the lowest concentration was 0,02  $\mu$ g/cm<sup>2</sup>. The concentrations were determined based on the OECD (Organisation for Economic Co-operation and Development) TG 487 guidelines (OECD, 2022) for testing manufactured nanomaterials in a way that the concentration of 40  $\mu$ g/cm<sup>2</sup> equaled the concentration of 100  $\mu$ g/mL. Usually, it is recommended that the top dose is restricted to 100  $\mu$ g/mL or 100  $\mu$ g/cm<sup>2</sup>, whichever is higher, because doses higher than this are not physiologically relevant, and can result in interference with scoring due to high deposition on cells (OECD, 2022). As a positive control, 15 % DMSO



was used, since currently, there are no suitable nanoparticles that can be used as positive controls for the *in vitro* assays (OECD, 2022).

#### 4. Conclusion

In general, the toxicity of nanoparticles can be assessed with several different approaches, among which, the most beneficial ones in terms of cost and time saving are the *in vitro* studies. *In vitro* studies are essential to identify biochemical and molecular mechanisms of nanoparticles' cyto- and genotoxicity and are also the first step in identifying potentially harmful effects for humans and the environment. When it comes to *in vitro* studies, 3D cell models also known as spheroids are a powerful tool for studying the genotoxic effects of chemicals/materials. Not only do they mimic *in vivo* conditions better, but their usage allows us to minimize testing on animals, thus following the 3R (Replacement, Reduction, and Refinement) principle. Furthermore, spheroids represent a promising model for nanoparticle (geno)toxicity assessment and regarding that, significant progress has already been made. However, a lot of inadequate information is available and that is why more research needs to be done to identify the most appropriate approach for assessing nanoparticle toxicity.

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

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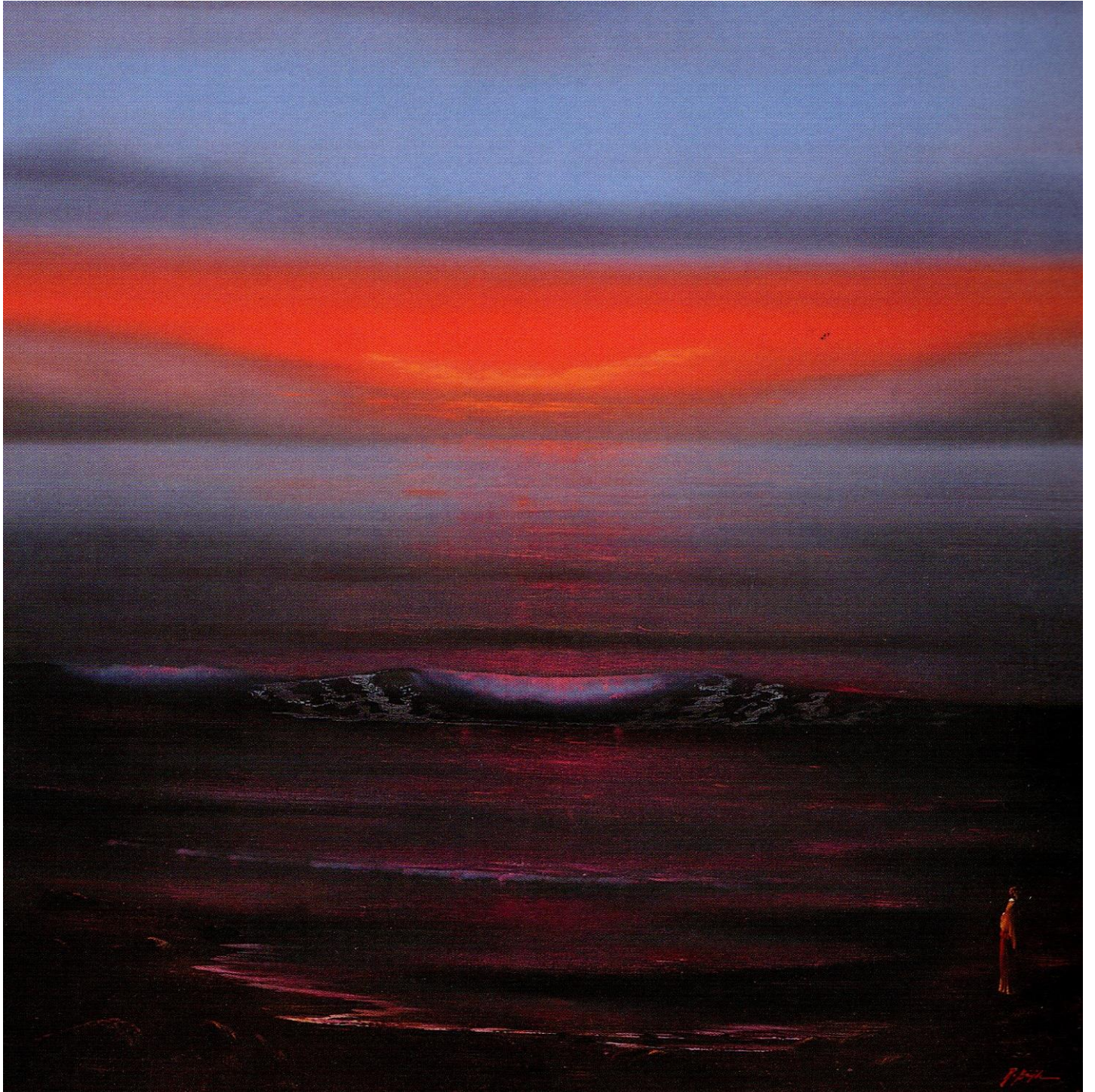


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# The Effect of Size and Surface Treatment of Nucleating Agents on Polyamide 6 Morphology Studied by Flash Differential Scanning Calorimetry

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## Abstract:

The use of recycled polymer materials with low carbon footprint, which enables circular economy, is becoming increasingly interesting for commercial use in the world. The main reasons for this are the accumulation of waste after the use of polymer products and the warming of the atmosphere due to the overloading of the environment with greenhouse gases produced during the extraction of these materials. We were interested in the differences in properties between differently modified samples of thermoset waste in a thermoplastic matrix. Our main focus was on the influence of the modification of the interface on the stiffness and strength of the prepared material. The results of tensile and bending tests showed that with the right combination of compatibilizers, we were able to successfully increase the stiffness and strength of the composites. The simultaneous increase in stiffness and strength is a very good indicator that with the right combination of compatibilizers (polypropylene grafted maleic anhydride (PP-g-MA) and modified thermoplastic polyurethane (TPU) co-polymer) we were able to ensure good surface interaction between the fibres and the polymer matrix in these samples. Waste paper was added to the thermoplastic matrix as a reference. The difference in the performance of the composites can be explained by the better wettability of the waste paper with the SEBS-g-MA compatibilizer and the better interfacial interactions of the modified TPU copolymer with the thermoset and the PP-g-MA with the glass fibres in the case of the waste thermoset composite.

**Keywords:** waste thermoset, compatibilizer, thermoplastics, different fraction of waste thermosets, composites



## 1. Introduction

The recycling of plastic waste, especially post-consumer recycling (PCR), has begun and is still developing very slowly. Compared to other materials such as paper, glass and metals, recovery and recycling rates of plastics are generally low. Even in countries with advanced waste management systems and years of experience in recycling, the recycling rates of plastics are generally much lower than those of the other materials mentioned above. This is partly due to the wide variety of uses of plastics, types of additives and types of polymer composites. This diversity of uses is one of the main advantages of plastics, but it also poses a problem for recycling (Shen and Worrell, 2014).

The high melting point of polyamides is the result of strong hydrogen bonding in the crystals. This also enables stiffness above the glass transition temperature and almost up to the melting point. The effect is further enhanced when reinforcing materials such as glass fibres are added. The melting point is determined by the number of hydrogen bonds between the chains, which depends on the density of the amide groups. The melting temperature decreases with increasing length of the aliphatic chain between the amide groups. Polyamides crystallize quickly, which can be advantageous for small particles to further accelerate this process; this can shorten the cycle time and increase productivity. Nucleating agents are substances in the form of fine particles that accelerate the crystallization of polyamides. Nucleation increases tensile strength and stiffness, but makes the material more brittle (Wypych, 2016) (Palmer, 2000).

The glass transition temperature depends on the cold crystallization of polyamide 6 (PA6) and is influenced by the cooling conditions. The ratio between the rigid and the mobile amorphous fraction in PA6 is determined by the thermal history (Parodi et al., 2017).

Nucleating agents are additives used to increase the crystallization rate during processing by controlling the morphology of the polymers. For this reason, nucleating agents are often specific to a particular polymer or family of polymers and should be carefully selected depending on the desired change in physical properties. Nucleating agents at low cooling rates (10 K/min) cause a shift of crystallisation temperature ( $T_c$ ) to higher temperatures. The crystallization rate of PA6 is not affected by nucleating agents up to 90 °C, as the density of nuclei is very high at temperatures close to the glass transition temperature. In order to measure the effectiveness of nucleating agents, Flash differential scanning calorimetry (DSC) measurements must be carried out by rapidly cooling the melt to a crystallization temperature between the glass transition temperature ( $T_g$ ) and melting temperature ( $T_m$ ) (Mileva et al., 2012). The reported crystallization rate for PA6 is lower compared to PA66, which is due to the odd (PA6)/even (PA66) number of CH<sub>2</sub> groups (Poel, Istrate and Mathot, 2016).

With Flash DSC the fast-scan chip-calorimeter measurements in a broad temperature range were conducted, also in low temperature region. In the case of nylon, the crystallization rate showed a parabolic shape depending on the crystallisation temperature with a maximum between  $T_g$  and  $T_m$  (Li et al., 2020).

## 2. Methods & materials

Commercially available PA6 (Akulon K222-D) was purchased from DSM, Netherlands (PA6 in **Table 1**). Commercially available antioxidant (AT 10 – AO in **Table 1**) was purchased from AMIK ITALIA, Italy. Commercially available CaCO<sub>3</sub> was provided by Calcit, Slovenia. Trade names of CaCO<sub>3</sub> are Calplex Extra (NA<sub>01</sub> in **Table 1**), Calplex 5 (NA<sub>02</sub> in **Table 1**), Calplex 40 (NA<sub>03</sub> in **Table 1**), Polyplex Extra (NA<sub>11</sub> in **Table 1**), Polyplex 5 (NA<sub>12</sub> in **Table 1**) and Polyplex 40 (NA<sub>13</sub> in **Table 1**). Commercially available talc (Plustalc H15 – NA T in **Table 1**) was purchased from Elementis Minerals, Netherlands. Commercially available nucleating agent (Prisma AD PE Nucleant 91225 – NA AD in **Table 1**) was purchased from Frilvam, Italy and the only material in the pellet form.

The composition of the samples is listed in **Table 1**. For all tests, the materials were mixed separately and extruded on the Labtech LTE 20-44 twin screw extruder. The screws had a diameter of 20 mm, an L/D ratio of 44:1, a screw speed of 600 rpm and an increasing temperature profile from the hopper (245 °C) to the die (260 °C) (Pracella et al., 2010).



The first sample (Sample 0) was pure PA6, which we extruded and injection moulded. Then we produced a PA6 sample with 0.5 % antioxidant (Sample 1, with AO). Composites with 0.1 % calcium carbonate were produced in 6 different versions; in three the calcium carbonate was uncoated (Samples 2-4, with NA<sub>01</sub>, NA<sub>02</sub> and NA<sub>03</sub>, respectively) and in three it was coated with stearic acid (Samples 5-7, with NA<sub>11</sub>, NA<sub>12</sub> and NA<sub>13</sub>, respectively). We prepared two more versions: in one we added 0.1 % talc (Samples 8, with NA T) and in the last one we added 1 % nucleating agent based on polyethylene with 50 % talc (Samples 9, with NA AD). All composites contained 0.5 % antioxidant.

**Table 1.** Sample composition.

Sample	AO (%)	NA <sub>01</sub> (%)	NA <sub>02</sub> (%)	NA <sub>03</sub> (%)	NA <sub>11</sub> (%)	NA <sub>12</sub> (%)	NA <sub>13</sub> (%)	NA T (%)	NA AD (%)	PA6 (%)
0	0	0	0	0	0	0	0	0	0	100
1	0.5	0	0	0	0	0	0	0	0	99.5
2	0.5	0.1	0	0	0	0	0	0	0	99.4
3	0.5	0	0.1	0	0	0	0	0	0	99.4
4	0.5	0	0	0.1	0	0	0	0	0	99.4
5	0.5	0	0	0	0.1	0	0	0	0	99.4
6	0.5	0	0	0	0	0.1	0	0	0	99.4
7	0.5	0	0	0	0	0	0.1	0	0	99.4
8	0.5	0	0	0	0	0	0	0.1	0	99.4
9	0.5	0	0	0	0	0	0	0	1	98.5

The injection moulding was carried out on a Krauss Maffei 50-180 CX with a screw diameter of 30 mm. The temperature profile increased from the hopper (220 °C) to the nozzle (230 °C). The mould temperature was set to 80 °C and the cooling time to 20 s. Injection moulded pieces were shaped according to ISO 527 (for tensile tests and DMA) and ISO 179 (for impact test) (Jazani et al., 2011). The tensile tests were carried out with the Shimadzu AG-X plus in accordance with ISO 527-1 (Huang et al., 2013). Tensile modulus ( $E_t$ ), tensile strength ( $\sigma_m$ ), elongation at tensile strength ( $\epsilon_m$ ) and elongation at break ( $\epsilon_{tb}$ ) were evaluated in the tensile tests. Five measurements were carried out for each sample. The thermomechanical properties were analysed using a Perkin Elmer DMA 8000 (Wunderlich, 2005). The samples were heated at 2 °C/min from 25 °C to 210 °C in an air atmosphere. A frequency of 1 Hz and an amplitude of 20  $\mu$ m were used in dual cantilever mode (Jazani et al., 2011). Thermogravimetric analyses (TGA) were performed using a Mettler Toledo TGA/DSC 3+ thermal analyser. Analyses were carried out in a nitrogen atmosphere (20 mL/min) from 40 to 550 °C with a heating rate of 10 °C/min, followed by an isothermal segment in an oxygen atmosphere (20 mL/min) at 550 °C for 10 minutes using an Al crucible. Changes in the weight are evaluated as degradation in % and peak of the first derivative of the measured curve as degradation temperature. Thermal measurements were performed with a Differential Scanning Calorimeter (DSC 2, Mettler Toledo) in a nitrogen atmosphere (20 mL/min). The temperature of the samples was raised from 0 to 260 °C at a heating rate of 10 °C/min and held in the molten state for 5 minutes to clear the thermal history. After cooling at 10 °C/min, the samples were reheated at 10 °C/min to 260 °C.  $T_c$ , the crystallisation enthalpy ( $\Delta H_c$ ),  $T_m$  and the melting enthalpy ( $\Delta H_m$ ),  $T_g$  and the change in specific heat capacity ( $\Delta c_p$ ) were determined from the cooling and the second heating scan. The crystallinity ( $W_{c,h}$ ) was calculated from melting enthalpy, divided by the melting enthalpy of 100 % crystalline PA6 (Wunderlich, 2005).

Flash DSC 1 with Huber TC45 intercooler and nitrogen purge gas (50 mL/min) was used to preformed crystallization kinetics measurements. The test samples were heated (230 °C) and cooled (20 °C) three times at a high heating and cooling rate (2.000 °C/s) so that



most of the nuclei that formed during cooling from previous processing have melted. During the first heating, the sample melts and spreads over the entire sensor. During the second heating the thermal history is erased and during the third heating we obtain realistic results of the material. The sample is cooled to different temperatures and held there for 5 seconds. The temperatures at which the measurement stopped were between 55 °C and 180 °C in 5 °C increments. After the measurements were completed, we evaluated the rate of crystallization as an exothermic peak during this isothermal segment.

The impact tests were performed using the Dongguan Liyi Test Equipment pendulum, type LY-XJJD5 (Lin et al., 2011), according to ISO 179. The distance between the supports was 60 mm and a 5 J pendulum was used. Impact strength was determined as the quotient between the energy required to fracture the sample divided by the cross-section of the sample.

### 3. Results & Discussions

#### 3.1. Tensile tests

The results of the tensile tests are shown in **Table 2**. Sample 0 is the reference material, pure PA6, with a tensile modulus of 2.29 GPa, tensile strength of 70.0 MPa, elongation at tensile strength of 4.52 % and elongation at break of 114 %. In Sample 1, only the antioxidant was mixed into the matrix. The addition of the antioxidant reduced the tensile modulus  $E_t$  by 15.7 %, the tensile strength  $\sigma_m$  by 0.1 %, the elongation at break  $\epsilon_{tb}$  by 24.6 % and increased the elongation at tensile strength  $\epsilon_m$  by 3.5 % (**Table 2**).

Samples 2, 3 and 4 consisted of matrix, antioxidant and uncoated calcium carbonate, which differ in particle size. Sample 2 and 3 showed a decrease in tensile modulus  $E_t$  at tensile strength  $\sigma_m$  by 1.3 % and 6.1 %, respectively; Sample 4 showed an increase in tensile modulus  $E_t$  at tensile strength  $\sigma_m$  by 3.9 %. All three samples showed a decrease in tensile strength  $\sigma_m$  by 1.2 % for Sample 2, 2.7 % for sample 3 and 2.7 % for Sample 4. The elongation at tensile strength  $\epsilon_m$  did not change drastically. The elongation at break  $\epsilon_m$  decreased by 15.2 % for sample 2, 52.5 % for sample 3 and 42.1 % for sample 4 (**Table 2**).

Samples 5, 6 and 7 consist of a matrix, an antioxidant and coated calcium carbonate with different particle sizes. For all three samples, we observe a decrease in tensile modulus  $E_t$ , tensile strength  $\sigma_m$  and elongation at tensile strength  $\epsilon_m$  and an increase in elongation at break  $\epsilon_{tb}$ . Of these three samples, Sample 5, which contained Polyplex Extra with the smallest particles, achieved the highest stiffness, strength and toughness. It was as we expected: the smaller the particles, the better the effect of adding calcium carbonate (**Table 2**).

To improve the tensile modulus  $E_t$ , we need to use Calplex 40, which is uncoated and has larger particles than Calplex Extra and Calplex 5. Other calcium carbonates reduced the tensile modulus  $E_t$  (**Table 2**). All samples showed a reduction in tensile strength  $\sigma_m$  compared to neat PA6, but this was lowest for Calplex Extra, which is uncoated and contains the smallest particles. The Calplex additives are optimal for slight changes in tensile strength  $\sigma_m$ , which hardly change it at all; the Polyplex additives reduce it slightly (**Table 2**). Elongation at break  $\epsilon_{tb}$  increased the most with Polyplex 40, which contains larger particles and is coated.

Talc is added to Sample 8. Talc reduced the tensile modulus  $E_t$  by 3.5 % compared to pure PA6, while the tensile strength  $\sigma_m$  increased slightly (by 0.3 %). The elongation at tensile strength  $\epsilon_m$  decreased by 6.2 %; the elongation at break  $\epsilon_{tb}$  decreased by 71.6 % compared to the reference (**Table 2**).

A nucleating agent consisting of 50 % talc and 50 % LDPE reduced the tensile modulus  $E_t$  by 2.2 %, the tensile strength  $\sigma_m$  by 2.6 %, the elongation at tensile strength  $\epsilon_m$  by 1.6 % and the elongation at break  $\epsilon_{tb}$  by 33.1 % (**Table 2**).

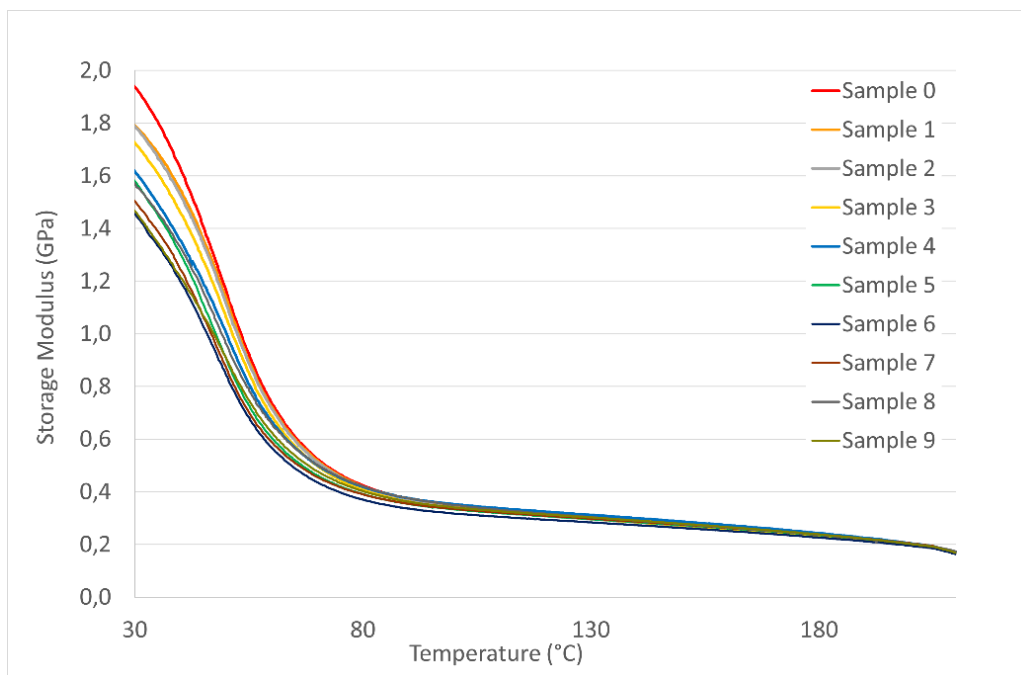


**Table 2.** Tensile tests results with recycled PP matrix.

Sample	E <sub>t</sub> (GPa)	σ <sub>m</sub> (MPa)	ε <sub>m</sub> (%)	ε <sub>tb</sub> (%)
0	2.29 ± 0.12	70.0 ± 1.1	4.52 ± 1.14	144 ± 36
1	1.93 ± 0.34	70.0 ± 0.5	4.68 ± 0.07	86 ± 57
2	2.26 ± 0.15	68.1 ± 0.2	4.52 ± 0.04	173 ± 46
3	2.15 ± 0.03	68.1 ± 0.6	4.52 ± 0.09	173 ± 41
4	2.38 ± 0.14	68.1 ± 0.6	4.51 ± 0.14	66 ± 42
5	2.25 ± 0.06	65.7 ± 0.4	4.39 ± 0.08	174 ± 52
6	2.01 ± 0.01	65.3 ± 0.5	4.47 ± 0.11	186 ± 0
7	2.02 ± 0.01	64.1 ± 1.2	4.46 ± 0.07	208 ± 37
8	2.21 ± 0.01	70.2 ± 0.4	4.24 ± 0.08	32 ± 9
9	2.24 ± 0.02	68.2 ± 0.5	4.45 ± 0.13	76 ± 22

3.2. Dynamic mechanical analysis tests

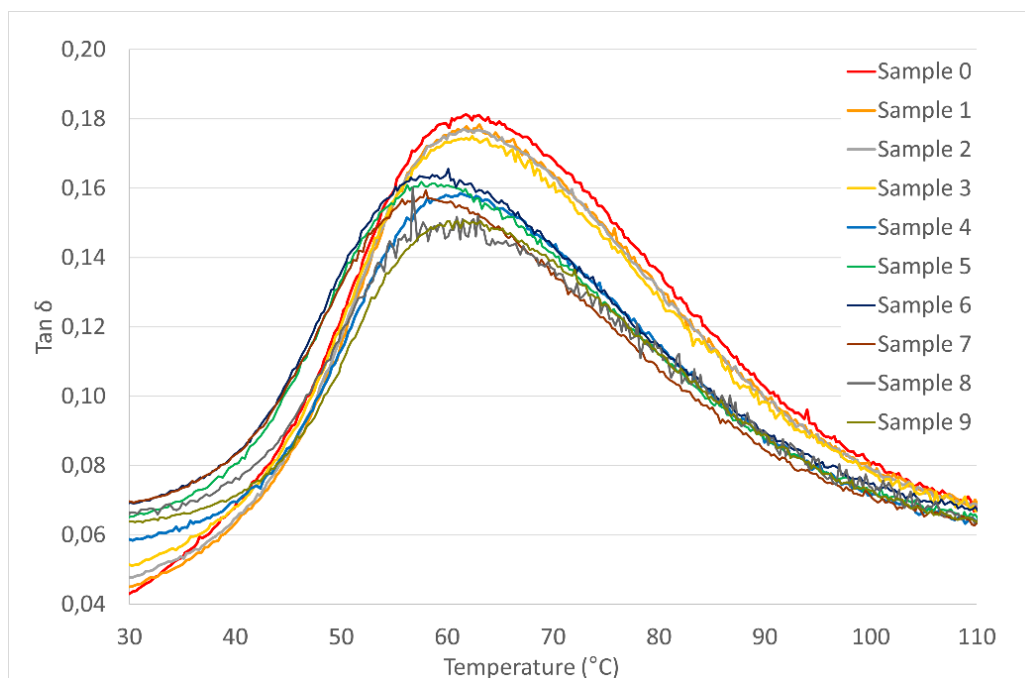
The storage modulus (**Figure 1**) of the samples agrees with the results of the tensile test, with the exception of Sample 4. Sample 0 has the highest storage modulus, while Sample 6 has the lowest storage modulus, measurement of stored energy representing the elastic proton. Uncoated calcium carbonate lowers the storage modulus, namely Calplex Extra by 7.7 %, Calplex 5 by 10.1 % and Calplex 40 by 16.6 %. Coated calcium carbonate reduces the storage modulus more, namely by 18.5 % for Polyplex Extra, 24.9 % for Polyplex 5 and 22.4 % for Polyplex 40. Talc lowers the storage modulus by 19.0 %, while the combination of talc and LDPE lowers it by 24.4 %. The curve is constantly decreasing, which means that we did not observe any cold crystallization during heating.



**Figure 1.** Storage modulus vs. temperature for the samples.



The peak of the loss factor curve (**Figure 2, Table 3**) indicates the glass transition. If the peak is lower, it means that the material has a higher elastic response, i.e. a higher degree of crystallinity. This can be observed in Sample 8, to which talc was added, and is consistent with the results of the tensile tests. The loss factor, quotient between viscous and elastic portion, is highest for Sample 0 and lowest for sample 9. Uncoated calcium carbonate lowers the loss factor. The smallest particle size (Calplex Extra) reduces the loss factor by 2.2 %, the medium size (Calplex 5) by 3.3 % and the largest size (Calplex 40) by 12.2 %. Coated calcium carbonate or Polyplex Extra, Polyplex 5 and Polyplex 40 reduce the loss factor by 10.5 %, 8.3 % and 12.2 % respectively. Talc reduces the loss factor by 11.6 % and the mixture of talc and LDPE by 16.6 %. From this we can conclude that the loss factor decreases with the addition of talc and calcium carbonate.



**Figure 2.** Loss factor (Tan  $\delta$  (-)) vs. temperature ( $^{\circ}\text{C}$ ) for the samples.

**Table 3.** Loss factor results at DMA measurements for the samples.

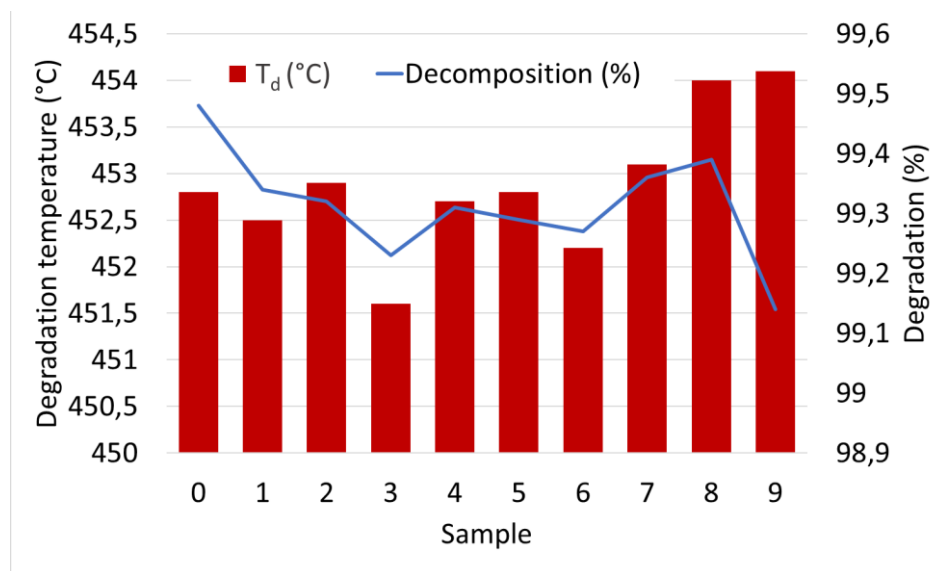
Sample	tan $\delta$ (-)	T <sub>g</sub> ( $^{\circ}\text{C}$ )
0	0.181	62.1
1	0.177	62.1
2	0.177	62.0
3	0.175	62.4
4	0.159	61.2
5	0.162	57.6
6	0.166	60.1
7	0.159	58.0
8	0.160	56.8
9	0.151	61.6





### 3.3. Thermogravimetric analysis tests

The degradation temperature of the samples varied within less than 0.3 % (**Figure 3**), which means that the additives we used did not drastically affect the degradation temperature. The inorganic filler content changed (**Figure 3**), which is completely logical as we have added inorganic fillers. Pure PA6 has 0.04 % inorganic fillers. The addition of an antioxidant increases the proportion of inorganic fillers to 0.08 %. In Samples 2 to 6, the inorganic fillers were 0.1 % - as much as we have added calcium carbonate. Sample 7 had only 0.04 % inorganic fillers. Sample 8 had 0.07 % inorganic fillers. Sample 9 had 0.34 % inorganic fillers, which means that some of the nucleating agent, which is low density polyethylene (LDPE), has degraded. Since the scattering of the results are smaller than the amount of inorganic residues in neat PA6 we can conclude that the distribution of the fillers is homogeneous and constant.



**Figure 3.** Degradation temperature and degradation amount at TGA measurements for the samples.

### 3.4. DSC tests

The results of the DSC analysis are shown in **Table 4**. The melting temperature  $T_m$  of sample 0 is at 220.1 °C. Most of the additives had no drastic influence on the melting temperature. The glass transition temperature  $T_g$  did not change drastically due to the additives (less than 2 % with the exception of Calplex 5, which lowered it by 3.7 %). In theory, it was stated that nucleating agents have no effect on the melting temperature (Poel, Istrate and Mathot, 2016), which is consistent with our results.

The melting enthalpy ( $\Delta H_m$ ) was most strongly affected by talc, which increased it by 5.2 % (**Table 4**, Sample 8). This was followed by Sample 9, where  $\Delta H_m$  was 4.4 % higher than that of pure PA6.  $\Delta H_m$  was reduced only by antioxidants (by 2.3 %) and Calplex 40 (by 2.6 %); all other additives increased it by between 0.8 % (Calplex 5) and 5.2 % (talc) (**Table 4**, Samples 3 and 8, respectively).

The degree of crystallinity of pure PA6 is 31.5 %. The addition of antioxidants reduced it by 1.8 %. Calplex Extra increased it by 4.2 %, Calplex 5 by 1.4 % and Calplex 40 reduced it by 2.1 % (**Table 4**). This indicates that smaller particles are more effective because they have a larger surface area in the case of uncoated calcium carbonate. The addition of Polyplex Extra increased the degree of crystallinity by 2.0 %, Polyplex 5 by 3.5 % and Polyplex 40 by 4.0 %. With coated calcium carbonate it is exactly the opposite as with uncoated calcium carbonate. Larger particles achieve a higher degree of crystallinity. The most optimal inorganic filler for increasing the degree of crystallinity is talc. It increases it by 5.9 %, while the combination of talc and LDPE increases it by 4.9 %.



The crystallization temperature is an indicator of heterogeneous crystallization, which is modelled by the addition of nucleating agents. Sample 8 has the highest crystallization temperature, followed by sample 2, which is consistent with the tensile tests and the DMA results.

**Table 4.** DSC tests results for the samples.

Sample	T <sub>g</sub> (°C)	T <sub>m</sub> (°C)	ΔH <sub>m</sub> (J/g)	W <sub>ch</sub> (%)	T <sub>c</sub> (°C)
0	55.5	220.1	72.5	31.5	191.7
1	54.9	220.8	70.8	31.0	191.4
2	54.5	220.0	75.1	32.9	192.1
3	53.5	220.7	73.1	32.0	191.5
4	54.3	220.4	70.6	30.9	191.8
5	54.6	220.3	73.5	32.1	191.8
6	55.3	220.7	74.6	32.6	191.5
7	55.1	220.6	75.0	32.8	191.5
8	54.4	220.5	76.3	33.4	193.0
9	54.6	220.0	75.7	33.1	191.9

### 3.5. Flash DSC tests

The Flash DSC analysis (**Figure 4**) shows the peak temperature of the crystallization and the crystallization rate. The highest is peak temperature, the fastest is the crystallization. The height of the graph represents the rate of crystallization. If the graph is higher, it means that the material crystallizes faster. All materials were tested under the same conditions. If the peak of the crystallization curve is shifted towards higher temperatures, this means that smaller crystals are formed (Poel, Istrate and Mathot, 2016). This gives the material greater transparency and toughness (Poel, Istrate and Mathot, 2016).

Sample 8, where talc was added into PA6 matrix, crystallized the fastest. From this we can conclude that Sample 8 had the highest degree of crystallinity, which was confirmed by our DSC analysis results (**Table 4**). Sample 2 crystallized fastest at a higher temperature, which means that smaller crystals were formed and it was more transparent after injection moulding. From this we can conclude that Calplex Extra provides nuclei for crystallization because it increases the crystallization temperature.

For calcium carbonate, Calplex Extra proved the best product because it is uncoated and contains the smallest particles. It increased the degree of crystallinity, the rate of crystallization when cooling PA6 and the temperature at which the material crystallizes fastest. Stiffness increased and strength was reduced. Coated calcium carbonate also increased the degree of crystallinity, but the other properties were not improved as much. With sufficiently small nucleating agents, no steric hindrances occurred, which is why the degree of crystallinity also increased.

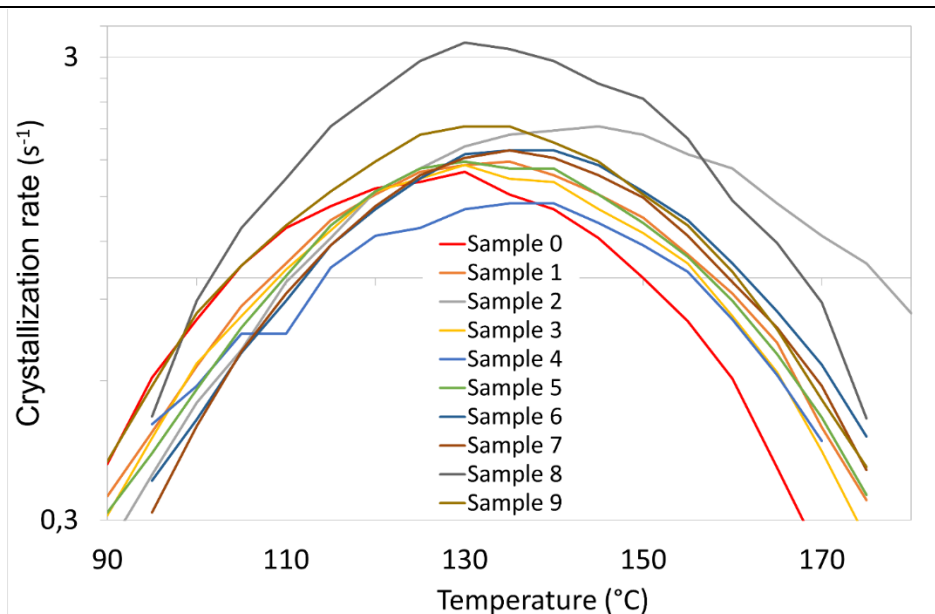


Figure 4. Flash DSC crystallization rate evaluation for the samples.

### 3.6. Impact tests

Figure 5 presents the impact strength of the samples. The impact strength of Sample 0 was 114 kJ/m<sup>2</sup>; the sample did not crack. The impact strength decreased with all additives except talc. Antioxidant decreased the impact strength by 5.5 %; Calplex Extra decreased it by 7.0 %; Calplex 5 decreased it by 3.3 %; Calplex 40 decreased it by 9.0 %. Polyplex Extra had the greatest effect on impact strength, reducing it by 17.1 %. Polyplex 5 reduced it by 5.4 % and Polyplex 40 by 2.0 %. Talc was the only agent that increased impact strength by 0.6 %. The mixture of talc and LDPE reduced the impact strength by 1.6 %. All additives that act as nucleating agents (except talc) reduced the impact strength.

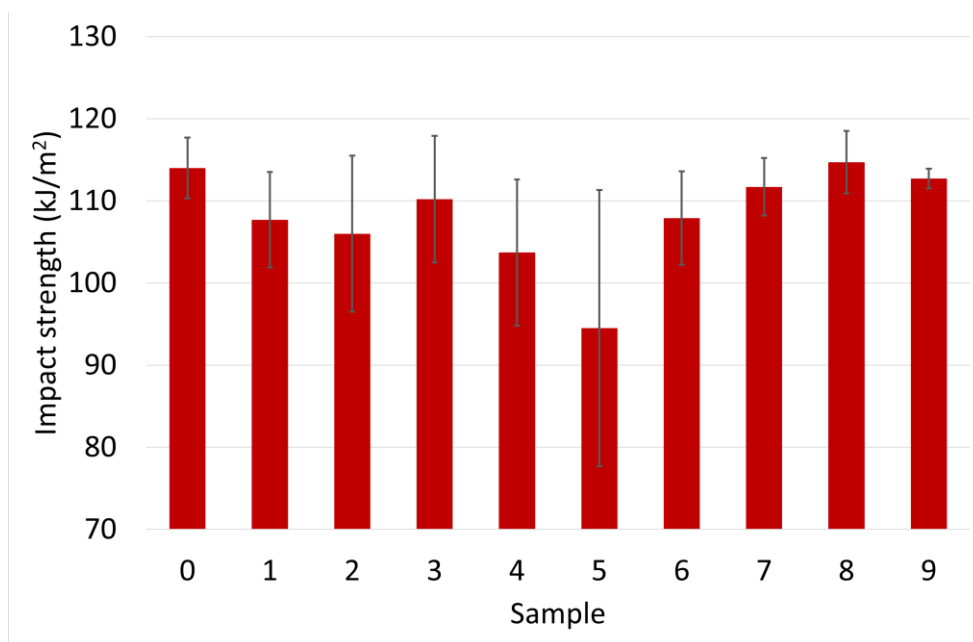


Figure 5. Impact strength for the samples.



#### 4. Conclusions

Generally, it was observed that the type, size, and surface treatment of the nucleating agents influenced the morphology behaviour of PA6. Among them, NA<sub>01</sub> was the best, as it increased the degree of crystallinity the most. The degree of crystallinity increased with all variants of added calcium carbonate, except for NA<sub>03</sub>. Uncoated particles larger than 90 μm already hinder crystallization. The highest crystallization temperature was achieved with Calplex Extra, which increased productivity. The difference between coated and uncoated calcium carbonate was large. Our results showed that it is better to use smaller particles as they have a larger specific surface area. The addition of calcium carbonate lowered the tensile modulus and tensile strength of PA6. The addition of coated calcium carbonate increased the elongation at break, while the addition of uncoated calcium carbonate decreased it. The impact toughness was reduced the most by Polyplex Extra and the least by Polyplex 40. The inorganic filler that increases the degree of crystallinity of PA6 the most was talc (Sample 8). It increased it by almost 6 % without changing the melting point of PA6. PA6 with added talc is also characterised by its crystallization rate, but crystallizes at a low temperature (Sample 8), compared to other tested samples. Talc lowered the tensile modulus and the elongation at break and slightly increased the tensile strength of PA6. Of the versions of calcium carbonate tested, Calplex Extra proved to be the most effective nucleating agent. Although talc increased the degree of crystallinity most successfully, Calplex Extra achieved the highest crystallization temperature. For further investigations, we would recommend determining the optimum concentration of calcium carbonate. The effect of the particle size and concentration of the nucleating agent should be further investigated. Based on the results of our study, we would suggest an investigation in the direction of even smaller particle size of nucleating agents. From the present study it is evident, that too high concentration has no positive effect on the degree of crystallinity. The initial concentration would be 0.1 %; it would be increased in steps of 0.1 %; the final concentration would be 1.0 %.

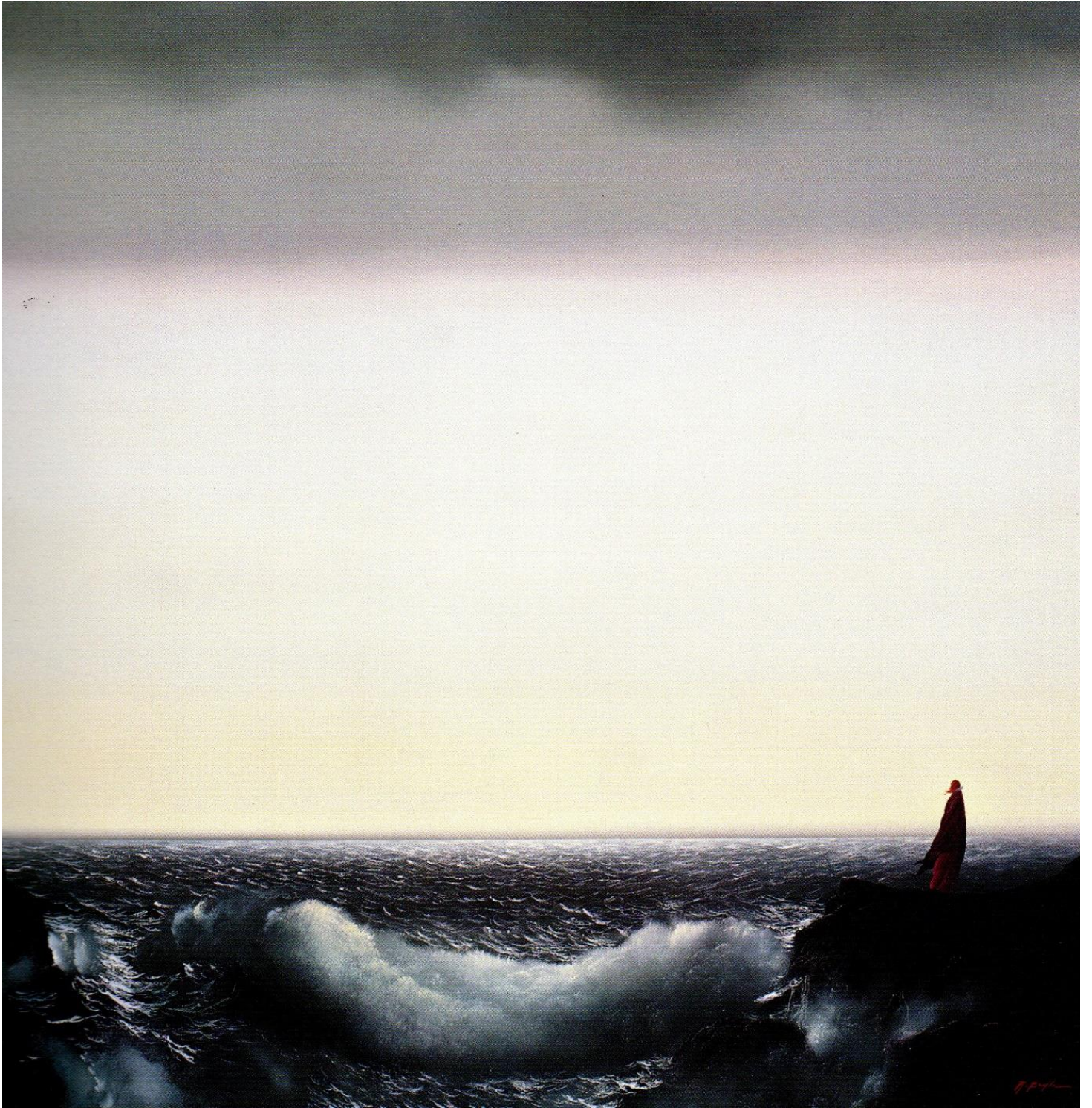
**Conflicts of Interest:** The authors declare no conflict of interest.

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# The Use of Different Fractions of Waste Thermosets for Ther-moplastic Composites

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## Abstract:

The use of recycled polymer materials with a low carbon footprint, which enable a circular economy, is becoming increasingly interesting for commercial use in the world. The main reasons for this are the accumulation of waste after the use of polymer products and the warming of the atmosphere as a result of the overloading of the environment with greenhouse gases produced during the extraction of these materials. We were interested in the differences in properties between differently modified samples of thermoset waste in a thermoplastic matrix. We paid the most attention to the influence of the modification of the interface on the stiffness and strength of the prepared material. The results of tensile and bending tests showed that with the right combination of compatibilizers, we could successfully increase the stiffness and strength of the composites. The simultaneous increase in stiffness and strength is a very good indicator that with the right combination of compatibilizers (PP-g-MA and modified TPU co-polymer) we were able to ensure a good surface interaction between the fibres and the polymer matrix in these samples with the right combination of compatibilizers (PP-g-MA and modified TPU copolymer). Waste paper was added to the thermoplastic matrix as a reference. The difference in the performance of the composites can be explained by the better wettability of the waste paper with the SEBS-g-MA compatibilizer and the better interfacial interactions of the modified TPU copolymer with the thermoset and the PP-g-MA with the glass fibres in the case of the waste thermoset composite.

**Keywords:** waste thermoset, compatibilizer, thermoplastics, different fraction of waste thermosets, composites



## 1. Introduction

The recycling of plastic waste, especially post-consumer recycling (PCR), has started and is still developing very slowly. Compared to other materials such as paper, glass and metals, recovery and recycling rates are generally low. Even in countries with advanced waste management systems and years of experience in recycling, the recycling rates of plastics are generally much lower than those of the other materials mentioned above. This is partly due to the wide variety of uses of plastics, types of additives and types of polymer composites. This diversity of uses is one of the main advantages of plastics, but it also poses a problem for recycling (Shen and Worrell, 2014).

Industrial waste is generally better separated than municipal waste, which is why municipal waste is sorted much more frequently than industrial waste. The same applies to washing, as municipal waste is usually much more contaminated. It is therefore easier to recycle industrial waste. As a result, we can produce recyclable materials of significantly higher quality and with better or, above all, more homogeneous properties (mechanical, chemical, optical) from this waste (Ragaert et al., 2017).

Polyolefins are a type of polymer that can be perfectly mechanically recycled, modified and reused in a variety of applications with suitable separation processes from industrial and municipal waste. The most common polyolefins include polypropylene (PP) and polyethylene (PE). Precisely because of their mechanical recycling properties and the possibility of modification, polyolefins are very often modified by adding various fillers in order to reduce the price and increase rigidity and strength. Due to their low cost, low density and high stiffness, natural fibres such as wood, cellulose, jute, bamboo, conifers and, last but not least, waste paper have attracted the attention of researchers. Thermoset waste and waste paper make up a large proportion of municipal waste which, if properly separated and processed, can be an excellent addition to the polymer matrix as a reinforcing agent, improving above all the mechanical properties of the biocomposite. The incorporation (dispersion) of fibres and the interfacial adhesion of thermoset waste and waste paper with the polyolefin matrix can be problematic. Therefore, improving the interfacial interactions between hydrophilic glass and natural fibres and hydrophobic polyolefins is an important research task, as the interfacial adhesion between glass and natural fibres and polyolefins plays an important role in determining the properties of composite materials. In this way, we can reduce the impact on the environment and realise the idea of transforming waste materials into valuable, commercially viable biocomposites (Bolka et al., 2020).

Thermoset composites are cured by a chemical reaction or by heat or radiation (Boquillon and Fringant, 2000; Hay and O'Gara, 2006; Walczyk and Kupperts, 2012). Their main advantage is excellent properties at the same time low production cost when combining the thermoset matrix with reinforcing fibers (Gore and Kandasubramanian, 2018; Blanco et al., 2021; Caydamli et al., 2021). At the end of the life of the composites, the waste stream can be divided into three main streams. Waste stream of smaller products that are not systematically collected at national or European level and end up as mixed waste. The second waste stream consists of large composite parts (parts of wind turbines, aeroplanes, ships). The third waste stream consists of industrial waste from the production of composite materials. For this waste stream, the composition and quantity of the waste are known (Colledani, 2022).

There are mechanical, thermal and chemical recycling technologies for composites. Regardless of the technology, the reinforcement fibres obtained cannot completely replace the virgin fibres, but can only be added to the virgin fibres to a certain percentage in order to maintain the properties of the composites. Mechanical recycling always takes place in several steps. Firstly, large composite parts are cut into smaller pieces that can be shredded. This is followed by shredding and, if necessary, screening, during which fractions of different sizes are obtained. The smallest fraction in the form of dust can be used as filler or reinforcement (Bernardeau et al., 2018), but is usually utilised for energy by incineration. Larger fractions are usually used in the manufacture of new composite products (Pickering, 2006; Oliveux et al., 2015).





This paper presents a two-pronged review of thermoset composite waste. In the first part, the use of the smallest fraction of waste thermoset composites in combination with a thermoplastic polyamide matrix (Bernardeau et al., 2018) is presented. In the following, the use of a larger fraction as reinforcement for a thermoplastic recycled polypropylene matrix (Bream and Hornsby, 2000; 2001) is presented, using composites with waste paper for comparison (Bourmaud and Baley, 2007; Zhidan et al., 2011; Xiaolin, Xiangfeng and Rumin, 2013; Akbulut et al., 2016; Scholten and Meiners, 2019).

## 2. Methods & materials

Commercially available PA 6 (Badamid B70) was purchased from Bada, Germany (PA6 in Table 1). Commercially available recycled polypropylene was donated by TAB-IPM, Slovenia in ground form (rPP in Table 2). Commercially available antioxidant (AT 10 – AO in Tables 1 and 2) was purchased from AMIK ITALIA, Italy. Commercially available lubricant (Crodamide ER – SA in Table 1) was purchased from Croda, Italy. Commercially available ethylene elastomer modified with compatibiliser (Fusabond N416) was purchased from DuPont, Switzerland (C<sub>1</sub> in Table 1). The commercially available compatibilizer PP-g-MA (Exxelor PO 1020) was purchased from Exxon Mobil, Netherlands (C<sub>2</sub> in Table 2). The commercially available compatibilizer SEBS-g-MA (Taipol 7126) was purchased from TSRC Corporation, Taiwan (C<sub>3</sub> in Table 2). Commercially available compatibilizer modified TPU copolymer (Kuramiron U TU-S5265) was purchased from Kuraray Europe, Germany (C<sub>4</sub> in Table 2). The waste paper was donated by Papirnica Vevče, Slovenia in ground form (WP in Table 2). The thermoset composite waste in dust form was donated by the company Iskra ISD plast, Slovenia (rTC<sub>1</sub> in Table 1).

**Table 1.** Sample composition with PA6 matrix.

Sample	PA6 (wt.%)	AO (wt.%)	SA (wt.%)	C <sub>1</sub> (wt.%)	rTC <sub>1</sub> (wt.%)
PA6	95.5	0.5	0	0	0.0
PA6 AO rTC <sub>1</sub> 0.1	99.4	0.5	0	0	0.1
PA6 AO rTC <sub>1</sub> 0.5	99.0	0.5	0	0	0.5
PA6 AO rTC <sub>1</sub> 1.0	98.5	0.5	0	0	1.0
PA6 AO SA1 C <sub>1</sub> 5 rTC <sub>1</sub> 30	63.5	0.5	1	5	30.0

Thermoset composite waste as a fraction sieved through a 4 mm sieve was donated by Technol, Slovenia (rTC<sub>2</sub> in Table 2). The composition of the samples is listed in Tables 1 and 2. For all tests, the materials were mixed separately and extruded on the Labtech LTE 20-44 twin-screw extruder. The screws had a diameter of 20 mm, an L/D ratio of 44:1, a screw speed of 600 rpm and an increasing temperature profile for PA6 and rPP from the hopper (165 °C and 220 °C, respectively) to the die (190 °C and 250 °C, respectively) (Pracella et al., 2010).

The injection moulding was carried out on a Krauss Maffei 50-180 CX with a screw diameter of 30 mm. The temperature profile for PA6 and rPP increased from the hopper (175 °C and 220 °C) to the nozzle (190 °C and 250 °C, respectively). The mould temperature for PA6 and rPP was set to 80 °C and 45 °C, respectively and the cooling time to 10 s (Jazani et al., 2011). The tensile tests were carried out with the Shimadzu AG-X plus in accordance with ISO 527-1 (Huang et al., 2013). Tensile stiffness (Et), tensile strength (om), elongation at yield (em) and elongation at break (etb) were evaluated in the tensile tests. Five measurements were carried out for each sample. The thermomechanical properties were analysed using a Perkin Elmer DMA 8000 (Wunderlich, 2005).

**Table 2.** Sample composition with recycled PP matrix.

Sample	rPP (wt.%)	AO (wt.%)	C <sub>2</sub> (wt.%)	C <sub>3</sub> (wt.%)	C <sub>4</sub> (wt.%)	WP (wt.%)	rTC <sub>2</sub> (wt.%)
A	100.00	0.00	0.0	0.0	0.0	0	0
B	69.62	0.38	0.0	0.0	0.0	30	0
C	64.62	0.38	0.0	0.0	5.0	30	0
D	64.62	0.38	2.5	0.0	2.5	30	0
E	64.62	0.38	0.0	2.5	2.5	30	0
F	69.62	0.38	0.0	0.0	0.0	0	30
G	64.62	0.38	0.0	0.0	5.0	0	30
H	64.62	0.38	2.5	0.0	2.5	0	30
I	64.62	0.38	0.0	2.5	2.5	0	30

The samples were heated at 2 °C/min from 25 °C to 210 °C and 170 °C under air atmosphere for PA6 and rPP matrix based composites, respectively. A frequency of 1 Hz and an amplitude of 20 μm were used in dual cantilever mode (Jazani et al., 2011). Thermogravimetric analyses (TGA) were performed on a Mettler Toledo TGA/DSC 3+ thermal analysis instrument. The analyses were carried out in a nitrogen atmosphere (20 mL/min) from 40 to 550 °C with a heating rate of 10 °C/min, followed by an isothermal segment in an oxygen atmosphere (20 mL/min) at 550 °C for 30 min. using an Al<sub>2</sub>O<sub>3</sub> crucible. Thermal measurements were performed using a differential scanning calorimeter (DSC 2, Mettler Toledo) under a nitrogen atmosphere (20 mL/min). The temperature of the samples was raised from 0 to 260 °C for PA6 and 0 to 180 °C for rPP samples at a heating rate of 10 °C/min and kept in a molten state for 5 minutes to extinguish the thermal history. After cooling at 10 °C/min, the samples were reheated to 260 °C for PA6 and to 180 °C for rPP at 10 °C/min. The crystallisation temperature ( $T_c$ ), the crystallisation enthalpy ( $\Delta H_c$ ), the melting temperature ( $T_m$ ) and the melting enthalpy ( $\Delta H_m$ ), the glass transition temperature ( $T_g$ ) and change in the specific heat capacity ( $\Delta c_p$ ) were determined from the cooling and the second heating scan. (Wunderlich, 2005). The impact tests were performed with the Dongguan Liyi Test Equipment pendulum, type LY-XJJD5 (Zhidan et al., 2011), in accordance with ISO 179. The distance between the supports was 60 mm and a 1 J pendulum was used.

### 3. Results and Discussion

#### 3.1. Tensile tests

A comparison of  $E_t$  in **Table 3** shows that the addition of waste thermoset composite powder as nucleating agent greatly increases  $E_t$  and that 0.5 wt.% of added waste thermoset composite powder is the optimum amount. When 30 wt.% of waste thermoset composite is added and in combination with a compatibilizer,  $E_t$  even decreases slightly. The tensile strength is also increased when waste thermoset powder is added as a nucleating agent, and the highest increase in  $\sigma_m$  is also achieved with 0.5 wt.% waste thermoset powder added. At an addition of 30 wt.% and in combination with a compatibilizer, the tensile strength decreases drastically, as this is a consequence of the shape of the particles, which only act as a filler and not as a reinforcing agent. In terms of elongation, both  $\varepsilon_m$  and  $\varepsilon_{th}$ , the trend is that elongation decreases with increasing addition of thermoset composite powder. However, they decrease drastically when 30 wt.% of waste thermoset composite powder is added and in combination with a compatibilizer.

**Table 3.** Tensile tests results with PA6 matrix.

Sample	$E_t$ (GPa)	$\sigma_m$ (MPa)	$\varepsilon_m$ (%)	$\varepsilon_{tb}$ (%)
PA6	$2.31 \pm 0.20$	$66.3 \pm 0.4$	$4.3 \pm 0.1$	$158.6 \pm 64.8$
PA6 AO rTC <sub>1</sub> 0.1	$2.78 \pm 0.25$	$68.1 \pm 0.2$	$4.4 \pm 0.1$	$15.3 \pm 7.2$
PA6 AO rTC <sub>1</sub> 0.5	$2.97 \pm 0.20$	$69.9 \pm 1.5$	$4.2 \pm 0.3$	$6.2 \pm 3.0$
PA6 AO rTC <sub>1</sub> 1.0	$2.90 \pm 0.17$	$65.9 \pm 6.5$	$3.9 \pm 0.7$	$5.1 \pm 2.2$
PA6 AO SA1 C <sub>15</sub> rTC <sub>1</sub> 30	$2.25 \pm 0.27$	$47.0 \pm 0.2$	$3.5 \pm 0.2$	$3.8 \pm 0.4$

We can conclude that the waste thermoset composite powder acts as a nucleation agent and that the optimum amount to add is 0.5 wt.%. The waste powder of the thermoset composite could also be used as a filler, but the  $\sigma_m$  and strains are drastically reduced.

The  $E_t$  comparison shown in **Table 4** clearly shows that the rPP samples with added fibres achieved significantly higher  $E_t$  values than the pure rPP polymer matrix samples. This is of course the expected result, as  $E_t$  increases when fibres are added to the polymer, regardless of whether they are synthetic or natural fibres. The  $E_t$  value of the pure rPP matrix was 0.9 GPa. Among the rPP samples with added waste paper fibres, the highest  $E_t$  value was achieved by the D sample and the lowest by the C sample. The  $E_t$  values of the rPP and waste paper samples with various additives (modifiers) were between 1.26 GPa and 1.69 GPa. Among the samples containing waste thermoset composite with glass fibres and various additives, the highest  $E_t$  value was achieved by the F sample and the lowest by the G sample. The values of the tensile modulus of the samples containing the thermoset composite with glass fibres were between 1.52 GPa and 1.98 GPa. We can conclude that the addition of fibres to the polymer matrix improves the  $E_t$  of composite. Even better properties can be achieved by modifying the interface between matrix and fibres, namely by adding antioxidants, modifiers and compatibilizers. Improving the interface between fibres and matrix means better interfacial interactions, as the modifiers act as binders both on the side of the polymer matrix and on the side of the fibres.

A comparison of  $\sigma_m$  and  $\varepsilon_m$  is shown in **Table 4**. **Table 4** for  $\sigma_m$  shows that  $\sigma_m$  of the pure rPP sample is 25.7 MPa. In the following, we can observe a slightly higher  $\sigma_m$  of the rPP materials to which paper fibres and additives were added, while at the same time a decrease in  $\varepsilon_m$  can be observed for all samples, which is due to the added fibres, as their elongation is much more limited than that of the rPP matrix. It can be observed that the B sample, which contained rPP and paper fibres but no additives, reached the same  $\sigma_m$  value (27.7 MPa) as the samples made of pure rPP and that at the same time its  $\varepsilon_m$  value decreased significantly, namely to an elongation of 5.4 %. For all other rPP samples with added paper and additives, we can observe a concrete increase in the  $\sigma_m$  value and at the same time a decrease in the  $\varepsilon_m$  value compared to the samples made of pure rPP. The highest  $\sigma_m$  value was achieved by the sample D, which reached 33.3 MPa at an elongation of 5.7 %. The worst tensile properties of the rPP samples with added paper and additives were exhibited by the sample C, which achieved a value of  $\sigma_m$  of 25.6 MPa at an elongation of 5.3 %. It can be concluded that all combinations of additives in the samples proved to be effective, as all combinations achieved better results than the rPP sample with added paper without additives. The exception was the addition of modified TPU copolymer to the C sample, which achieved comparable results to the B sample. The combination of the compatibilizers PP-g-MA and modified TPU copolymer had the greatest influence on the improvement in tensile strength. The  $\sigma_m$  values of the rPP samples with the addition of a waste thermoset composite with glass fibres and additives decreased and were between 19.8 MPa and 21.7 MPa. The only exception was the sample H, which reached  $\sigma_m$  of 28.0 MPa. In addition,  $\varepsilon_m$  decreased for all samples. The elongations of the samples varied between 3.1 % and 4.7 %. The lower tensile strengths can be attributed to poorer interfacial interactions between the matrix and the waste thermoset composite with glass fibres, the lower elongations are the result of significantly lower elongations of the glass fibres and the proportion of waste thermoset composites compared to cellulose fibres. Similar to the rPP samples with added waste paper, the combination of the compatibilizers PP-g-MA and modified TPU copolymer proved to be the best additive.



The comparison of  $\epsilon_{tb}$  shown in **Table 4** is extremely revealing as we can see how the addition of fibres to the matrix of pure rPP significantly affects the ability of the composite to elongate before it collapses. The elongation at break decreased from 289.5 % for the pure rPP sample to a minimum of 6.4 % to a maximum of 7.4 % for the rPP samples with added waste paper and additives and to a minimum of 3.8 % to a maximum of 11.2 % for the samples with added waste thermoset composites with glass fibres and additives. The decrease in elongation at break can be attributed to the limited tensile properties of the added waste paper fibres and glass fibres, which allowed less plastic deformation (more brittle fracture) due to their presence in the matrix.

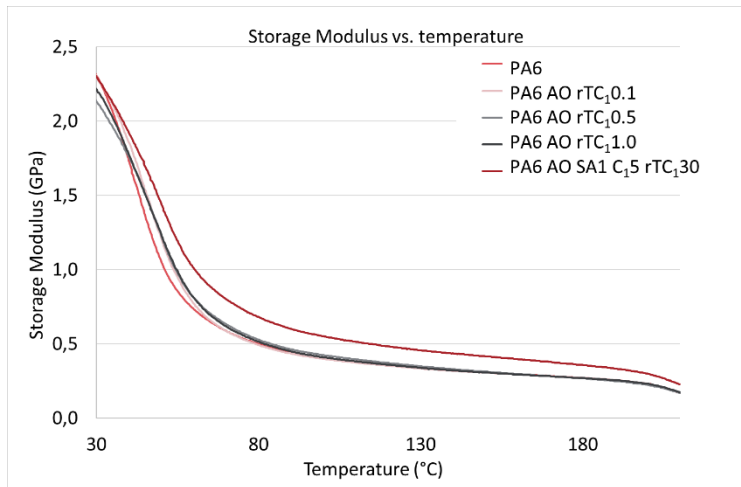
**Table 4.** Tensile tests results with recycled PP matrix.

Sample	$E_t$ (GPa)	$\sigma_m$ (MPa)	$\epsilon_m$ (%)	$\epsilon_{tb}$ (%)
A	$0.90 \pm 0.12$	$25.7 \pm 0.7$	$7.8 \pm 0.4$	$289.5 \pm 128.4$
B	$1.64 \pm 0.24$	$24.7 \pm 0.5$	$5.4 \pm 0.3$	$7.3 \pm 0.4$
C	$1.26 \pm 0.17$	$25.6 \pm 0.3$	$5.3 \pm 0.2$	$6.5 \pm 0.7$
D	$1.69 \pm 0.37$	$33.3 \pm 0.3$	$5.7 \pm 0.1$	$6.4 \pm 0.3$
E	$1.64 \pm 0.20$	$29.5 \pm 0.3$	$5.9 \pm 0.2$	$7.0 \pm 0.5$
F	$1.98 \pm 0.18$	$21.6 \pm 0.2$	$3.2 \pm 0.4$	$7.6 \pm 2.4$
G	$1.52 \pm 0.14$	$19.8 \pm 0.2$	$4.7 \pm 0.2$	$11.2 \pm 1.8$
H	$1.83 \pm 0.19$	$28.0 \pm 0.2$	$3.1 \pm 0.2$	$3.8 \pm 0.3$
I	$1.54 \pm 0.21$	$21.7 \pm 0.1$	$3.2 \pm 0.1$	$8.9 \pm 1.4$

With the right combination of compatibilizers, we were able to successfully increase the stiffness and strength of the composites, and in all cases the elongation at break was drastically reduced. We were able to increase the stiffness of the composites with waste paper by 82 % and the strength by 33 %, and the stiffness of the waste thermoset composites by 120 % and the strength by 9 %. The simultaneous increase in stiffness and strength is a very good indicator that we have succeeded in ensuring good surface interactions between the surface of the fibres and the thermoplastic matrix in these composites with the right combination of compatibilizers. By using different combinations of compatibilizers, we can produce a composite material with precisely defined properties. The lower strength increase in composites with a waste thermoset composite is to be expected because the waste thermoset composite contains approx. 60 % cured thermoset, which is very brittle, and only 40 % glass fibres. For composites with added waste paper, the partial volumes containing the waste paper particles are much smaller than in the waste thermoset composites, so the partial strains at the interface between the waste paper and the rPP matrix are also much smaller compared to the waste thermoset composite. The size of the partial volumes is reflected in the differences in the tensile strength of these composites.

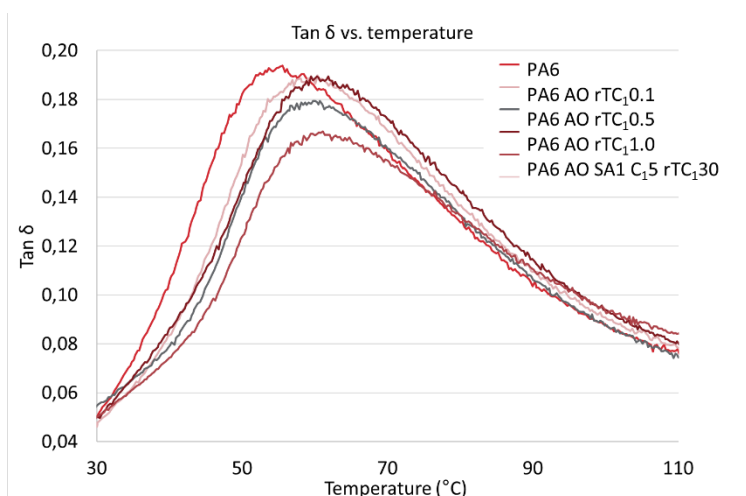
### 3.2. DMA tests

The storage modulus (**Figure 1**) in the range from 40 to 90 °C for samples with PA6 matrix is consistent with the results of the tensile test. The more waste thermoset composite powder is added, the higher the storage modulus. The highest storage modulus is found in the entire temperature range for the composite PA6 AO SA1 C<sub>15</sub> rTC<sub>130</sub>, to which 30 wt.% of waste thermoset composite powder was added. All samples show drastic drop in the storage modulus (around 50 °C) in the area of the glass transition of PA6, although this depends on the amount of waste thermoset powder added.



**Figure 1.** Storage modulus vs. temperature for the composites with PA6 matrix.

The glass transition increases with the amount of waste thermoset powder added. This can also be seen in **Figure 2**, which shows the loss factor. The level of the loss factor illustrates the elastic response of the material and is lowest at 0.5 wt.% for the versions to which the waste thermoset composite powder is added. The result is consistent with the tensile tests, in which the PA6 AO rTC<sub>i</sub>0.5 sample showed the highest stiffness and strength. The PA6 AO SA1 C<sub>1.5</sub> rTC<sub>1.30</sub> sample has the peak of loss factor at the highest temperature and the lowest peak height. This means that the sample has the highest glass transition and reacts most elastically of all the samples measured. This is due to the waste thermoset composite powder added at 30 wt.% which acts as a filler but still influences the stiffness and dynamic behaviour of the composite.



**Figure 2.** Loss factor vs. temperature for the composites with PA6 matrix.

The storage modulus for the samples with rPP matrix (**Figures 3 and 4**) is significantly higher for composites with waste paper and waste thermoset composites in the entire temperature range than for the pure rPP matrix. The highest storage modulus is exhibited



by the composite samples to which a combination of PP-g-MA compatibilizers and modified TPU copolymer was added. The storage modulus is lower for composites with waste paper than for composites with waste thermoset composites. This result was expected, as glass fibres have a much higher stiffness than cellulose fibres in waste paper, despite their low proportion in the waste thermoset composite. Regardless of the composition, the temperature resistance of the composites is also drastically improved compared to the pure rPP matrix. For composites with waste thermoset composite, the F sample, to which no compatibilizer has been added, is comparable to the H sample. This means that the waste thermoset composite is homogeneously mixed with the rPP matrix. At a temperature of 70 °C, the storage modulus of the F sample starts to decrease compared to the H sample, as there are no good interfacial interactions between rTC<sub>2</sub> and the rPP matrix.

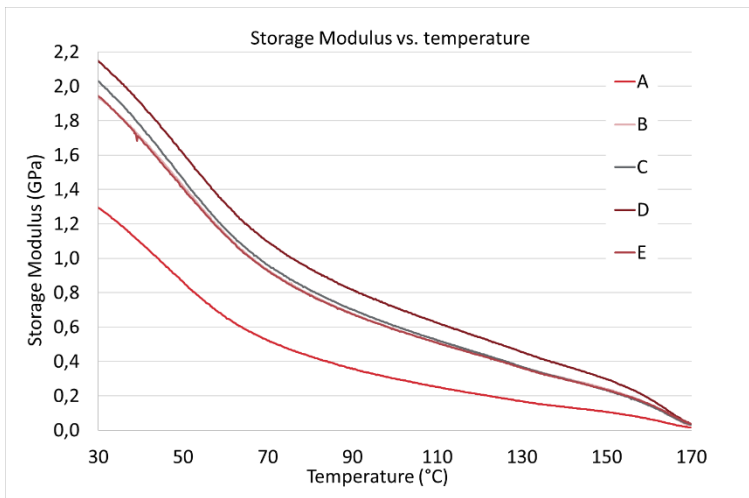


Figure 3. Storage modulus vs. temperature for the composites with rPP matrix and waste paper.

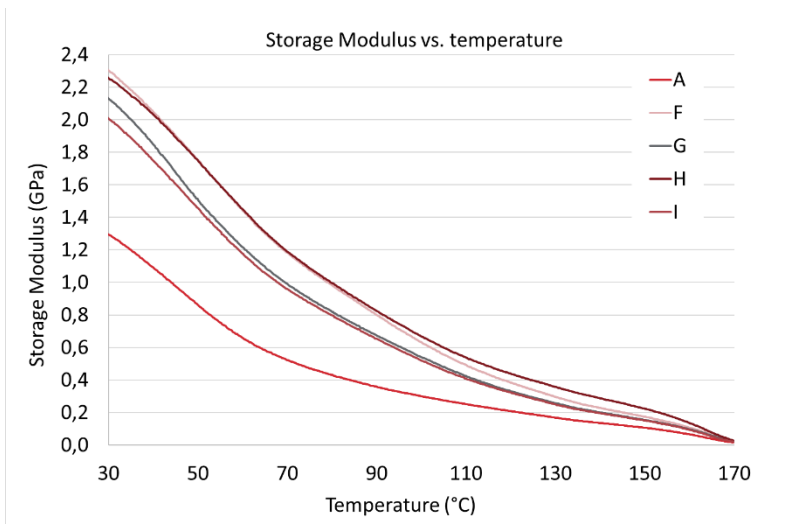
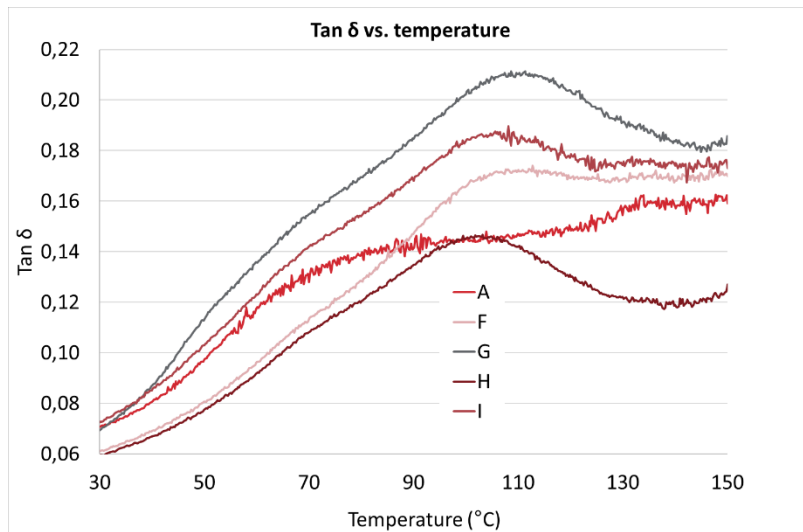


Figure 4. Storage modulus vs. temperature for the composites with rPP matrix and recycled thermoset composites.

The loss factor (Figure 5) in the rPP sample does not show a pronounced peak. The samples with added rTC<sub>2</sub> show pronounced peaks, which can be attributed to the glass transition of the thermoset resin in the source of the waste thermoset composite. The peak of the glass transition for the samples is between 100 °C and 110 °C, because this means that the compatibilizers in the samples are differently compatibilized for both the rPP matrix and the thermoset resin. The combination of the compatibilizers PP-g-MA and modified



TPU copolymer shows the best compatibility, as the glass transition for the thermoset resin has moved to lower values (closest to the glass transition for the rPP matrix).

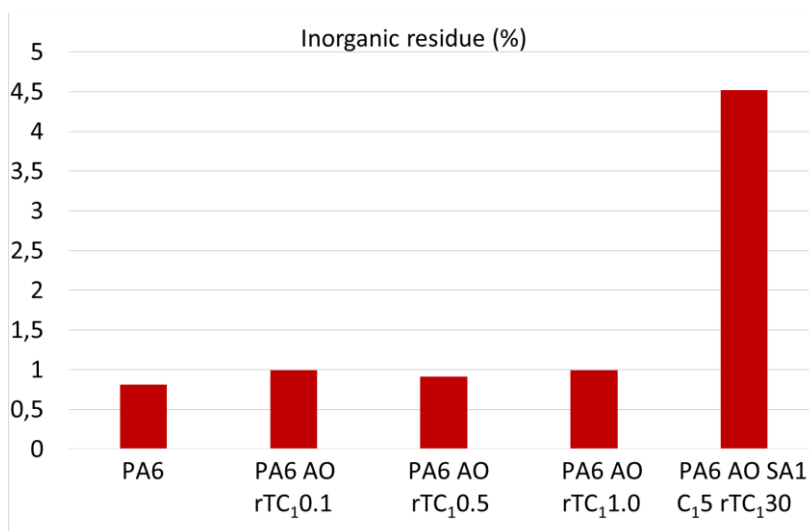


**Figure 5.** Loss factor vs. temperature for the composites with rPP matrix and recycled thermoset composites.

From the results, we can conclude that the compatibilizer PP-g-MA has good interactions between the glass fibers and the rPP matrix and has a great influence on the mechanical properties of the composite, while the modified TPU copolymer compatibilizer has good interactions between the thermoset resin and the rPP matrix and has great influence on the thermal properties of the composite, but has a weaker influence on the mechanical properties of the composite.

### 3.3. TGA tests

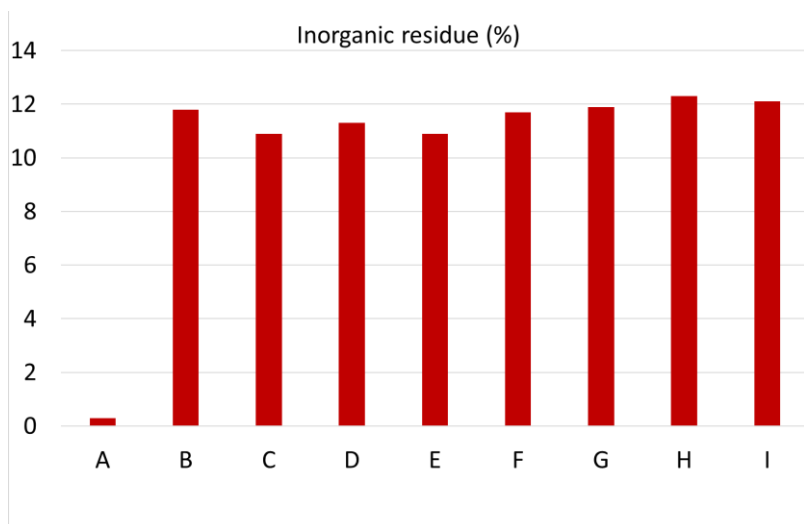
The characterization of the inorganic residues for the samples with PA6 matrix (**Figure 6**) after TGA annealing shows that there are quite a lot of inorganic additives in the pure PA6 matrix, which are probably processing additives. In the versions to which 0.1 to 1.0 wt.% rTC<sub>1</sub> was added, there were no differences in the inorganic residues after TGA annealing. In the PA6 AO SA1 C<sub>15</sub> rTC<sub>1</sub>:30 sample, the inorganic residue was just under 4 wt.%, which means that only about 13 wt.% glass fibres are contained in the rTC<sub>1</sub>.





**Figure 6.** Inorganic residues after TGA measurements for the samples with PA6 matrix.

The characterization of the inorganic residues for the samples with rPP matrix (**Figure 7**) after tempering on the TGA shows that 0.3 wt.% of inorganic impurities are contained in the rPP matrix. In the samples of composites with waste paper, the inorganic residues are between 10.9 and 11.8 wt.%. In samples of composites with rTC<sub>2</sub>, the inorganic residues are between 11.7 and 12.3 wt.%. In composites with recovered paper, there is an inorganic residue of CaCO<sub>3</sub>, which is added to the paper as a filler and is present in the recovered paper between 36 and 39 wt.%. In rTC<sub>2</sub>, the inorganic residue consists of glass fibres, which account from 39 to 41 wt.% in rTC<sub>2</sub>.



**Figure 7.** Inorganic residues after TGA measurements for the samples with rPP matrix.

### 3.4. DSC tests

For samples with a PA6 matrix (**Table 5**), the addition of small percentages of rTC<sub>1</sub> has practically no effect on the glass transition or crystallization on cooling. From this we can conclude that we cannot detect any change in the morphology of the composites with DSC measurements. In the PA6 AO SA<sub>1</sub> C<sub>15</sub> rTC<sub>1</sub>30 sample, however, the glass transition is significantly higher, the crystallization temperature, the melting enthalpy and also the melting point decrease slightly.

**Table 5.** DSC tests results for samples with PA6 matrix.

Sample	T <sub>g</sub> (°C)	C <sub>p</sub> (J/gK)	T <sub>c</sub> (°C)	ΔH <sub>c</sub> (J/g)	T <sub>m</sub> (°C)	ΔH <sub>m</sub> (J/g)
PA6	55.2	0.05	192.2	31.1	220.9	38.5
PA6 AO rTC <sub>1</sub> 0.1	55.6	0.06	191.9	32.4	221.1	36.8
PA6 AO rTC <sub>1</sub> 0.5	53.6	0.03	191.8	30.0	221.1	37.4
PA6 AO rTC <sub>1</sub> 1.0	55.0	0.05	192.1	32.6	220.7	38.5
PA6 AO SA <sub>1</sub> C <sub>15</sub> rTC <sub>1</sub> 30	61.4	0.02	190.7	21.0	220.0	24.5

For the samples with rPP matrix (**Table 6**), the first melting point is the melting of the low PE content in the rPP matrix and thus the crystallization temperature for the PE content. The melting point and crystallization temperature for the rPP matrix are in the temperature range of 165.4 °C to 166.4 °C and 121.5 °C to 124.4 °C respectively. Composites with a combination of PP-g-MA compatibilizers and modified TPU copolymer exhibit the high-





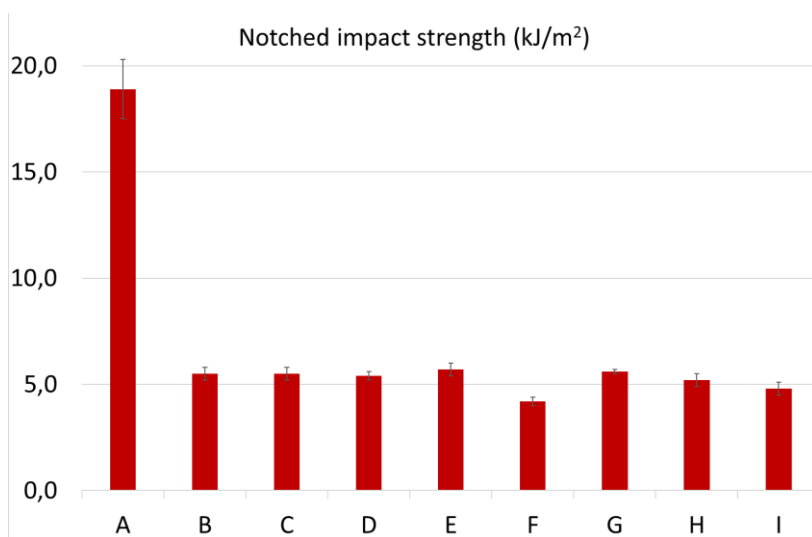
est crystallization temperatures, which means that this combination triggers heterogeneous crystallization of the rPP matrix during cooling. These two composites also achieve the highest degree of crystallinity in the versions with added compatibilizers.

**Table 6.** DSC tests results for samples with recycled PP matrix.

Sample	2 <sup>nd</sup> Heating				Cooling			
	T <sub>m1</sub> (°C)	ΔH <sub>m1</sub> (J/g)	T <sub>m2</sub> (°C)	ΔH <sub>m2</sub> (J/g)	T <sub>c1</sub> (°C)	ΔH <sub>c1</sub> (J/g)	T <sub>c2</sub> (°C)	ΔH <sub>c2</sub> (J/g)
A	126.4	11.4	165.6	79.0	112.5	9.5	124.0	76.5
B	126.1	8.1	165.8	52.3	112.6	6.9	123.6	51.0
C	126.0	6.9	165.9	49.9	112.3	6.0	122.7	48.9
D	125.9	8.9	166.2	55.3	111.1	6.3	124.4	52.7
E	125.6	6.3	165.4	47.7	112.3	5.5	121.9	46.7
F	126.5	6.8	166.4	54.9	112.4	6.2	123.1	54.2
G	126.3	7.5	166.1	52.5	112.3	6.0	122.6	51.5
H	125.9	8.8	165.8	53.7	111.2	6.6	123.3	50.8
I	125.8	6.0	165.6	50.4	112.0	5.5	121.5	50.2

3.5. Notched impact tests

With regard to the notched impact strength (**Figure 8**), a drastic decrease in all composites samples can be seen, both with waste paper and with added rTC<sub>2</sub>. The notched impact strength is slightly higher for composites with waste paper than for composites with rTC<sub>2</sub>, which is due to the low glass fibre content of rTC<sub>2</sub>. Compatibilizers increase the notched impact strength in all variants. This increase in notched impact strength is more pronounced in composites with rTC<sub>2</sub>, as the polyester resin without compatibilizer does not have good interfacial interactions with the rPP matrix. The sample G has the highest notched impact strength, which means that the modified TPU copolymer with the compatibilizer improves the interactions between the rPP matrix and the polyester resin the most. The results are in very good agreement with the results of the tensile and DMA tests.



**Figure 8.** Notched impact strength for the composites with rPP matrix and recycled thermoset composites.



#### 4. Conclusions

The tests carried out have shown that with the right choice of thermoplastic matrix, both fine powder from thermoset waste and larger fractions can be used to improve the properties of the newly created composite. The fine powder from thermoset waste works perfectly as a core in the PA6 matrix and the composite material improves stiffness, strength, temperature resistance and glass transition, but drastically reduces elongation at break and toughness. Larger proportions of thermoset waste, which in our case contained only about 40 wt.% of glass fibres, improve the stiffness, strength and temperature resistance of the composite in combination with the right compatibilizers and the rPP matrix, but drastically reduce the elongation at break and toughness. For the rPP matrix, PP-g-MA and modified TPU copolymer proved to be the best combination of compatibilizers, with PP-g-MA providing good interfacial interactions between glass fibers and rPP matrix and modified TPU copolymer between thermoset matrix particles and rPP matrix. The results provide a good basis for further research into the mechanical recycling of thermoset waste as nucleating agent or reinforcement for thermoplastic matrices. In this way, mechanical recycling could be used to utilise the entire amount of waste thermoset composite, as fine dust is a by-product or waste from grinding for larger fractions.

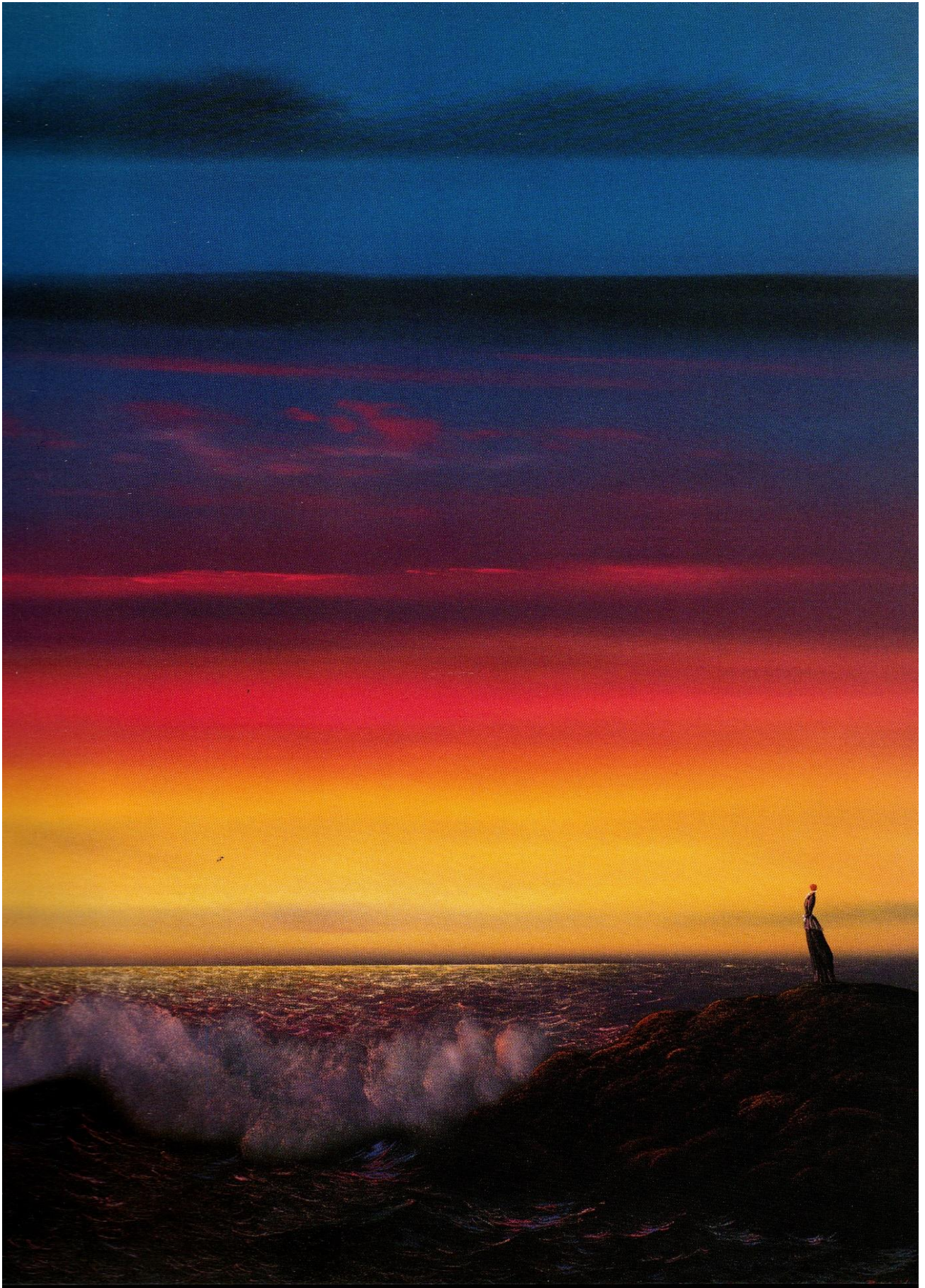
**Conflicts of Interest:** The authors declare no conflict of interest.

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# Dark Energy and Dark Matter

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## Abstract:

Study of space is never ending quest of humanity. An emerging question regards dark energy and dark matter which are the subjects of this contribution. We will consider the difference between the dark energy and dark matter, history of matter and contemporary efforts to find experimental evidence on dark energy and dark matter. We will briefly consider galaxy rotation curves, gravitational lensing and cosmic microwave background.

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## 1. Introduction

Dark matter theories focus on questions such as how did stellar systems and galaxies evolve throughout time, and how is universe changing with time. Despite its significance, knowledge on cosmology is yet rudimentary and better understanding of dark energy and dark matter presents a challenge.

Dark matter is defined as a substance that pulls galaxies together, while dark energy pushes them apart (causes universe expansion). It was estimated that roughly 27% of the matter in the universe is dark matter and 68% of the energy in the universe is dark energy (NASA, 2024). Normal matter was estimated to form about 5% of the matter in the universe (NASA, 2024).

Normal matter, also known as baryonic matter is composed of particles call baryons, which include protons and neutrons. These baryons make up atoms, which interact with electromagnetic field and can emit, absorb and reflect light. Therefore, normal matter is observable. Dark matter on the other hand is a form of matter that does not interact with electromagnetic field and does not emit, absorb or reflect light. Despite its invisibility dark matter has observable effects on the motion of galaxies (Bertone et al., 2005).

## 2. History of dark matter

Throughout history, philosophers have been speculating about what are we, what is everything around us made of, and what is the nature of the matter. The ancient Greeks were thinking about these things and they indicated that there might be forms of matter that are imperceptible or not noticeable, simply because it was too far away or invisible and undetectable by our senses. The oldest records on the matter were found from 5th century Before the Christian Era (BCE) (Bertone and Hooper, 2018). Leucippus and Democritus were convinced that all matter was made of the same fundamental and invisible building blocks called atoms and that these atoms were infinite in numbers (Bertone and Hooper, 2018). Sir Isaac Newton contributed significantly to the history of cosmology; in 1687 he published »Philosophia Naturalis Principia Mathematica«. He developed scientific tools leading to discoveries about universe. John Michell and Simon Laplace found that there could exist objects so massive that even light would not be able to escape the gravitational pull (Montgomery et al., 2009). This was considered the first mention of black holes (Bertone and Hooper, 2018). In particular after development of astronomical photography, it was acknowledged that stars were not evenly distributed in the sky. In dense stellar fields, dark regions were observed (Bertone and Hooper, 2018). Two explanations were suggested: either stars were lacking or – an absorbing matter were present along the line of sight (Bertone and Hooper, 2018). The second explanation was considered the more probable one. Lord Kelvin estimated the amount of dark matter in the Milky Way galaxy (Bertone and Hooper, 2018). He claimed that »if stars in Milky Way can be described as a gas of particles, acting under the influence of gravity, then one can establish a relationship between the size of the system and the velocity dispersion of the stars« (Bertone and Hooper, 2018).

The Coma Cluster of galaxies was observed with the telescope (Morrison and Zwicky, 2015, Primack, 2024) and reported on thousands of galaxies. A question was posed what would happen if all these galaxies were stationary? (Morrison and Zwicky, 2015). The answer led to inevitable collapse due to gravity (Morrison and Zwicky, 2015). On the other hand, if the galaxies would be moving above some threshold velocity, they would fly apart (Morrison and Zwicky, 2015). It was concluded that since they are still there after 8000 million years after they were formed, there must exist a fine balance between energy of motion (kinetic energy) and the gravitation attraction (potential energy). If one could measure the kinetic energy, mass of the respective volume of space could be calculated. The result of the calculation indicated that there



is about 6 or 7 times more mass in The Coma Cluster than it was observed (Bertone and Hooper, 2018; Morrison and Zwicky, 2015; Primack, 2024).

### 3. Evidence of dark matter

#### 3.1. Galaxy rotation curves

Galaxy rotation curves are rotational velocities of the stars and gas in the galaxy as a function of their distance from the galactic centre. Under some reasonable simplifying assumptions, it is possible to infer the mass distribution of galaxies from their rotation curves.

In 1960s and 1970s, image tube spectrograph was developed ( Bertone and Hooper, 2018) to perform spectroscopic observations of the Andromeda Galaxy. The results on the mass in outer parts of some galaxies disagreed with rotation curves predicted from photometry (Bertone and Hooper, 2018 ). The stars at the edge of galaxies were moving so fast that they should be (according to the theoretical estimations) flung off into space. Instead, the stars stayed in their orbits. This was explained by the gravitational pull exerted by something undetectable (NOVA, 2018).

Newton's law of gravity describes the gravitational force  $F_g$  acting on an object with mass  $m$  in rotational orbit:

$$F_g = G M_{enc} m/r^2 \quad , \quad (1)$$

where  $G$  is gravitational constant,  $M_{enc}$  is the mass enclosed within the orbit and  $r$  is the distance from the centre of mass.

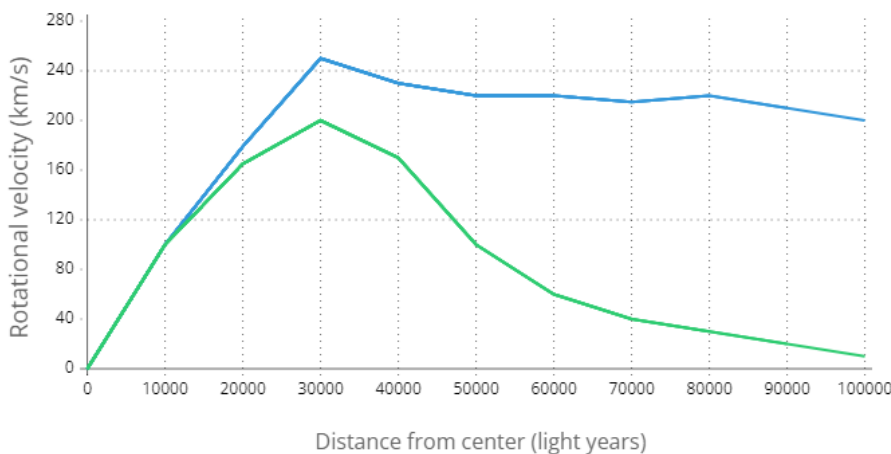
The gravitational force acts as the centripetal force  $F_c$  needed to keep the object in rotational motion,

$$F_c = mv^2/r \quad , \quad (2)$$

where  $v$  is the orbital velocity. When we set these two forces equal to each other and express  $v$ , we get

$$v = (G M_{enc}/r)^{1/2} \quad . \quad (3)$$

The calculated velocity was however considerably smaller than the measured velocity (**Figure 1**). Agreement however be obtained by assuming the presence of the additional mass, or dark matter, within galaxies. The equations are fundamental to understanding galaxy rotation curves and indicate the existence of dark matter in galaxies.



**Figure 1:** Rotation curves of the Andromeda Galaxy. Measured rotational velocities of the outer stars are the blue line, while velocities that would be expected from the estimated mass of the visible matter in the galaxy are the green line. Adapted from (Nerich, 2011).



### 3.2 Gravitational lensing

Gravitational lensing is a phenomenon, where the gravitational field of a massive object, such as a galaxy or a cluster of galaxies, bends and distorts the light from distant objects, like background galaxies.

Already Newton considered the possibility that light could be deflected by gravity (Bertone and Hooper, 2018). But within the general relativity theory published by Einstein, this phenomenon was quantitatively predicted (Bertone and Hooper, 2018). The empirical test of gravitational lensing and of the general relativity theory was conducted in 1919 by Arthur Eddington who organized expedition to observe solar eclipse (Bertone and Hooper, 2018). Experiment consisted of measurement of the deflection of light by the gravitational field of the massive object (sun). Bending of light lead to the distortion or magnification of distant objects like background galaxies or quasars. The results of measurement were in favour of Einstein’s predictions (Bertone and Hooper, 2018).

After the confirmation, the gravitational lensing became a powerful tool for cosmologists and astrophysicists to study the distribution of matter in universe including dark matter and dark energy. It was used for discovery and study of distant galaxies and for estimation of the expansion rate of the universe (Bertone and Hooper, 2018).

Following Bovy (2023), we describe the deflection of light due to gravity as illustrated in **Figure 2** where a two dimensional representation of the three-dimensional configuration is shown. In the figure, the source is on the left, the lens is in the middle, and the observer is on the right. The extent of the lens are considered much smaller than the distances between the source, lens, and observer so that the lens is taken as very thin in the direction perpendicular to the light ray. The perturbed light path is taken to be a straight line until it reaches the lens at  $\xi$  where it changes its direction by the deflection angle  $\alpha$  and continues its path in a straight line until being observed. The angle  $\beta$  represents the direction of light in the absence of the lens while the angle  $\theta$  represents the observed direction. All the angles in **Figure 2** are taken to be small and the relation between the angles is (Bovy, 2023)

$$\beta = \theta - \alpha(\xi) D_{LS}/D_s \tag{4}$$

where  $\beta$  is the observed angular position of the source,  $\theta$  is the observed angular position of the image of the source,  $\alpha$  is the deflection angle,  $D_{LS}$  is the distance between the lens and the source and  $D_s$  is the distance to the source (**Figure 2**). It follows from the general theory of relativity that in case that point mass is acting as a gravitational lens,

$$\alpha(x) = 4GM / (D_L \theta c^2) \tag{5}$$

where  $G$  is the gravitational constant,  $M$  is the mass of the lensing object and  $c$  is the speed of light (Einstein, 1916). Eqs.(4) and (5) relate the apparent position of a distant source  $\theta$  to its observed position by taking into account the gravitational influence of the mass ( $M$ ) causing the lensing.



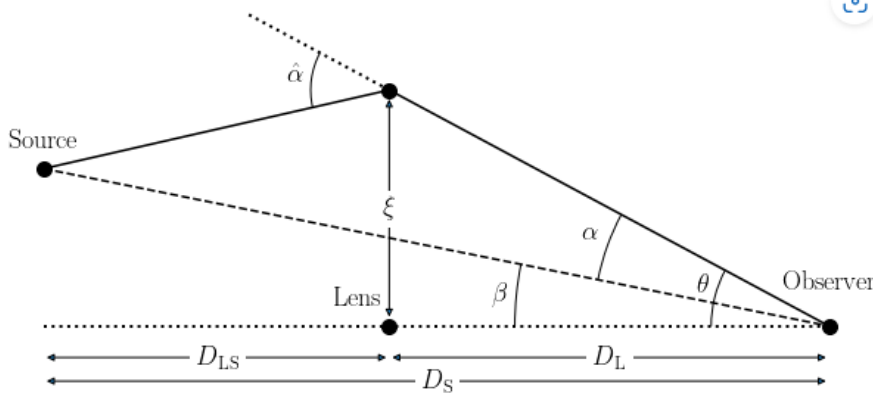


Figure 2: Geometry of lensing system. From (Bovy, 2023).

There are two types of gravitational lensing. Strong lensing: This occurs when the gravitational field is strong enough to produce clearly visible distortions or multiple images of the background object. The lens equation is crucial for understanding and predicting the positions of these multiple images. Weak lensing: In this case, the gravitational distortion is subtle and doesn't produce multiple images but instead causes a slight stretching or shearing of the background object.

### 3.3 Cosmic microwave background

Cosmic Microwave Background (CMB) is a faint glow of radiation that dates from the very beginning of everything. It's what's left of the early stages of the universe, from the era known as »recombination era«, which occurred 380 000 years after Big Bang (Bertone and Hooper, 2018). At that point the universe cooled down enough for electrons and protons to combine and form different atoms. The first atoms were hydrogen atoms, which made universe transparent for radiation. The light particles (called photons) that were present at that time have been traveling freely through space ever since and we observe them today as cosmic microwave background (Peebles, 1993). CMB supports the Big Bang theory and has provided information about the scale, composition and evolution of the universe, in particular early universe's structure and dynamics.

The radiation spectrum of CMB is given by the Planck's law which law describes the spectral density of electromagnetic radiation emitted by a blackbody in thermal equilibrium at a given temperature  $T$ , when there is no net flow of matter or energy between the body and its environment,

$$I(\nu) = \frac{2h\nu^3}{c^2(e^{h\nu/kT} - 1)} \quad , \quad (6)$$

where  $I(\nu)$  is the intensity of radiation,  $\nu$  is the frequency of the electromagnetic radiation,  $h$  is the Planck constant,  $c$  is speed of light,  $k$  is the Boltzmann constant (Peebles, 1993). The temperature  $T$  of CMB is approximately 2.73 K.

To describe CMB, we also need to consider Wien's displacement law that determines at what wavelength  $\lambda$  the intensity of radiation emitted from a blackbody reaches its maximum,

$$\lambda = b/T \quad (7)$$

where  $b$  is the Wien's displacement constant.

### 3.4 Other evidences

Large scale formation refers to the distribution of matter in the universe on a scale much larger than galaxies such as galaxy clusters and superclusters. By studying these



structures scientists can test the prediction of different cosmological scenarios. The consistency between theoretical models or advanced computer simulations incorporating dark energy and the observed large scale structure may provide evidence for the existence of the dark energy (Blumenthal et al., 1984).

Baryon Acoustic Oscillations (BAO) are unique features imprinted on the distribution of matter in the early universe (Bertone and Hooper, 2018). They originate from acoustic waves in the hot, dense plasma of the early universe, before the formation of atoms. These waves created regions of over-dense and under-dense matter and can be observed in the universe today.

#### 4 Conclusions

The possible realms of dark matter and dark energy continue to captivate and challenge our understanding of the universe. There are evidences and theories that provide explanation of the observations that indicate existence of dark matter and dark energy. This is reflected in accelerated expansion of the universe, gravitational lensing and cosmic microwave background. The pursuit of knowledge regarding dark matter and dark energy, not only expands our scientific comprehension but also pushes us to re-evaluate or re-assess the fundamentals of our description by space and time. Technological advances will likely play an important role; we expect a step forward in the field of exploring the universe as in January 2025 Large Synoptic Survey Telescope (LSST) in Chile is expected to start operating (Primack, 2024).

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# Artificial Intelligence in Law Enforcement: Current State and Development Prospects

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## Abstract:

This article provides an analysis of the current state and future prospects of Artificial Intelligence (AI) implementation in law enforcement. As advancements in technology continue to reshape various sectors, the integration of AI in policing has become a focal point, revolutionizing traditional methods and offering new opportunities. The article begins by outlining the contemporary landscape of AI applications in law enforcement, encompassing predictive policing, facial recognition, data analysis, and crime pattern identification. The discussion delves into the benefits and challenges associated with these technologies, addressing concerns related to privacy, bias, and ethical considerations. Furthermore, the article explores the evolution of AI in law enforcement, examining how machine learning algorithms enhance predictive capabilities, streamline investigative processes, and contribute to proactive crime prevention. It also highlights successful case studies and real-world implementations, showcasing the positive impact AI has had on solving complex criminal cases and optimizing resource allocation. In exploring development prospects, the article considers emerging trends such as explainable AI, human-AI collaboration, and continuous advancements in data analytics. The importance of responsible AI deployment is emphasized, emphasizing the need for transparent and ethical frameworks to guide law enforcement agencies. The article concludes by envisioning a future where AI technologies are seamlessly integrated into law enforcement practices, fostering improved crime detection, community safety, and overall operational efficiency. The insights presented aim to contribute to informed discussions surrounding the responsible and effective use of AI in the evolving landscape of law enforcement.

**Keywords:** Artificial intelligence; Data analysis; Digital automation; Law enforcement; Facial recognition systems



## 1. Introduction

### 1.1. Background

The rapid evolution of technology has permeated every facet of contemporary society, revolutionizing traditional approaches and introducing innovative solutions. One such transformative force is Artificial Intelligence (AI), which has progressively found its way into the realm of law enforcement. As law enforcement agencies worldwide seek to enhance their capabilities, AI emerges as a potent tool, offering unprecedented opportunities to tackle evolving challenges in crime prevention, investigation, and public safety.

### 1.2. Scope of the article

This article explores the current state and development prospects of AI in law enforcement, aiming to provide a comprehensive understanding of the technological landscape and its implications. From predictive policing to facial recognition and data analytics, we delve into the diverse applications of AI that have reshaped the modus operandi of law enforcement agencies.

### 1.3. Current state of AI implementation

To comprehend the present scenario, it is essential to dissect the various AI applications currently integrated into law enforcement practices. This article will scrutinize predictive policing algorithms, the use of facial recognition technologies, data-driven crime analysis, and the incorporation of AI in identifying complex crime patterns. Additionally, we'll analyze benefits accrued and challenges encountered in the adoption of these technologies.

### 1.4. Challenges and ethical considerations:

While AI offers immense potential, it is not without its challenges. Privacy concerns, algorithmic bias, and ethical considerations have emerged as critical issues that demand careful examination. This article will delve into the multifaceted challenges associated with AI implementation in law enforcement and discuss the ethical frameworks required to navigate this rapidly evolving landscape.

### 1.5. Evolution:

The evolution of AI in law enforcement has been marked by technological advancements, particularly in machine learning. This segment will explore how these advancements enhance predictive capabilities, streamline investigative processes, and contribute to proactive crime prevention. Through this exploration, we aim to contribute valuable insights that foster a nuanced understanding of AI's current role in law enforcement and illuminate the path forward in harnessing its potential for future development.

## 2. Methods

The article employs a multifaceted approach to achieve its objectives. The research utilizes both theoretical and empirical methods, combining a comprehensive analysis of specialist literature with practical investigations.

The theoretical framework involves an in-depth examination of the existing literature pertaining to the problem of integrating artificial intelligence into law enforcement activities. This includes a detailed analysis of scholarly works addressing various aspects of AI implementation in the field.

On the empirical front, the research incorporates interviews conducted with students, cadets, and teachers from Donetsk State University of Internal Affairs and specialists of law enforcement agencies of Ukraine. This qualitative approach aims to gather valuable insights and perspectives regarding the practical implications and challenges of incorporating artificial intelligence into law enforcement practices. This combination of theoretical and empirical methods ensures a comprehensive exploration of the current state and future prospects of artificial intelligence in law enforcement.



### 3. Results

The current state and future prospects of Artificial Intelligence (AI) implementation in law enforcement represent a dynamic landscape with significant implications for the future of policing. Current state is reflected in the works of such Ukrainian scientists as Zachek O., Dmytryk Yu., Senyk V., Kovtun V., Rvachov O., Haborets O., Karchevsky M. and other researchers. Zachek O., Dmytryk Yu. and Senyk V. (Zachek O, et al. 2023) note that one of the problems that need to be solved is the lack or imperfection of legal regulation of Artificial Intelligence both in Ukraine and abroad. Kovtun and Rvachov (2020) write that in law enforcement, the use of artificial intelligence is the process of endowing law enforcement activities with systematic intellectual properties. This process should serve as a factor for intensive development and a tool for enhancing the efficiency of law enforcement through the development and implementation of new technologies in the following directions: information-analytical support for law enforcement activities; information-reference support for law enforcement activities; creation of specialized information intelligence systems for operational-search purposes; advancement of intelligent video surveillance systems; establishment of departmental specialized intelligent information systems; implementation and development of intelligent information educational systems.

Most scientists note that use of AI in identifying crime patterns contributes to more effective and efficient crime-solving, leading to improved public safety.

There are many examples of successful implementation of artificial intelligence in domestic and international law enforcement practice. So, for example, in the USA, the Federal Bureau of Investigation (FBI) uses artificial intelligence to recognize criminals on video from surveillance cameras in real time. With the help of artificial intelligence, FBI agents can access and analyze large amounts of data to identify criminals and predict the place and time of the next crime (Zachek et al. 2023). In Great Britain, the police use artificial intelligence to analyze social networks and other open sources of information to identify possible threats to national security. In Germany, the police use artificial intelligence to detect crimes related to financial transactions.

In various countries AI-powered facial recognition systems analyze facial features to identify and verify individuals, providing law enforcement with a powerful tool for surveillance and identification. Facial recognition is used in public spaces and airports for law enforcement investigations, assisting in suspect identification and tracking. As an example, the Miami Police Department uses Clearview AI software to investigate crimes. This technology allows law enforcement agencies to check a person's photo for a match in the database, then providing links to relevant images on the Internet. But at the same time, there are controversies surround the ethical implications of mass surveillance, potential misuse of facial recognition, and the need for stringent privacy regulations. In March 2022, the company Clearview AI provided its technology to Ukraine to assist in defense against the Russian invasion. Initially, this technology was given to the Ministry of Defense of Ukraine, and later, many other agencies, including the National Police of Ukraine, joined the project (Zachek et al. 2023). Clearview AI's technology is applied for checking individuals at checkpoints, identifying deceased soldiers and prisoners of war, and searching for missing persons. Additionally, facial recognition technology can be used to investigate military crimes by identifying the faces of criminals in photos and videos that the criminals themselves proudly share on the Internet.

AI algorithms process vast amounts of data, extracting valuable insights to aid law enforcement in decision-making. Data analysis helps law enforcement agencies identify trends, anticipate criminal activities, and streamline investigative processes. But striking a balance between utilizing data for crime prevention and protecting individual privacy remains a critical challenge.

Current state and development prospects of Artificial Intelligence in law enforcement are considered also by Foreign scientists, such as Rademacher T, Wischmeyer T, Raaijmakers S, Apostolakis K, Dimitriou N, Margetis G, Surden H, Safdar N, Banja J, Meltzer C and other researchers.



Scientists Rademacher and Wischmeyer (2020) claim in their works that in certain domains, AI already fulfills the task of detecting suspicious activities better than human police officers ever could. Safdar et al. (2020) in their research pay special attention to ethical considerations in artificial intelligence.

In conclusion, the contemporary landscape of AI applications in law enforcement showcases the transformative potential of predictive policing, facial recognition, data analysis, and crime pattern identification. While these technologies offer unprecedented benefits, addressing ethical concerns, ensuring privacy, and fostering public trust are imperative for responsible implementation in the evolving realm of law enforcement.

#### 4. Discussion

Let's analyze the key aspects, including existing applications, benefits, challenges, and the anticipated trajectory of AI integration in the law enforcement domain.

1. Predictive policing algorithms: AI is increasingly employed for surveillance through facial recognition, object detection, and behavioral analysis. Predictive policing algorithms leverage historical crime data to identify potential hotspots, aiding law enforcement in proactive measures.
2. Automation of routine tasks: AI technologies automate routine tasks such as data analysis, paperwork, and evidence processing. This not only enhances efficiency but also allows human officers to focus on more complex aspects of policing.
3. Crime Pattern Recognition: AI algorithms can identify intricate patterns within large datasets, aiding law enforcement in recognizing trends, modus operandi, and potential threats. This contributes to more effective crime prevention and investigation.
4. Virtual assistants and chatbots: police departments are integrating AI-powered virtual assistants and chatbots to enhance communication with the public, provide information, and streamline administrative tasks.
5. Enhanced efficiency: AI streamlines processes, reducing the time and resources required for tasks such as data analysis and paperwork, allowing law enforcement agencies to operate more efficiently.
6. Improved decision-making: AI systems, when trained on relevant data, can assist law enforcement in making data-driven decisions, contributing to more informed and objective policing.
7. Public safety: predictive policing and surveillance technologies contribute to enhanced public safety by enabling proactive responses to potential threats and criminal activities.

As for the issues open for discussion, we can include:

1. Bias and discrimination: AI systems may inherit biases present in training data, leading to discriminatory outcomes. Ensuring fairness and mitigating bias in AI algorithms poses a significant challenge.
2. Privacy concerns: the extensive use of AI in surveillance raises concerns about privacy infringement. Striking a balance between public safety and individual privacy is a complex issue.
3. Ethical considerations: the ethical implications of AI in law enforcement, including accountability, transparency, and the potential misuse of technology, require careful consideration. As AI continues to evolve, there will be a growing emphasis on developing and adhering to ethical frameworks to address biases, privacy concerns, and other ethical considerations.

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



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# Corporate Social Responsibility as a Key Factor for Development of Sustainable Business

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## Abstract:

Responsibility is an organizational position that ensures discipline and the most effective attitude of the Company to its responsibilities to the public. Corporate social responsibility contributes to the creation of a favorable environment for business development, interaction between business and the state, where the interests of the population are represented by public organizations, which in turn turns into social partnership. Social partnership depends on the balance of three important systems, such as: social, economic and environmental. In one of the companies in Kazakhstan that takes an active part in the development of corporate social policy a program is being used within the framework of social responsibility that the company is actively pursuing. Within the framework of this program, the construction of socially significant facilities is being implemented, such as: construction of a central hospital in the city of Kulsary, construction of a central stadium in the city of Kulsary, construction of a pre-school in the Koktem microdistrict in the city of Atyrau, construction of a school named after Zhumabayev in the city of Atyrau. The company carries out partnership with higher educational institutions of the Republic of Kazakhstan, one of such educational institutions is Kazakh-British Technical University (KBTU) University. The company is actively developing a corporate social policy in the field of environmental protection, supporting social programs and improving the lives of the local population. Considering the above, we can conclude that corporate social responsibility is an integral part of the development of the company's sustainable business in Kazakhstan.

**Keywords:** Corporate culture; Corporate social policy; Responsibility; Ecology; Social program; Environment.



## 1. Introduction

### 1.1. Purpose of the scientific article

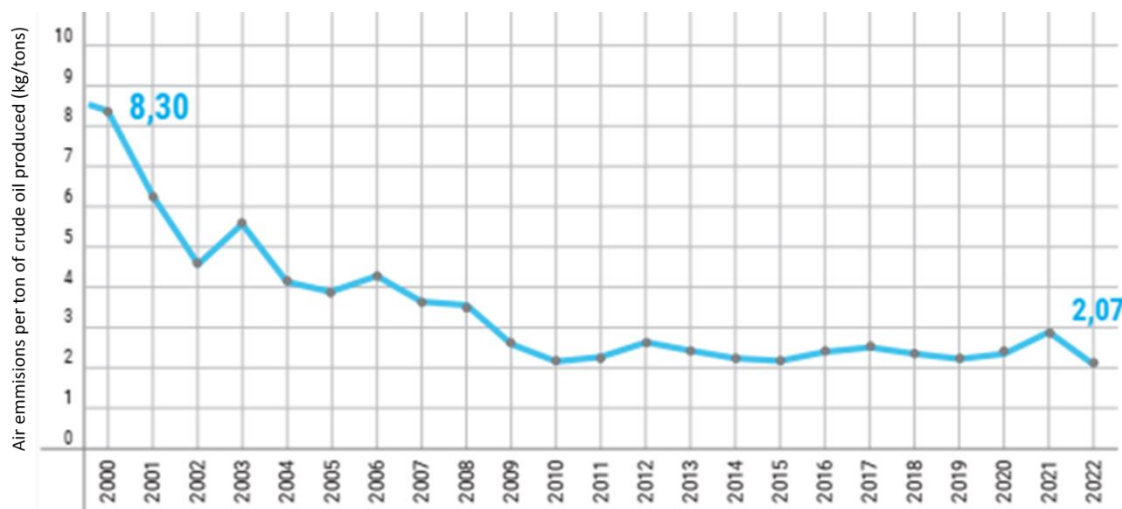
The purpose of this scientific article is to systematically and in-depth analyze the role of Corporate Social Responsibility (CSR) as a major factor influencing the development of sustainable business (Green Alternative Energy Assets, n.d.). Speaking about the experience of applying CSR in the company's activities in order to sustainable development, we note that in Kazakhstan there are a number of large socially responsible companies. The benefits of CSR are practically demonstrated by such companies. The specific focus of the article is aimed at studying a particular company (Company A) in the field of CSR and identifying specific practices that contributed to the creation of a sustainable socially corporate model.

### 1.2. Goals of the research

The key tasks to achieve the goal were:

- Study of CSR practices in Company A: Assessment of current programs and strategies related to social responsibility implemented in the company's activities;
- Community and Environmental Engagement Analysis: Assessing the impact of CSR on relationships with local communities, including the impact on social structures, support for education and charitable initiatives. Also considering the impact on environmental sustainability.

Total Cost of Ownership (TCO) rigorously complies with legislative requirements of Republic of Kazakhstan (RoK) and performs extensive air protection activities through state-of-art technologies resulting in the continuous reduction of air emissions while oil production volumes have increased significantly over the years. It should be emphasized that the company's emissions are currently at an extraordinarily low level as a result of the execution of several significant projects aimed at improving operational reliability. These have allowed the company to lower its air emissions by 75% per ton of oil produced during the past 22 years (**Figure 1**).



**Figure 1.** Air emissions of Company A with respect to time from 2000 to 2022.

## 2. Methods

To write this article, a quantitative research method was used, based on the use of available statistical data related to the social and environmental activities of the Company from the official website. The basis was a survey method among former colleagues who are directly residents of the Atyrau region and had the opportunity to observe the changes that were described in this article.

## 3. Results

Having analyzed open sources that disclose Company A's social programs aimed at stimulating the potential of the region as a whole and its individual resident, the following



results were discovered in the field of sustainable support for the corporate social responsibility program and opinions of my colleagues who was able to provide oral feedback the following conclusions were drawn.

Company A contributes to environmental protection, environmental monitoring, social investment and the development of social and infrastructure development programs. So, according to the environmental report for 2022, the main environmental indicators for 2022 are:

- Reduction of emissions intensity from 2000 to 2022 by 75%;
- Reduction of gas flaring over the past 5 years by 57%;
- Increase of water reuse rates by 49%;
- Increase of waste recycling rates by 63%.

As a part of the implementation of the Roadmap for a comprehensive solution to environmental problems in the Atyrau region, TCO is carrying out landscaping work in the city of Kulsary. In 2022, spring-autumn planting of elm, ash, apple, maple, and island seedlings was carried out on an area of 15 hectares in the city of Kulsary for a total of 9,030 trees on the street. R. Balmukhanova, st. Akzhar and along the Atyrau-Kulsary road, in the micro-district Atyrau.

The survival rate averaged 69.7%. In the places where 32.3% of seedlings did not take root, an additional 3,030 seedlings were planted in October and November 2022. The company continues to plant trees in Kulsary and cares for seedlings.

In 2022, under the program, Company A began to implement the construction of facilities such as: Central Hospital in the city of Kulsary, Central Stadium in the city of Kulsary, Pre-school in the Koktem microdistrict, Atyrau, Pre-school in the microdistrict. Zhuldyz, Atyrau, Construction of a school named after Zhumabayev in Atyrau. The program gave a powerful impetus to the infrastructural development of the Atyrau region. New, modern buildings have appeared that have beautified the regional center and Zhylyoi district, multifunctional medical clinics and centers, well-equipped schools and other educational institutions. Decisions on which projects are to be included in the program are made jointly with the Akimat of the Atyrau region.

The main criteria: sustainability, focus on the priority needs of the local population, benefits for the majority of the population, and most importantly, projects should create opportunities to unlock the potential of residents of the Atyrau region (ibid.).

The Social Investment Program, which was launched in 2010, is also being implemented. It is aimed at supporting education, healthcare, community involvement in the volunteer movement and economic development. Within the framework of this program in 2010-2022, 108 projects were implemented for a total amount of about 13.4 million US dollars. In 2022, the budget for this program was 2.3 million US dollars.

#### 4. Discussion

Supporting social corporate responsibility has given the Company A a number of advantages such as:

- Enhanced Reputation: Adopting social responsibility can significantly enhance a company's reputation. This creates a positive perception in the eyes of the public and government;
- Support for public policy: creating tax incentives for companies that build strong social responsibility;
- Improved relationships with regulators: Companies that demonstrate transparency, ethics, and social responsibility often interact better with regulators, which can reduce the likelihood of regulatory issues arising.

Thus, corporate social responsibility not only contributes to the well-being of society, but also benefits companies in their relations with the government.

#### 5. Conclusion

CSR has grown from a simple term to a fundamental principle that plays a key role in the development of sustainable business. In the course of analyzing the role of CSR using the example of the Company A, it becomes clear that the integration of the principles of social responsibility into the strategy of an enterprise has an important impact on its sustaina-



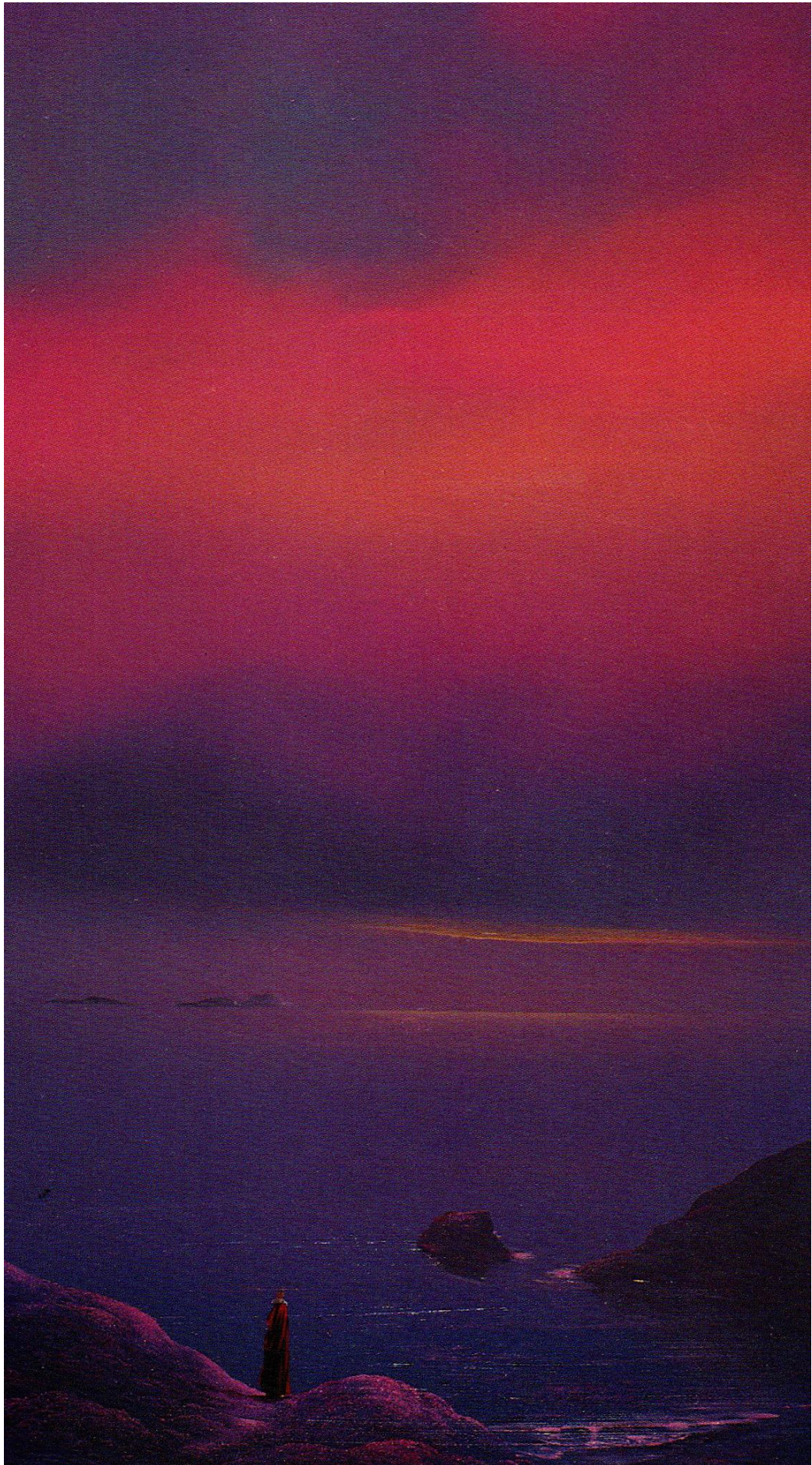
bility and prosperity. First of all, CSR is an important tool for establishing long-term relationships with stakeholders. Engaging with employees, customers, suppliers and the community through ethics, transparency and respect for human rights strengthens the company's reputation and creates the basis for lasting partnerships.

CSR also has a significant impact on financial stability. Companies that integrate social and environmental aspects into their strategy demonstrate not only social responsibility, but also a higher degree of efficiency and innovation, which ultimately impacts their financial performance. In addition, socially responsible practices have a positive impact on the internal dynamics of the company. Participation in social and educational projects, creating favorable working conditions and supporting diversity in the team help attract and retain talented employees. Thus, CSR is not just a fashionable trend, but is becoming an integral part of the sustainable development strategy of modern companies. Company A, as an example of successful implementation of the principles of social responsibility, confirms that investing in social welfare and respect for the environment are key factors not only for sustainable business, but also for leadership in the industry. In the future, business management based on ethics and responsibility represents a promising prospect for achieving harmony between corporate interests and the needs of society.

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

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# Challenges of Modernising European Universities

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## Abstract:

One of the paradoxes of the 21st century is that while most countries are striving to build knowledge societies and economies, the majority of universities remain conservative in modernizing their curricula. This lack of modernization includes issues such as balancing knowledge, competencies, and skills, implementing student-centered and interactive teaching methods, and involving practical experts in the study process. Awareness of this paradox is still limited not only within academia but also among governments, professional sectors, and the general public. The media, too, could play a more active role in promoting necessary changes. One key reason for the slow modernization of universities is the conservative interpretation of academic freedom and institutional autonomy. This stems from several factors, including the misinterpretation of academic freedom, the career patterns of university academic staff, the prevalent use of traditional lecture-based teaching methods, inadequate research funding, and limited inter-university collaboration. As a result of these conditions, countries struggle to fully develop their human capital, hindering their transition into innovation-based knowledge economies and impeding their international competitiveness. To address these challenges, all societal actors must play a role. Governments should stimulate the development of modern tertiary education systems, business leaders should clearly articulate the competencies and skills they expect from graduates, academia should advocate for sufficient funding for their modernization efforts, and NGOs and the media should raise awareness and urge governments to actively support the development of knowledge economies.

**Keywords:** Modern university; Knowledge society and economy; Importance of competencies and skills; Innovation ecosystem; Inter-university collaboration



## 1. Introduction

### 1.1. Tertiary education's division between the classical and higher professional education

The post-industrial society based on the quality of human capital is much richer in knowledge, as well as in competencies and skills, than earlier societies. That puts much higher and qualitatively different demand on the entire educational system – including on universities. This has been properly understood by only a few countries, who have reflected pragmatically, therefore divided tertiary education into classical and higher professional education.

The first remained general, knowledge-based academic upbringing of future intellectuals - expected to perform various jobs in spheres of management, administration, education, and other social services. The second, however, was designed to provide the background and skills for students who were expected to perform engineering as well as technical jobs. These programmes are usually carried out by “Technical Universities” or Colleges – normally taking 3 years (while university programmes normally run for 4 years).

This is a practical answer to the issues opened, with classical universities still facing many of the issues introduced by the knowledge society.

The questions being faced by universities in most countries around the world are not easy to answer, taking into account the changes having happened in modern societies, where knowledge is not any more the privilege of the few, and when diversification of knowledge has grown beyond anything, we have been used to, even only 2-3 generations ago.

When looking for the main causes of this challenge, we need to go some two centuries back. Namely, that was the time, when the principal role of modern universities in Europe has been to prepare the future elite of society. Therefore, it is not surprising that in that period, less than estimated 5% of youngsters entered university, and they mostly came from the upper classes. Therefore, they were actually predetermined to become part of the elite in their respective societies. For those circumstances the elite university made perfect sense, anything else would not have been accepted.

### 1.2. The concept of modern university:

The concept of the modern university dates back to the year 1809 when the Prussian government appointed one of its leading intellectuals, Friedrich Wilhelm Heinrich Alexander von Humboldt, to be responsible for education. He performed excellently, and his system was adopted by most European countries due to its elaborateness, systematic approach, and effectiveness in achieving objectives. This was the case two centuries ago when the majority of working people were illiterate. Now, the situation is, of course, completely different: all working people are not only able to read and write, but have also completed at least 9 years of education. About 1/3 of youngsters even graduate from university, implying that they have been educated for 17-18 years, yet they are not automatically considered the elite of their respective societies.

The following quote from von Humboldt's text indicates that he himself was much more modern than generally interpreted: “Just as primary instruction makes the teacher possible, so he renders himself dispensable through schooling at the secondary level. The university teacher is thus no longer a teacher, and the student is no longer a pupil. Instead, the student conducts research on his own behalf and the professor supervises his research and supports him in it” (Clark, 2006). This clearly indicates that von Humboldt is often misunderstood, and that he was more modern than commonly believed. In fact, this perspective taken by von Humboldt is quite different, indeed even contrary, to what is often considered the “Humboldt-type” university, which still serves as the mental background for the concept of most present-day European universities. However, this is not the case in the USA, which von Humboldt visited several times and influenced their academia.

While von Humboldt operated in the period of industrial society, we are now experiencing the achievements as well as the challenges of the digital age in the mature innovative, knowledge society. Many work operations that traditionally depended on responsible

<sup>1</sup> Clark CM, 2006. Iron kingdom: the rise and downfall of Prussia, 1600-1947, Harvard University Press, p. 333.





workers' reasoning and decisions are now digitized and increasingly controlled by artificial intelligence. At the same time, people's roles have shifted to primarily guiding and controlling computer-guided processes and decisions.

Unfortunately, this shift is not properly reflected in the concept of present-day European and many other universities, which have not recognized the changes experienced over the last 50-60 years and still adhere to a traditional approach in fulfilling their mission of building intellectuals. In essence, they still see their mission as enriching students with scientific knowledge to make them intellectuals and a kind of societal elite. Most academics, and consequently, also governments, therefore accept the position that teaching practical competences and skills does not fit into the world of academia and should not be part of post-secondary education curricula

## 2. Methods

In preparation for the article, observation research has been undertaken. A reduced interest in studying the Bologna experience has been observed since 2015. What could be the reasons for this unexpected trend? Probably the main reason could be that very little is happening in terms of the Bologna Process impacting university education. Namely, out of the 49 subscribing countries, even one third of them did not introduce the two-cycle university study concept, while there is very limited information about the implementation of the other Bologna targets.

Therefore, our approach remain mainly interpretative: it is based on published documentation, combined with some observations of the private and public universities in Slovenia. Observations are accumulated over decades from active involvement at various Slovenian universities.

## 3. Results

Our principal observation on the achievements of the Bologna Process is that – after 20 years since the adoption of the Declaration – the results achieved are very modest, to say the least. This qualification is justified by the fact that modern circumstances more than justify the need to update and adjust university studies, including equipping students much more with competencies and skills required in today's working environment. Obviously, one cannot blame only the universities – though their responsibility cannot be underestimated or even ignored. The more they emphasize their academic autonomy, the higher is their responsibility for failing to accept the need to change! The fundamental responsibility, however, remains with governments, which in most countries continue paying inadequate attention to education, including university education.

This fits into a more general weakness preventing many governments from accepting primary responsibility for formulating policies for building a knowledge economy and society as a building block of national development strategy. Leaving the strategies and declared policies aside, there is one indication of how much they are willing to undertake to support building a knowledge economy. This is the share of GDP devoted to financing research and innovation from the budget (GERD, Gross domestic expenditure on R&D). Governments of countries which do care, maintain GERD at 3% or more, and those which do not, achieve GERD below 2%. Of course, it's not only about how much funding is available; what matters as well is how the financial support is offered and provided. If public funding is available as a source of supporting institutions' budgets – this is simpler, but not most effective. It should be available for seriously selected and promising projects, which is procedurally more demanding, but definitely much more effective and productive. Equally important for innovation performance of a country is the development of its financial market, offering support not only to interesting research projects, but also for opening new companies (start-ups) and supporting the growth of small and young companies (scale-ups). As a share of available risk capital in GDP, the US is five times stronger than the EU average. Without important changes in the entrepreneurial environment, Europe is not facing a promising future in the global innovation-based competition! This is related also to the general awareness of the critical importance of the quality of education, science, and research – which is in most countries around the globe far from sufficient.



#### 4. Discussion

What are the major causes for the dissatisfactory situation in implementing Bologna?

Let us first look at the universities themselves, and identify the key factors responsible for the problems preventing progress along Bologna model:

- (1) A major contributing factor is the traditional career pattern of most European university professors, who predominantly remain at the university from entering as a student, distinguishing themselves, being invited to join the staff, and normally experiencing their entire professional career at the university, up to their retirement. Therefore, they remain encapsulated in this particular environment, depriving themselves of the opportunity to experience "real-life conditions." Being gradually recognized as a problem, in some countries they are now starting to take measures to prevent this "deformation." For example, in Germany, this is now prevented by law, as well as by university rules for professors' re-elections, according to which the candidate for re-election has to provide evidence of at least some out-of-university work experience. This approach is very slowly gaining acceptance around the world, and therefore reaching beyond the old-fashioned academic tradition will obviously take some time<sup>2</sup>.
- (2) Indeed, a major change from "teaching theory" to "preparing students for future work" is needed, and that is in itself rather demanding. This is not only an issue at the university level – many experts in pedagogy claim that generally and in most countries teachers are not sufficiently stimulating creativity and critical thinking in their students. This is a problem throughout the whole educational system, contradicting what is needed by people working in the 21st century in any sector. This goes particularly for workers who are surrounded by and actively assisted by high technology, including artificial intelligence. The main function of most workers nowadays is to control and guide the machines and make decisions based on options offered by the HITECH equipment. If not enabled to function in this fashion, even a highly knowledgeable and intellectually capable individual will not necessarily be very productive in a modern work environment<sup>3</sup>.

In these circumstances, besides knowledge, what determines the quality of workers is their attitude towards co-workers, their ability to communicate productively and flexibly, as well as develop critical thinking – with the objective to find solutions accepted by the respective teams. In the last British Employers Association's survey among 72,000 employers even 23% of newly engaged workers were declared to be lacking relevant skills. 3)

When commenting on this result, one needs to take into account that British universities are more pragmatic than their colleagues on the continent. The government has categorized public universities into 3 categories by the quality of their performance and differentiated levels of funding accordingly: only the best could receive more money, while the worst experienced serious cuts, and some even had to merge with others or close their doors.

- (3) A major factor in developing relevant curricula and maintaining its quality is the ability of universities to conduct good basic and applied research, which implies receiving appropriate level of funding. The average official data for European countries' on GERD (Gross Expenditures on R&D) - where universities participate with about 1/4 to 1/3 – is around 2.2% of GDP – while many member states remain below 1.5%, demonstrate that neither public, nor the private funding come close to what is required. As a logical consequence, for example, Slovenian universities have lost over the last 20 years about a quarter of their researchers due to insufficient funding. Facing this situation – which

<sup>2</sup> Employer Skills Survey 2022 – Research Report, December 2023, London, 216 pages

<sup>3</sup> Nic Mitchell -- "University World News" Online Conclusions, 2023



undermines also the quality of teaching -- the public universities should raise this question with governments more energetically, as it is a condition for their normal functioning.

- (4) A bit of the theory-practice curriculum balance can be achieved where universities invite experienced practitioners to contribute some lectures and seminars/workshops. It is an excellent opportunity for students to hear from distinguished people from companies and other practical environments about the problems experienced in real life, understand their origin, dimensions and impact. It is important that this is well integrated into the curriculum, and treated as its valuable component. Experience shows that these "guest lectures" are highly appreciated by students, which speaks for itself.
- (5) Connected and related to the above issues is also the modest inter-university collaboration – at national and at international level.

As Swedish Professor Charles Edquist – an internationally recognized authority on innovation – has proven, the innovation contributes at least 60% to the GDP growth, and that modern innovation is not any more following the traditional linear pattern (from basic to applied research, to development, and finishing at the market). The innovation has become much more complex, involving practically all segments of society – including the government. This is particularly important through direct funding, as well as supporting domestic innovation activities through public procurement (which comes on average to 13-20% of GDP – and representing globally an impressive amount of 9.5 trillion \$).

The most undisputable indicator about the innovation awareness and commitment of a country is GERD. According to the Global Innovation Index reports 2018 – 2022, in the first category the following 20 countries appear on the top: Switzerland, Sweden, USA.

Neither most of the European universities' mind-frame, nor the relevant regulatory environments are very collaboration-oriented. Therefore, the modest extent, and the impact of national and international university collaboration is not fully in line with the intensity of present-day globalization - affecting also research and innovation activities. Limitations to productive collaboration are reflected also in the slow and modest achievements of the objectives of the Bologna Declaration – as recognized for example in the Rome Communiqué of 2020. It is even noticed that the interest for doing research on Bologna implementation seems to be decreasing after 2015.

One very graphic illustration of the above assessment is also the number of European universities actively involved in the existing alliances – for example in the European University Alliance, with only 130 members from the total of 1,706 European universities. Realizing these problems, the European Commission has created in September 2020 (with some delay – compared to other domains), the European Research Area – as a logical component of the European integration process. On this basis, and with rising awareness of the importance of creating conditions for research and innovation collaboration, the next step was undertaken in November 2021 by the creation of the Pact for R&I. The European Commission seems to be aware that - in times of knowledge economy - lack of integration in R&I domain has serious negative consequences for the European international competitiveness. Therefore, the new ERA monitoring system, including a scoreboard, a dashboard and an online policy platform, has also been created in 2023. All this is rather promising, but it is still too early to expect tangible effects. With the bulk of high quality research potential being concentrated at universities and their affiliated institutes, the prime responsibility to activate fully this potential remains with the universities, and in particular with their leadership.

Fortunately, many innovation actors tend to collaborate more intensely from year to year, but too many of the European universities remain almost unaffected by this important and promising trend. There are, however, also some good examples, and they should be identified at all continents, presenting their achievements and benefits, as well as trying to identify what are the causes behind the respective universities' collaboration orientation, and the good results achieved.



What are the reasons for slow adjustments by most universities? There is general understanding that the following factors have an impact on universities' collaboration:

- Modestly international composition of teaching & research staff;
- Funding instruments promoting insufficiently international collaboration;
- Share of international students;
- Mechanisms stimulating teachers for developing international collaboration;
- Impact of graduates' employers (wanting their future workers to have an internationally open mindset, and appropriate skills).

### Conclusions

In summary, European universities are less internationalized than those in other parts of the globe – particularly in North America and in many Asian countries. In present-day circumstances exceptional achievements in training and research can hardly be achieved without intensive links among national and international colleagues (sharing and evaluating experience and challenges), and understanding wider, global trends and problems.

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

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# STEAM Approach: SMS (Stories Based on Music about Scientists) on Artificial Intelligence Created by Jacob Bruce (1669–1735)

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## Abstract:

The proposed theme of this paper is how "Arts" and "Science," with the inclusion of the topic of "Health", can be combined through a story based on research about innovation in the Past. The goal of this paper is threefold. *First*, to present a research-based story about Jacob Bruce (1669–1735), a scholar of Scottish descent born in Moscow, and his innovation – the prototype of Artificial Intelligence, humanoid robot. *Second*, to illustrate the new experimental interdisciplinary method of storytelling - SMS (Stories based on Music about Scientists), a novel approach that integrates Arts (Music, Poem, Digital images), Science (Historical Research about Innovations of the Past), and Culture (cultural heritage with metaphor of a philosophical soul development). *Third*, to explain how this method can be applied. SMS method created in 2012 uses the Avant-garde genre of Music with the integration of interdisciplinary knowledge, holistic approach, and implies a deeper level of integration of various disciplines. The theme of this SMS is a song-story about the beautiful humanoid girl-robot (but with a different technology of the 17<sup>th</sup> century), which serves as a prototype of the modern homunculus or AI created by Jacob Bruce. AI humanoid already resembled a natural human being by the end of the 17<sup>th</sup> century. The author explains and discusses how and why SMS can be used as an Innovative Teaching and Learning method, related to Science and Arts (similar to STEAM approach), with the power of Lifelong Learning (LLL), and pedagogical didactics across branches of sciences, thus stimulating innovations and discoveries.

**Keywords:** Arts and Science; Innovations; STEAM/SMS (Stories based on Music about Scientists) method; Jacob Bruce (1669–1735); Innovative Teaching and Learning; Health



*“Only Art and Science can raise men to the level of gods.”*

Ludwig van Beethoven (1770 – 1827)

*Music is beneficial for everyone, both to cure diseases and to maintain health.*

*Its power should not be underestimated*

Al-Farabi (870-950 AD) (The Great Book of Music)

## 1. Introduction

### 1.1. Research: Story-telling about Jacob Bruce, a Scientist of Scottish descent

A central figure in this “new type” of storytelling’s research is Jacob Bruce (1669-1735), who created AI in the 17<sup>th</sup> century. Jacob Bruce was born in Moscow and descended on his father’s side from the Bruces of Clackmannan in Scotland (Collis, 2012). Bruce’s biographer M.D. Khmyrov (М. Д. Хмыров) emphasizes the encyclopedic nature of Bruce’s knowledge, and writes about him<sup>1</sup>: “Astronomer and mathematician, artilleryman and engineer, botanist and mineralogist, geographer, author of several and translator of many scientific works” (Луппов, 1973). Luppov (1973) also added that Count Bruce was the first prominent figure in the field of Russian pedagogy and science of that time, and a companion of Peter the Great (Comments: this name of the tsar is commonly used in the West, while in the East, he is better known as Peter the First, - “Петр Первый”).

The ratio of the number of books on different branches of knowledge in Bruce's library characterizes the area of his primary interests. Noteworthy is the large number of literature on physical and mathematical sciences (233 books), medicine (116 books), geological and geographical sciences (71 books). Bruce was at the forefront of Russian printing, and rich practice in mint reforms (after visiting Isaac Newton in England and discussing it), travelling, the literature on Arts and religions and military sciences (91 books) in artillery, and fortification (Копанева, 2020). He was a leading figure in the Russian science, and the head of mining and industry, the head of the Mathematical and Navigation School based in the Sukharev Tower. Bruce also established the first observatory of Russia on the third floor of this Tower in 1699. **Figure 1** (below) demonstrates his portrait and pictures of the legendary Suharev Tower, where Jacob Bruce worked on his innovations.

Bruce was also one of the leading diplomats, esteemed for his great learning, and Russia’s first Newtonian, who created a housemaid from flowers (Collis, 2012). This housemaid was an android or AI, robot-type girl, created in the Sukharev Tower, who looked like a genuine girl: she cleaned the bedroom, served coffee, but she could not speak. This bears a resemblance to a legend about the medieval alchemist Albertus Magnus, in which he reputedly created an android in his secret workshop (Collis, 2012, p. 53). In medieval and early writings, there are stories about medieval AI or an artificial construct in the shape of a human body animated for the purpose of divination, associated with Gerbert of Aurillac, Albertus Magnus, Robert Grosseteste, Roger Bacon (Kang and Halliburton, 2020, p. 72).

In this story, the author refers to the 17<sup>th</sup> century time frame which was a crucial period in the development of modern science in Europe. It saw advancements in various fields such as astronomy, physics, mathematics, and biology, and this period is often referred to as the Scientific Revolution. The Royal Society of London for Improving Natural Knowledge, commonly known as the Royal Society, was founded on November 28, 1660, when a group of prominent scientists and philosophers gathered at Gresham College in London. It was granted a royal charter by King Charles II in 1662, officially establishing it as The Royal Society. Similarly, in 1666, Louis XIV established learned societies to encourage the spirit of French scientific research, promoting academic disciplines such as the arts and sciences.

<sup>1</sup> Луппов С.П. Книга в России в первой четверти XVIII в. Л., 1973. Глава пятая. Частные книжные собрания. Translated from Russian, the original text is in the source: <https://www.alib.ru/bouluppov52.phtml>



## Сухарева Башня и Яков Брюс the Sukharev Tower and Jakob Bruce: research of 1862



**Figure 1.** From the left to the right: Photo of Suharev Tower. Portrait of J.V.Bruce by lithography of F. Mayer. XIX century. From the book: Primitive Calendar of Bruce, from the 1<sup>st</sup> edition during the lifetime of Bruce, with his portrait and biography. Kharkiv, 1875. MAE Russian Academy of Sciences. On the right: Suharev Tower in Moscow (Moscow, 1802).

### 1.2. **Health:** A healing power of Arts (Storytelling in Poetry, Music, and Virtual Arts)

In this paper, the author introduces the new method of research with a story telling (based on research about Jacob Bruce) - mechanism, which embeds Arts and Science. In facts, it includes many related fields: **Arts, Culture, Science**, Innovations and Health. The applied SMS (Stories based on Music about Scientists) is a novel method, a STEAM approach which integrates **Science** (Historical Research stories about Innovations of the Past), **Arts** (Music, Poem, Digital images), and **Culture** (cultural heritage with metaphor of philosophical soul development). In order to understand these concepts we briefly define them.

- **Stories** are a universal language loaded with symbolism and significance, allowing us to unravel the mysteries of the world: they seek to discover the meaning of human existence and consider our own individual purpose within it (Rooney et al., p. 147).

Kant in "Critique of the Power of Judgment" gives the definition of **Art**, which is defined:

- **Art** is "a kind of representation that is purposive in itself and, though without an end, nevertheless promotes the cultivation of the mental powers for sociable communication" (Adajian, 2007). "The cultivation of the mental power" in this Kant's definition is very important, because of similarities with a broader concept of "**Culture**".

- **Culture** as a word originates from the Latin root "cultura" or "cultus" meaning to "inhabit, cultivate, or honour". Thus, culture includes ideas, language, beliefs, customs, codes, institutions, tools, techniques, works of art, rituals, and ceremonies.

The origins of the word "poem" trace back to the Greek word "poiema," which means "a thing made" or "a creation", and a poet, as defined in ancient terms, is essentially "a maker of things." This concept underscores the creative act of crafting poetry.

- **Poetry** has a special place and meaning in the history of healing: shamans and medicine men and women chanted poems as a part of their *healing art* (Chavis, 2011, p. 19). In ancient Greece, Appolo, the patron God of **music and poetry** (poetry: light, sun and prophecy; drama: inspiration and catarsis for entire community), is also recognised as the divinity of **medicine and healing** (Chavis, 2011), thus, directly linked to "health" issues. Al-Farabi, the Central Asian scientist who wrote on the perfect city, logic, astronomy, linguistics, politics, mathematics, geometry, medicine, optic, philosophy, and music, known as the 'second teacher' (after Aristotle, the first teacher) discussed the importance of **music**





(Nurysheva & Tercan, 2021). Music was not only as an art form, but also as a tool for cultivating the soul and promoting physical and mental well-being in the 9th AD (Nurysheva & Tercan, 2021). Al-Farabi who raised most of his philosophy and theory on Greek philosophers such as Aristotle, Pythagoras and Plato, wrote in his Great Book of Music: - **Music** is beneficial for everyone, both to cure diseases and to maintain health”.

He acknowledges the therapeutic effects of music on the soul and its ability to induce states of calmness and relaxation. Al-Farabi believed that music had the power to influence both the body and the soul, depending on type of music: *rational, ethical, and customary*. Rational music (closely linked to philosophical and intellectual pursuits), had the most profound effect on the soul, leading to intellectual and spiritual development, ethical music aimed to instill virtuous qualities in listeners, and customary music was associated with entertainment and social customs. SMS incorporates the rational music qualities, and might go even beyond it due to the additional and intentional components of Arts and Poetry. Al-Farabi shared the story of 'Alqama ibn 'Abda (sixth century), the poet who was refused a hearing by the Ghassanid king Al-Harith ibn Abi Shamir (529-69) until he had melodized his verse and sung it to him (Farmer, 1929, p.18). Thus, music and arts in the Past played integral roles in promoting health and well-being by addressing the emotional, cognitive, social, and physical aspects of human experience, and contributing to a multitude of additional qualities and abilities.

## 2. Methods

SMS (Stories based on Music about Scientists) method was developed as a music experiment on a “hobby” research in 2012-2013 years in Europe (Slovenia), with the idea to create the author project “Education for all”, when STEAM approach was not known. Following the inspiring European ideas of Leonardo da Vinci (1452-1519), an Italian Renaissance painter, scientist and inventor with “principles for the development of a complete mind”: “*Study the science of art. Study the art of science. Develop your senses – especially learn how to see. Realize that everything connects to everything else*” (Haesen and Van De Put, 2018, p. 9). Therefore, it was created as the European novel and pioneering approach that integrates Arts (Music, Poem, Visual Arts (digital images), Science (Research about Innovations) and Culture (Language, Ideas, and Beliefs). The author has been using SMS since 2012 year and received 13 awards from the International Music festivals. The author uses this approach with the both components of Arts and Science as a STEAM Ambassador (volunteer in the Deloitte & European Commission’s ESTEAM project) to promote the power of innovations through Arts and incorporate the digital competences of women and girls in STEM disciplines.

### 2.1 SMS (Stories based on Music about Scientists): Innovative Teaching and Learning method

Storytelling here is present as a Methodological Approach in Research. The other key inspiring method, which is based on a storytelling, is the method of SMS (Stories based on Music about Scientists) which embeds both the Arts and Sciences. The description of SMS method is presented and explained here through: “What (1-2)-Why (3)-How (4-7)” questions. The SMS method has the following conceptual framework:

1. Research about the scientist of the Past time
2. Story about a Scientist-Polymath of the Past time (mainly, the 17<sup>th</sup>-20<sup>th</sup> centuries)
3. Key idea of Innovation, its Ethical issue or inspiring facts from the life of Scientist
4. Lyrics (a): written based on research)/or Poem (b) written by Scientist himself with some elements of phantasies (in 3a) or construction of the key shorter version (3b)
5. Music (emphasising the idea of lyrics)
6. Visual Arts (digital images /pictures, video, etc.) to explain the key ideas to those who do not understand the original languages of lyrics (English, Russian, Slovenian, or Latin).
7. Performance (live) or Music Notes or Recording (digital) placed on Youtube/Facebook

There are two types of SMS, which can be used as a method of Innovative Teaching and Learning, the interdisciplinary method with the intersection of Arts and Science:



- First type of SMS – written based on research with the initial poem about scientist of the Past time based on research facts about the life of scientist or his innovation
- Second type of SMS – written based on the poem of scientist himself (his original song), and the composed music is added to the poem with the digital images

The project “Heritage of Science” in “Education for All” is a personal author’s artistic non-funded initiative, which was set up as an attempt to share the knowledge about Science through the poetic stories about scientists of the past centuries based on the volunteering author’s research. The author promotes the power of Science and Arts in the method SMS (Stories based on Music about Scientists) which can be used as Innovative Teaching and Learning method (the Bologna initiative), Life-Long Learning technique and a holistic and health approach.

### 2.2 SMS (Stories based on Music about Scientists) vs. STEAM approach

The SMS principle was developed independently, and approximately at the same time as the STEAM approach was developed in the United States (in about 2013, described in the history of its development in “STEM to STEAM” (Istieulova, 2022)). STEAM (science, technology, engineering, the arts and mathematics approaches to education have been recently launched in the EU to improve the relevance and quality of higher education with a holistic and dynamic educational experience with integration of concepts and methods from multiple disciplines, fostering interdisciplinary learning. The European Commission is championing the STEAM approach through the work of the EU STEM Coalition and the actions outlined in the European Skills Agenda.

In 2013, John Maeda, the founder of STEAM, an Asian American computer scientist, educator, and professor at the MIT Media Lab, answered the question: “What does it mean to add Art to turn STEM into STEAM?” (Lamont, about 2013) He announced that art and design transform the American economy, and the STEAM movement is an opportunity for America to sustain its role as an innovator of the world through ‘problem-solving, fearlessness, and critical thinking’ (Maeda, 2013).

### 3. Results

The Romance about Alchemist (with the dedication to Jacob Bruce) – is the title of SMS, and this song was written in Spring, 2015. It has the following elements:

1. Historical research about Jacob Bruce on AI of the Past time, but different technology with the additional elements of phantasy related to the “technology of creation” (which is a “black box”) suggesting that one element was made with the inclusion of cultural heritage (Slovenian Neanderthal flute, about 60-67 mln. years, the oldest instrument): [https://www.youtube.com/watch?v=EaN\\_NLOvYb0](https://www.youtube.com/watch?v=EaN_NLOvYb0)
2. Inspiring Story about a Scientist-Polymath of the Past time who introduced innovation.
3. Key idea of Innovation (AI in its perfect form) and its Ethical issue in transformed into the SMS (song) entitled “Romance about Alchemist”.
4. Lyrics (a): written based on research with some elements of phantasies dedicated to the act of woman’s creation. For instance, in a poem, there was a metaphor to depict the creation of woman through musical performance on the ancient flute (referring to the Neanderthal flute, the oldest instrument in the world, found in Slovenia).
5. Music (emphasising the idea of lyrics) – Musical Notes (below, the introduction).
6. Visual Arts (digital images /pictures, video, etc.) – should be published in YouTube channel (author/under her other name of Aleona von Sultanova – used in Music/SMS): <https://www.youtube.com/channel/UCI2j8TiTOj1cwQpHWD8Ngg>
7. Performance (live, in the hostel Celica), and/or Music Notes <https://www.facebook.com/1346167803/videos/pcb.10220238261770494/10220238242370009>. Typically,

The link presents a live performance, dedicated to the memory of Ljuben Dimkaroski, a dear friend, and musician, who played tidldibab, as he wished to call it or the oldest Neanderthal flute (which was found in Divija baba, Slovenia). The act of creating the artificial ‘Jashkina Baba’ (or AI of the 17th century, a girl-android) is described in the song, where



one of Jacob Bruce's rituals involved playing the oldest instrument (the oldest flute), hence the numerous analogies. Although there are some elements of phantasies, but they are also linked to some legends and real stories. Ljuben Dimkaroski, shared one of the real stories. During his interview in 2015 to the author of this article, he shared a story, that after 2.5 years of having the replica of this flute in his apartment in Ljubljana, during one night, he saw himself in the night dream. In that dream, someone was showing to him how to play this ancient flute (from the bone).

It should also be noted, that the concept of AI is broader than that of a robot, and the concept of a robot is broader than that of an android, which specifically refers to robots designed to resemble humans. Thus, according to these three concepts, it would probably be more precise to use the term 'android' for a humanoid robot. At the same time, the author uses the general term AI to illustrate that AI models could be more advanced in the 17th century compared to those created today, yet ethical questions remain the same. The text of SMS lyrics is provided below:

### Романс об Алхимике (Romance about Alchemist)

В конце 17-го столетья алхимик формулу искал:

Искал он формулу совершенства, чтобы создать свой идеал.

Мечтал увидеть он новый облик – свой женский Евы идеал,

Спускаясь с башни в пещеру, алхимик романс свой тайный напевал:

Припев:

Как вверху, так и внизу, как сейчас, так и тогда,

При полной луне и в маске от блаженства

Как Парацельс, сотвори ту алхимию любви

И прошепчи ты формулу Трисмегиста.

Что такое душа? Как ее сотворить?

Духов ты вызывал, с ангелами говорил,

книгу Еноха читал, в шар хрустальный смотрел,

Мудрости меч рассекал, и душой постарел:

Чернокнижником став, золото из меди создав,

омолодив живою водой о сокровенном мечтал,

Чтобы душу вдохнуть – это не плоть омолодить:

равносильно творцу то уметь творить

Из рая иль ада, и что же ты создал?

И формулу жизни вложил в свой идеал,

Глаза - как изумруды, копна волос в цветах.

Восточные глаза и совершенный стан.

Но образ твой прекрасный немой и без души,

И ты не знаешь точно, что чувствует внутри,

И движется прекрасно, и кофе подает, -

улыбка ж безучастна, глаза ее грустны.

Книга Соломона указала некий знак,

Фиалками и розами гомунгул осыпал,

И каплями росы и дуновением весны

и, до сих пор, ты поисках алхимии души

Маг и алхимик славянской страны,

Древнюю флейту в руки возьми

И совершенство ты сотвори:

В танце суфиста движенье планет пробуди, прошептав:



Припев:

Как вверху, так и внизу, как сейчас, так и тогда,  
При полной луне и в маске от блаженства  
Ты во сне увидал, как играть на флейте той,  
При полной луне на восток в танце суфиста.

**Romance about Alchemist (translated in English language).**

At the end of the 17th century, an alchemist sought a formula:  
He searched for perfection to create his ideal.  
He dreamed of witnessing a new manifestation - his feminine ideal of Eve.  
Descending from the tower into the cave, the alchemist sang his secret romance:

Chorus:

As above, so below, as now, so in the Past,  
Under the full moon wearing a mask of bliss  
You, like Paracelsus, create that alchemy of love  
And whisper the formula of Trismegistus.  
What is the soul? How does one create it?  
You called upon your spirits and conversed with angels,  
You delved into the book of Enoch and gazed into the crystal ball,  
You cleaved the air with the sword of wisdom, yet your soul aged further:

You became a warlock, transmuting copper into gold, rejuvenated by living water,  
You dreamed of the internal secret:  
to 'breathe in' a soul is not to rejuvenate your flesh  
but to embody the Creator's ability to create  
Whether from heaven or hell, what have you created?

And he infused the formula of life into his ideal:  
Her eyes akin to emeralds, a head adorned with lovely flowers,  
Oriental eyes and a perfect figure.  
Yet your beautiful image is mute and soulless,  
And you do not understand what resides within.  
It moves gracefully and serves coffee, -  
Her smile is indifferent, her eyes are sad.

The Book of Solomon indicated a certain sign,  
you showered the homungul with violets and roses,  
with drops of dew and a breath of spring and, somewhere there,  
you are still in search of the alchemy of the soul

Magician and alchemist of the Slavic lands,  
take the ancient flute in your hands,  
and strive for perfection:  
In the dance of a Sufi, awaken the movement of the planets, whispering:

Chorus:

As above, so below, as now, so in the Past,  
Under the full moon and wearing a mask of bliss,  
In your dream, you saw how to play the ancient flute of the one  
With the full moon to the east in a Sufi dance.



## Romance about Alchemist

Intro  $\text{♩} = 236$  Words & Music by Yelena Istileulova

Bb/D A Dm

Piano

5

10 E|maj7 Abm

14 B7

Pno.

Figure 2: Excerpt from the Introduction of the Musical Composition on Jacob Bruce (Romance about Alchemist).

### 4. Discussion

In the modern higher education of the third decade of the 21<sup>st</sup> century there are a lot of attempts to integrate STEAM approaches in the inter- and transdisciplinary higher education through developing a comprehensive methodological framework through, speculative design, art thinking, process-based research and participatory practice and the methods to implement STEAM pedagogical approaches (Carter et al., 2021). The SMS method is easily associated with the STEAM approach. However, the STEAM approach does not incorporate methods similar to SMS.

According to discussions by Carter (2021), HEIs refer to the ideal approach to STEAM, which is (potentially) involving:

- a culture of the Arts and Sciences on an equal footing
- operating within a process-driven paradigm, student-centred, holistic



- being collaborative, diverse and delivered through safe spaces
- establishing a mind of openness, reflection, experimentation and curiosity
- generating qualities that promote learning, cooperation and multi-modality
- supporting transdisciplinary practices and emphasise prototyping
- developing competencies of critical thinking, creativity and communication whilst investigating how to generate solutions.

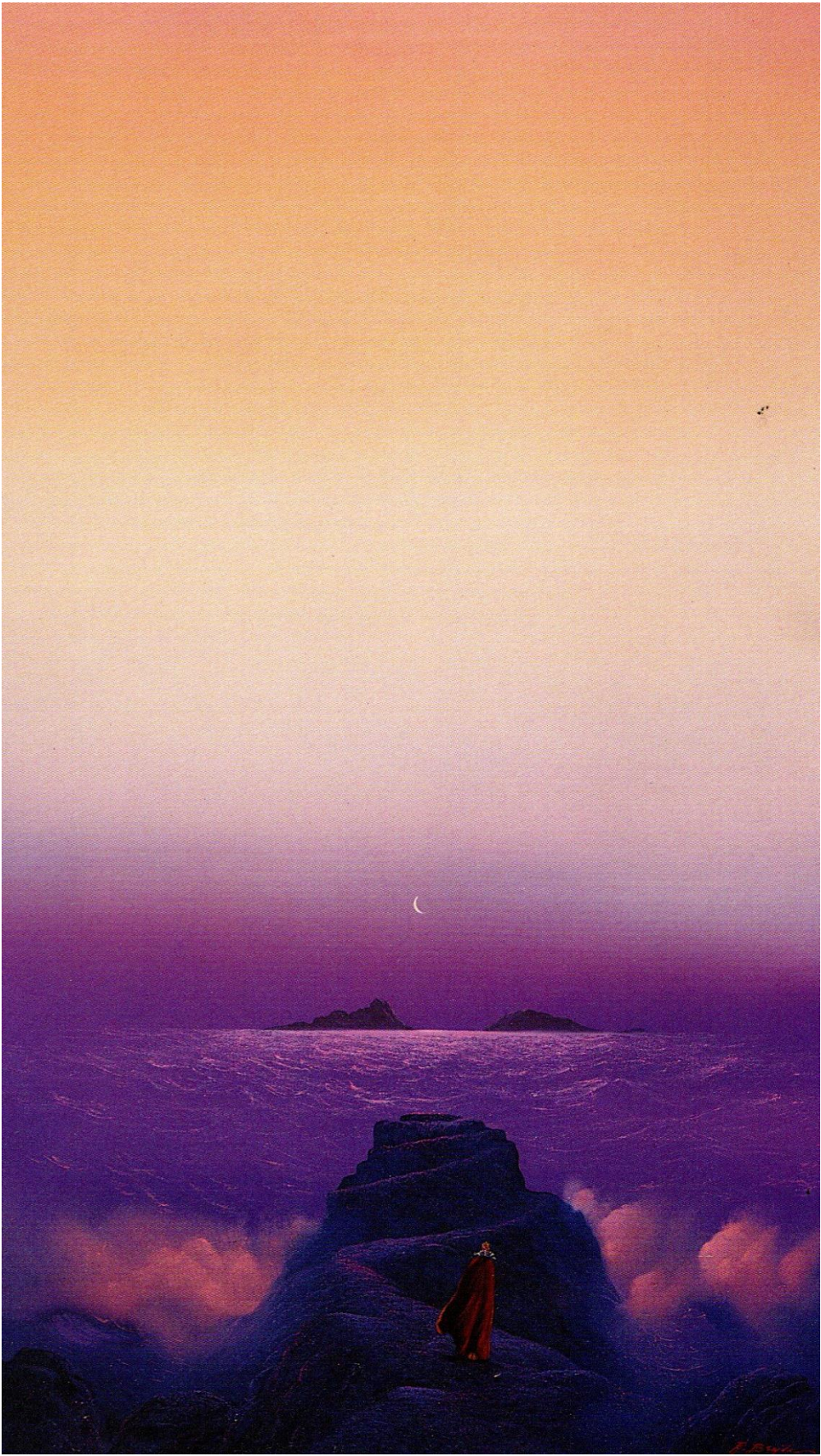
The SMS method easily meets all the criteria set forth by prominent HEIs. It is the Avant-garde & Experimental genre of music which embeds the key fields of knowledge: Science (History of Science/Historical research), Arts (Music, Poetry, and Digital Images), and Culture (Language, Ideas, Beliefs). Additionally, it aligns with innovative teaching and learning methods, as well as lifelong learning (LLL) practices, which can be associated with STEAM Ambassadorship. Returning to the elements of art and science cited by Beethoven, when music contributes to health benefits and the cultivation of mental faculties for social communication, as an element of culture, along with music and poetry, provides a holistic approach. Rational music, with its power to influence both the body and the soul through philosophical and intellectual pursuits, leads to intellectual and spiritual development. Further effects is to be studied through Al-Farabi and Avicenna, who wrote about the influence of music on health, including its effects of curiosity and stimulation for new discoveries in science.

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# The Slovenian Model of Scientific Research Work of Secondary School Students and their Participation in Competitions as Motivation for Career Choice

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## Abstract:

Research in education plays an important role in maintaining curiosity and training skills. It is primarily used in the classroom, but can also be found in everyday life without us even realising it. The research work includes the views of young researchers from Slovenian secondary schools who participated in a research assignment within the Association for Technical Culture of Slovenia (ATCS) between the years 2010 and 2017 and won the gold award. We found that carrying out the research assignment helped the young researchers on their path in life and on their way to success. The correlation test of the chosen research pool showed that there is no statistically significant relationship between the chosen research area in secondary school and further university or professional orientation.

**Keywords:** Research work, Young researchers, Motivation, Education, Career choice, Association for Technical Culture of Slovenia





## 1. Introduction

It is common to come into contact with research or project work at some point (Petek, 2012). Research work in itself means gathering one's own thoughts and ideas, collecting data and dealing with a specific problem. When young people decide to undertake a more extensive project work, which means additional success and progress for them in the future, it is important that they are encouraged by the surrounding society. Various sources of support include relatives, peers, but also mentors and various institutions that help young people to achieve excellent results. Through the research approach two fundamental goals of education are pursued: to maintain the individual's curiosity and continuing interest in knowledge and to develop the skills required for independent problem solving (Petek, 2012). To achieve these goals, one of the key factors is motivation, i.e. encouragement, maintenance and direction of the individual's activities to achieve the goal (Kompore, 2009). In addition to motivation, self-confidence is also necessary, which shows us what we want to achieve and how we will achieve it. A key role in motivation in research is played by the manager, who must positively encourage the researchers, listen to them and find out what the researcher wants to achieve. A good manager also encourages motivation by helping to solve problems, by acknowledging also negative results, by boosting self-confidence and by making the work enjoyable. Both, praise and reprimand are extremely important in research work (Uranič, 2008). As mentioned earlier, institutions are also responsible for a large part of motivation by supporting designers in various ways. Such method was demonstrated at a local school in Ljubljana region (Biotechnical Educational Centre Ljubljana, General Upper Secondary School and Veterinary Technician School), where a project was launched in 2015 to further develop the knowledge on water. The project enabled students to expand their knowledge in various areas and acquire new skills at the same time.

General conditions for participation in the national competition are the ranking in the regional (local) competition and the maximum number of research projects (contributions/articles) from each region that can qualify for the national competition. This is determined on the basis of the average number of research papers in the regional competition in the previous three years, which is set each year in the announcement for the national competition of the current school year. The topics of the research papers are freely selectable. Current topics, also from the local environment, are recommended, especially in cooperation with the community, the business community or other interested parties, who can also announce the topics of the research papers via the Association for Technical Culture of Slovenia (ATCS). Participants must submit their research papers on time and in the form specified in the call for proposals for the respective school year. The research papers must be written in Slovenian and in a format that meets the current standards in the respective research fields. Foreign-language research papers that are reviewed by the main committee are exempt from the use of the Slovenian language. The use of a foreign language is permitted for such research papers. A foreign-language paper that does not deal with the use of a foreign language may be rejected by the committee. The papers are assessed by the members of the panel of experts responsible for the respective area. The panel is chaired by a chairperson. In the first round, the members of each expert panel review and evaluate the research papers that have qualified for the national competition and rank them according to points based on the criteria specified in the Call for Papers for the respective school year. During the presentation in the national competition, the members of the expert panel examine the independence of the candidate in the conception of the research project, the research approach, the methods and results, the systematics and significance of the presented material as well as the general knowledge (ATCS, n. d.).

At the end of the state competition, the participants receive awards and prizes depending on the results achieved. On the recommendation of the technical committees, the best-rated



projects are selected by the national committee to take part in international competitions for young researchers (ATCS, n. d.).

Our work involves qualitative and quantitative data analysis of research assignments conducted by secondary school students between 2010 and 2017. We focused on areas such as chemistry, biology and interdisciplinary fields. We invited former winners of the Association for Technical Culture of Slovenia (ATCS) who were secondary school young researchers to complete a questionnaire (ATCS, n. d.). Using a correlation analysis, we looked for a relationship between the chosen research area at the secondary school level and the choice of further studies at university level.

## 2. Methods and hypotheses

The survey (using a questionnaire (**Appendix**)) was completed by young researchers that were awarded the Gold Award for Young Scientists of Slovenia by ATCS during their previous education in the secondary school. The survey focused on competition in the fields of chemical and biological research projects as well as interdisciplinary scientific projects.

The questionnaire was created on the 1KA website (1 KA Enklik anketa, n. d.) and then distributed to the winners via social media (Facebook) and electronic messages using Gmail. After all respondents completed the questionnaire, we collected all data using the analytical tools via Microsoft Excel (Microsoft Corporation, version 2018). The collected data was then presented using pie charts created with the same programme.

Before the empirical part of the work, we established hypotheses that guided us throughout the study. We hypothesized that individuals who had a natural science focus of the project (chemistry, biology or interdisciplinary project) in the secondary school would also have a natural science focus in their further studies and profession. We hypothesized that the interdisciplinary subject will be most strongly represented among the respondents. As the research work was voluntary in most cases, we hypothesized that the students would not find it difficult, but on the contrary would enjoy it and were able to gain new experiences. We hypothesized that the majority would perform the research work again if they had the opportunity to do so.

### 2.1. Sample

Young researchers from secondary schools and grammar schools who received the ATCS Gold Award for secondary school students during their previous education between 2010 and 2017 were included. We were assisted by the organization ATCS, the main organizer of this competition. Some individuals of the researchers on our list of names were repeated and sometimes even tripled because they applied for and worked on the competition more than once during the observation period. We treated these researchers as one person who completed the questionnaire once. Of the 75 people invited, 35 successfully answered the questionnaire, some of whom are studying or working.

### 2.2. Instrument

We composed a questionnaire and sent it out via social media platforms (Facebook and Gmail). We opted for a closed questionnaire, which means that the respondent does not write the answer to the question, but selects one of the already prepared answers that seem the most appropriate. The questionnaire comprised a total of 14 questions, which were compiled in such a way that we could confirm or refute the hypotheses after the evaluation and find out the personal opinion of the respondents.

### 2.3. Statistical analysis

Pearson's chi-square ( $\chi^2$ ) is a basic statistical measure that compares observed and expected frequencies and evaluates how these frequencies differ. Larger values of Pearson's  $\chi^2$  indicate larger differences between the observed and expected frequencies. To interpret the results, the p-value was used, which represents the value and measures the probability that the observed difference between observed and expected frequencies is purely random if there is no real relationship between the variables. If the p-value is below

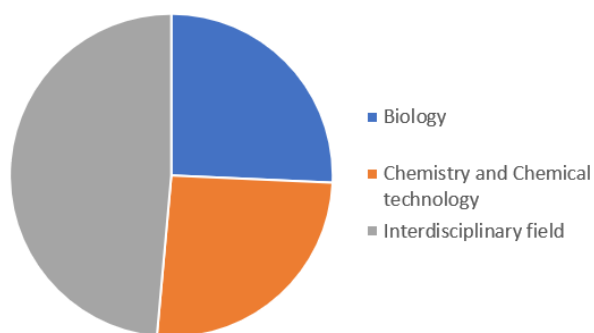


the selected significance level (usually 0.05), the null hypothesis is rejected and a statistically significant correlation is concluded.

The correlation analysis was carried out using the SPSS programme. We analysed correlations with the  $\chi^2$ -test (hi-square) by using the programme IBM Corp. Released 2010, IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp., New York, USA. The results of the analysis (**Table 2**) were presented in various parameters. The degree of freedom (df) indicates the number of independent variables in the analysis. With the statistical programme SPSS, the output table contains a sig. value (2-tailed). Asymptotic p-values are useful for sample sizes where it is difficult to calculate an exact p-value. This value represents the 2-sided p-value of the test (Asympt. Sig. 2-sided p-value). It is roughly assumed that the sample size is large enough for the test statistic to converge to the corresponding marginal normal or chi-squared distribution. The p-value calculated using the approximate true distribution is called the asymptotic p-value (Analyse-it Software, n. d.). In general, the term association refers to coefficients that measure the strength of a relationship. The coefficients in this section are designed for use with nominal data. Phi and Cramer's V are based on adjusting chi-squared significance for sample size (approximate significance, Approx. Sig.). Phi is a chi-squared based measure of association. The chi-squared coefficient depends on the strength of the relationship and the sample size. Phi eliminates the sample size by dividing the chi-square by n, the sample size, and taking the square root. In purely mathematical terms, phi is the square root of chi-square divided by n, the sample size:  $\phi = \text{SQRT}(\chi^2/n)$ . Phi therefore measures the strength of the relationship, defined as the number of cases on one diagonal minus the number on the other diagonal, adjusted for the marginal distribution of the variables (Virginia Commonwealth University, n. d.). Cramer's V is the most popular of the chi-squared based measures of nominal association because it gives a good normalisation from 0 to 1 regardless of table size when the row edges are equal to the column edges. V is equal to the square root of the chi-square divided by the sample size n times m, i. e. the smaller of (rows-1) or (columns-1):  $V = \text{SQRT}(\chi^2/nm)$ . SPSS and other important programmes indicate the significance level of the calculated V value (Virginia Commonwealth University, n. d.).

### 3. Results

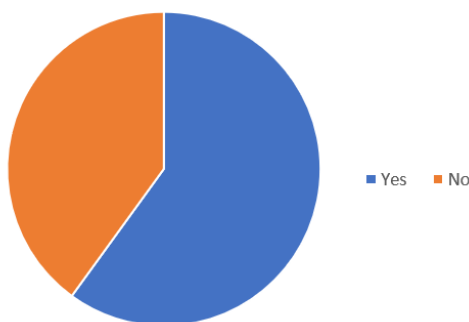
With the first question of the questionnaire, we collected information about the gender of our participants. Of the 35 respondents, 24 identified themselves as male and 11 as female. The second question related to the respondents' current education, and it turned out that the largest percentage of respondents (77%) were natural science students. The smallest percentage of respondents were social science students (6%), and only a few of them (6%) were working on a secondary school degree. The third question related to the field of study in which the respondents were researching. One percent less than half of the respondents did research in an interdisciplinary field, and the rest did research in chemistry and biological engineering or biology (**Figure 1**).



**Figure 1.** Choice of research area of the young people included in the sample. Distribution of choice within the natural sciences.

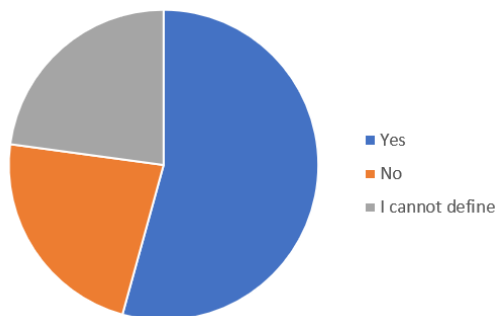


The fourth question, which was answered in the affirmative by 97% of respondents, was: "If you were a young researcher in secondary education, would you attend the young researchers competition again". With the fifth question, we wanted to find out whether the respondents thought that research is a good way to gain new knowledge, and we received the answer that they all agreed. The next question related to the selection or creation of the topic for the research paper and its elaboration. Of the 35 participants, 11 were without record of help (31%), 6 were supported by a school-based mentor (17%), 15 had help from an external mentor (43%) and 3 had received help from a school and an external mentor (9%). With the seventh question, we wanted to find out which part of the research the respondents enjoyed the most. Most (97%) found the practical part of the research work the most enjoyable, only one found the preparation of the written documentation the best. In the eighth question we asked about their impressions of the experimental part of the work and in the ninth question about their impressions of the written part of the work. When asked about the experimental part, 33 respondents chose the answer: "I liked it, I was motivated and had fun". The same answer was given by 20 respondents to the ninth question. In the 9<sup>th</sup> question, the participants were asked for their opinion on the role of the mentors (school or external). Most (29 respondents) felt that the role of the mentor was important as it added value to the research and the candidate gained a lot from the research process; some felt that the role of the mentor was important but that the author of the research was responsible for everything, or that the role of the mentor was not important. The 10<sup>th</sup> question related to the current relationship with their mentor. The answers showed that more than half (60%) of the researchers have remained in contact with their mentor to this day. Regarding the 11<sup>th</sup> question: "Do you think your research has helped you develop other skills not related to the experimental part of the research (e. g. public speaking, overcoming stage fright, courage to stand by your results, personal growth, responsibility, etc.)?" 89% of respondents answered affirmative. In 12<sup>th</sup> question, we wanted to know if respondents were aware that research can help them in their future job search, and it turned out that 60% were aware of this fact (**Figure 2**).



**Figure 2.** Question: Did you know that research can increase your chances of getting a job in the future?

In the 13<sup>th</sup> question, we asked the respondents whether the research work they had carried out had helped them in their choice of further education. More than half (54%) answered "yes", 8 individuals answered "no" and the same number of individuals did not specify ("I cannot define") (**Figure 3**).



**Figure 3.** Question: Was the research helpful for your further choice of education (if you are a student) or employment or to establish yourself?

In the last (14<sup>th</sup>) question, we asked where the respondents see themselves in 10 years' time. 51% of respondents answered that they see themselves in the role of a researcher in public institutions (e. g. as a researcher at an institute or university), 23% in a company where they are involved in the development of products, 17% in a university, 9% as an independent entrepreneur and none in the role of a teacher or university professor. Once collecting the data from the questionnaire, we also performed the correlation analysis (**Table 1**). The correlation of statistical differences we were interested in resulted from the second (2<sup>nd</sup>) and third (3<sup>rd</sup>) questions. We were interested in the statistical compatibility between the choice of subjects in secondary level and the later choice of educational or professional pathway.

**Table 1.** Correlation between responses to Question 2 and responses to Question 3.

				Question 3			Total
				Chemistry and chemical technology	Biology	Interdisciplinary field	
Question 2	Natural science student	Count		<i>0</i>	<i>1</i>	<i>3</i>	4
		Expected count		<b>1</b>	<b>1</b>	<b>2</b>	4
Social sciences student	Count		<i>6</i>	<i>8</i>	<i>13</i>	27	
	Expected count		<b>7</b>	<b>7</b>	<b>13</b>	27	
Employed in the natural sciences	Count		<i>1</i>	<i>0</i>	<i>1</i>	2	
	Expected count		<b>0.5</b>	<b>0.5</b>	<b>1</b>	2	
Employed in the social sciences	Count		<i>2</i>	<i>0</i>	<i>0</i>	2	
	Expected count		<b>0.5</b>	<b>0.5</b>	<b>1</b>	2	
<b>Total</b>	Count		<i>9</i>	<i>9</i>	<i>17</i>	35	
	Expected count		<b>9</b>	<b>9</b>	<b>17</b>	35	

The bolded values indicate the correlation of mutually exclusive results (if the quantities were completely independent of each other) (*i. e.* there would be no correlation). The values in italic indicate the values determined by the crosstabulation.



**Table 2.** Correlations between the choice of research topic in secondary school and the subsequent choice of studies or employment (Q2 and Q3 questions) as calculated by Chi-square test.

	Value	df	Asymptotic significance (2-sided) (Significance test)
Pearson Chi-Quare	8.646	6	0.194
Likelihood ratio	9.659	6	0.140
Linear-by-linear association	5.633	1	0.018
Number of valid cases	35		

	Value	Approximate significance
Symmetric measures		
Nominal by nominal	Phi	0.497
	Cramer's V	0.351
Number of valid cases	35	

According to the results in **Table 2**, the p-value is greater than 0.05, which means that there is not enough evidence to reject the null hypothesis. This indicates that there is no statistically significant relationship between the choice of research topic (biology, chemistry and interdisciplinary field) and further education or employment path for our sample (further studies or career choice).

#### 4. Discussion

We found that the completed research work helped the young researchers finding the path of their further career, which has at the time already taken shape. The young researchers were convinced that research is an excellent way to gain new knowledge. It is therefore of utmost importance that young individuals in primary and secondary schools are introduced to the research method for solving problems. The correlation analysis ( $\chi^2$ -test) of the collected sample has shown that there is no statistically significant relationship between the chosen research field and the further educational or employment path. Our results indicate that the motivation of the individuals and their interest in any field of study are crucial. However, it should be borne in mind that the population comprised only 35 people and the lack of statistical significance could be due to an insufficient number of participants. The study will be continued.

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## Appendix 1 Questionnaire

1. Select your gender.
  - a) Male
  - b) Female
2. Please select your current level of education.
  - a) Secondary school student.
  - b) Student of natural sciences.
  - c) Student of social sciences.
  - d) Employed in the natural sciences.
  - e) Employed in the social sciences.
3. What is your field of research? Please select the appropriate answer.
  - a) Chemistry and chemical technology.
  - b) Biology.
  - c) Interdisciplinary field.
4. As a young researcher at a secondary school, would you take part in the Young Scientists Competition again?
  - a) Yes.
  - b) No.
5. Do you think that research is a good way to acquire new knowledge?
  - a) Yes.
  - b) No.
6. How did you choose or create the topic and the development of the research paper?
  - a) I knew what I wanted to do, so I was completely independent.
  - b) The topic and general development of the research paper was suggested to me by my school supervisor.
  - c) The topic and general development of the research paper was suggested to me by an external mentor.
  - d) The topic and the general development of the research paper was created jointly by the school and the external mentor.
7. Which phase of the research work did you like best?
  - a) Carrying out the practical work.
  - b) Preparing the written documentation.
8. What are your impressions of the experimental part of the research work?
  - a) I enjoyed it, I was motivated and I enjoyed it.
  - b) I did not care.
  - c) The experimental work was superfluous, but I did it.
  - d) I have unpleasant memories of the experimental work.
9. What are your impressions of the written part of the survey?
  - a) I enjoyed it, I was motivated and I enjoyed it.
  - b) It did not bother me.
  - c) The writing was superfluous, but I did it.
  - d) I have unpleasant memories of the written part.
10. In your opinion, what role does a (school or external) mentor play?
  - a) Very important because the work has added value and the candidate benefits a lot from the research process.
  - b) Important, but the author of the thesis is responsible for everything.
  - c) Not important.
  - d) I have never thought about that.
11. What is your current relationship with your supervisor?
  - a) I have no contact with him/her.
  - b) I respect him/her because he/she put me on the right path, but we do not see/hear each other often.
  - c) I respect him/her because he/she put me on the right path, but we see/hear each other.





d) I am still in contact with him/her.

12. Do you feel that you have developed other skills through your research work that are not specifically related to experimental work (e. g. fluency, overcoming fears, standing up for your own results, personal development, responsibility, etc.)?

- a) Yes.
- b) No.
- c) I have never dealt with this specifically.
- d) I do not think research work involves other skills.

13. Were you aware that your research work could lead to future employment opportunities?

- a) Yes.
- b) No.

14. Did your research help you to choose a different course of study (if you are a student) or a job or to start your own business?

- c) Yes.
- a) No.
- b) I can not say.

15. Where do you see yourself in 10 years?

- a) In a career as a researcher or research professional.
- b) I will be working in a company where I can help develop products.
- c) I see myself as a teacher or professor encouraging young people to be creative.
- d) I see myself as a faculty member.
- e) As an independent entrepreneur.





# Cat Facial Action Coding System (CatFACS) and Scientific Illustration

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## Abstract:

This study explores the complex world of feline communication, focusing on analyzing facial expressions. It utilizes the Cat Facial Action Coding System (CatFACS) with art theory for scientific illustration, presenting a novel approach in this field. CatFACS is employed for a detailed, objective analysis of feline facial expressions. However, as CatFACS does not inherently examine the emotional component, art theory is integrated to interpret these expressions. This combination provides a comprehensive understanding of cats' emotional states by focusing on both observable physical cues and inferred emotional contexts. Integrating CatFACS with scientific illustration reveals intricate insights into feline emotions and communication. This approach uncovers complex behavioural patterns, enhancing the accuracy of behavioural interpretation. This study significantly contributes to feline welfare and understanding of human-cat relationships. Highlighting the importance of nuanced approaches in animal behaviour studies provides practical insights for pet owners and veterinary professionals, aiding in better understanding and caring for cats.

**Keywords:** Feline; Facial expression; Communication; Illustration



## 1. Introduction

Research into feline facial expressions is pivotal in unravelling the complexities of feline behaviour and enhancing human-cat interaction. Recognized as vital members of households worldwide, cats employ a variety of facial expressions as a means of communication. Deciphering these expressions is crucial for gaining deeper insights into their emotional states and fostering closer bonds between humans and their cats. This study specifically delves into the importance of studying feline facial expressions, utilizing scientific illustrations and advanced methods, such as the Cat Facial Action Coding System (CatFACS) (Caiero et al., 2017), decoding the nuanced language of cats. The face serves as a critical tool for visual communication in felines. Facial expressions, encompassing elements such as posture, gestures, and movement, provide critical indicators of a cat's internal state. Various visual tools are employed to analyze these expressions in depth. For instance, the Facial Grimace Scale (FGS) (Evangelista et al., 2019) and Geometric Morphometrics (GM) (SLice, 2007) offer detailed assessments. In pain evaluation in felines, professionals often rely on instruments like the UNESP-Botucatu Multidimensional Composite Pain Scale (Belli et al., 2021) and the Glasgow Composite Measure Pain Scale-Feline (rCMPS-F) (Reid et al., 2017). The Feline Grimace Scale© application is also available for cat owners, providing an accessible means to interpret their pets' discomfort or distress.

The primary aim of this article is to demonstrate how integrating CatFACS with scientific illustration can significantly enhance our understanding of feline facial expressions. This study advances welfare practices and deepens human-cat relationships by emphasizing the importance of nuanced approaches in animal behaviour studies, which are crucial for enhancing the well-being of domestic cats and strengthening their bonds with humans.

## 2. Methods and Results

### 2.1 Cat Facial Action Coding System (CatFACS)

The CatFACS manual is used to analyze the intricacies of feline facial movements. Like ethograms (Stanton, 2015), CatFACS is not an emotion labelling system. It adopts an anatomically-based, standardized, and systematic approach to studying feline facial behaviour. This manual enables the meticulous coding of individual cat movements on their faces, utilizing a comparative and evolutionary framework (Caiero et al., 2013). The Facial Action Coding System (FACS) provides a standardized coding system for individual movements or action units (AUs) involving specific muscle groups (Figure 1), generating various facial changes or cues. Action Descriptions (ADs) are also used to code broader movements or non-mimetic actions. Fifteen individual facial movements (Action Units), six miscellaneous movements (Action Descriptors), and seven Ear Action Descriptors have been identified in the domestic cat.

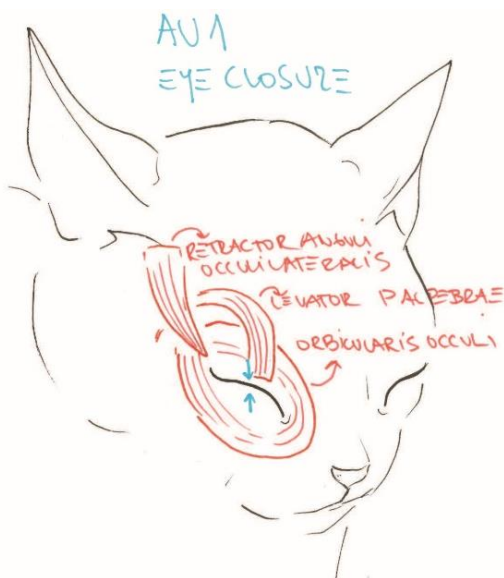


Figure 1: Illustration of the Action Unit 143 eye closure and muscles (P. Kovačič).



2.2 Observation and Measurement

Our observational and measurement procedures involve estimating time and distance and constructing ethograms to delineate behaviour patterns before, during, and after specific events involving objects or subjects in various locations. Initially, we establish a baseline for what is considered normal or neutral for each cat breed and individual. The system utilizes facial landmarks for coding to account for individual differences in facial morphology, such as the cephalic index, fatty deposits, and wrinkles. We acknowledge that visual communication is limited when studied as unimodal; thus, we combine it with other communication modes like olfactory, auditory, gustatory, and tactile. Furthermore, certain forms of signalling, such as vocalization, are adapted for bimodal or multimodal communication, especially with visuals.

Contextualizing the situation (**Figure 2**) allows us to measure various parameters, including the duration of emotions and emotional states, the time required for a cat to return to a normal state or behavioural homeostasis, and the spatial distance relative to the situation. Emotions manifest in the face; hence, the facial muscular plan is adapted to encompass muscle origin, insertion, fibre direction, and movement. We then focus on the natural face and spontaneous facial behaviour, explicitly targeting the head (neck, face, and ears). Spatial representation (cranial-caudal, dorsal, ventral, rostral-caudal; medial-lateral; proximal-distal) (**Figure 3**) mirrors planes in a spatial cross in art, albeit with slightly different terminology (frontal plane, medial plane, ocular plane, and ground plane) (**Figure 4**). This approach is significant for dividing the cat's face into planes and directions, facilitating a more straightforward interpretation.



Figure 2. Illustration of affiliative context.

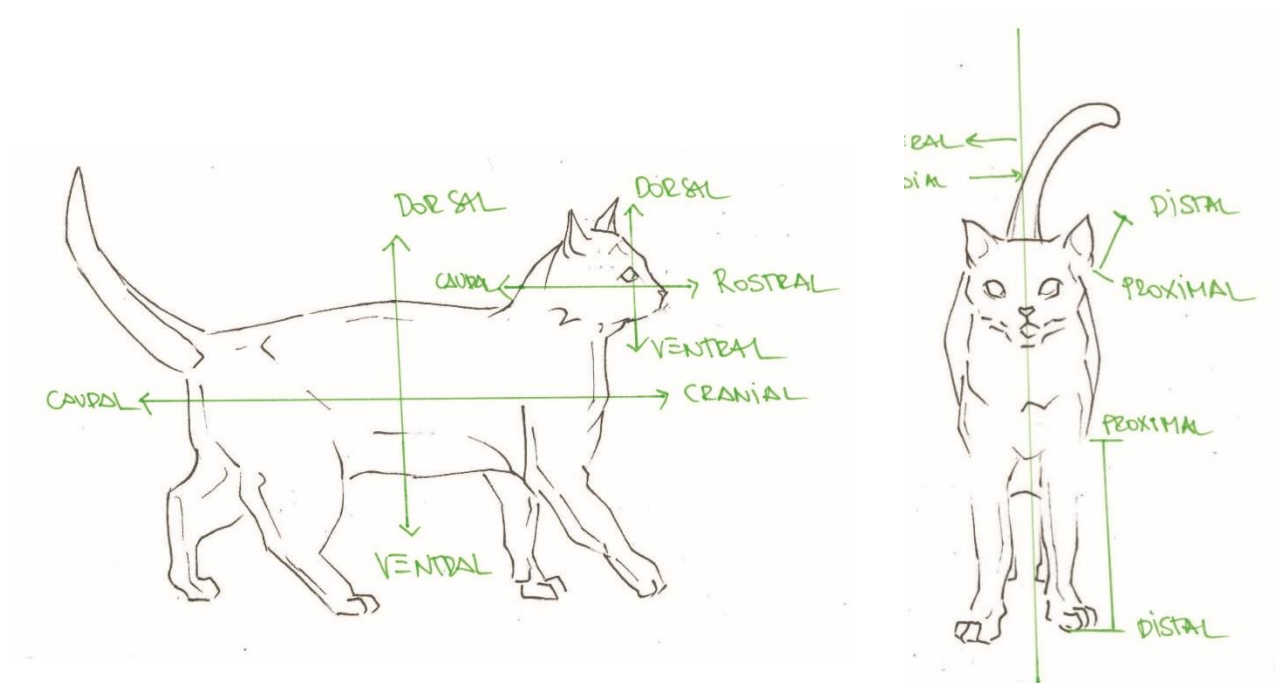


Figure 3. Illustration of spatial representation side view (left) and front view (right).

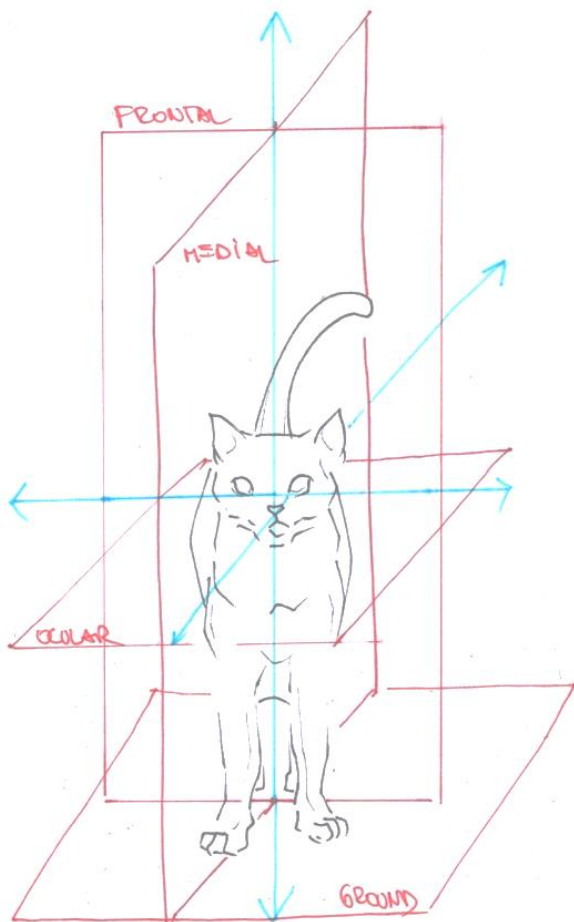


Figure 4. Illustration of the spatial cross.



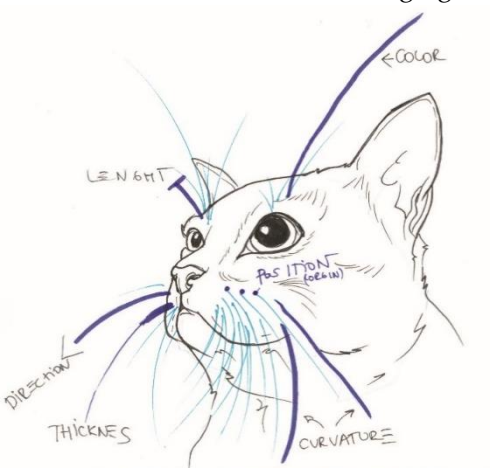
### 2.3 Scientific Illustration

The study employs scientific illustration for the rigorous analysis of feline facial expressions, body posture, limb gestures, movement, and spatial aspects, including location and distance (Case, 2010). This method enables a detailed focus on specific elements or a broader perspective through sketches and visual analyses. Techniques employed include highlighting, detailing, caricature, simplification, omission, cropping, enlargement, and comparison. Male (2017) states that such illustrations fall under non-fiction categories like documentary, referential, and instructional. Christiansen (2018) further describes these illustrations as both informative and metaphorical. The versatility of these methods allows for realistic, informative, and diagrammatic approaches, making scientific illustration indispensable for studying emotional states, motivational systems (Montag et al., 2017), behaviour, and communication in felines.

Analyzing social behaviour and visual communication through scientific illustration enables a nuanced understanding of feline communication. Artists achieve a connection between art theory and facial anatomy, viewing facial features as visual elements adhering to foundational art principles such as point, line, shape, geometric body, light-dark, and colour. Facial attributes like ears, eyes, nose, muzzle, and whiskers (Illustration 6) encompass various visual variables, including position, distance, direction, size, shape, weight, density, abundance, importance, and texture (Butina, 2003). Wrinkles, muscle tension, piloerection, colours, and patterns are also considered for comprehensive communication analysis. Combining these facial parts and movements with emotional components and contextual situations gives us an overview of felines' expressive repertoire and emotional spectrum. This approach allows us to transition seamlessly from analyzing individual facial features to understanding their role and relationships within the social-physical environment. Through this method, we utilize art as a conduit for scientific communication, harmonizing creativity with accuracy (Gould, 2023) and making the invisible aspects of feline communication visible (Christiansen, 2018).

### 2.4. Integration of Methodologies

Integrating the Cat Facial Action Coding System, observational and measurement techniques, and scientific illustration forms the backbone of our research approach. CatFACS provides a detailed and systematic method for coding feline facial movements. At the same time, our observational and measurement procedures offer a broader context for these expressions, considering the cat's overall behaviour and environment. Scientific illustration further enhances our understanding by visually representing these complex behaviours and expressions. Together, these methodologies create a comprehensive framework for analyzing and interpreting the multifaceted aspects of feline communication. A more complex approach is possible with this integrated approach to understanding feline behaviour, bridging the gap between scientific analysis and visual interpretation.



**Figure 5.** Illustration of art theory and anatomy.



### 3. Discussion

Integrating CatFACS with observational techniques and scientific illustration has provided a comprehensive understanding of feline communication. This study faced challenges in standardizing assessments across different breeds, highlighting the complexity of feline behaviour. However, these challenges were mitigated through a rigorous methodology and artistic interpretation. The findings have practical applications in veterinary practice and enhance pet owners' understanding, contributing significantly to animal behaviour research.

#### 3.1 *Integration of Methodologies and Their Implications*

The seamless transition from observational and measurement procedures to scientific illustration underscores the comprehensive nature of our study. We have gained a profound understanding of feline behaviour by meticulously constructing ethograms and establishing baselines for normal or neutral behaviours in various cat breeds. This detailed observation and contextual analysis have allowed us to measure various parameters crucial for interpreting feline emotions and reactions.

The integration of scientific illustration has significantly enhanced this process. The study has bridged the gap between cat visual communication and artistic expression by employing diverse artistic elements, variables, and compositions. This approach captures physical and physiological aspects and delves into the psychological realm of feline communication. The illustrator's role in organizing and presenting this information artistically has been instrumental in enriching the understanding of both researchers and viewers alike. This aspect of our study highlights the profound connection between scientific observation and artistic representation, as Butina (2020) emphasized, where scientific accuracy and artistic interpretation converge to provide a deeper understanding of the subject matter.

#### 3.2 *Enhancing Viewer Understanding Through Artistic Interpretation*

The illustrative aspect of our research plays a pivotal role in making complex scientific data more accessible and understandable to a broader audience. By translating intricate behavioural patterns into visual formats, we facilitate a better comprehension of feline behaviour among pet owners, veterinary professionals, and the general public. This approach contributes to academic discourse and has practical implications for improving human-feline interactions and welfare.

#### 3.3 *Future Directions*

This study's methodologies and findings open avenues for further research. Future studies could explore applying these techniques in different contexts or with diverse feline populations. The potential for developing more refined behavioural assessment tools and exploring other aspects of feline communication also presents exciting opportunities for continued research in this field.

### Conclusions

This study highlights the integral role of CatFACS and scientific illustration in advancing our understanding of feline behaviour and communication. By integrating these methodologies, we have gained novel insights into feline communication, significantly impacting welfare and human-cat relationships. This research contributes to academic knowledge and offers practical applications for veterinary professionals, feline behaviourists, and cat owners, fostering a deeper appreciation and understanding of our feline companions.

Future research directions include

- applying these techniques in different contexts or with diverse feline populations,
- aiming to develop more refined behavioural assessment tools and
- exploring other aspects of feline communication.

This study serves as a foundation for further exploration of felines' rich emotional lives, bridging the gap between scientific analysis and visual interpretation.





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

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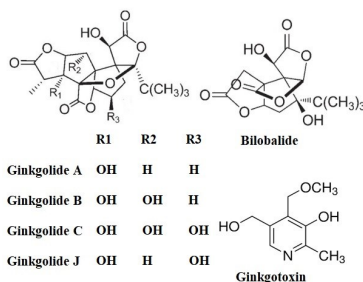
## A multiplex GC-MS/MS method for the quantitative analysis of active ingredients in *Ginkgo biloba* derivatives

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 Contact: Gabriella Pócsfalvi: gabriella.pocsfalvi@ibbr.cnr.it

### OUTLINE AND BACKGROUND

*Ginkgo biloba* (GB) is among the most studied herbal drugs. GB leaf extract standardized to contain 24% Ginkgo Flavone Glycosides and 6% Terpene lactones represents the only herbal alternative to synthetic antedementia drugs in the therapy of Alzheimer's disease and cognitive decline. GB seeds are used in traditional Chinese medicine to heal stomach pain, asthma, tuberculosis, and cognitive dysfunction, and in everyday life as a food snack. Here, our goal was to develop a multiplex analytical method based on gas chromatography-tandem mass spectrometry (GC-MS/MS) for the simultaneous quantitation of five terpene lactones (Ginkgolide A, B, C, J and Bilobalide) and Ginkgotoxin (Figure 1) in different *Ginkgo biloba* extracts.



**Figure 1:** Chemical structures of Ginkgolides A, B, C, J, Bilobalide and Ginkgotoxin

This study investigates the quantities of these primary ginkgo-specific ingredients in the methanol extract of ginkgo nuts, ginkgo cells from suspension and callus cultures, micro- and nanovesicles isolated from seeds as well as a commercial supplement (Figure 2). A previous study compared different brand products and found Bilobalide content 2.8-3.2 mg and Ginkgolides (A, B, C) content 2.9-3.2 mg using LC-ESI-MS/MS [1]. Another study analysed Ginkgotoxin content in *Ginkgo biloba* seed by LC-MS/MS and determined 174 ppm Ginkgotoxin values [2]. While there have been LC-MS/MS-based studies on terpene lactones that investigate commercial pharmaceutical products containing standardized extracts, no GC-MS/MS method is available for the simultaneous analysis of terpene lactones and ginkgotoxin.

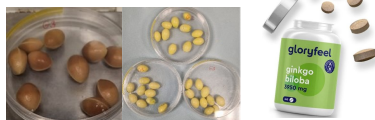
### EXPERIMENTAL METHODS

Callus and cell suspension cultures were established using GB seed's embryo (Figure 2). Micro- and nanovesicles (MVs and NVs) were isolated by differential ultracentrifugation from the homogenate of GB seeds (Figure 3). GB supplement was from Gloryfeel (Figure 4). Five biological replicates were prepared of the suspension and callus cultures, seeds and tablet, and triplicates for MVs and NVs samples. Dry and wet samples from suspension culture and callus were also prepared. Sample homogenization was performed in methanol using a Tissuelyser (Qiagen) at 30 Hz, 5 x 1 min. Samples were centrifuged at 15000 x g for 20 min and the supernatants were vacuum dried and then further dried under nitrogen flow.

Stable isotope labelled sugar alcohol, D-Mannitol-<sup>13</sup>C6 (600 ng) was added as internal standard and samples were derivatized using 50 µL silylation agent BSTFA:TMCS=99:1, 35 µL pyridine at 70 °C for 1 hour at 500 rpm.



**Figure 2:** GB seed's embryo, callus and cell suspension cultures



**Figure 3:** GB seeds



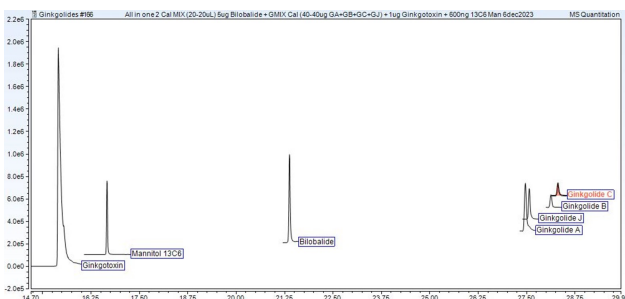
**Figure 4:** Gloryfeel GB supplement

GC-MS/MS analysis were performed using a Thermo Scientific Trace 1300 GC coupled to a TSQ 8000 Duo mass spectrometer instrument equipped with an electron ionization (EI) ion source, triple quadrupole ion analyzer (Figure 5). Separation was performed using a TG-SQC 30 m, 0.25 mm x 0.25 µm capillary column. GC parameters; measurement time: 35 minutes, column flow: 1.2 ml/min (helium), injection volume: 1.0 µL, injection type: splitless, inlet temperature 230 °C. MS parameters; transfer line temperature: 280 °C, ion source temperature: 250 °C.

GC temperature ramp; initial temperature: 70 °C hold for 1 min, ramp rate 1: 10 °C/min to 280 °C, ramp rate 2: 6 °C/min to 313 °C, ramp rate 3: 0.6 °C/min to 314 °C, ramp rate 4: 6 °C/min to 316 °C, ramp rate 5: 2.4 °C/min to 318 °C, ramp rate 6: 6 °C/min to 320 °C hold for 2 min.

### RESULTS

- ✓ We have set up a multiplex GC-MS/MS MRS quantitative analysis (Figure 5 and 7) for the simultaneous determination of six GB secondary metabolites (Figure 1) related to the GB bioactivity. The results of our quantitative analysis are reported in Table 3.
- ✓ Based on our analysis we found that the commercial supplement contained 7.77% terpene lactones per tablet.
- ✓ The seed samples contained 50 ppm Ginkgolide A and 14 ppm Ginkgolide B, but Ginkgolide C and J were below LOQ.
- ✓ Methanol extracts of *Ginkgo biloba* cells from callus and cell suspension, MVs and NVs contained considerably less bilobalides and ginkgolides than the seed and supplement extracts.
- ✓ we found that microvesicles have 269 ppm, nanovesicles 61 ppm, suspension culture in dry condition 3 ppm, seed 266 ppm and tablet 37 ppm Ginkgotoxin.



**Figure 6:** Representative MRM spectra of Ginkgotoxin, Bilobalide, Ginkgolide A, B, C, J and internal standard, the D-Mannitol-<sup>13</sup>C6 (MS quantitation)



**Figure 5:** GC-MS/MS

Compound	m/z Precursor / Product Ion	Collision Energy (eV)
Ginkgotoxin	280,1 / 206,1	10
	295,1 / 280,1	10
Mannitol <sup>13</sup> C6	426,1 / 264,1	10
	426,1 / 336,14	10
Bilobalide	299,1 / 271,1	10
	398,3 / 223,1	10
Ginkgolide A	537,2 / 187,0	25
	537,2 / 391,2	10
Ginkgolide B	625,3 / 479,2	10
	625,3 / 597,2	5
Ginkgolide C	713,3 / 567,1	10
	713,3 / 595,1	10
Ginkgolide J	478,3 / 264,2	10
	478,3 / 422,2	10

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

Compound	RT (min)	Calibration points	Range (ug)	R <sup>2</sup>	Slope
Ginkgolide A	27,53	5	0,50-50	0,9939	16,15
Ginkgolide B	28,20	5	0,50-50	0,9965	14,44
Ginkgolide C	28,35	5	0,50-50	0,9982	14,55
Ginkgolide J	27,60	5	0,50-50	0,9804	14,76
Bilobalide	21,41	6	0,05-30	0,9917	343,02
Ginkgotoxin	15,40	7	0,01-20	0,9944	13502,73

**Table 2:** Calibration data (retention times, ranges, R<sup>2</sup>, slopes)

Sample	Weight (mg)	Ginkgotoxin (ppm)	Bilobalide (ppm)	Ginkgolide A (ppm)	Ginkgolide B (ppm)	Ginkgolide C (ppm)	Ginkgolide J (ppm)
Nanovesicle (wet)	0,3	61	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Microvesicle (wet)	0,3	269	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Suspension culture (wet)	123,3-190,9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Suspension culture (dry)	11,97	3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Callus (wet)	127,8-182,9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Callus (dry)	12,41	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Seed (dry)	49,20-52,04	266	<LOQ	50	14	<LOQ	<LOQ
Tablet (dry)	274,05	37	11303	55525	4309	5008	1514

**Table 3:** Results of the analysis of nanovesicle, microvesicle, suspension culture, callus, seed and tablet – average values

### CONCLUSION

The ginkgolides are known to inhibit platelet aggregation, while the bilobalide protects against neuronal death caused by brain ischemia. On the other hand, ginkgotoxin is a neurotoxin structurally related to vitamin B6, naturally occurring in GB nuts and leaves. These natural ingredients are present at very different quantities in GB derivatives (cells, MVs, NVs, seeds and supplement) and their simultaneous quantitation is neither trivial nor easy. To achieve our goals, we have improved the separation of 4 ginkgolides in the GC timescale by the careful setting of temperature ramping. We have selected and optimized the SRM precursor and product ions transitions as well as the collision energy for each analyte. We found that the terpene lactone level of in vitro grown ginkgo cells, MVS and NVs is lower than the detection limit. In MVs we detected more ginkgotoxin than in NVs.

### References:

- [1] DOI: 10.1016/j.jpba.2009.05.040  
 [2] DOI: 10.1093/jaoac/83.6.1313

# Production and morphological properties of water-based cellular particles containing biologically active molecules from European spruce (*Picea abies*) needle homogenates by experimental and theoretical approaches



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## OUTLINE

## EXPERIMENTAL METHODS

Plants have the incredible ability to synthesize many of the active ingredients on which today's medicines and therapies are based. They are able to defend themselves against external influences (enemies) by releasing the corresponding molecules in individual parts of the plant. The European spruce (*Picea abies*) (Figure 1) contains a different composition of biologically active and complex substances. The composition of these substances depends on various factors, e.g. the geological composition of the soil, the climate, the time of harvest, the availability of nutrients, etc. (Jeran, et al. 2021).

Due to the accelerated development of medical diagnostics and the development of delivery systems, such as vesicles (liposomes), researchers began to look for alternative sources in the field of natural sources (Figure 2). Since cell particles of smaller size can also be found in plant samples and represent an alternative to biological fluids, research today also focuses on the development of cost-effective and reproducible methods for the isolation and characterization of these particles.

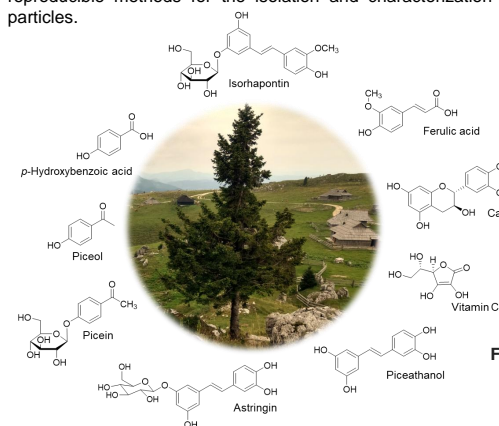


Figure 1: Example of structures of biologically active compounds in European spruce (Jeran, et al. 2021).

The samples of spruce needles were collected and processed by mechanical deformation of the plant tissue. The homogenates (in distilled water (dH<sub>2</sub>O), saline (0.9% NaCl) and 9.0% NaCl) were aged at room temperature and after achieving boiling point using microwaves. The formation of the resulting particles was investigated (Figure 3).

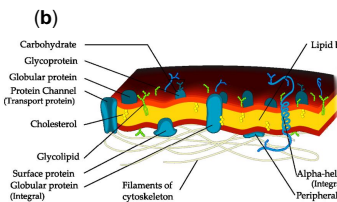
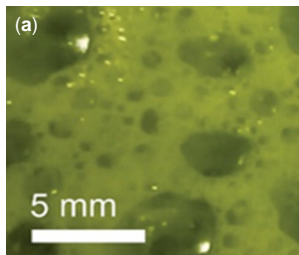


Figure 2: (a) Lipid content of the foam after homogenization of spruce needles (Jeran, et al. 2023). (b) A phospholipid bilayer as the main component of the cell membrane forming cellular vesicles (de Reggi, et al. 2021).

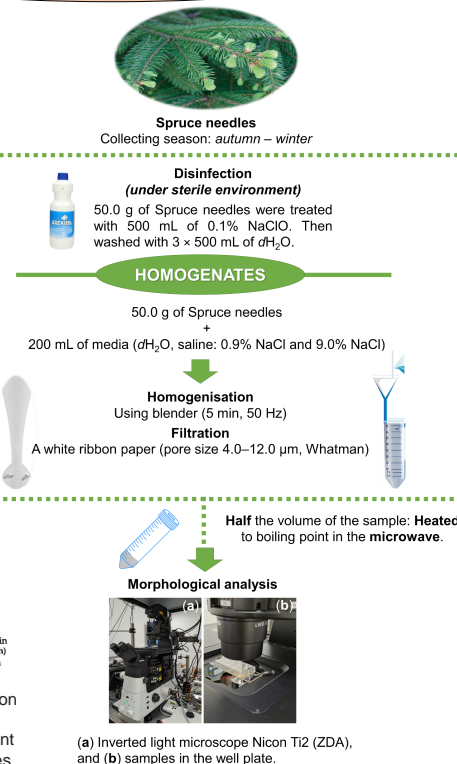
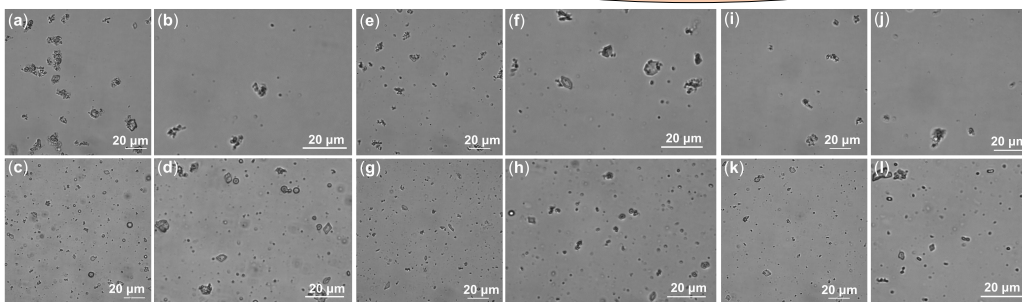


Figure 3: Flow chart of the experimental design for the preparation of homogenates from European spruce needles.

## RESULTS



double membrane resembling a cellular vesicle can also be seen. (i)-(j): The 0.9% and 9.0% NaCl solution allowed the opening of larger agglomerated particles and aggregates. (e)-(f): A lower concentration of cell particles and many particle clusters and agglomerates with the formation of mainly spherical particles with small dimensions can be seen. (k)-(l): A lot of material with smaller dimensions was formed under microwave conditions, slightly less if physiological conditions are included.

**We discovered mainly morphologically heterogeneous structures of different sizes and (mathematical) shapes.**

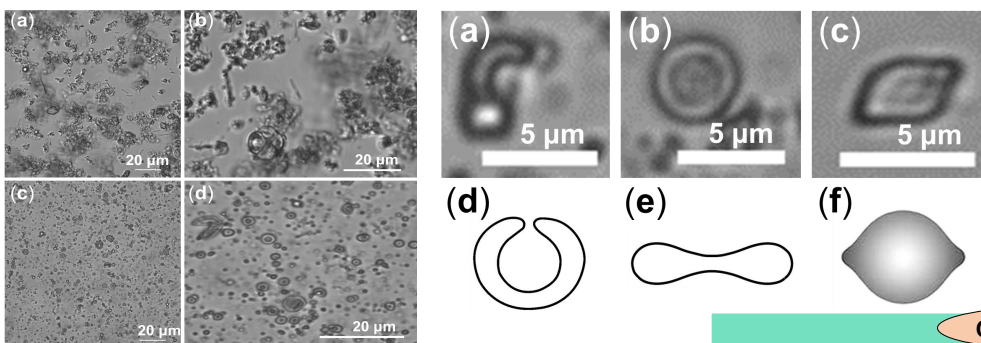


Figure 6: Spruce needle homogenate "aged" for 1 week at room temperature ((a)-(b)) showed an increased number of small cellular particles. An increased concentration of membrane-enclosed cell particles was found in the "aged" homogenate sample that was treated under microwaves before aging ((c)-(d)). They exhibited different morphological shapes and heterogeneous dimensions.

Figure 7: Cellular particles with smooth contours: (a)-(c) in a spruce needle homogenate on a model of a 9.0% NaCl solution, using microwaves, and (d)-(f) models of theoretical shapes corresponding to minimal energy membrane shapes (Jeran et al., 2023). The shapes of the particles formed in the homogenates of the spruce needles, which correspond to the theoretical shapes, were particularly pronounced in the homogenates prepared with the aid of microwaves. With appropriate addition of salt (0.9 and 9.0% NaCl), the above particles were also formed at room temperature. In general, several stomatocytes and discocytes were observed.

## CONCLUSION

The salt concentration has a decisive influence on the formation and morphology of the formatted cell particles. Compared to NaCl media, the dH<sub>2</sub>O media shows the formation of aggregates in the form of particle agglomerates, which can be easily broken down into smaller units with the assistance of thermal energy via microwaves. The formation of particles with a double-membrane was observed in all media used as well as in a one-week "aged" sample prepared in dH<sub>2</sub>O. Spruce needle homogenates contain particles with a minimal membrane energy that are similar in shape to particles of blood cells.

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The authors are thankful to Anja Jančič, Frida Benedek, Polonca Krasovec, and Milena Žohar for their technical support.



# MINIMALLY INVASIVE SURGERY : ENDOSCOPIC SPINE SURGERY

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## INTRODUCTION

Minimally invasive surgery (MIS) of the spine is an essential component of spine care. One type of MIS is endoscopic spine surgery (ESG), which allows surgeons to use a percutaneous approach to treat spinal pathology rather than the wide-open surgical exposure. The main objective of ESG is to minimize tissue trauma while achieving equivalent results to open spine surgery.

## PROCEDURE

The approach should be carried out percutaneously with a small working cannula, which serves two important purposes - This allows the use of local anesthesia or conscious sedation, thereby minimizing anesthesia risks and minimizing tissue damage. Second, access to spinal pathology should be gained via a working window to permit the endoscope and cannula to pass through to the spinal canal. Additionally, endoscopic visualization can provide a clear view of the spinal pathology.

## BENEFITS

First, the procedure involves less tissue trauma due to a smaller skin incision, less tissue dissection, and less bone removed to access the spinal canal. Second, reduced operative time, quicker recovery due to less tissue damage, less use of narcotic medication after surgery, fewer wound complications, and quicker return to work. The mean hospital stay was 12 hours and average time to return to work was 6.79 weeks.



FIGURE 1: Intraoperative view of an endoscopic disectomy at L5-S1 below a previous L5-S1 fusion



FIGURE 2: A large disc fragment is removed through a working sheath

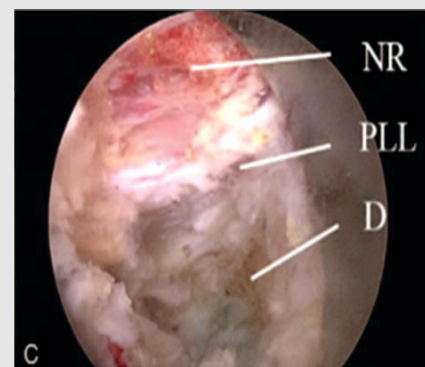


FIGURE 3: Anatomical details are well demonstrated including decompressed NR, the PLL and the maternal disc

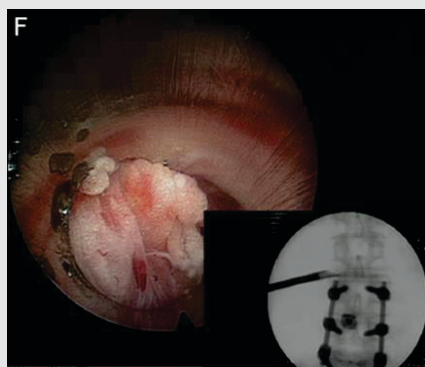
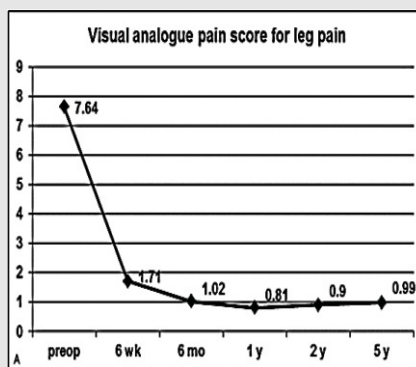
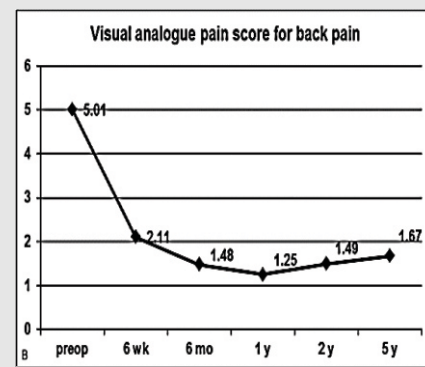


FIGURE 4: Anteroposterior fluoroscopic image of the area of the decompressed exiting nerve



GRAPH 1: Visual analogue pain score for leg pain



GRAPH 2: Visual analogue pain score for back pain

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# Decoding the facial expressions of cats: Insights through scientific illustration

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OUTLINE	METHODS	CONCLUSION
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The prospective doctoral thesis delves into the intricate realm of feline visual communication. This poster centers on the analysis of facial expressions employing scientific illustrations and advanced research methods. The objective is to elucidate the complexities associated with how cats convey emotions and intentions through facial cues. The examination of feline facial expressions (Illustration 1) contributes to the fields of neuroethology and psychobiology, fostering a more nuanced comprehension of feline behavior. By addressing the scientific facets of feline visual communication, the endeavor aims to bridge the divide between empirical research and practical insights into interactions with cats.

**Preparation for Illustration: Blending Art and Science in Capturing Cat Expressions**  
 Engaging in scholarly discussions, collaborating closely with scientists and peers, and understanding the intricacies of feline behavior lay the foundation for this artistic endeavor. Before illustrating cat facial expressions, the imperative step involves gathering photo-video references curating a comprehensive repository capturing cats in various emotional states. Illustrators employ resources like anatomy books, 3D models, and animation apps, complemented by firsthand observation of real cats (Illustration 2) or exploration of taxidermed specimens. This amalgamation ensures the sustained vitality of the project over months or even years.

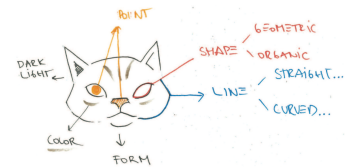
Our research significantly contributes to understanding feline behavior, emphasizing the critical role of advanced methods and scientific illustrations, particularly in exploring facial expressions. Thorough research is imperative due to the complex nature of feline communication. Achieving compassionate coexistence requires an interdisciplinary approach, merging science and art to educate and foster harmony among individuals passionate about felines. In conclusion, our study deepens the understanding of feline behavior, highlighting the urgency for in-depth exploration, especially in facial expressions, utilizing advanced analytical methods and scientific illustrations. Feline communication, with its captivating yet intricate nature, calls for a nuanced approach to comprehending their subtle nonverbal signals.



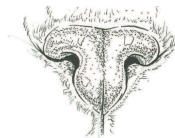
**Illustration 1:** Portrait of domestic cat (P. Kovačič).

**From Cat Visual Communication to Art Representation**  
 The focus can be on shapes (2D), bodies (3D), or forms, representing the highest level of organization of visual elements or how something is expressed. Visual variables provide constant sensory characteristics and emotional qualities relating to size, weight, position, direction, and the relationship between position and weight. These elements assist in examining and interpreting observations, facilitating the transformation of these observations into visual expressions (Illustration 3). They are crucial in simplifying complex information and identifying parallels and patterns, enabling an effective explanation through suitable visual language.

**Example:**  
 The cat's face is a form composed of visual elements (Illustration 4) or facial organs: ears, eyes, nose, muzzle, whiskers, fur, and wrinkles, interconnected in relationships and distances. Each element possesses its position, distance, shape, size, thickness, direction, weight, structure, texture, contrast, and gestures or expressions. These elements transform and move in response to external stimuli or internal emotional states, revealing the cat's soul's manifestation in the body. By sketching, simplifying, or using diagrams, we interpret expressions, deciding how to present a part (Illustration 5) or the entire face based on our study's essential aspects.

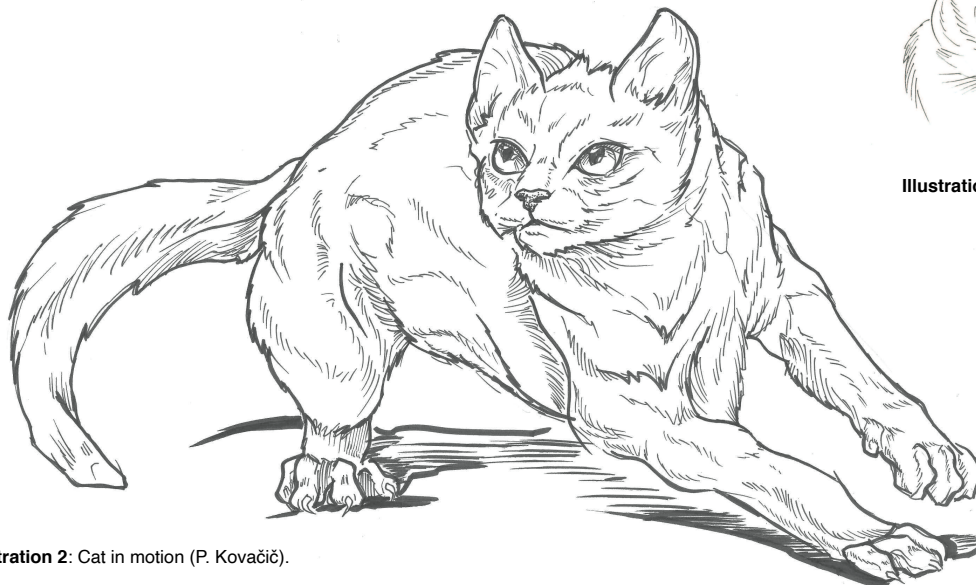


**Illustration 4:** Visual elements (P. Kovačič).



**Illustration 5:** Nose texture and form study (P. Kovačič).

**RESULTS ARE ILLUSTRATIONS**



**Illustration 2:** Cat in motion (P. Kovačič).



**Illustration 3:** Cat facial expression (P. Kovačič).

# Sodium disulfonate salt as a water-soluble fluorescence-active fluorescein analogue and its activity in labelling urothelial urinary bladder cancer cells



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## OUTLINE

## EXPERIMENTAL DESIGN

**Bladder cancer** is the ninth most common cancer in the world. Its detection is difficult, as the clinical symptoms can be very similar to other not so severe bladder diseases. Often it is detected too late, and can consequently prove fatal. In addition to the latter, bladder cancer cells appear multifocally, i.e. in many separate spots in the bladder, so it is difficult to completely remove them by operation. The described fact indicates a high probability of a further recurrence of the disease (Zupančič, 2011). Some cases include *in situ* and non-invasive papillary carcinoma (the Ta and Tis stages). The Register of Cancer of the Republic of Slovenia (RRRS) for all years follows a rule that T1-T4 bladder cancers are included in the incidence of cancer, especially in situ cases with non-invasive papillary carcinoma (Zupančič, 2011).

In the **urinary bladder**, urine is only temporarily stored until being conveniently excreted. Since its composition should not change during rest, the bladder has a specifically built epithelium – **urothelium**. Urothelium covers most of the lower part of the urinary tract, i.e. proximal urethra, urinary bladder, ureter and renal pelvis (Romih *et al.*, 2005). Its task is to form a blood-urine barrier that prevents the returning of toxins, water, ammonia and ions from the urine into the blood (Erdani Kreft, *et al.* 2010).

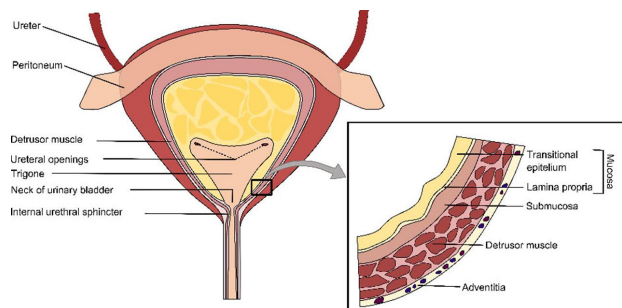


Figure 1: Urinary bladder.

The aim of this study was to synthesize the fluorescent active substance SUF (sodium 3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-4',5'-disulfonate (according to reference (Kostaskova, 2010)) and to test its application in medicine. The aim of the work is to find new methods and reagents that could replace expensive and time-consuming procedures for obtaining fluorescent dyes in the field of cell biology. According to the innovative synthetic approach, SUF is suitable for large-scale production because the production route is fast, simple and very efficient (high yield). We believe that the proposed method could improve efficiency in tumor detection and advance cell biology in general. We hypothesized that the compound SUF could be used to distinguish cancerous urothelial cells from healthy (normal) cells by differences in fluorescence intensity and/or distribution (Jeran, *et al.* 2019).

### Synthesis of fluorescence activity SUF

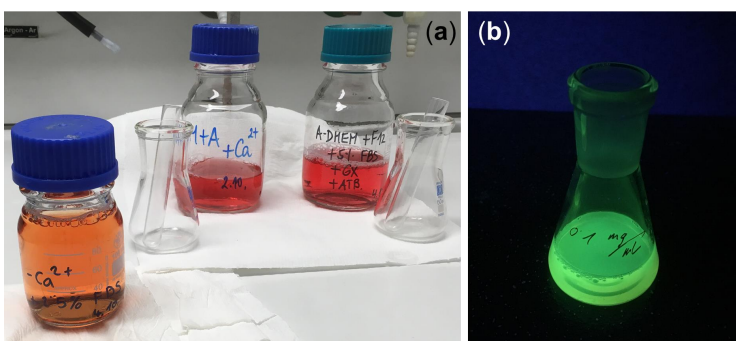
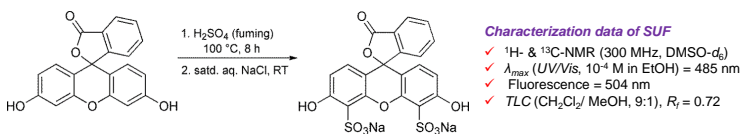


Figure 2: SUF is soluble in polar and non-polar media. (a) SUF solution in water-based (polar) media before application to the cells, and (b) its fluorescence under UV-light. The fluorescence of SUF was only detected in polar solvents and was stronger at lower concentrations (0.1 mg/mL or 100 µg/mL). The light emission was stronger under UV-light at 366 nm.

## RESULTS

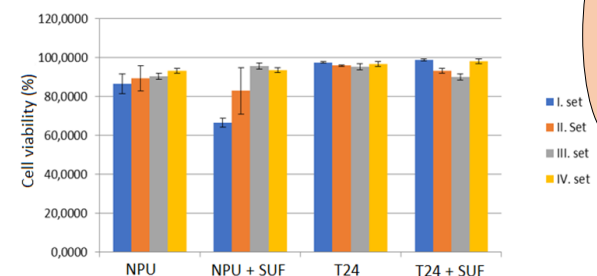


Figure 3: Comparison of the viability of normal (NPU) and cancer cells (T24) treated with SUF dye. Each set represents an independent experiment. A repetition of the viability test.

We repeated the counting with the hemocytometer method and the analysis sixty times, as NPU cells grow slowly and are more demanding to cultivate. Due to the relatively small number of cells, the deviation of the results can be very large, which in our case was solved by calculating the mean values of several repetitions. The average values of all four cell viability analyses indicate that the SUF dye at a concentration of 100 µg/mL does not affect the viability of the urothelial cells and may be suitable for further *in vitro* and *in vivo* applications.

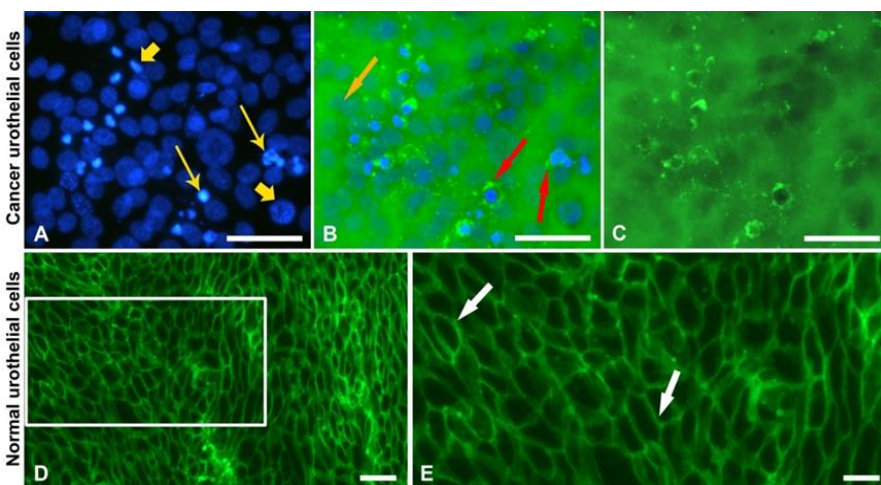


Figure 4: Fluorescence microscopy. Cancer and normal urothelial cells labelled with SUF. A: Cells labelled with DAPI (blue nuclei), fragmented nuclei (yellow thin arrows; chromosomes of cells in mitosis (yellow thick arrows). B: Cells labelled with DAPI (blue nuclei) and SUF (green). Cancer urothelial cells with fragmented nuclei (yellow thin arrows) and membrane compartments with the compound SUF (red arrows), a cancer urothelial cell with normal nucleus and diffuse labelling with SUF (orange thin arrow). C: The same field of view as in A and B, but only SUF is visible. D and E: Differentiated normal urothelial cells labelled with SUF (green). In E, the area within the white box is enlarged. The white arrows show the SUF labelling of the apicolateral part of the cells. Scale bar 50 µm in A, B, C and E, and 100 µm in D.

SUF mostly accumulated in the areas between the apical and basolateral cell membranes, resulting in a contrast between the fluorescent light emitted from inside and outside the cell. Since SUF does not fluoresce in non-polar media, we ruled out the possibility of binding to the lateral cell membranes. In cancer cells, we assumed that the dye enters their interior by endocytosis and causes labelling of the endocytotic compartments. From this we concluded that under *in vitro* conditions it is possible to distinguish between cancer cells and normal urothelial cells.

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## CONCLUSION

The compound showed potential for use in the field of medical applications. Due to its chemical structure, it was found to be non-toxic and can be used as a cell marker. We have shown experimentally that the compound does not damage the cells and can differentiate between normal and cancerous urothelial cells. The results of the viability tests showed no differences between the controls (cells grown without SUF) and the samples (cells grown in the presence of SUF). Examination of the cells with a fluorescence microscope, which showed cells at different stages of cell division, confirmed that SUF does not inhibit cell division. Based on the results obtained, we assume that the compound SUF is not cytotoxic. However, further studies are required for a definitive answer.



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# Lithium-ion battery project: ESTEAM perspectives

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## OUTLINE

This poster was initiated within the framework of the ESTEAM Mentorship programme, with the objective of fostering coachability, self-awareness, and allocating time to understand each other's needs.

ESTEAM stands for Entrepreneurship, Science, Technology, Engineering, Arts and Mathematics) have been and will be organised in 19 EU Member States with the aim to boost women and girls' competences, inspire them, and give them the chance to connect with like-minded peers. ESTEAM online communities for women and girls are being built to sustain the momentum of the events and to allow them to keep learning and connecting with their peers online. Throughout the communities' workshops and activities, participants learn about ESTEAM career paths and are inspired by women role models who thrive in these domains, while developing not only their digital and entrepreneurial competences but also their collaboration, creativity, problem-solving and public speaking skills.

## EXPERIMENT

The participants were engaged in discussions on various topics of lithium through the lens of Mendeleev's table of elements that are mutually beneficial for the mentor and mentee's development.

The inception of the experiment was driven by the aspiration to uncover connections between Science and Art. Lithium-ion batteries, ubiquitous in our daily lives, powering smartphones and laptops that have transformed modern society, are now poised to revolutionize the transportation sector through electric cars, buses, and bikes.

The research focused on a pivotal component of batteries – lithium – tracing its origins and discovery back to Mendeleev's Periodic Table.

## CONCLUSION

Throughout the experiment, a fascinating revelation emerged: just as Mendeleev's Periodic Table delineates the elements in Chemistry, a comparable structure can be identified in Music.

Upon closer examination of these systems, it becomes apparent that, by adhering to certain principles, Mendeleev's lithium corresponds to a measure in the Periodic Table of Music Notation.



## RESEARCH

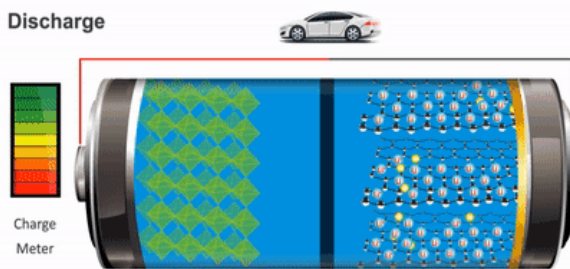


Figure 1. How lithium-ion battery works?

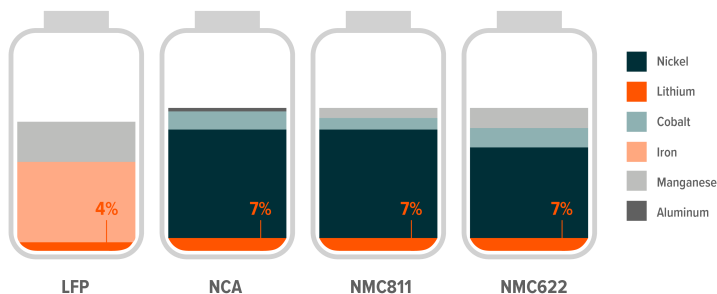


Figure 2. Metal content by battery chemistry

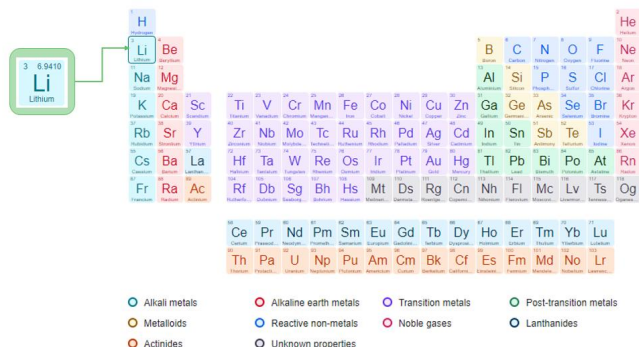


Figure 3. Mendeleev's Periodic Table.



Figure 4. Periodic Table of Music Notation

*There's antimony, arsenic, aluminum, selenium  
And hydrogen and oxygen and nitrogen and rhenium  
And nickel, neodymium, neptunium, germanium  
And iron, americium, ruthenium, uranium  
Europium, zirconium, lutetium, vanadium  
And lanthanum and osmium and astatine and radium  
And gold protactinium and indium and gallium  
And iodine and thorium and thulium and thallium  
There's yttrium, ytterbium, actinium, rubidium  
And boron, gadolinium, niobium, iridium  
And strontium and silicon and silver and samarium  
And bismuth, bromine, lithium, beryllium, and barium  
There's holmium and helium and hafnium and erbium  
And phosphorus and francium and fluorine and terbium  
And manganese and mercury, molybdenum, magnesium  
Dysprosium and scandium and cerium and cesium  
And lead, praseodymium and platinum, plutonium  
Palladium, promethium, potassium, polonium  
And tantalum, technetium, titanium, tellurium  
And cadmium and calcium and chromium and curium  
There's sulfur, callifornium and fermium, Berkelium  
And also mendelevium, einsteinium, nobelium  
And argon, krypton, neon, radon, xenon, zinc and rhodium  
And chlorine, carbon, cobalt, copper, tungsten, tin and sodium  
These are the only ones of which the news has come to Piggy  
And there may be many others but they haven't been discovered*

Tom Lehrer – The Elements (Live Film from Copenhagen in 1967)

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# Hybridosomes from *Dunaliella tertiolecta* and *Phaeodactylum tricornutum* sludge

P7

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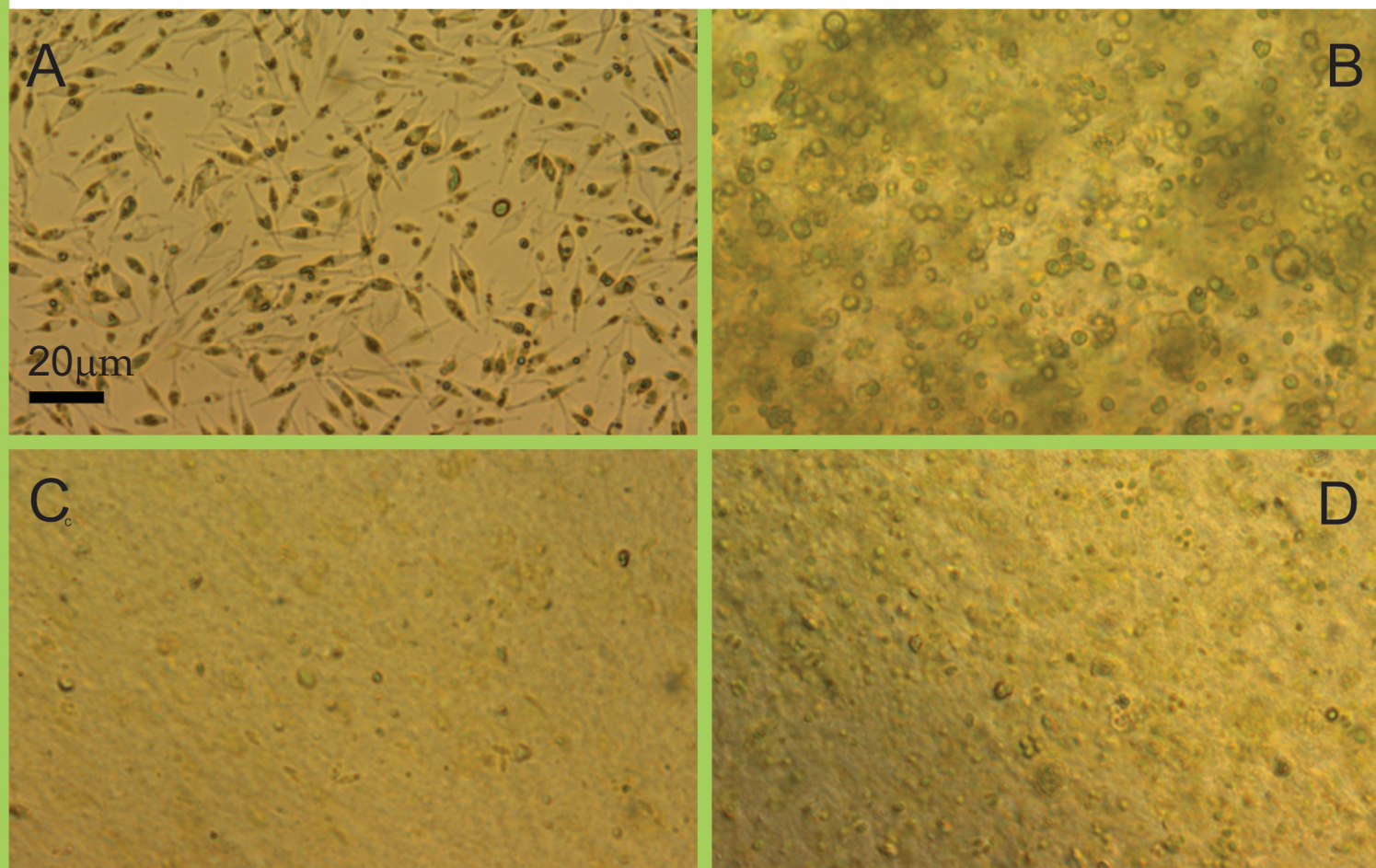


Figure 1 A: Sludge #2 (*Phaeodactylum tricornutum*), B: Sludge #7 (*Dunaliella tertiolecta*), C: Hybridosomes from sludge #2, D: Hybridosomes from sludge #7.

## Methods

### 2.1. Cultivation of microalgae

Cultures of *D. tertiolecta* CCAP 19/22, and *P. tricornutum* CCAP 1052/1A from the Culture Collection of Algae and Protozoa (CCAP) of SAMS (Oban, Scotland) were grown in mineral water Radenska naturelle supplemented with Guillard's (F/2) Marine Water Enrichment Solution (ref. G0154, Sigma Aldrich, USA) in proportion 20 mL of F/2 per one litre of mineral water [1]. Culture was grown in a room at 18 °C with natural light.

### 2.2. Design of the experiment with microalgae

About 500 ml of conditioned media with microalgae was aliquoted into 50 mL falcon tubes and centrifuged at 300g and 18°C for 10 minutes to sediment microalgae. The pellets were gathered and centrifuged at 2000g and 18°C for 10 minutes to concentrate the sludge. The sludge was spread on petri dishes and left in the digestorium for 1 hour under the UV light. Then a part of the sludge was saved at 4 °C while a part was used to prepare hybridosomes.

### 2.3. Preparation of hybridosomes

Hybridosomes were prepared by mixing appropriate proportions of lyophilized soya lecithin granules, glycerol and microalgae sludge, at room temperature. Soyabean lecithin granules were placed into the falcon tubes. Sludge was added and the suspension was left at room temperature for 1 hour. Glycerol was added and the samples were mixed mechanically (manually) with metallic stick until the ingredients formed a uniform cream-line consistency. The samples were kept at room temperature.

### 2.4. Interferometric light microscopy of hybridosomes [2]

The average hydrodynamic diameter ( $D_h$ ) and the number density of EPs were determined by ILM using Videodrop (Myriade, Paris, France). Signal from the medium (physiological saline) was under the detection limit. The threshold value of 3.8 was used. Seven microliters of sample were placed between cover glasses and illuminated by 2 W of blue LED light. The light scattered on the particle was imaged by a bright-field microscope objective and allowed to interfere with the incoming light. The image was recorded by a complementary metal-oxide-semiconductor high-resolution high-speed camera. Interference enhances the information in the scattered light. The contribution of the incident light was subtracted from the detected image. The obtained pattern, which includes contrasting black and white spots, was recognized as a particle, and its position in the sample was assessed. The number density of the particles is the number of detected particles within the detected volume, which depends on the microscope characteristics and the particles' size. The typical detection volume was 15 pL. Hydrodynamic diameter  $D_h$  was estimated by tracking the position of the imaged particle within the recorded movie. The diffusion coefficient  $D$  of the motion of the particle is taken to be proportional to the mean square displacement  $d$  of the particle between two consecutive frames taken in the time interval  $\Delta t$ ,  $\langle d^2(\Delta t) \rangle = \langle 4D \Delta t \rangle$ , while the hydrodynamic diameter was estimated by assuming that the particles were spherical and using the Stokes-Einstein relation  $D_s = kT/3\pi\eta D$ . Each particle that was included in the analysis was tracked and processed individually, and the respective incident light signal was subtracted from each image. Processing of the images and the movies was performed by using the associated software, QVIR 2.6.0 (Myriade, Paris, France). We measured each sample 3 times. Samples were measured undiluted.

### 2.5. Light microscopy

Samples were put on cover glasses and observed under optical microscope Nikon microscope Nikon Eclipse TE2000S with CCD 512 Digital Camera System SPOT BOOST (Visitron Systems, Germany).

### References

- Božič D, et al. Ultrastructure and stability of cellular nanoparticles isolated from *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* conditioned media Open Res Europe 2022, 2:121
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## Results

Sample	Number density ( $10^9/\text{ml}$ )	SD ( $10^9/\text{ml}$ )	Dh (nm)	SD (nm)	N tracked
Sludge #7	102.00	7.00	332.00	227.00	688.00
Hybridosomes #7	27,000.00	1,680.00	269.00	129.00	1,031.00
Sludge #2	95.00	8.00	356.00	164.00	320.00
Hybridosomes #2	18,700.00	2,350.00	295.00	179.00	645.00
Liposomes	25,200.00	655.00	287.00	147.00	622.00

## Conclusion

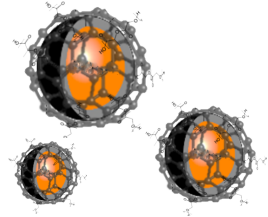
Creation of hybridosomes from microalgae sludge increases the number density of particles composed of natural source for two orders of magnitude.



# New Approaches for Testing the (Geno)Toxic Activity of Nanoparticles *In Vitro*

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## OUTLINE

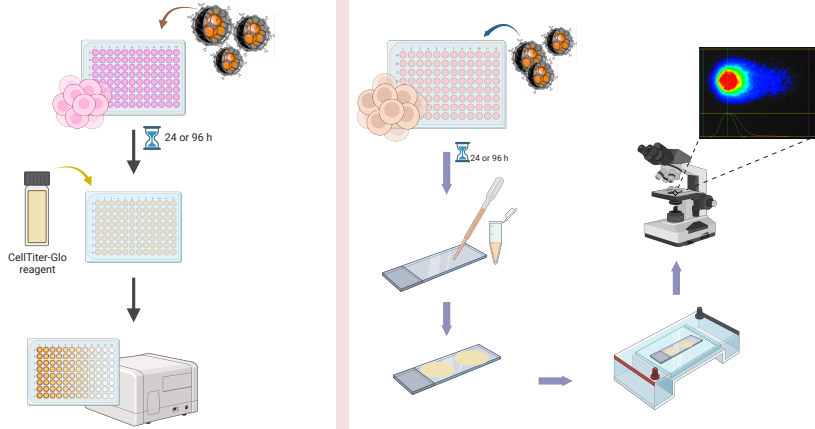
In the last decade, the production and use of nanomaterials has grown tremendously, and as a result, so has human exposure to these materials. Since human exposure to nanoparticles is inevitable much attention has been drawn to nanoparticle toxicology – especially to the potential acute and chronic adverse effects that nanoparticles may cause on humans – mostly because nanoparticles due to their high surface-to-volume ratio, high reactivity, and unique physical, chemical, and biological properties exhibit a greater risk of toxicity than the corresponding bulk material.

The study of nanoparticle adverse effects and toxicity is referred to as nanotoxicology and even though exposure to nanoparticles is increasing, information on their toxicological properties remains inadequate, especially when it comes to the question which *in vitro* model would be most appropriate for nanoparticle toxicity assessment.

Within the Department of Genetic Toxicology and Cancer Biology at the National Institute of Biology and as part of my PhD research related to the H2020 project NESTOR (GA: 101007629) and J1-4395 project NaNoZymSafe we develop 3D cell models as a new *in vitro* methodological approach for nanoparticle (geno)toxicity assessment to better understand the impact nanomaterials have on environmental and human health.

## Methods

The *in vitro* cyto- and genotoxicity of tested iron-based core-shell nanoparticles were assessed in the HepG2 cell line, using the CellTiter-Glo® assay (left) and the comet assay (right).



## Results

The results cannot be published due to still ongoing testing and certain reservation about data publishing since the data gathered from this research are part of a still unpublished article.

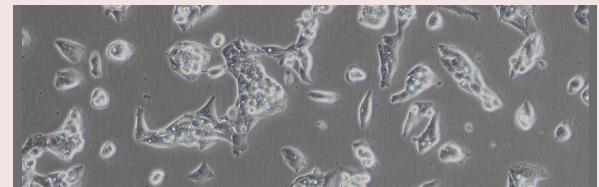
## *In Vitro* Cell Models

### Advantages of a 2D cell model:

- inexpensive
- well established model
- easier maintenance
- easy performance of functional tests

### Disadvantages of a 2D cell model:

- lacking complex cell-cell and cell-environment interactions
- unrestricted access to essential compounds
- no cellular microenvironment
- different cell morphology and molecular mechanisms compared to *in vivo*



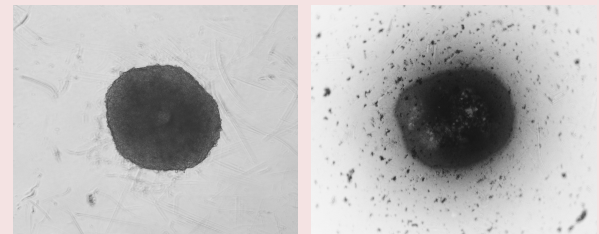
HepG2 cells in a monolayer.

### Advantages of a 3D cell model:

- production of a matrix that promotes tissue-specific cell binding
- direct cell-to-cell interactions
- cell-to-extracellular matrix interactions
- preservation of natural morphology
- preservation of high viability for several weeks
- gene and protein expression levels better resemble levels found *in vivo*

### Disadvantages of a 3D cell model:

- slower culture formation
- more complex procedures
- higher reagent cost
- fewer commercially available tests
- more demanding analysis



Spheroid in cell media (no exposure to nanoparticles).

Spheroid exposed to nanoparticles at a concentration of 10 µg/mL.

# Discovery of a New Candidate Type I Toxin-Antitoxin System in the Cyanobacterium *Microcystis aeruginosa* PCC 7806

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## Introduction

Toxin-antitoxin (TA) systems are genetic elements that encode a **stable toxin** and a **labile antitoxin**. In type I systems, the antitoxin is a short non-coding RNA that prevents toxin translation by forming base pairs with the toxin mRNA (Fig. 1). The majority of type I toxins are short membrane proteins. Some disturb membrane integrity through pore formation while others cause nucleoid condensation<sup>1</sup>.

The notorious bloom-forming cyanobacterium *Microcystis aeruginosa* is especially interesting in terms of TA systems as it contains the highest number of type II TA loci of all bacteria<sup>2</sup>. Despite this, only one locus has been experimentally studied up to now<sup>3</sup> while type I TA systems have not been found in this species so far.

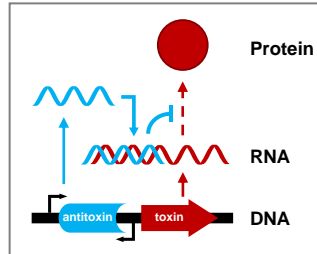


Figure 1: Schematic representation of toxin translation control in type I TA systems.

## Aim of the study

Since *M. aeruginosa* contains the highest number of type II TA systems of all bacteria, we set out to figure out whether it also contains type I systems. Our aim was to:

1. Perform a bioinformatic search for hypothetical type I TA systems in *M. aeruginosa* PCC 7806.
2. Test the effect of expression of predicted type I toxins on *Escherichia coli* cell growth.
3. Indirectly confirm the existence of the predicted cognate type I antitoxins by confirming the biologic activity of their promoters.

## Bioinformatic search

Our bioinformatic analysis (Fig. 2) revealed **fifteen candidate type I TA loci**.

We chose the **six most prospective loci** for experimental characterisation: BH695\_0311, BH695\_0320, BH695\_3336, BH695\_4017, BH695\_4989, and BH695\_5020.

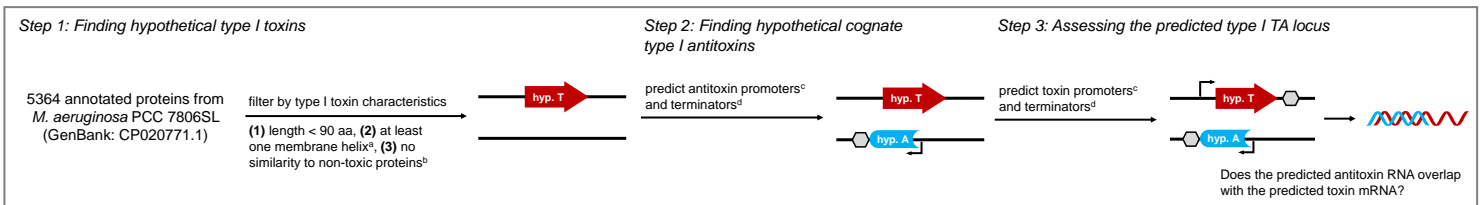


Figure 2: Schematic representation of our bioinformatic search for hypothetical type I TA systems in *M. aeruginosa* PCC 7806. \*predicted using TMHMM 2.0, †using BLASTP, ‡predicted using bTSSfinder and BPROM, §predicted using ARNold and FindTerm.

## Expression of six predicted type I toxins

Recombinant *E. coli* BL21(DE3) pLysS were cultured in M9CK minimal medium. IPTG was added at OD<sub>600</sub> 0.4 – 0.6 to induce predicted type I toxin expression.

Expression of BH695\_0320 and BH695\_4017 has a **negative effect on *E. coli* cell growth**. Expression of the remaining four predicted type I toxins has **no effect** (Fig. 3).

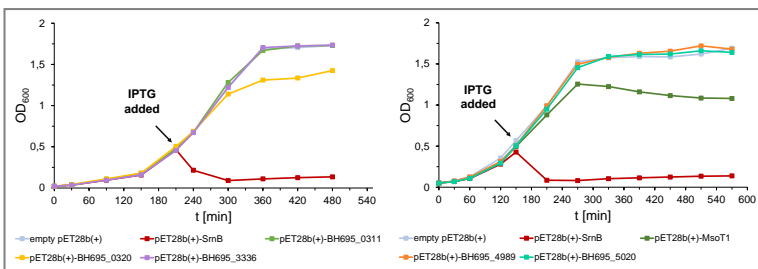
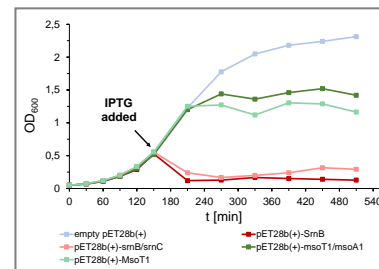


Figure 3: Growth curves of *E. coli* BL21(DE3) pLysS expressing different predicted type I toxins under the control of the inducible P<sub>77</sub> promoter. Cells carrying empty pET28b(+) served as a negative control (light blue). Cells expressing the Smb type I toxin from *E. coli* under the control of P<sub>77</sub> served as a positive control (red).

## Further characterisation of MsoT1/MsoA1

Focus on BH695\_4017 → renaming to **MsoT1** and its predicted cognate antitoxin to **MsoA1**.

Expression of MsoT1 has a **delayed toxic effect** when the plasmid also contains MsoA1 under the control of its predicted P<sub>MsoA1</sub> promoter (Fig. 4).



→ **MsoA1 is expressed under the control of P<sub>MsoA1</sub>**.

Figure 4: Growth curves of *E. coli* BL21(DE3) pLysS expressing MsoT1 under the control of the inducible P<sub>77</sub> promoter with the plasmid also containing MsoA1 under the control of its predicted P<sub>MsoA1</sub> promoter (dark green) and without the presence of MsoA1 (light green). Cells carrying empty pET28b(+) served as a negative control (light blue). Cells expressing the Smb type I toxin from *E. coli* under the control of P<sub>77</sub> served as a positive control (red). Cells expressing Smb under the control of P<sub>77</sub> with the plasmid also containing its cognate antitoxin Smb under the control of its native P<sub>Smb</sub> promoter served as an additional positive control (pink).

## Biologic activity of MsoA1 antitoxin promoters

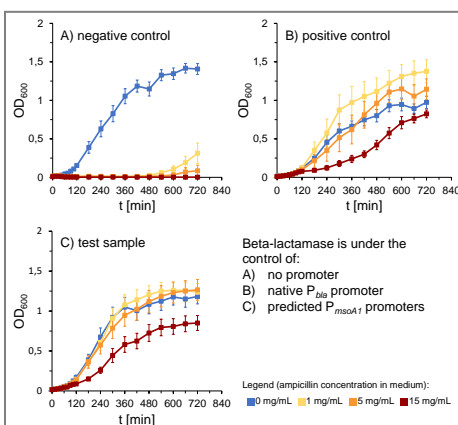


Figure 4: Growth curves of *E. coli* BL21(DE3) in LBC media with different concentrations of ampicillin.

Reporter: beta-lactamase (grants cells resistance to the antibiotic ampicillin).

Recombinant *E. coli* BL21(DE3) were cultured in LBC medium with different concentrations of ampicillin.

Cells carrying the plasmid where beta-lactamase is under the control of the predicted P<sub>MsoA1</sub> antitoxin promoters grow in media containing high concentrations of ampicillin (Fig. 4).

→ **P<sub>MsoA1</sub> antitoxin promoters are biologically active.**

## Conclusion

Our results suggest MsoT1/MsoA1 to be a novel type I TA system as well as the first type I TA system discovered in cyanobacteria.

## Future prospects

Test the effect of expression of predicted type I toxins on cyanobacterial cell growth → compare the results with the ones from *E. coli*. Confirm the existence of the predicted cognate type I antitoxins directly (RT-PCR, *in vitro* translation).

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2. Y. Xie, Y. Wei, Y. Shen, X. Li, H. Zhou, C. Tai, Z. Deng, H.-Y. Ou: TADB 2.0: an updated database of bacterial type II toxin-antitoxin loci. *Nucleic Acids Res* 2018, 46, D749-D753.
3. M. Klemenčič, A. Halkizan Vasile, M. Dolinar: The cysteine protease MaOC1, a prokaryotic caspase homolog, cleaves the antitoxin of a Type II toxin-antitoxin system. *Front Microbiol* 2021, 12, 1-13.

# Characterization of EVs Subpopulations From CIMmultus<sup>®</sup> EV Using PATfix<sup>®</sup> System

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## Introduction

Cells release extracellular vesicles (EVs) of different sizes and intracellular origin. Due to their heterogeneity, the isolation of the target EV population from a mixture of supernatant-derived particles can be challenging. Anion exchange chromatography (AEX) exploits the negative charge on EV surface molecules for binding to the positively charged solid phase. CIMmultus<sup>®</sup> EV, an AEX chromatography monolith column, can separate EVs in subpopulations based on charge and offers insight into the heterogeneity of particles.

Besides the availability of preparative tools for separation, combining multiple orthogonal and complementary characterization tools is crucial for defining the EV product of interest. In this work, we used a multiple-detector PATfix<sup>®</sup> system for the analysis of CIMmultus EV-fractionated samples. Samples were analyzed for the presence of EV-related tetraspanins using the fluorescence detector. PATfix MALS 3609 detector was used for the analysis of particle-containing samples and calculation of particle sizes.

## 1. Sample preparation

Native HEK293T cells and engineered HEK293T CD63-eGFP cells were cultivated on FACT III (Sartorius) microcarriers in Ambr250 bioreactor (Sartorius) in DMEM growth media containing 10% FBS, then switched to production media without FBS for 2 days. For harvesting, the vessels were removed from the system and microcarriers were allowed to settle by gravity. Conditioned media was collected and filtered through Sartopure PP3 capsule 1.2 μm (Sartorius).

100 mL of each conditioned media sample was treated with nuclease and re-buffered, then applied to a CIMmultus EV column with a column volume (CV) of 1 mL and 2 μm channels. The chromatographic separation was performed on an Akta Pure 25 M system (Cytiva), equipped with a MALS detector. The loading buffer composition was 25 mM BTP 100 mM NaCl 2% sorbitol pH 7.5 and the elution buffer 25 mM BTP 2 M NaCl 2% sorbitol pH 7.5. The columns were loaded at 10 CV/min flow rate and eluted at 2 CV/min. EVs were eluted at different salt concentrations and collected as shown in Figure 1.

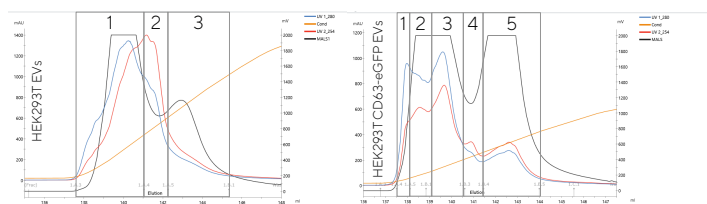


Figure 1: Preparative chromatograms from CIMmultus EV column. Vesicles were separated in salt gradient and collected in fractions labeled HEK293T EV fraction 1-3 and HEK293T CD63-eGFP EV fraction 1-5.

## 2. PATfix analytical setup

Samples were analyzed using the PATfix system (Sartorius BIA Separations) and size exclusion chromatography (SEC). HEK EVs, engineered to express CD63-eGFP on the surface were analyzed unlabeled. Native HEK EVs were labeled with anti-CD9, anti-CD63, and anti-CD81 FITC-conjugated antibodies (BioLegend). Each sample was injected to a SEC column TSKGEL G4000SWXL (Tosoh Bioscience) where the labeled EVs were separated from the excess antibody. The analysis was performed on the PATfix system with the following detectors: UV cell with 50 mm optical path length, fluorescence detector, and MALS 3609 detector. Absorbance was monitored at 260 and 280 nm, fluorescence was set to excitation and emission peaks of the used fluorophores, and light scattering was monitored at 9 angles. The running buffer was 50 mM MES, 150 mM NaCl, 0.05% Poloxamer, pH 6.5, and the flow rate was 1 mL/min.



Figure 2: PATfix system setup used in these experiments (Created with BioRender.com). Samples are contained in an autosampler, before being injected into a SEC column. Absorbance, fluorescence, and light scattering are monitored and the data is interpreted in PATfix software. An example SEC chromatogram shows the void volume peak where particles are eluted.

## 3. Evaluation of EV distribution in chromatographic samples

SEC chromatograms were analyzed in PATfix software. Fluorescence (FL) and light scattering (LS) peak area were calculated from SEC void volume peak area, sample volume, and dilution. The distribution of FL and LS peaks in chromatographic samples was plotted in Figure 3. Light scattering shows the particles are distributed in two main peaks in both the native and engineered cell lines. The EVs with the highest fluorescence signal response were eluted in the first fraction.

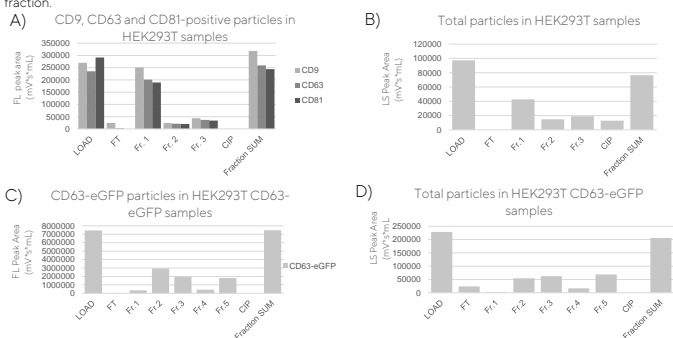


Figure 3: Distribution of FL and LS peak area in representative samples LOAD (total sample loaded to CIMmultus EV column), FT (flow-through, the unbound fraction), Fr. 1-5 (elution fractions containing EVs) and CIP (cleaning-in-place); A) and B) HEK293T samples, C) and D) HEK293T CD63-eGFP samples.

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## 4. Determination of EV size using PATfix MALS

The radius of particles was determined from SEC chromatograms in PATfix software (Figure 4). Additionally, NTA analysis was performed on Nanosight 300 (Malvern Analytical) (Figure 5). Despite the fact that fractions vary in particle charge and surface antigens, all fractions contained particles in the size range of EVs, emphasizing the importance of using multiple approaches for the characterisation these particles.

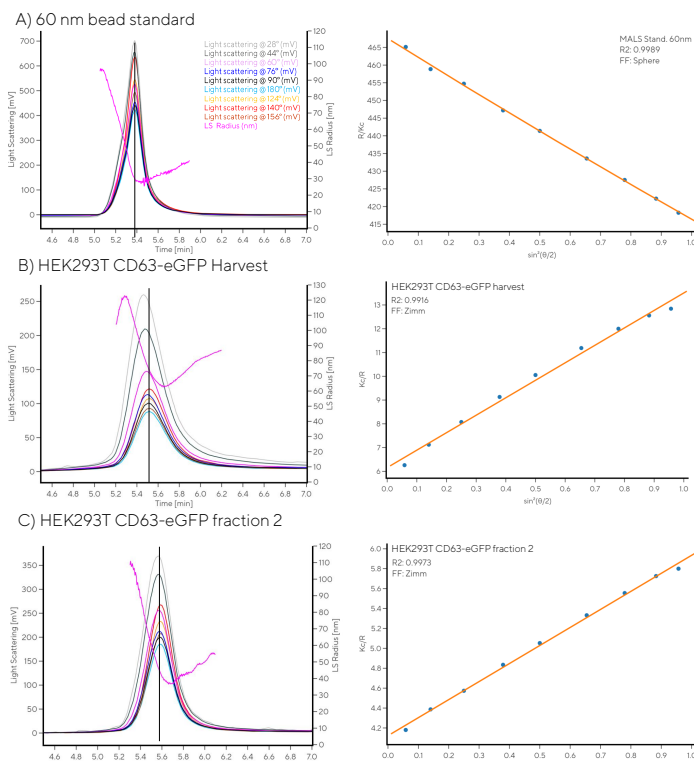


Figure 4: Light scattering chromatograms of the SEC void volume and corresponding LS charts. The particle radii were measured at the LS 90° peak. (A) SEC chromatogram of 60 nm polystyrene spherical bead standard (Postnova, Part No. Z-PS-POS-000-0,06) and the corresponding LS chart using form factor sphere. The standard was used for detector normalization. (B) SEC chromatogram of HEK 293T CD63-eGFP EV Harvest and the corresponding LS chart using Zimm plot. All chromatographic samples were characterized using this plot. (C) SEC chromatogram of HEK 293T CD63-eGFP fraction 2.

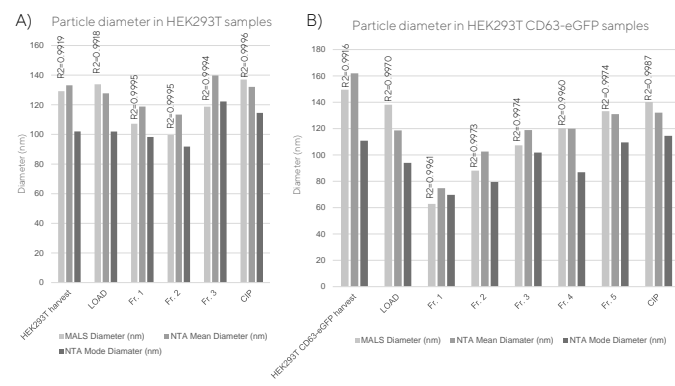


Figure 5: Particle diameter determined by MALS and NTA for A) HEK293T samples and B) HEK293T CD63-eGFP samples.

## 5. Conclusions

- CIMmultus EV chromatographic column fractionates supernatant-derived vesicles in multiple populations that differ in size and surface antigen composition
- PATfix triple detector setup enables rapid sample characterization for both native and engineered EV samples
- Fluorescence detector allows for relative quantification of fluorescent EVs or EVs labeled with a fluorescent antibody
- Combining MALS detector with SEC analytics enables relative quantification of particles in samples and determination of particle size

## 6. Acknowledgements

Acknowledgements: We would like to thank Bernd Giebel and his group from University Hospital Essen, Institute of Transfusion Medicine, for kindly providing the HEK293 CD63-eGFP cell line.