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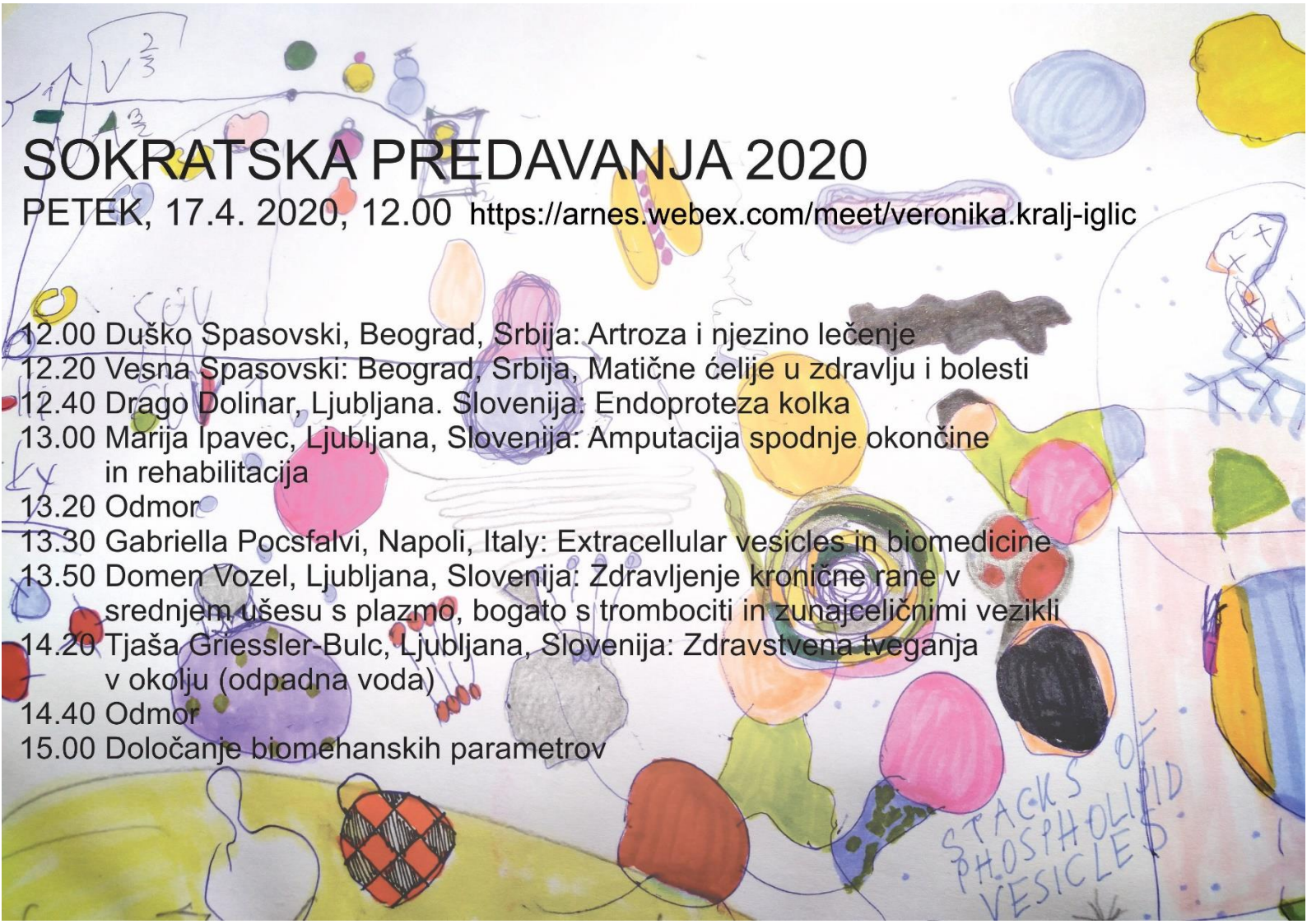

Kulturni spored med odmori:

1. Bach JS, Siciliana iz sonate za violino (flavto) in klavir v Es duru, flavta: Anita Prelovšek
2. Jeena Jeena, poje: Niharika Rawat
3. Jansmo J, Kastelein J: Skladbe za pozavno in klavir, pozavna: Emil Somun
4. Horner J, Tema iz Titanika, piano: Elena Startseva-Somun

EDITORIAL

It was recorded that Socrates conversed with his fellow citizens in the Agora of Athens, ironically confronting them with unusual questions and urging them to explain and justify their claims in this regard. The dialectical conversation assumes a considerable importance, it is a work that teaches active participation, listening and respect for the thought of others. Socratic dialogue is considered as a group activity, the aim of which is to clarify a concept and seek a common benefit. It is a 'get-together' in order to share. We are striving for that in our »Socratic lectures«; participants are encouraged to present the fruit of their work and share according to each one's best possibilities.

Symposia »Socratic lectures« with accompanying cultural and social events are taking place from 2008. Until 2019, the symposia followed the spirit of focusing on the contents and were for that purpose kept in silence and off the record, although participation of excellent scientists and professionals as well as of the students was always the main feature. In 2018, the event was recorded officially and in 2019, the proceeding was published for the first time. Cultural events included musical insertions and movies while social events were dedicated mainly to the presenting guests. The events were held at Museum of Architecture and Design at Fužine castle.

This year COVID-19 triggered fear of physical contact, however, the encounters took place at a distance through the media and online web technology, which in this period revealed their great qualities and potentials. The Symposium was held online at April 17, 2020. The record of the present proceedings has however an additional quality with respect to the previous one: to save the memory of the darkness in which we were waiting to see the stars again.

The proceedings features 25 contributions labeled as Invited lectures (contributions 1-4), Scientific contributions (4-6), Reviews (1-3,5-12), Student contributions (13-18), Reflections (19-25) and COVID-19 related contributions (1,10,21). We outline the contribution of doc. Duško Spasovski, M.D. who was a guest speaker at the symposium and went through personal experience with COVID-19 which he described in his contribution. Students have distinguished themselves by participation in the symposium; for some, it was their first scientific record. We are thankful to the participants and authors and hope to we will keep revival of Socratic ideas also in the future.

Veronika Kralj-Iglič and Anna Romolo

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Invited lecture

A CORONA EXPERIENCE

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Abstract

We usually tend to consider risky events as something that happens to someone else. It is a rather effective self-defense mechanism and at first also I thought so about COVID-19. But it crushed like a sand castle after I got touched by a twitch of destiny. In the presented contribution I describe my experience with COVID-19.

Infection

I have no idea how I got infected, it was really unexpected, since I followed all the preventive measures for weeks: wearing the mask outside the home, keeping a safe distance, cleaning hands with 70% alcohol-based solution. All the patients and staff at my hospital were wearing masks too. At that very morning (day 1) I had my routine sport session - and dragonboat is all about large boats crowded with people. There were 38 of us in three boats. None of the others got infected.

It was a sunny and warm day, as Sundays in June usually are. But in the afternoon I started to feel a bit cold, as well as I sensed a mild fatigue. There were no other symptoms. I thought it was either a sunstroke, or an onset of some enteral virus because these things are common in summer.

Testing

On the Day 2 I woke up with more fatigue, dry cough, stiff and painful neck and I was subfebrile. It was then clear that instead of going to work at the hospital I should be going to the local COVID-19 ambulance – the primary care checkpoint for suspected COVID-19 patients. It was crowded with people who had similar symptoms, waiting to be called in, and cutting time in endless "Fb expert" discussions stuffed with conspiracy theories, unproven news and beliefs remotely related to science. I couldn't tell which was harder: trying to stand for almost 4 hours in growing exhaustion, or struggling not to take part into surrounding brainstorming.

Finaly I got inside. It was the first time to closely see a doctor in full equipment - tight-sealed plastic suit, face shield with two masks plus protective glasses, and two pairs of sterile surgical gloves. She was exhausted to, not from disease but from the package she was sealed in. I was still subfebrile, and O2 saturation was measured to be 96%. Auscultation revealed no signs of lung impairment. Along with blood for leukocyte count and CRP, they also performed a nose and throat swab for RT-PCR analysis. I also got a chest radiography, where no pneumonia was detected.

I got prescribed perusal antibiotic (Azithromycin), supplementation (Vitamins C and D, Selenium and Zync), antiaggregation prophylaxis by low doses of Aspirine, Paracetamole as Antipyretic, and I was instructed to continue with home treatment until I would get PCR results. The next two days I have spent in home isolation, separated from wife and children who all were healthy.

Home treatment

In the next 6 days temperature rised slowly, and varied between 38 and 39 oC all the time. I completely lost the sense of smell, and partially the sense of taste. Such an unusual feeling: you have to actually look at the food in order to know what you eat! This however had a more serious consequence - due to impaired senses, water was so tasteless and repulsive that I barely managed to force myself to drink. Other senses were altered as well: I could not stand bright light, and hearing was so turned on that slightest background sounds were like unpleasant loud noise.

Day by day I slowly got dehydrated, so paracetamol was less and less effective. I was so exhausted that I could hardly walk, and could only do so by holding the nearby furniture! Constant care, help and support provided by my wife was something I could not do without.

PCR result came positive on day 4, and then I went to check-up with infectology specialist. Physical examination and blood tests were still suggesting home treatment. Honestly, the relief that I seem to be away from Intensive Care Unit and intubation meant very much to me and the family. But it lasted only four days. Late in the evening of day 8, I got a surge in temperature to 39,6 oC, cough hot worse and breathing got difficult. It was the anticipated worsening that a fraction of COVID-19 patients experience between Days 7 to 10.

1g of Paracetamole was ineffective. For the first time, I felt fear. I was then wrapped by achochol-soaked drapes, and it took couple of hours to eventually lower the fever.

Hospital

The next day (day 8) X-ray showed bilateral interstitial pneumonia, so I was admitted to a COVID-19 hospital, one of many that were established in Belgrade. Antibiotics switched to full dose of meropenem i.v., thromboprophylaxis was achieved with full dose of enoxaparin, and dehydration was corrected by i.v. infusions of saline, along with high doses of Vitamin C. Chloroquine was introduced in the dose of 2x400 mg throughout the hospital treatment. All other supplemenetation and therapy was continued.

I spent 11 days in hospital. I had several blood analyses and at the peak of the disease, my blood iron concentration dropped to 3 mmol/L, with ferritin above 4000 mg/L (15x normal values) and CRP close to 40 mg/L indicating inflamation. I used oxygen support nostrils almost all the time, with blood oxygen saturation up to 94% (and without oxygen it was under 90%). Two chest X-rays showed slow reduction of pneumonia signs. After four days of hospital treatment, fever was gone and I could feel slight improvement in breathing. Slow but persistent recovery followed. I regained senses too.

Home recovery

I was discharged with several kilos of weight loss, able to walk for up to 5 minutes, but happy and full of confidence! After discharge I did another RT-PCR COVID-19 test which was negative, and also serologic tests for antibodies, which was positive for both IgM and IgG. Liver enzymes were elevated 8-fold, due to both, virus and medications, and got normalized after more that one month. The restoration of strength and aerobic capacity were the slowest. It took weeks to try to exercise a little bit. I continued with angiaggregation prevention with 2x500 mg of aspirin along with proton pump inhibitor for gastric ulcer prevention, for two additional weeks. I believe that's why my breething was further improving.

Final thoughts

SARS-CoV-19 virus affects not only an individual, but the whole family and close contacts. In my case, after I got initial positive PCR result, I spent whole day calling all the contacts in past two weeks, trying to find out where I got it. Everybody I called were healthy. We than tested close contacts in my family. It turned out that I transferred COVID-19 to my wife (thank God she had no symptoms) and mother, who had serious symptoms and after 20 days of hospitalization, is now recovering well.

SARS-CoV-19 virus connects people in several ways. With each other, through support and help in everyday activities (shopping, transportation etc.) that one cannot accomplish when taken ill. But it also connects a person with its own internal mindset, in a way that important personal postulates and decisions seem to be more clearly visible when projected at the white ceiling overhead the patient bed. Besides rest and breathing and introspection, there is not much that one can do in long COVID-19 days. Achieving the balance of self-image, inner drives and future expectations are very important aspects in recovery.

Lastly, the great factor that contributed to my recovery was the confidence. I witnessed that medical doctors, unlike at the beginning of pandemic, knew precisely what to do and when to take which kind of action. A detailed treatment protocol has been established. They enriched the information given from international scientific authorities by their own actual clinical experience. The team of medical doctors who treated me included experts of many kinds of subspecialities (infectous diseases, cardiology, pulmology, endocrinology) simply because in COVID-19 hospitals all the medical doctors are engagged in treating COVID-19, with fascinanting energy to cooperate and to do their best. In a situation where doctors and nurses were overwhelmed with patients and were left with little time to rest, as a physician I am fascinated by their effort that I personally witnessed, and as a patient I feel deep gratefulness and appreciation.

Invited lecture

EXTRACELLULAR VESICLES IN BIOMEDICINE: FROM BIOMARKERS TO TISSUE REGENERATION

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Abstract

Extracellular vesicles (EVs) were initially regarded as useless cellular waste carriers, while recent studies demonstrate that EVs play vital biological roles in many aspects. EVs derived from mesenchymal stem/stromal cells and other probable sources are one of the options to regenerate damaged tissues, but their roles and uses have not been fully elucidated yet. EVs have been found in most body fluids, and numerous studies have shown that they play important roles in both normal physiological and pathological situations, such as respiratory diseases, cardiovascular disorders, inflammatory diseases, kidney injuries, chronic wounds, muscular dystrophies, bone and cartilage defects and cancers. Several protein and RNA expression biomarkers have been proposed for the monitoring of various diseases through EV-based liquid biopsies. EVs may participate in multiple regenerative processes, including induction of tubular cell survival, proliferation, suppression of inflammation, and inhibition of fibrosis. The transfer of biological cargo (proteins, mRNAs, miRNAs and DNAs) of EVs into target cells have been considered fundamental in the enhancement of proliferation and survival of cells. Here, we describe some basic fundamentals of EVs in relationship with their application in biomarker discovery and tissue regeneration.

1. Introduction

Extracellular vesicles (EVs) are a variety of membrane bound structures secreted by cells and characterized by the presence of a lipid bilayer. Their size varies from 30 nm to 1000 nm in diameter. EVs can be classified into different groups based on their secretion pathway, cells of origin and molecular content, however, the lack of suitable tools and their overlapping physicochemical properties often makes the assessment difficult. There are three main types of EVs groups, namely, exosomes, microvesicles and apoptotic bodies. Exosomes are typically 30-200 nm in diameter, formed through the endolysosomal route, and are released by the fusion of the late multivesicular body with the plasma membrane. Cargo sorting in exosomes is associated with the endosomal sorting complex required for transport (ESCRT) complexes, such as ALG-2-interacting protein X (ALIX) and tumor susceptibility gene 101 protein (TSG101) that are commonly used as marker proteins for exosomes. Microvesicles arise from the outward budding of the plasma membrane and have a size range of 100-1000 nm, and are associated with the GTP-binding protein ARF6, which are often used for their identification (1,2). Apoptotic bodies are formed through fragmentation of the cell membrane of apoptotic cells and have a broad size range between 50–2000 nm in diameter. The molecular composition of EVs is extremely rich and dynamic. They carry a range of different lipids, proteins, metabolites and nucleic acids. Importantly, this biocargo can be transferred to close or distant recipient cells (3). By carrying and transferring bioactive molecules including enzymes and genetic material, EV as a vehicle participates in cell-to-cell communication. In addition, EVs also participate in the removal of cellular waste.

With the outstanding role in intercellular communication, EVs represent an attractive source for the development of new strategies for drug delivery, immunomodulatory or regenerative therapies, and biomarker discovery. The interest in EVs is continuously growing and has expanded over various fields. A huge number of studies address the challenges to identify EV-based biomarkers for disease diagnosis, prognosis and monitoring of certain diseases. More recently, the application of EVs in therapeutics has also gained field and this will further increase the clinical utility of EVs. Here, we review recent developments in these two promising areas, biomarker research and tissue regeneration.

2. EV-based biomarkers

EVs are present in all body fluids such as blood, urine, saliva, cerebrospinal fluid, breast milk, sweat, etc. The biocargo of EVs isolated from biofluids has been proposed as a resource of biomarkers in a variety of diseases (4). EVs contain a representative set of molecular signatures of their cell of origin. Moreover, specific biocargo in the EVs has been shown to be associated with various physiological and pathological conditions. Importantly, the composition and the expression level of the different constituents of EV biocargo change in a characteristic way during pathogenesis (5). For example, *in vitro* studies have demonstrated that tumorigenic cells

release more EVs than a cell in normal condition, this overproduction of EVs has been associated with the acidic pH of the tumor microenvironment (6).

Due to their potential as a resource of cell and disease specific biomarkers, EVs isolated from biofluids have been proposed as cell free liquid biopsy. A liquid biopsy is a new diagnostic and monitoring tool, and has the main advantage of being noninvasive in comparison to the classical surgically removed biopsy specimens that are used to assess different pathologies including cancer. Liquid biopsy using circulating biomarkers in biofluids such as the blood — has been receiving considerable attention nowadays. Moreover, they have been proposed to evaluate the efficiency of certain treatments. In cancer studies, circulating tumor cells (CTCs) and/or circulating tumor DNA (ctDNA) are collected and analyzed. Recent progress in EV-based biomarker research raised the possibility to use circulating tumor-derived EVs in liquid biopsy because it may offer advantages in terms of abundance, stability, and accessibility with respect to CTCs and ctDNA (7). Blood and urine are the most frequently exploited biofluids for EV-based liquid biopsy.

3. Blood-derived EVs

Blood represents a biofluid that is of easy access, routinely isolated and provides general information on the overall condition of patients. Moreover, blood is the most commonly studied body fluid, thus it has been the choice for most of the biomarker studies focusing on EVs. EVs are isolated either from plasma or serum (8). The isolation of blood derived EVs is challenging because of i.) blood is a viscous matrix of multiple components and ii.) the presence of lipoprotein structures. Lipoproteins are complex particles that have a central hydrophobic core of non-polar lipids, primarily cholesterol esters and triglycerides surrounded by a hydrophilic membrane consisting of phospholipids, free cholesterol, and apolipoproteins (9). The main lipoproteins in blood are chylomicrons, very-low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). They are particles responsible for the transportation of lipids throughout the body. Lipoproteins have similar characteristics to EVs; they are similar in size and density, and their concentration is highly variable and is dependent on parameters such as age, diet and metabolism (10).

It is important to note that the vast majority of the EV studies have not paid sufficient attention for the removal of lipoproteins. It should be underlined that a single ultracentrifugation, filtration or chromatography separation-based purification method to the removal of lipoproteins results in a high portion of non-EV components, mostly lipoproteins (11). As a consequence, the data deposited to EV databases like Vesiclepedia (12) contains a high percentage of information related to co purified contaminants. Hence, it is of great importance to develop or improve isolation protocols. Currently, there is no isolation method, which is able to provide EV samples that are free of lipoproteins and soluble proteins. However, the different

protocols that have been developed can reduce the concentration of these co-isolating molecules considerably. Of special interest in the isolation of blood derived EVs is the combination of different methods to obtain a “purer” EVs isolate.

For biomarker studies, the downstream analytical approach is usually omics-based, i.e. proteomics, transcriptomic, lipidomics or metabolomics. To obtain meaningful qualitative and quantitative information relating to the EV biocargo, and not to the associated impurities, usually highly purified EV isolates are required. Some protocols, in fact, apply consecutive isolating steps each removing different contaminants. Figure 1 shows a tree consecutive step-based process recently used for the purification of EVs from human plasma (13). The first step is based on differential ultracentrifugation (dUC). Particles of different densities or sizes in a suspension will sediment at different rates during dUC, with the larger and denser particles sedimenting faster (A). Thus dUC pellets EVs together with the bigger particles in the bottom of the tube, whereas the smaller lipoproteins and soluble proteins mainly remain in the supernatant. In a second step, the EVs isolated by dUC are further purified by density gradient ultracentrifugation (GUC). GUC separates the components based on their densities (B). The large lipoproteins (chylomicrons and VLDL) are expected to be removed in this step. In a final step, the EV enrich fraction selected by density is further purified by size exclusion chromatography (SEC). SEC uses porous beads to allow large particles, which cannot enter the pores, to rapidly pass through a column gel, while smaller sample constituents are slowed down by the tortuous path through the pores (C).

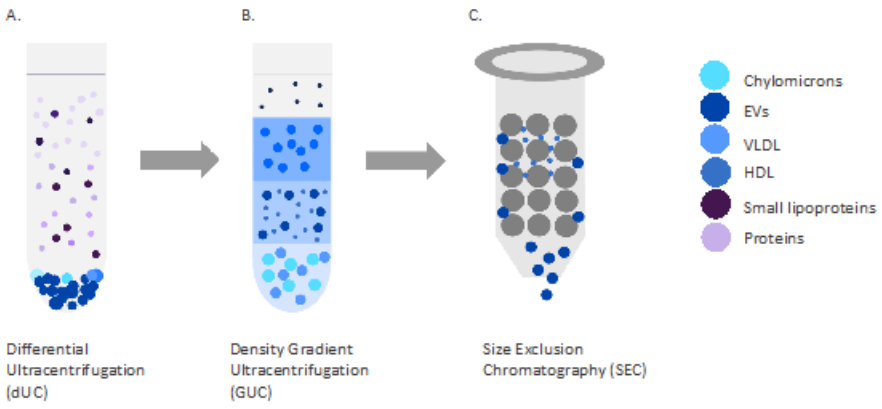

Figure 1. A three-step workflow for the isolation of EVs from human plasma

4. Isolation of blood-derived EVs for biomarker discovery

Downstream analysis of blood-derived EVs can be used for biomarker discovery studies. A biomarker is a molecular attribute that can be quantitatively measured and evaluated to assess normal physiology, pathologic progression, or reaction to a therapeutic treatment. Blood-derived EVs have been proposed for the evaluation of a wide range of different diseases including neurodegenerative (14), cardiovascular (15), renal (16), and especially cancer (17). Consequently, the clinical evidence on the relevance of EVs as disease biomarkers is rapidly increasing.

A number of studies have underlined an increase in the number of circulating EVs during pathogenesis in comparison with the healthy state. There are mainly two ways to use blood-derived EVs as biomarkers, one is based simply on the number of EVs and another is on the alteration of the expression level of proteins and nucleic acids, especially small RNAs, related to specific diseases. For example, it has been observed that melanoma patients with advanced disease presented significantly higher levels of plasmatic EVs when compared to healthy volunteers, moreover, the increase in the amount of plasmatic caveolin-1 and CD63-positive EVs was associated to stage III and IV melanoma patients (18).

PubMed shows an exponential growth in the EV field. A search using the term “extracellular vesicle” resulted in a total of 15,576 articles in the last 10 years (data refers to April 2020). Many EV-based potential biomarkers have been developed in recent years, and in fact, when the keyword “biomarker” is added to the above search still a considerable number of articles (4,094) are shown. Further narrowing the search to “blood extracellular vesicle biomarkers” a total of 1834 articles are displayed showing that roughly half of EV biomarker research uses blood. Consulting the clinical trials website from the National Institutes of Health (NIH) we have found 105 studies, from which 37 studies are focused in the use of specific EV biocargo(s) as biomarkers or in the measurement of the number of EVs in different diseases. 9 Studies have a status of completed, 18 are recruiting, 2 are active and not recruiting and 6 are not recruiting yet. Table 1 reports the different biomarkers used in the completed clinical studies. These data show that the discovered potential biomarkers are slowly but steadily translated into the clinics.

Table 1. Ten completed clinical trials retrieved from www.clinicaltrials.gov of US National Library of Medicine database related to blood-derived extracellular vesicles biomarkers.

Disease	Aim	Specific biomarker	Name of the trial	ReOnline access
Cancer	Biomarker and assess treatment	HSP70	Pilot Study With the Aim to Quantify a Stress Protein in the Blood and in the Urine for the Monitoring and Early Diagnosis of Malignant Solid Tumors	https://ClinicalTrials.gov/show/NCT02662621
Cancer	Biomarker and assess treatment	Stain vesicles with Pimonidazole	Pimo Study: Extracellular Vesicle-based Liquid Biopsy to Detect Hypoxia in Tumours	https://ClinicalTrials.gov/show/NCT03262311
Cancer: Pancreatic Ductal Adeno-carcinoma (PDAC)	Biomarker	GLP-1	Diagnostic Accuracy of Circulating Tumor Cells (CTCs) and Onco-exosome Quantification in the Diagnosis of Pancreatic Cancer - PANC-CTC	https://ClinicalTrials.gov/show/NCT03032913
Cancer: Non Small Cell Lung Cancer	Searching for ICD markers	Immunogenic cell death such as HSP70, HSP90, CRT, HMGB1	Detection of Circulating Biomarkers of Immunogenic Cell Death	https://ClinicalTrials.gov/show/NCT02921854
Cardiovascular: Overweight Children With Type 2 Diabetes Risk	Searching for new profile mRNAs in EVs	None	Prevention of Diabetes in Overweight/Obese Preadolescent Children	https://ClinicalTrials.gov/show/NCT03027726
Ginecology: Preeclampsia	Biomarker	miRNAs 136, 494 and 495 genes	microRNAs Role in Pre-eclampsia Diagnosis	https://ClinicalTrials.gov/show/NCT03562715
Infectology: Sepsis	Biomarker	miRNA Expression and PGRN	Validation of Progranulin as a Biomarker for Sepsis	https://ClinicalTrials.gov/show/NCT03280576
Neurodegenerative: Parkinson's Disease	Biomarker and assess treatment	LRRK2	LRRK2 and Other Novel Exosome Proteins in Parkinson's Disease	https://ClinicalTrials.gov/show/NCT01860118
Neurodegenerative: Parkinson's Disease	Biomarker	LRRK2	Fox BioNet Project: ECV-003	https://ClinicalTrials.gov/show/NCT03775447

Heat shock protein 70 (HSP70), Glypican-1 (GLP-1), Immunogenic cell death (ICD), Heat shock protein 90 (HSP90), Calreticulin (CRT), High mobility group box 1 (HMGB1), Progranulin (PGRN), Leucine-rich repeat kinase 2 (LRRK2)

5. Examples of EV-based biomarkers in blood

5.1. *Neurodegenerative diseases*

A longitudinal study, with the largest number of case-control samples, demonstrated the role of certain proteins related to Alzheimer's disease enclosed in blood derived vesicles. Participants at early stage of Alzheimer's disease showed higher levels of phosphorylated p-tau181, p-tau23, pY-IRS-1 and pSer312-IRS-1 in blood derived EVs than controls. Moreover, it was shown that higher p-tau181 was associated with worse verbal memory, attention, executive function, visuospatial function cross-sectionally and was more present in elderly subjects. Higher pSer312-IRS-1 was associated with worse verbal memory and executive function cross-sectionally. Hence, the use of EV biomarkers for prognosis and diagnosis for individual and model derived risk score is very promising (19).

5.2. *Cancer*

One of the best-studied EV-derived protein biomarkers for the early diagnosis of cancer is glypican-1 (GPC1). Plasma EV GPC1 levels of patients with pancreatic cancer were shown to be considerably higher than normal (12). Moreover, GPC1 level was considerably lower in healthy subjects and patients with benign pancreatic disease and from patients with early-stage pancreatic cancer. Clinical trials are ongoing with promising results (20).

Several candidate biomarkers present on the EV surface were successfully tested *in vitro* and pilot clinical settings. For example, a high amount of CD47, CD71 and EpCAM were detected on ovarian cancer-derived EVs (21). Similarly, a high amount of CD147 was detected on the colorectal cancer-derived EVs (22), anti-prostate-specific membrane antigen (PSMA) was found to be increased in EVs in patients with prostate cancer (23)(24), as well as ephrin A2 (25). In another study, CKAP4 was suggested as an EV biomarker for pancreatic cancer (26). Serglycin, tropomyosin 3, thrombospondin were proposed for lung cancer (27). These are only some examples of the progress made and many other possible protein biomarkers are being studied.

5.3. *Liver disease*

In liver disease, annexin A2 (ANXA2) has been found to be elevated in hepatocellular carcinoma (HCC) with cirrhosis background compared with cirrhosis and healthy controls. Arbelaiz et al detected high levels of galectin-3-binding protein (LG3BP) in serum EVs of HCC patients as compared with cholangiocarcinoma (CCA), primary sclerosing cholangitis (PSC), and healthy controls (28). Also, EV-based non alcoholic fatty liver disease (NAFLD) diagnosis performed in

2012 by Kornek et al in humans, showed that EVs from several subsets of immune cells were differentially enriched in NAFLD (CD14þ and iNKTþ EVs) and chronic hepatitis C patients (CHC; CD4þ and CD8þ MVs) enabling an almost complete separation among these patients (29).

5.4. *Cardiovascular disease*

Increased levels of circulating EVs have been reported in cardiovascular diseases too (30). For example, alpha-2-Macroglobulin (A2MG), fibrinogen (α, β, and γ chains), complement component 3 (C3), complement component 4A (C4A), CD5 antigen like protein (CD5L), haptoglobin (HP), clusterin, and immunoglobulin heavy constant mu, were found to be involved in acute coronary syndrome (ACS). Moreover, these proteins have an important role in the complement and coagulation cascades. Recently, some studies have focused on the possible role of EVs in cardiometabolic-related diseases for early prevention of cardiovascular events. These represent a wide array of diseases that usually start with insulin resistance in early periods of life and progress into conditions hypertension, prediabetes and type 2 diabetes mellitus (T2DM) (15).

6. Biomarkers for the assessment of treatment

EVs can also be helpful to assess the effectiveness of a therapy in patients under cancer treatments. For example in a preclinical setting, treatment with proton pump inhibitors in xenograft with human melanoma has shown that EVs plasmatic levels are consistent with reduction of the tumour size (31). Another study showed that the presence of program death ligand 1 (PD-L1) on metastatic melanoma-derived EVs to be a potential predictive marker of anti–PD-1 therapy response (32). Demonstrating that plasmatic levels of existing tumour markers may change in different stages of the disease, as well as produce side effects of current therapies.

7. Uses of EVs in tissue regeneration

EVs isolated from stem or progenitor cells and especially from mesenchymal stem cells (MSCs) have shown promising regenerative effects (33). The MSCs are multipotent and can be isolated from different tissues, such as bone marrow, umbilical cord, placenta, fat tissue, hair follicle, synovium, and periodontal ligament. Due to their de-differentiation potential, self-renewal ability, low immunogenicity and paracrine effects, MSCs were elected as an excellent biomaterial for tissue repair. However, some studies have shown a low survival and grafting rate of MSCs in injured areas, indicating that these characteristics might compromise the use of MSCs in some specific areas of tissue regeneration. Recently, an increasing interest has focused on MSCs derived EVs, as they can imitate most of the biological characteristics of MSCs, such as differentiation, maturation, and self-renewal, while can avoid degradation under the protection of the biomembrane, enabling long-distance transportation of the biocargo (34,35).

Interestingly, there are some studies demonstrating that EVs derived from other than MSCs sources (renal tissues, fibroblasts) can promote tissue regeneration too. Grange, C., *et al.* propose a possible therapeutic use of renal-derived EVs isolated from urine (urine-derived EVs, uEVs) in acute kidney injury. uEVs promote renal recovery, stimulate tubular cell proliferation, decrease the expression of inflammatory and injury markers, and restore endogenous Klotho which were lost due to renal injury. Similarly, ineffective fibroblast derived EVs were shown to obtain reno-protection potential when they are engineered with human recombinant Klotho (36).

Figure 2 schematically shows how stem cell derived EVs act as vehicles for transmitting genetic information between stem cells and injured renal cells and by this way activating regenerative programs in damaged cells and stimulate them to dedifferentiate into healthy normal cells. Besides, it is believed that EVs secreted by injured cells can alternatively transmit mRNAs and miRNAs to stem cells and cause their proliferation and dedifferentiation to normal renal cells and favor renal recovery (37).

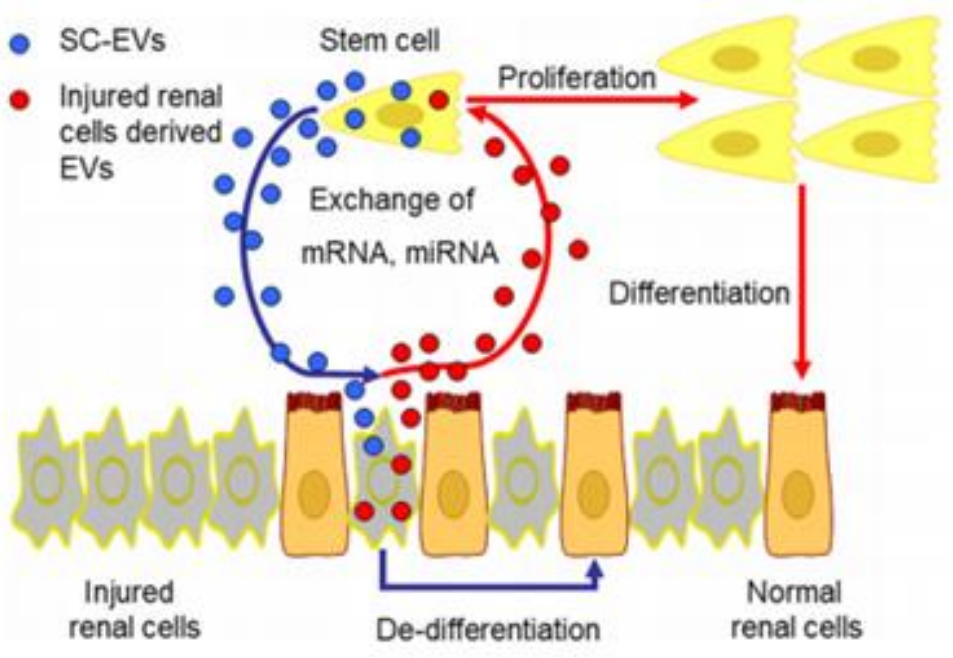

Figure 2. Importance of EVs as vehicles for genetic communication between stem cells and injured renal cells during AKI (37). SC-EVs, stem cells derived EVs; EVs, extracellular vesicles. Reproduced from Ref 37 under the Creative Commons Attribution License, CC-BY 4.0.

Table 2. Regenerative potential of EVs – Selected examples reported based on target organs, source of EVs and regenerative potential.

Target organ	Source of EVs	Reported regenerative potential	Ref.
Nerves	Human BMSCs	Enhancing endogenous angiogenesis and neurogenesis; attenuating neuroinflammation in rats with traumatic brain injury	(39)
Lungs	Swine bone marrow-derived MSCs	MSC-EVs inhibited influenza virus replication and virus-induced apoptosis in lung epithelial cells	(40)
Skin	Platelet-rich plasma	Induce proliferation and migration of endothelial cells and fibroblasts to promote angiogenesis and re-epithelia-lization in chronic cutaneous wound healing process	(41)
Liver	Hepatocyte	Promote the proliferation of hepatocyte *in vitro* and liver regeneration *in vivo*	(42)
Cartilage	Human embryonic MSCs	Restore cartilage and subchondral bone with good surface regularity, and accomplish bonding to adjacent cartilage	(43)
Kidney	Human Wharton's Jelly Mesenchymal Stromal Cells	Renal tubular injury was alleviated and renal function was improved; cell apoptosis was reduced; oxidative stress in injured kidney tissues decreased	(44)
	Human urine-derived EVs	Renal recovery was promoted; tubular cell proliferation was improved; the expression of inflammatory and injury markers was reduced; and endogenous Klotho loss was restored	(36)
Heart	Human UCMSCs	Protect myocardial cells from apoptosis; improve cardiac systolic function; reduced cardiac fibrosis and promote angiogenesis in rat model with acute myocardial infarction	(45)
	Acellular gelatinous Wharton's jelly of the human umbilical cord	Protect myocardial cells from apoptosis, improve cardiac systolic function, reduced cardiac fibrosis and promote angiogenesis in rat model with acute myocardial infarction	(46)

Mesenchymal stem cells (MSCs), bone marrow derived mesenchymal stem cells (BMSCs), umbilical cord mesenchymal stem cells (UCMSCs)

EVs can be used in combination with various tissue-engineered scaffolds or other therapeutic surfaces to improve the efficiency of tissue regeneration and healing processes. Alternatively, EVs have been exploited as a carrier for the delivery of therapeutic agents such as RNAs and small drugs (47). There are two main strategies for EV engineering: i.) post isolation engineering using chemistry targeting molecules on the EV surface and ii) manipulation of parent cells either through genetic or metabolic engineering. By these ways, vesicles with different or more pronounced biological functions can be generated, or engineered, and can boost the yield of EV production. Each technique has its pros and cons which should be considered for choosing a suitable technique for EV engineering (48,49).

Conclusions

EVs have been shown to be implicated in disease progression, immune response, inflammation, intercellular communication and other biological phenomena. Hence, EVs have emerged as a novel tool in the study of diseases. During the last decade, EVs have evolved as tools for diagnosis and treatment. A huge number of potential biomarkers have been identified for various diseases and several U.S. Food and Drug Administration (US FDA) approved trials in clinical applications are in progress. ExoDx Prostate IntelliScore (EPI) (50) test is the first liquid biopsy based on urinary EVs that received breakthrough status from the U.S. FDA. However, it should be noted that there is none blood-EV liquid biopsy-based test approved for clinical use yet. Similarly, although the potential therapeutic properties of EVs clearly represent new opportunities for tissue repair and regeneration, there are some limitations in the use of EVs in regenerative medicine, such as problems related to immunological rejection and tumorigenicity, but also, isolation and purification methods and long term effects.

To advance EVs in clinical daily use, it is important to prepare highly pure isolates that could provide a blueprint of the normal state of the EVs, which would facilitate the detection of alterations related to pathological processes. To obtain purer isolates new methods and protocols need to be developed and/or the existing ones should be improved. The International Society for Extracellular Vesicles (ISEV) prompts researchers to improve the reporting methodology so the information available can be translated to clinical trials and clinical use.Plant-derived vesicles have been also used for tissue repair (38). The use of nanovesicles (NVs) isolated from wheatgrass juice on cutaneous wound healing process was studied *in vitro* wound healing model. Annexin V staining of apoptotic cells further accompanied with the cell cycle analysis revealed that the apoptotic cell number is reduced with no dispersion to the cell cycle analysis while plant derived NVs have also promoted tube-like structure formation of the endothelial cells. Wheatgrass NVs possess wound healing features due to related gene expression, stimulation of fibroblast, modification and coordination in vascularization.

Moreover, plant-derived vesicle therapy strategies would be safer and an economical alternative for clinical wound healing.

Some examples of the regenerative potentials of EVs reported in the literature are listed in Table 2 according to different target tissues/organs and the sources of EVs.

References

1. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol [Internet]. 2018;19(4):213–28. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29339798

2. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles [Internet]. 2018 Dec 1;7(1):1535750. Available from: https://doi.org/10.1080/20013078.2018.1535750

3. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol [Internet]. 2007;9(6):654–9. Available from: https://doi.org/10.1038/ncb1596

4. Fais S, O'Driscoll L, Borras FE, Buzas E, Camussi G, Cappello F, et al. Evidence-Based Clinical Use of Nanoscale Extracellular Vesicles in Nanomedicine. ACS Nano. 2016;10(4):3886–99.

5. Pollet H, Conrard L, Cloos A-S, Tyteca D. Plasma Membrane Lipid Domains as Platforms for Vesicle Biogenesis and Shedding? [Internet]. Vol. 8, Biomolecules. CELL Unit. 2018 Sep; 8(03). Available from: http://europepmc.org/abstract/MED/30223513

6. Logozzi M, Mizzoni D, Angelini D, Di Raimo R, Falchi M, Battistini L, et al. Microenvironmental pH and Exosome Levels Interplay in Human Cancer Cell Lines of Different Histotypes. Cancers (Basel) [Internet]. 2018 Oct 5 [cited 2020 Apr 18];10(10):370. Available from: http://www.mdpi.com/2072-6694/10/10/370

7. Chang L, Ni J, Zhu Y, Pang B, Graham P, Zhang H, et al. Liquid biopsy in ovarian cancer: recent advances in circulating extracellular vesicle detection for early diagnosis and monitoring progression. Theranostics [Internet]. 2019;9(14):4130–40. Available from: http://www.thno.org/v09p4130.htm

8. Wiklander OPB, Brennan M, Lötvall J, Breakefield XO, Andaloussi SEL. Advances in therapeutic applications of extracellular vesicles. Sci Transl Med. 2019;11(492):1–16.

9. Feingold R Kenneth GC. Introduction to Lipids and Lipoproteins. South Dartmouth MDText.com, Inc [Internet]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK305896/

10. Simonsen JB. What are we looking at? Extracellular vesicles, lipoproteins, or both? Circulation Research. 2017.

11. Sódar BW, Kittel Á, Pálóczi K, Vukman K V, Osteikoetxea X, Szabó-Taylor K, et al. Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection. Sci Rep [Internet]. 2016;6(January):1–12. Available from: http://dx.doi.org/10.1038/srep24316

12. Pathan M, Fonseka P, Chitti S V, Kang T, Sanwlani R, Van Deun J, et al. Vesiclepedia 2019: a compendium of RNA, proteins, lipids and metabolites in extracellular vesicles. Nucleic Acids Res [Internet]. 2018 Nov 5;47(D1):D516–9. Available from: https://doi.org/10.1093/nar/gky1029

13. Karimi N, Cvjetkovic A, Jang SC, Crescitelli R, Hosseinpour Feizi MA, Nieuwland R, et al. Detailed analysis of the plasma extracellular vesicle proteome after separation from lipoproteins. Cell Mol Life Sci [Internet]. 2018;75(15):2873–86. Available from: https://doi.org/10.1007/s00018-018-2773-4

14. Li T-R, Wang X-N, Sheng C, Li Y-X, Li FZ-T, Sun Y, et al. Extracellular vesicles as an emerging tool for the early detection of Alzheimer's disease. Mech Ageing Dev [Internet]. 2019;184:111175. Available from: http://www.sciencedirect.com/science/article/pii/S0047637419301800

15. Barrachina MN, Calderón-Cruz B, Fernandez-Rocca L, García Á. Application of Extracellular Vesicles Proteomics to Cardiovascular Disease: Guidelines, Data Analysis, and Future Perspectives. Proteomics [Internet]. 2019 Jan 1;19(1–2):1800247. Available from: https://doi.org/10.1002/pmic.201800247

16. Thongboonkerd V. Roles for Exosome in Various Kidney Diseases and Disorders [Internet]. Vol. 10, Frontiers in Pharmacology . 2020. p. 1655. Available from: https://www.frontiersin.org/article/10.3389/fphar.2019.01655

17. Schaffner F. Tumor Liquid Biopsies [Internet]. Vol. 215. 2020. Available from: http://link.springer.com/10.1007/978-3-030-26439-0

18. Logozzi M, De Milito A, Lugini L, Borghi M, Calabrò L, Spada M, et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. PLoS One. 2009 Apr 17;4(4).

19. Kapogiannis D, Mustapic M, Shardell MD, Berkowitz ST, Diehl TC, Spangler RD, et al. Association of Extracellular Vesicle Biomarkers with Alzheimer Disease in the Baltimore Longitudinal Study of Aging. JAMA Neurol. 2019;76(11):1340–51.

20. Moutinho-Ribeiro P, Santos A, Batista I, Adem B, Silva S, Morais R, et al. Exosomal Glypican-1 and Pancreatic Adenocarcinoma: Prime Time for Early Diagnosis?: 79. Am J Gastroenterol [Internet]. 2018;113. Available from: https://journals.lww.com/ajg/Fulltext/2018/10001/Exosomal_Glypican_1_and_Pancreatic_Adenocarcinoma_.79.aspx

21. Zaborowski MP, Lee K, Na YJ, Sammarco A, Zhang X, Iwanicki M, et al. Methods for Systematic Identification of Membrane Proteins for Specific Capture of Cancer-Derived Extracellular Vesicles. Cell Rep [Internet]. 2019 Apr 2;27(1):255-268.e6. Available from: https://doi.org/10.1016/j.celrep.2019.03.003

22. Tian Y, Ma L, Gong M, Su G, Zhu S, Zhang W, et al. Protein Profiling and Sizing of Extracellular Vesicles from Colorectal Cancer Patients via Flow Cytometry. ACS Nano [Internet]. 2018 Jan 23;12(1):671–80. Available from: https://doi.org/10.1021/acsnano.7b07782

23. Park YH, Shin HW, Jung AR, Kwon OS, Choi Y-J, Park J, et al. Prostate-specific extracellular vesicles as a novel biomarker in human prostate cancer. Sci Rep [Internet]. 2016;6(1):30386. Available from: https://doi.org/10.1038/srep30386

24. Logozzi M, Angelini DF, Iessi E, Mizzoni D, Di Raimo R, Federici C, et al. Increased PSA expression on prostate cancer exosomes in in vitro condition and in cancer patients. Cancer Lett [Internet]. 2017;403:318–29. Available from: http://www.sciencedirect.com/science/article/pii/S0304383517304184

25. Li S, Zhao Y, Chen W, Yin L, Zhu J, Zhang H, et al. Exosomal ephrinA2 derived from serum as a potential biomarker for prostate cancer. J Cancer [Internet]. 2018;9(15):2659–65. Available from: http://www.jcancer.org/v09p2659.htm

26. Kimura H, Yamamoto H, Harada T, Fumoto K, Osugi Y, Sada R, et al. CKAP4, a DKK1 Receptor, Is a Biomarker in Exosomes Derived from Pancreatic Cancer and a Molecular Target for Therapy. Clin Cancer Res [Internet]. 2019 Jan 4; Available from: http://clincancerres.aacrjournals.org/content/early/2019/02/03/1078-0432.CCR-18-2124.abstract

27. Vykoukal J, Sun N, Aguilar-Bonavides C, Katayama H, Tanaka I, Fahrmann JF, et al. Plasma-derived extracellular vesicle proteins as a source of biomarkers for lung adenocarcinoma. Oncotarget; Vol 8, No 56 [Internet]. 2017; Available from: https://www.oncotarget.com/article/20748/text/

28. Arbelaiz A, Azkargorta M, Krawczyk M, Santos-Laso A, Lapitz A, Perugorria MJ, et al. Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. Hepatology [Internet]. 2017 Oct 1;66(4):1125–43. Available from: https://doi.org/10.1002/hep.29291

29. Kornek M, Lynch M, Mehta SH, Lai M, Exley M, Afdhal NH, et al. Circulating microparticles as disease-specific biomarkers of severity of inflammation in patients with hepatitis C or nonalcoholic steatohepatitis. Gastroenterology [Internet]. 2012 Aug 1 [cited 2020 Apr 19];143(2):448–58. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0016508512006130

30. Oggero S, Austin-Williams S, Norling LV. The Contrasting Role of Extracellular Vesicles in Vascular Inflammation and Tissue Repair [Internet]. Vol. 10, Frontiers in Pharmacology . 2019. p. 1479. Available from: https://www.frontiersin.org/article/10.3389/fphar.2019.01479

31. Federici C, Petrucci F, Caimi S, Cesolini A, Logozzi M, Borghi M, et al. Exosome Release and Low pH Belong to a Framework of Resistance of Human Melanoma Cells to Cisplatin. PLoS One [Internet]. 2014 Feb 6;9(2):e88193. Available from: https://doi.org/10.1371/journal.pone.0088193

32. Chen G, Huang AC, Zhang W, Zhang G, Wu M, Xu W, et al. Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response. Nature [Internet]. 2018;560(7718):382–6. Available from: https://doi.org/10.1038/s41586-018-0392-8

33. Lamichhane TN, Sokic S, Schardt JS, Raiker RS, Lin JW, Jay SM. Emerging Roles for Extracellular Vesicles in Tissue Engineering and Regenerative Medicine. Tissue Eng Part B Rev [Internet]. 2014 Jun 23;21(1):45–54. Available from: https://doi.org/10.1089/ten.teb.2014.0300

34. Zhang B, Tian X, Hao J, Xu G, Zhang W. Mesenchymal Stem Cell-Derived Extracellular Vesicles in Tissue Regeneration. Cell Transplant [Internet]. 2020 Jan 1;29:0963689720908500. Available from: https://doi.org/10.1177/0963689720908500

35. Casado-Díaz A, Quesada-Gómez JM, Dorado G. Extracellular Vesicles Derived From Mesenchymal Stem Cells (MSC) in Regenerative Medicine: Applications in Skin Wound

Healing [Internet]. Vol. 8, Frontiers in Bioengineering and Biotechnology . 2020. p. 146. Available from: https://www.frontiersin.org/article/10.3389/fbioe.2020.00146

36. Grange C, Papadimitriou E, Dimuccio V, Pastorino C, Molina J, O'Kelly R, et al. Urinary Extracellular Vesicles Carrying Klotho Improve the Recovery of Renal Function in an Acute Tubular Injury Model. Mol Ther [Internet]. 2020 Feb 5;28(2):490–502. Available from: https://doi.org/10.1016/j.ymthe.2019.11.013

37. Zhao L, Hu C, Zhang P, Jiang H, Chen J. Genetic communication by extracellular vesicles is an important mechanism underlying stem cell-based therapy-mediated protection against acute kidney injury. Stem Cell Res Ther [Internet]. 2019;10(1):119. Available from: https://doi.org/10.1186/s13287-019-1227-8

38. Şahin F, Koçak P, Güneş MY, Özkan İ, Yıldırım E, Kala EY. In Vitro Wound Healing Activity of Wheat-Derived Nanovesicles. Appl Biochem Biotechnol [Internet]. 2019;188(2):381–94. Available from: https://doi.org/10.1007/s12010-018-2913-1

39. Zhang Y, Chopp M, Zhang ZG, Katakowski M, Xin H, Qu C, et al. Systemic administration of cell-free exosomes generated by human bone marrow derived mesenchymal stem cells cultured under 2D and 3D conditions improves functional recovery in rats after traumatic brain injury. Neurochem Int [Internet]. 2017;111:69–81. Available from: http://www.sciencedirect.com/science/article/pii/S0197018616302510

40. Khatri M, Richardson LA, Meulia T. Mesenchymal stem cell-derived extracellular vesicles attenuate influenza virus-induced acute lung injury in a pig model. Stem Cell Res Ther [Internet]. 2018;9(1):17. Available from: https://doi.org/10.1186/s13287-018-0774-8

41. Tao SC, Guo SC, Zhang CQ. Platelet-derived extracellular vesicles: An emerging therapeutic approach. Int J Biol Sci. 2017;13(7):828–34.

42. Nojima H, Freeman CM, Schuster RM, Japtok L, Kleuser B, Edwards MJ, et al. Hepatocyte exosomes mediate liver repair and regeneration via sphingosine-1-phosphate. J Hepatol [Internet]. 2016 Jan 1;64(1):60–8. Available from: https://doi.org/10.1016/j.jhep.2015.07.030

43. Zhang S, Chu WC, Lai RC, Lim SK, Hui JHP, Toh WS. Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration. Osteoarthr Cartil [Internet]. 2016 Dec 1;24(12):2135–40. Available from: https://doi.org/10.1016/j.joca.2016.06.022

44. Zhang G, Zou X, Huang Y, Wang F, Miao S, Liu G, et al. Mesenchymal Stromal Cell-Derived Extracellular Vesicles Protect Against Acute Kidney Injury Through Anti-Oxidation by

Enhancing Nrf2/ARE Activation in Rats. Kidney Blood Press Res [Internet]. 2016;41(2):119–28. Available from: https://www.karger.com/DOI/10.1159/000443413

45. Zhao Y, Sun X, Cao W, Ma J, Sun L, Qian H, et al. Exosomes Derived from Human Umbilical Cord Mesenchymal Stem Cells Relieve Acute Myocardial Ischemic Injury. Sluijter J, editor. Stem Cells Int [Internet]. 2015;2015:761643. Available from: https://doi.org/10.1155/2015/761643

46. Bakhtyar N, Jeschke MG, Herer E, Sheikholeslam M, Amini-Nik S. Exosomes from acellular Wharton's jelly of the human umbilical cord promotes skin wound healing. Stem Cell Res Ther [Internet]. 2018;9(1):193. Available from: https://doi.org/10.1186/s13287-018-0921-2

47. Ramasubramanian L, Kumar P, Wang A. Engineering Extracellular Vesicles as Nanotherapeutics for Regenerative Medicine. Biomolecules [Internet]. 2019 Dec 28 [cited 2020 Apr 22];10(1):48. Available from: https://www.mdpi.com/2218-273X/10/1/48

48. Kim H, Kim D, Nam H, Moon S, Kwon YJ, Lee JB. Engineered extracellular vesicles and their mimetics for clinical translation. Methods [Internet]. 2019 Oct 15 [cited 2020 Apr 22]; Available from: https://www.sciencedirect.com/science/article/pii/S104620231930221X

49. Jing H, He X, Zheng J. Exosomes and regenerative medicine: state of the art and perspectives. Transl Res [Internet]. 2018 Jun 1;196:1–16. Available from: https://doi.org/10.1016/j.trsl.2018.01.005

50. Minciacchi VR, Zijlstra A, Rubin MA, Di Vizio D. Extracellular vesicles for liquid biopsy in prostate cancer: where are we and where are we headed? Prostate Cancer Prostatic Dis [Internet]. 2017;20(3):251–8. Available from: https://doi.org/10.1038/pcan.2017.7

Invited lecture

REDUCTION OF ENVIRONMENTAL AND HEALTH RISK DUE TO WASTEWATER REUSE IN AGRICULTURE BY NATURE-BASED SOLUTIONS

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Abstract

The use of treated wastewater is one of the solutions to reduce water scarcity in agriculture and to conserve water resources. However, irrigation with wastewater due to pathogenic microorganisms, multidrug resistant bacteria, and contaminants of emerging concern that remain in treated effluent of wastewater treatment plants can pose environmental and health risks. Nature-based solutions (NBS), such as constructed wetlands, high-rate algal ponds, evapotranspirative willow systems can recover nutrients via biomass production and produce treated water for reuse while reducing environmental and health risks. The results from algae photobioreactors showed that removal of contaminants of emerging concern varied between the culture of mixed algae-bacteria biomass and microalgae *Chlorella vulgaris*, while other NBS were most efficient in nutrient recovery.

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1. Wastewater reuse

A growing urbanisation, increasing resource consumption, climate change and the transition to a low carbon society require increasing flexibility of the urban environment, more sustainable wastewater (WW) management in addressing re-use/recycling of valuable resources such as water, nutrients, biomass, and energy (1) . World's water resources are increasingly coming under stress due to over extraction of water and higher water demand, leading to water scarcity and deterioration of its quality. WW is rich in nutrients and because it is independent of seasonal and weather variations, it can be considered as a reliable water and nutrient supply for agriculture (2). The environment could benefit from its reuse in the sense of lowering the pressure on freshwater extraction from resources and decreasing the discharge from wastewater treatment plants (WWTPs) to sensitive areas (2). The integrated water resource management can be a potential way to cope with future challenges regarding water and nutrients (e.g. phosphorus) availability as well as food scarcity (1) . Irrigation with treated WW is of significant importance in dry areas with scarce or decreasing freshwater resources. The practice is especially prevalent in Northern Mediterranean countries, where the agricultural use of freshwater is estimated to be between 70% - 80% of all water use. Due to this, the use of WW for agricultural irrigation has already become widespread in this area (3). In Spain, standards for WW reuse were set in the Royal Decree 1620/2007 establishing the quality criteria (e.g., limits for nematodes, *Escherichia coli*, suspended solids and turbidity) for reusing water according to different uses (4).

2. Problems to be tackled by wastewater reuse

After treatment, concentrations of organic substances, salts, nutrients and other contaminants in WW can be higher than desired. Irrigation with such water can have a negative impact on the soil and agricultural yield, therefore monitoring of the treated WW quality is necessary, in order to prevent degradation of soil in agricultural land (5). Treated WW can also still contain microorganisms, pathogenic and/or multidrug resistant bacteria (MDB), and contaminants of emerging concern (CECs) such as plasticizers, antimicrobial agents, pharmaceuticals, pesticides, etc. and pose a risk to natural ecosystems (6,7,8). CECs in treated WW, used for irrigation, can accumulate in soil and affect the soil biota, be taken up by plants, like alfa-alfa (9) and lettuce (10), and enter the food chain, therefore if consumed, can also pose a threat to human health (11,12) .

Treated WW is known to contain pharmaceuticals like carbamazepine, metropolol and sulfamethoxazole, especially in the treated WW from hospitals, and plasticizers, such as bisphenol A and its analogues (13,14). Carbamazepine and its metabolites were found in the urine of individuals who had consumed crop, treated with WW (15), while bisphenol A was determined also in urine of children and adults (16). Antibiotic resistant bacteria can already be excreted and released into WW. Bacteria present in WW can develop resistance to

antibiotics due to the extensive use of antibiotics in hospitals, pharmaceutical industry and municipal effluents, and transfer of genes that make them resistant to antibiotics (17,18). In regard to viruses, it is known, that the SARS outbreak in 2003 (Amoy Gardens, Hong Kong) (19) evolved due to defects in inhouse WW plumbing system and ventilation systems and by indirect human contact with fomites. Several studies have detected the novel coronavirus SARS-CoV-2 in the infected patient's stool and in the municipal WW, but virus's infectivity potential in WW is so far not known (20,21,22).

Microplastic (MP) particles were found in treated WW as well. MP particles are an environmental pollutant and defined as all plastic particles in size lesser than 5 mm, that can enter the WW from personal care products and shedding of synthetic fibres from clothing during their washing (23,24). Majority of MPs are retained within the sewage sludge or removed by skimming during the treatment process, still some of them pass through WWTPs and end up in the WW effluents. Due to large volumes of WW passing through WWTPs, a great number of MPs is released in the environment, more precisely into the freshwater bodies; by irrigation with WW, these particles are deposited on agricultural land (25,26,27). MPs in soil can act as a vector for other contaminants, such as pesticides (28). Further, they and accumulate in soil, so that agricultural land acts as long-term sinks for MPs (26,29).

In a recent study (30) twas observed that the presence of MPs in soil decreases its retention capacity for tested pesticides, making them more available for transport through the soil. Study also confirmed the adsorption of pesticides onto MPs and fibres in soil, while the adsorption was dependent on the hydrophobic properties of the pesticide. The most highly adsorbed pesticides were the most hydrophobic ones, regardless of the type and morphology of the MPs (30)

3. The role of nature-based solutions in wastewater treatment and reuse

WW can be treated also using nature-based solutions (NBS). European commission defines NBS as solutions that a<re inspired and supported by nature, are cost effective and at the same time provide environmental, social and economic benefits. According to Langergraber et al. (31), NBS use organisms (e.g. microbes, algae, plants, insects, and worms) to achieve resource recovery. Examples of NBS treating municipal WW are constructed wetlands (CW), high-rate algal ponds (HRAP), evapotranspirative willow systems (EWS) and sludge treatment reed bed (STRB) which are all experiencing interesting advancements in their efficiency (e.g. removal of CECs, pathogens).

NBS for resource recovery have been studied at the central WWTP in Ajdovščina, Slovenia, where Climate Change Adaptation Centre (CCAC) was set up in 2019 (**Figure 1**). The WWTP provides the WW to be treated in a demonstrative pilot CW, HRAP and EWS, each of them

producing treated water and/or products to be used for irrigation or soil amendment in agriculture.

Moreover, the centre also has 25 lysimeters with integrated time domain reflectometry probes (TDR) for measuring the water content of the soil. The centre also includes units for sampling and quantification of leachate, which allows us to study the effect of irrigation with reclaimed WW on soil, cultivars, and of potential leaching of pollutants and nutrients into groundwater.

3.1. *Constructed wetlands*

CWs are engineered wetlands with regulated surface or subsurface flow of WW which is decontaminated with biodegradation, substrate adsorption, precipitation, plant uptake, photo- and hydrolysis processes (8). In comparison to conventional WWTPs, CWs are low cost systems with easy operation and maintaining (32). They are mainly in use for an on-site and a decentralized WW treatment; however bigger centralized systems for few 1000 PE are also applied. Regarding the removal of CEC and MDB, CWs have proven to be effective in removal of many CECs before effluent discharge in the environment, therefore coupling of CW and conventional WWTP offers more efficient strategy to remove CECs and reduce MDB (8) and therefore provide high quality effluent suitable for irrigation in agriculture.

The pilot CW at CCAC consists of a first (aerobic) stage vertical flow (VF) treatment beds and a second (mostly anaerobic) stage horizontal flow (HF) treatment beds. The CW reached steady removal of organic matter in terms of TSS, BOD and COD already during the first operation season, while in the second season we also observed removal of organic phosphorous and nitrification; however, inorganic nutrients like PO_4-P, NO_2-N and NO_3-N were not removed. Low removal of PO_4-P is expected in CW without additional chemical precipitation (33) while low removal of inorganic nitrogen can be explained by low abundance of denitrificators or lack of organic matter in the HF (34). In terms of nutrients recovery, a nutrient-rich effluent is beneficial for further use in agriculture.

Figure 1: The scheme of the Climate Change Adaptation Centre at the central WWTP in Ajdovščina, Slovenia.

3.2. *High-rate algal ponds*

Microalgae-based WWT systems (such as HRAP) are NBS, where the WW treatment is carried out by mixotrophic culture of microalgae and bacteria. Bacteria consume oxygen to metabolize organic compounds to inorganic nutrients and produce CO_2, which is taken up by microalgae and used for algae biomass growth (35). Microalgae have a great potential for removal of nutrients, BOD, heavy metals and pathogens from WW (35-37). (Benefits of WWT with microalgae are also CO_2 mitigation, oxygen production and production of valuable biomass, which can be used as a fertilizer, a feed, a source for production of chemicals, biofuels and other by-products (35,36).

HRAP at CCAC showed efficient removal of BOD, TP and PO_4-P, while removal of nitrogen compounds was moderate. The results indicate that phosphorous is probably the limiting factor for algae growth.

We researched the potential of microalga *Chlorella vulgaris* and mixed algae-bacteria biomass from HRAP to remove CECs on a laboratory scale (**Figure 2**). CECs spiked into photobioreactors were selected from the group of bisphenols, pharmaceuticals and substances from the EU watch list.

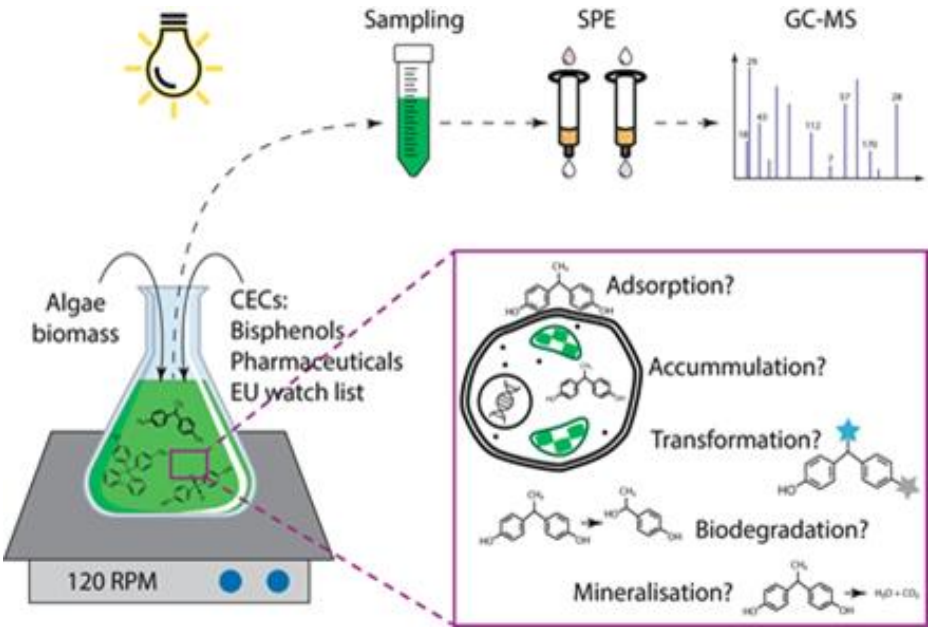

Figure 2: Experiments with *Chlorella vulgaris* and algae-bacteria mixed biomass on a laboratory- scale to study removal of contaminants of emerging concern.

Removal of CECs used in the experiments varied between the culture of mixed algae-bacteria biomass and *C. vulgaris.* Bisphenols were better removed in mixed algae-bacteria biomass culture, whereas certain compounds from the groups of pharmaceuticals and EU watch list were better removed in *C. vulgaris* culture. E.g., ibuprofen was removed by 75 % in 40 hours in a culture of *C. vulgaris*, whereas in a culture of mixed algae-bacteria biomass, it was removed by 12 %.

3.3. *Evapotranspirative willow systems*

Domestic WW contains organic matter and nutrients; therefore, it can be used as fertilizer and water source for production of non-edible products like wood biomass. In EWS all inflow WW is used for willow growth and evaporation; therefore, EWS have no discharge to the environment but produce wood biomass and nutrient rich media (38). EWS consist of waterproof bed filled with soil and planted with willows that are coppiced every 2-5 years in order to keep high evapotranspiration and biomass production. The pilot EWS at CCAC produces 33±14 to 59±14 t DM ha^{-1} or 5.8±1.0 to 8.5±0.6 kg of biomass per m^3 of WW in a 2-year rotation depending on the willow clone. The results indicate that applying specific willow clones selected for high biomass production enhances nutrient recovery from WW. The produced wood biomass is at harvest transformed to woodchip that can be used as an energy source for heating or as soil amendment in agriculture.

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References

1. Sarkodie SA, Strezov V, Economic, Social and Governance Adaptation Readiness for Mitigation of Climate Change Vulnerability: Evidence from 192 Countries. Sci Total Environ, 2019, 656: 150–164.

2. European Comission (2019), Water reuse. Updated August 2019. Accessed: 27.5.2020. Available from: https://ec.europa.eu/environment/water/reuse.htm

3. Juanico M, Salgot M, Water Reuse in the Northern Mediterranean Region. In: Jimenez B, Asano T, editors. Water Reuse: An International Survey on current Practice, issues and needs. IWA Publishing, 2008, pp. 3–26.

4. Torrens A, Folch M, Salgot M, Aulinas M, Recycling of carwash effluents treated with subsurface flow constructed wetlands. In: Stefanakis AI, editor. Constructed Wetlands for industrial wastewater treatment. New Jersey, USA, John Wiley & Sons, 2018, pp. 469-492

5. Zupanc V, Pintar M, Alternativni vir vode za namakanje - prečiščena odpadna voda. In: 29. Mišičev vodarski dan 2018, Mišičev vodarski dan: zbornik referatov,

6. European Environment Agency (EEA), Towards efficient use of water resources in Europe. EEA report No 1/2012, 2012. European Environment Agency, Copenhagen, Denmark.

7. Das S, Ray NM, Wan J, Khan A, Chakraborty T, Ray MB, Chapter 5 - Micropollutants in wastewater: Fate and Removal Process. In: Farooq R and Ahmad Z, editors. Physico-Chemical Wastewater Treatment and Resource Recovery. IntechOpen, 2017. Doi: 10.5772/65644

8. Garcia J, Garcia-Galan MJ, Day JW et al. A review of emerging organic contaminants (EOCs), antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARGs) in the environment: Increasing removal with wetlands and reducing environmental impacts. Bioresour Technol, 2020, 307: 123228

9. Dodgen LK, Li J, Parker D, Gan JJ, Uptake and Accumulation of Four PPCP/EDCs in Two Leafy Vegetables. Environ Pollut, 2013, 182: 150–156.

10. Calderón-Preciado D, Jiménez-Cartagena C, Matamoros V, Bayona JM, Screening of 47 Organic Microcontaminants in Agricultural Irrigation Waters and Their Soil Loading. Water Res, 2011, 45(1): 221-231.

11. Wu C, Spongberg AL, Witter JD, Fang M, Czajkowski KP, Uptake of pharmaceutical and personal care products by soybean plants from soils applied with biosolids and irrigated with contaminated water. Environ. Sci. Technol. 2010, 44, 6157–6161.

12. Shenker M, Harush D, Ben-Ari J, Chefetz B, Uptake of carbamazepine by cucumber plants – A case study related to irrigation with reclaimed wastewater. Chemosphere. 2011, 82(6): 905–910.

13. Radjenovič J, Petrovič M, Barceló D, Analysis of pharmaceuticals in wastewater and removal using a membrane bioreactor. Analytical and bioanalytical chemistry, 2007, 387(4): 1365–1377.

14. Česen M, Lenarčič K, Mislej V, et al. The occurrence and source identification of bisphenol compounds in wastewaters. Sci Total Environ, 2018, 616-617:744-752.

15. Paltiel O, Fedorova G, Tadmor G, Kleinstern G, Maor Y, Chefetz B, Human exposure to wastewater-derived pharmaceuticals in fresh produce: A randomized controlled trial focusing on carbamazepine. Environ. Sci. Technol. 2016, 50, 4476–4482

16. Tratnik JS, Kosjek T, Heath E, et al. Urinary bisphenol A in children, mothers and fathers from Slovenia: Overall results and determinants of exposure. Environmental research, 2019, 168: 32-40.

17. Barancheshme F, Munir M, Development of antibiotic resistance in wastewater treatment plants. In: Kumar Y, editor. Antimicrobial Resistance - A Global Threat. IntechOpen, 2019. doi: 10.5772/intechopen.81538.

18. Šunta U, Žitnik M, Finocchiaro N, Bulc T, Torkar K, Faecal indicator bacteria and antibiotic-resistant β-lactamase producing Escherichia coli in blackwater: a pilot study. Archives of Industrial Hygiene and Toxicology, 2019, 70(2): 140-148.

19. McKinney KR, Yu YG, Lewis TG, Environmental Transmission of SARS at Amoy Gardens. J Environ Health, 2006, 68(9): 26-30.

20. Holshue ML, DeBolt C, Lindquist S, et al. First Case of 2019 Novel Coronavirus in the United States. N Eng J Med, 2020, 382: 929-936.

21. Ong SWX, Tan YK, Chia PY, et al. Air, surface environmental, and personal protective equipment contamination by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from a symptomatic patient. Jama, 2020, 323(16): 1610-1612.

22. Lodder W, de Roda Husman AM, SARS-CoV-2 in wastewater: potential health risk, but also data source. Lancet Gastroenterol Hepatol, 2020, 5(6): 533-534.

23. GESAMP, Guidelines for the monitoring and assessment of plastic litter in the ocean. (Kershaw P, Turra A in Galgani F, ured.). (IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection), GESAMP, 2019, No. 99, 130p.

24. Conley K, Clum A, Deepe J, Lane H, Beckingham B, Wastewater treatment plants as a source of microplastics to an urban estuary: Removal efficiencies and loading per capita over one year. Water Res. X, 2019, 3: 100030.

25. Carr SA, Liu J, Tesoro AG, Transport and Fate of Microplastic Particles in Wastewater Treatment Plants. Water Res, 2016, 91: 174–182.

26. Horton AA, Walton A, Spurgeon DJ, Lahive E, Svendsen C, Microplastics in Freshwater and Terrestrial Environments: Evaluating the Current Understanding to Identify the Knowledge Gaps and Future Research Priorities. Sci. Total Environ, 2017a, 586: 127–141.

27. Horton AA, Svendsen C, Williams RJ, Spurgeon DJ, Lahive E, Large Microplastic Particles in Sediments of Tributaries of the River Thames, UK – Abundance, Sources and Methods for Effective Quantification. Mar Pollut Bull, 2017b, 114 (1): 218–226.

28. Bakir A, Rowland SJ, Thompson RC, Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions. Environ. Pollut, 2014, 185: 16–23.

29. Ng EL, Huerta Lwanga E, Eldridge S M, et al. An overview of microplastic and nanoplastic pollution in agroecosystems. Sci. Total Environ, 2018, 627: 1377–1388.

30. Šunta U, Prosenc F, Trebše P, Griessler Bulc T, Bavcon Kralj M (submitted), Adsorption of acetamiprid, chlorantraniliprole and flubendiamide on different type of microplastics present in alluvial soil. (not published)

31. Langergraber, G. et al. (2020). Implementing nature-based solutions for creating a resourceful circular city. Blue-Green Systems 2 (1): 173–185.

32. Álvarez JA, Ávila C, Otter P et al. Constructed wetlands and solar driven disinfection technologies for sustainable wastewater treatment and reclamation in rural India: SWINGS project. Water Sci Technol, 2017, 76(6): 1474-1489.

33. Kumar M, Singh R. 2017. Performance evaluation of semi continuous vertical flow constructed wetlands (SC-VF-CWs) for municipal wastewater treatment. Bioresour Technol. 232:321–330. doi:10.1016/j.biortech.2017.02.026.

34. Li H, Tao W. 2017. Efficient ammonia removal in recirculating vertical flow constructed wetlands: Complementary roles of anammox and denitrification in simultaneous nitritation, anammox and denitrification process. Chem Eng J. 317:972–979. doi:10.1016/j.cej.2017.02.143.

35. Abdel-Raouf N, Al-Homaidan AA, Ibraheem IBM, Microalgae and wastewater treatment. Saudi J Biol Sci, 2012, 19(3), 257-275.

36. Cai T, Park SY, Li Y, Nutrient recovery from wastewater streams by microalgae: Status and prospects. Renew Sust Energ Rev, 2013, 19: 360-369.

37. Žitnik M, Šunta U, Godič Torkar K et al. The study of interactions and removal efficiency of *Escherichia coli* in raw blackwater treated by microalgae *Chlorella vulgaris*. J Clean Prod, 2019, 238: 117865Abdel-Raouf N, Al-Homaidan AA, Ibraheem IBM, Microalgae and wastewater treatment. Saudi J Biol Sci, 2012, 19(3), 257-275.

38. Istenič D, Božič G, Aria CA, Griessler Bulc T, Growth dynamic of three different white willow clones used in a zero-discharge wastewater treatment system in the sub-Mediterranean region - an early evaluation. Desalination Water Treat, 2017, 91: 260-267.

THE ROLE OF PLATELET-AND EXTRACELLULAR VESICLE-RICH PLASMA IN THE TREATMENT OF TEMPORAL BONE CAVITY INFLAMMATION: A RANDOMIZED CONTROLLED TRIAL

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Abstract

Objective: To determine the efficacy of platelet- and extracellular vesicle-rich plasma (PVRP) for the treatment of chronic postoperative temporal bone cavity inflammation (CPTBCI) in a randomized controlled clinical trial. Methods: Patients with CPTBCI were recruited and randomly assigned to the group treated with PVRP or standard conservative treatment methods. Patients in the PVRP group received PVRP, prepared with plasma-based 2-stepped centrifugation method, at the first and second check-up. The outcome was measured with COMQ-12 (chronic otitis media questionnaire-12) at four check-ups. Results: The trial was completed by 11 cases of CPTBCI from the PVRP and 13 cases from the control group. Group assignment had a statistically significant effect on the COMQ-12 sum scores over the period of four check-ups ($p < 0.0005$, partial $\eta^2 = 0.304$). The difference in COMQ-12 sum scores between groups was statistically significant at the second ($p = 0.019$, partial $\eta^2 = 0.245$), third ($p = 0.014$, partial $\eta^2 = 0.267$) and fourth check-up ($p = 0.001$, partial $\eta^2 = 0.433$). The COMQ-12 sum score changed statistically significantly during the treatment within the PVRP group ($p < 0.001$, partial $\eta^2 = 0.494$), but not within the control group ($p = 0.339$). Discussion: The COMQ-12 sum scores were statistically significantly dependent on the mode of treatment (i.e. assigned group). Two PVRP applications had a beneficial effect on the quality of life, which persisted for at least two months after the second application. Also, treatment with PVRP improved the quality of life to a greater extent than established treatment.

1. Introduction

Platelet-rich plasma is a product, prepared from peripheral venous blood, which possesses beneficial immune, hemostatic, and regenerative effects. Since it is rich in extracellular vesicles (EVs), it can be called platelet- and extracellular vesicle-rich plasma (PVRP) (1). In addition to platelets, EVs are thought to be the main mediators of the regenerative effects of PVRP (2). EVs are a heterogeneous group of nanometrically-sized membrane structures that play an important role in intercellular communication and can, therefore, be used in diagnosis and treatment (3–5). Although PVRP has been used in medicine for tissue regeneration for decades, there is a lack of trials regarding EVs in association with PVRP so far (6).

Despite numerous clinical trials on the use of PVRP, research in otorhinolaryngology is currently scarce (7). A particular challenge is the treatment of chronic postoperative temporal bone cavity inflammation (CPTBCI). The postoperative temporal bone is most often the result of surgical treatment of cholesteatoma of the middle ear. Treatment of CPTBCI is often exhausted due to the failure of surgical procedures and standard conservative treatment methods. Up to 20 % of patients are expected to suffer from CPTBCI (8,9). Problems associated with CPTBCI not only impair quality of life, but also significantly impact the health care system (10–12).

We aimed to test the efficacy of PVRP in the treatment of CPTBCI after exhausted surgical and standard conservative treatment, in a prospective randomized controlled clinical trial. The outcome of treatment was assessed by comparing the quality of life of patients treated with PVRP and patients treated with standard conservative methods throughout four otorhinolaryngological examinations (i.e., check-ups).

2. Methods

National Medical Ethics Committee, Republic of Slovenia approved the trial (No. 0120-146 / 2019/5). The trial started by creating a list of patients or cases who met the inclusion criteria (i.e., recruitment) during visits at the Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre Ljubljana for six months (Table 1). CPTBCI on one ear (i.e., unilateral) was considered as one case of CPTBCI and CPTBCI on both ears (i.e., bilateral) as two cases of CPTBCI. Surgical treatment was considered as exhausted if an additional surgical treatment would not eliminate CPTBCI while preserving hearing. Standard conservative treatment was defined as exhausted if it was ineffective in treating CPTBCI for at least 8 weeks.

Table 1: Inclusion and exclusion criteria of the trial.

Inclusion criteria	**Exclusion criteria**
COM defined as a presence of ³ 1 of:	venepuncture site inflammation
• visible ear discharge	pregnancy or breastfeeding
• indirect ear discharge signs (e.g. on a pillow, clothes)	chronic use of immunomodulatory and / or antimicrobial drugs
• itching	presence of systemic infectious disease
• sensation of ear fullness	presence of malignancy
• clinical signs of inflammation exacerbation	other experimental attempts to treat the consequences of radical COM surgery
exhausted surgical and standard conservative treatment	inability and / or refusal of the patient to participate in the trial
age > 18 years	presence of autoimmune disease

COM – chronic otitis media.

After recruitment, patients were randomly allocated one of the two interventions: treatment with standard conservative methods (i.e., control group), and treatment with PVRP (i.e., PVRP group).
After recruitment and allocation, patients were examined at the Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre Ljubljana where they attended their regularly scheduled check-up. We informed each patient about the allocation and re-checked the same inclusion criteria (Table 1). If the patient met these criteria and wanted to participate in the trial voluntarily, he/she signed an informed consent, which meant the enrollment in the trial. If the patient refused to participate, he/she did not enter the survey, but this did not affect the regularly scheduled treatment. The latter included the continuation of standard conservative treatment methods, described below. Patients enrolled in the trial started the treatment according to the treatment protocol, either with standard conservative methods (i.e., control group) or with PVRP (i.e., PVRP group). Only patients who completed treatment according to the protocol were included in the final

analysis. The researchers who treated patients and the patients were informed about the allocation, therefore the trial was not blind.

2.1. *Treatment protocol*

The treatment included four check-ups by the leading researchers at the Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre Ljubljana. Four-week intervals between individual check-ups were planned, so the treatment was expected to last 12 weeks. The treatment protocol for both groups is shown in Table 2.

Table 2: Chronic postoperative temporal bone cavity inflammation treatment protocol (13,14).

<table>
<tr><th colspan="2"></th><th>Control group</th><th colspan="2">PVRP group</th></tr>
<tr><td rowspan="6">1</td><td>A</td><td colspan="3">review of inclusion and exclusion criteria</td></tr>
<tr><td>B</td><td colspan="3">invitation to participate in the trial</td></tr>
<tr><td>C</td><td colspan="3">enrollment in the trial</td></tr>
<tr><td>D</td><td colspan="3">completion of the COMQ-12 questionnaire</td></tr>
<tr><td>E</td><td colspan="3">toilet (i.e. cleaning) the middle ear</td></tr>
<tr><td>F_A</td><td>standard conservative methods</td><td>F_B</td><td>treatment with PVRP</td></tr>
<tr><td colspan="2">2_A (~ 4^{th} week)</td><td>1A, 1D, 1E, $1F_A$</td><td>2_B (~ 4^{th} week)</td><td>1A, 1D, 1E, $1F_B$</td></tr>
<tr><td colspan="2">3_A (~ 8^{th} week)</td><td>1A, 1D, 1E, $1F_A$</td><td>3_B (~ 8^{th} week)</td><td>1A, 1D, 1E</td></tr>
<tr><td colspan="2">4_A (~ 12^{th} week)</td><td>1A, 1D, 1E, $1F_A$</td><td>4_B (~12^{th} week)</td><td>1A, 1D, 1E</td></tr>
</table>

Numbers (1-4) mark each check-up. Capital letters (A-F) mark interventions performed during the check-up, alphabetically. A refers to the control group check-up, B refers to the PVRP group check-up. Example: 1FB stands for treating the patient in a PVRP group with a PVRP. 3A contains measures 1A, 1D, 1E, 1FA. The second examination was scheduled for 4 weeks, the third 8 weeks and the fourth 12 weeks after the first examination. PVRP – Platelet- and extracellular vesicle-rich plasma; COMQ-12 – chronic otitis media questionnaire 12.

Patients in the PVRP group received PVRP at the first and second check-up (Table 2). We performed a cleaning (i.e., toilet) of the ear at each check-up. The other standard conservative methods, described below, were not used in the PVRP group. At the third and fourth check-up, we performed the ear toilet only. The discontinuation of other standard conservative methods at the third and fourth check-up in the PVRP group did not pose any risk, as the patients' problems were chronic.

Patients in the control group continued the treatment with standard conservative methods during each check-up. These methods included ear toilet, use of topical antimicrobial drugs, anti-inflammatory drugs and antiseptic drugs (Table 2).

2.2. *Outcome measures*

The treatment outcome was measured at each check-up with Slovenian version of COMQ-12 (Chronic Otitis Media Questionnaire-12) (13) which is a chronic otitis media (COM)-related quality of life measure (12). The COMQ-12 was completed by the patient her/himself. The questionnaire comprises 12 questions scored 0-5. Seven questions relate to the severity of COM symptoms, two questions to the impact of COM on lifestyle and work, two questions to the burden on the health system due to COM, and one question is general. The maximum possible sum score is 60. Since the CPTBCI is a type of COM, the higher COMQ-12 sum score signifies the worse quality of life associated with COM or CPTBCI.

2.3. *Platelet- and extracellular vesicle-rich plasma preparation protocol*

Blood was taken (i.e., venepuncture) at the Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre Ljubljana from the cubital vein by a vacuum method by a medical technician. A 21 G wing needle (so-called butterfly) (Safety-Lok Blood Collection Set, BD Vacutainer, Becton Dickinson, USA) was used to draw blood into four 4.5 mL citrate tubes. (9 NC sodium citrate 0.105 M, BD Vacutainer, Becton Dickinson, USA). The tubes were stored at room temperature before and after venipuncture. PVRP was prepared according to the protocol described in Table 3 on the day of venepuncture in the Laboratory of Clinical Biophysics, Faculty of Health, University of Ljubljana, for each patient in PVRP group at the first and second check-up (Table 2).

Step 9 was followed by administration of PVRP in the ear via PVRP-soaked ear wick. Weighing was performed with balance Sartorius, T2145-OCE, Goettingen, Germany and centrifugation with Tehtnica, Centric 400R, Domel, d.o.o., Železniki, Slovenia. Termofisher scientific, USA (ref.: 225-1S, lot: 19100819) sterile pipettes and Simport scientific, Canada (ref.: T405-1A lot: 904175849) sterile tubes were used to prepare PVRP. PVRP – platelet- and extracellular vesicle-rich plasma.

PVRP was brought to a patient, waiting at the Department of Otorhinolaryngology and Cervicofacial Surgery, University Medical Centre Ljubljana. Immediately before administration, PVRP was resuspended, as it sedimented during the transport. Then the ear wick (i.e. gauze tamponade 1 cm x 10 m cut on a 7 cm long strip, Tosama d.o.o., Slovenia) was soaked with PVRP and inserted into the ear. The patient was instructed to pull the wick out of his ear after two days.

Table 3: PVRP preparation protocol (13).

Step	Description
1	Venipuncture of cubital vein to draw blood into four 4.5 mL sodium citrate tubes
2	Weighing of four 4.5 mL citrate tubes. If the weights differed > 0.02 g (incomplete blood collection), they were balanced by the addition of counterweight tubes.
3	First centrifugation step: 300 g, 5 min and 18 °C.
4	Transfer of the supernatant (i.e., plasma or yellow top layer without buffy coat) with a sterile pipette from four centrifuged citrate tubes into two sterile polypropylene tubes.
5	Second centrifugation step: 700 g, 17 min and 18 °C.
6	Removal of approximately half of the supernatant (i.e., platelet-poor plasma) from two sterile polypropylene tubes with a sterile pipette.
7	Resuspension or homogenization of the remaining halves in sterile tubes with a sterile pipette to obtain half of PVRP.
8	Fusion of two halves of PVRP.
9	PVRP resuspension.

2.4. *Statistical analysis*

We used the computer program Microsoft Excel for Mac (versions 16.9.0-16.36) to record and edit the trial data, and the computer program SPSS (statistical package 23, IBM Corp., Armonk, New York, USA) for statistical analysis. The difference between the groups was defined as statistically significant if the probability of rejecting the null hypothesis was greater than 95 % ($p < 0.05$).

3. Results

3.1 *Subjects*

25 patients or 27 cases of CPTBCI were recruited, as 2 patients had bilateral CPTBCI. Patients with bilateral CPTBCI were assigned to the control group. The remaining 23 recruited patients were randomly assigned to the control or PVRP group; 10 patients to control and 13 patients to the PVRP group. One patient assigned to the PVRP group refused enrollment in the trial. The trial included 24 patients or 26 cases of CPTBCI. The trial was completed by 11 patients from each group, i.e. 11 cases from the PVRP group and 13 cases (2 patients with bilateral CPTBCI) from the control group. These patients or cases were included in the final analysis. Due to the onset of the coronavirus disease 2019 (COVID-19) pandemic, we had to discontinue the trial in two patients (one patient from each group) prematurely. As these two patients attended only the first check-up, we did not include them in the final analysis.

Baseline demographic and clinical characteristics of patients included in the final analysis are shown in Table 4. In 21 patients (95 %) or 23 cases (96 %), CPTBCI occurred in the radical cavity after radical mastoidectomy (i.e., canal wall down mastoidectomy), in one patient (5 %) or one case (4 %) CPTBCI occurred in a cavity after subtotal petrosectomy.

3.2 *Check-ups and adverse events*

The time between the first and second check-up did not differ statistically significantly between the PVRP group (*Mdn* = 28) and the control group (*Mdn* = 28) according to the Mann-Whitney U-test (*U* = 45, *z* = –1,705, *p* = 0,332). This was also found for the time between the second and third check-up (Mdn = 28 for both groups, *U* = 51, *z* = –0.976, *p* = 0.519) and the time between the third and fourth check-up (*Mdn* = 28 for both groups, *U* = 63, *z* = 0.224, *p* = 0.898). No PVRP-related adverse events were detected.

Data are shown as a number and percentages of patients in the corresponding group. Time to treatment – time from the beginning of the problems due to chronic postoperative temporal bone cavity inflammation to the time of entering the trial; Time from surgery – the time from the last surgery required for the treatment of chronic middle ear infection to the time of entering the trial. PVRP – platelet- and extracellular vesicle-rich plasma; *p* – p-value; *M* – average value; *SD* – standard deviation, *Mdn* - median value; ‡ – independent samples t-test; * – Fisher's exact test; ** – Mann-Whitney U-test; p-value < 0.05 denotes statistically significant difference.

Table 4: Baseline demographic and clinical characteristics of patients that completed the trial (14).

	PVRP group (n = 11)	Control group (n = 11)	*p*
Gender			
male	9 (82 %)	9 (82 %)	1.000
female	2 (18 %)	2 (18 %)	
Age			
M ± *SD* [years]	47 ± 18	52 ± 24	0.632[‡]
Comorbidities			
yes (n, %)	6 (55 %)	6 (55 %)	1.000
no (n, %)	5 (45 %)	5 (45 %)	
Smoking			
yes (n, %)	2 (18 %)	5 (45 %)	0.361[*]
no (n, %)	9 (82 %)	6 (55 %)	
Alcohol consumption			
yes (n, %)	10 (91 %)	7 (64 %)	0.311[*]
no (n, %)	1 (9 %)	4 (36 %)	
Time to treatment			
Mdn [days – years]	2688 – 7.4	3172 – 8.7	0.116[**]
Time from surgery			
Mdn [days – years]	4644 – 12.7	5224 – 14.3	0.573[**]

3.3. COMQ-12 *sum scores during treatment*

Two-sided mixed analysis of variance (i.e., 2-way ANOVA) was used for statistical analysis of difference in COMQ-12 sum score during treatment. Group assignment (i.e., mode of treatment) had a statistically significant effect on the COMQ-12 sum scores over the period of four check-ups (F (3, 60) = 8.755 $p < 0.0005$, partial $\eta^2 = 0.304$). At the first check-up, there was no statistically significant difference between the groups in the COMQ-12 sum scores (F (1, 20) = 0.00, p = 0.949).

The difference in COMQ-12 sum scores between groups was statistically significant at the second check-up (F (1, 20) = 6.48, p = 0.019, partial $\eta^2 = 0.245$), third check-up (F (1, 20) = 7, 30, p = 0.014, partial $\eta^2 = 0.267$) and fourth check-up (F (1, 20) = 15.29, p = 0.001, partial η^2 =

0.433) (Figure 1) with the score being in favor (showing better clinical state) of the PVRP group.

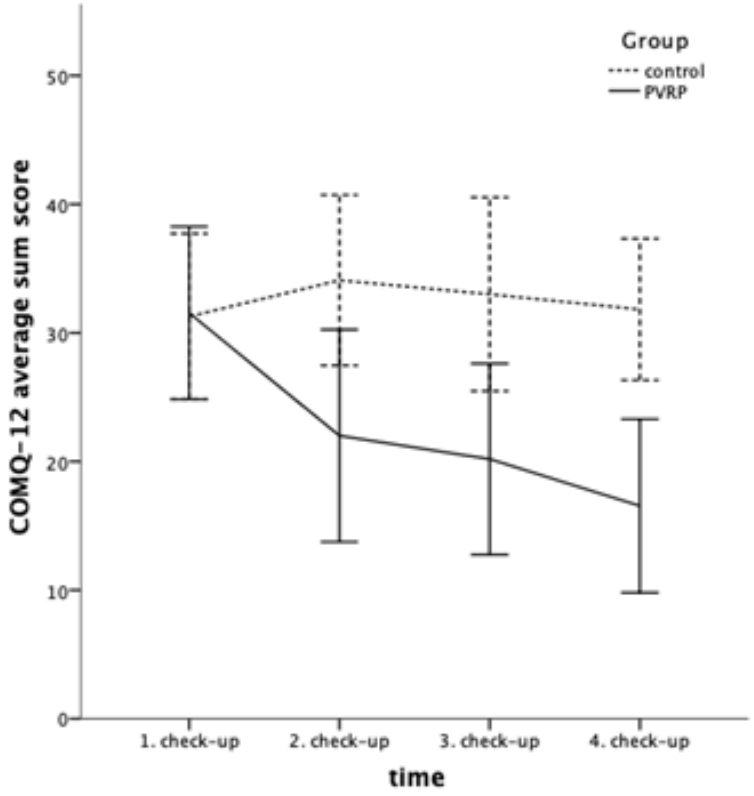

Figure 1: COMQ-12 average sum score during the treatment. 95 % confidence intervals are included in the PVRP and control group. There is a statistically significant difference in sum score in the PVRP group at the second, third and fourth check-up. Sum score decrement is statistically significant in the PVRP group, more accurately between the first and second check-up and first and fourth check-up. COMQ-12 – chronic otitis media questionnaire 12; PVRP – platelet- and extracellular vesicle-rich plasma. From (14).

The COMQ-12 sum score changed statistically significantly during the treatment within the PVRP group (F (3, 30) = 9.78, $p < 0.001$, partial $\eta^2 = 0.494$), but not in the control group (F (3, 30) = 1.17, $p = 0.339$). The COMQ-12 sum score decreased statistically significantly within the PVRP group from the first to the third check-up (M = 11 points, SE = 3 points, $p = 0.029$) and from the first to the fourth check-up (M = 15 points, SE = 2 points, $p = 0.001$). The decrease in the COMQ-12 sum score was not statistically significant between the first and the second check-up (M = 10 points, SE = 3 points, $p = 0.102$), between the second and the third check-up (M = 2 points, SE = 3 points, $p = 1.000$), between the second and the fourth check-up (M = 5 points, SE = 3 points, $p = 0.531$) and between the third and the fourth check-up (M = 4 points, SE = 3 points, $p = 1.000$) (Figure 1). The COMQ-12 sum score did not differ statistically significantly within the control group from the first to the second check-up (M = 3 points, SE = 2 points, $p = 0.632$), from the first to the third check-up (M = 2 points, SE = 2 points, p =

1.000), from the first to the fourth check-up (*M* = 1 point, *SE* = 1 point, *p* = 1.000), from the second to the third check-up (*M* = 1 point, *SE* = 2 points, *p* = 1.000), from the second to the fourth check-up (*M* = 2 points, *SE* = 2 points, *p* = 0.976) and from the third to the fourth check-up (*M* = 1 point, *SE* = 2 points, *p* = 1.000) (Figure 1).

Discussion

There were no differences in baseline demographic and clinical characteristics between the groups, which confirms the randomization. The median time to treatment confirms the longevity of the CPTBCI problems and the exhaustion of the standard conservative treatments used during this period. The median time from surgery means that problems with CPTBCI started at the median time of 6.1 years after surgery, calculated as the difference between the median time from surgery and the median time to treatment (13.8 –7.7 years).

The quality of life associated with CPTBCI was assessed with COMQ-12 in each patient and at each check-up (15). At the first examination, there were no statistically significant differences in COMQ-12 sum scores between groups, which confirms the randomization. During further check-ups, the COMQ-12 sum scores differed statistically significantly between groups. The COMQ-12 sum scores were statistically significantly dependent on the mode of treatment (i.e. assigned group) of CPTBCI. The COMQ-12 sum score decreased statistically significantly from the first to the third check-up and from the first to the fourth check-up in the PVRP group. Despite the reduction in the COMQ-12 sum score from the first to the second check-up, from the second to the third check-up and from the third to the fourth check-up, the differences were not statistically significant. The COMQ-12 sum scores did not differ statistically significantly in the control group during the check-ups. The decrease in the COMQ-12 sum score in the PVRP group, although statistically insignificant, and the predominantly unchanged score in the control group meant a statistically significant difference in scores between the groups for all compared check-ups. We can conclude that the improvement took place at small steps, but persistently.

The results show that two applications of PVRP have a beneficial effect on the quality of life, which persists for at least two months after the second application. Also, treatment with PVRP improves the quality of life to a greater extent than the currently standardized treatments. When interpreting the results of patients ’quality of life, we need to be aware of possible bias, as the research was not blind. Nevertheless, patients from the control group were treated actively, with standard conservative methods and at more frequent time intervals than in established clinical practice. The effectiveness of PVRP could be further evaluated by a placebo-controlled trial, but there are ethical concerns about placebo use. Based on statistically significant improvement in the quality of life of patients treated with PVRP and the absence of improvement in the quality of life of patients treated with standard conservative methods, we can state that the use of PVRP has beneficial effects due to potential immune and regenerative effects in surgically treated CPTBCI.

We did not detect PVRP-related adverse events, which is consistent with the findings of other trials (16). Thus, we suggest that PVRP is a safe and efficient way to treat CPTBCI.

Acknowledgments
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References

1. Uršič B, Vozel D, Šuštar V, Kocjančič B, Dolinar D, Kralj-Iglič V, Extracellular Vesicles from Platelet-Rich Plasma as Conveyors of Regeneration Potential in Orthopedics. J Hematol Thrombo Dis 2014, 2: 163.

2. Tao S-C, Guo S-C, Zhang C-Q, Platelet-derived Extracellular Vesicles: An Emerging Therapeutic Approach. Int J Biol Sci 2017, 13(7): 828–34.

3. van Niel G, D'Angelo G, Raposo G, Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol 2018, 19(4): 213–28.

4. Yáñez-Mó M, Siljander PR-M, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 2015, 14;4.

5. Ogorevc E, Kralj-Iglič V, Veranič P, The role of extracellular vesicles in phenotypic cancer transformation. Radiol Oncol 2013, 1;47(3): 197–205.

6. Alves R, Grimalt R, A Review of Platelet-Rich Plasma: History, Biology, Mechanism of Action, and Classification. Skin Appendage Disord 2018, 4(1): 18–24.

7. Vozel D, Božič D, Jeran M, et al. Treatment with platelet- and extracellular vesicle-rich plasma in otorhinolaryngology-a review and future perspectives. In: Pocsfalvi G, Bongiovanni A, Mauro M, Kralj-Iglič V, editors. ABLSA 32: Biological Membrane Vesicles: Scientific, Biotechnological and Clinical Considerations. Amsterdam, The Netherlands, Elsevier, in press.

8. Henatsch D, Alsulami S, Duijvestijn AM, Cleutjens JP, Peutz-Kootstra CJ, Stokroos RJ, Histopathological and Inflammatory Features of Chronically Discharging Open Mastoid Cavities Secondary Analysis of a Randomized Clinical Trial. JAMA Otolaryngol Neck Surg 2018, 144(3): 211–7.

9. Mastoidectomy: Canal Wall Down Techniques. In: Brackmann DE, Shelton C, Arriaga MA, editors. Otologic surgery. 3th ed. Philadelphia, W.B. Saunders Company, 1994. p. 239.

10. Maile EJ, Youngs R, Quality of life measures in otitis media. J Laryngol Otol 2013, 127(5): 442–7.

11. Bakir S, Kinis V, Bez Y, et al. Mental health and quality of life in patients with chronic otitis media. Eur Arch Otorhinolaryngol 2013, 270(2): 521–6.

12. Phillips JS, Haggard M, Yung M, A new health-related quality of life measure for active chronic otitis media (COMQ-12): development and initial validation. Otol Neurotol 2014, 35(3): 454–8.

13. Božič D, Vozel D, Jeran, M, et al., Elaboration of the platelet and extracellular vesicle-rich plasma preparation for wound healing, in preparation.

14. Vozel D, Božič D, Jeran M, et al., Platelet- and extracellular vesicle-rich plasma as an effective treatment modality for chronic postoperative temporal bone cavity inflammation: a randomized controlled clinical trial, submitted.

15. Vozel D, Steiner N, Božanić Urbančič N, Mladenov D, Battelino S, Slovenian cross-cultural adaptation and validation of health-related quality of life measures for chronic otitis media (COMQ-12), vertigo (DHI, NVI) and tinnitus (THI). Slov J Public Health. In press.

16. Dhillon RS, Schwarz EM, Maloney MD, Platelet-rich plasma therapy - future or trend? Arthritis Res Ther 2012, 14(4): 219.

Research

TOXICITY OF SURFACTANTS SODIUM DODECYL SULPHATE AND TRITON X-100 TO MARINE MICROORGANISMS

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Abstract

In this study, we investigated toxic effects of an anionic surfactant sodium dodecyl sulphate (SDS), and a non-ionic surfactant Triton X-100 (TX100) on a unicellular marine microalgae. Loss of motility was observed as one of the primary effects of both surfactants, with effective concentrations of 0.02 pmol/cell (16 µM) for TX100, and 0.35 pmol/cell (350 µM) for SDS which was determined after 30-minute exposure time at room temperature. Leakage of intracellular contents was observed at higher concentrations. Scanning electron microscopy revealed many sub-micron particles in the samples treated with the surfactant. No effect was observed for cells in the cystic state, which appeared intact even after 3 days of exposure at the concentrations of surfactant as high as 1 g/L. We found that surfactants may stimulate the microalgae to produce extracellular vesicles, however, the effect depends on the state of the microalgae as well as on the parameters of the surroundings.

1. Introduction

The onset of world-wide industrialisation in the course of the past 25 years has created a greater demand for man-made chemicals, which has led to an increase in the amounts of toxic contaminants that are being released into the environment (1). Consequently, the growing ecological and toxicological burden calls for the implementation of toxicity screening tests in order to assess the potential hazard of various compounds on microorganisms (1).

Marine microorganisms such as microalgae play an important role in global nutrient chains (2) and are considered as promising for the development of useful products in the area of industrial and pharmacological applications (3). The growing interest in microalgae draws attention to the possible effects of environmental pollutants on these organisms. Examining the interactions of different man-made chemicals with microorganisms provides an insight into their possible adverse effects on their growth and development. Synthetic surface-active agents or synthetic surfactants are man-made chemicals which are widespread in industrial and domestic settings (4). They are found in wetting and foaming agents, anti-adhesive solutions and agricultural chemical and household cleaning products (5). Discharging large amounts of surfactants into the environment, without proper treatment, can represent a serious risk to the ecosystem (5).

Enormous quantities of surfactants are already used daily for the industrial and household purposes, and the global annual production of synthetic surfactants is expected to continue growing along with the increasing demands for detergent and cosmetic products (6). After use, the surfactant residuals are released into sewage systems or directly into surface waters (5). If discharged into sewage systems, surfactants may be degraded in wastewater treatment plants. However, the degree of degradation depends on several factors, including the chemical structure of the surfactant and other conditions (e.g. pH, temperature, dissolved oxygen concentration) (5).

In terms of interactions with living organisms, the amphipathic nature of surfactants allows them to interact with proteins and lipid bilayers, causing a disruption of membrane organization and an increase in cellular permeability. This can result in the leakage and release of cellular contents into the extracellular environment (5). For microalgae, once inside the cell, the surfactants can disrupt protein arrangements and enzyme activity, which can affect the organization of the thylakoids and interfere with chlorophyll synthesis (7, 8).

Different surfactants differ in their characteristics and mechanisms of action; based on their structure they are classified as ionic (anionic, cationic and amphoteric) or non-ionic (9). Most surfactants are degraded to a certain extent in the environment by microbes, hence, the environmental concentrations of surfactants in aquatic environments are usually relatively low (measured in µg/L) (10). However, assessing the possible environmental risks that

surfactants and their degradation products may have at higher concentrations is necessary for the implementation of preventive measures, such as elimination of the surfactants in wastewater treatment plants.

In this study, we evaluated the effects of Triton X-100 (TX100) and sodium dodecyl sulphate (SDS) on morphology and physiology of a marine unicellular motile photosynthetic microalgae, the chlorophyte Tetraselmis chui (Figure 1). The toxicity of the two surfactants was assessed at five different concentrations of surfactants: 1 g/L, 0.1 g/L, 0.01 g/L and 0.001 g/L. The changes were assessed by light and scanning electron microscopy (SEM), and flow cytometry (FCM).

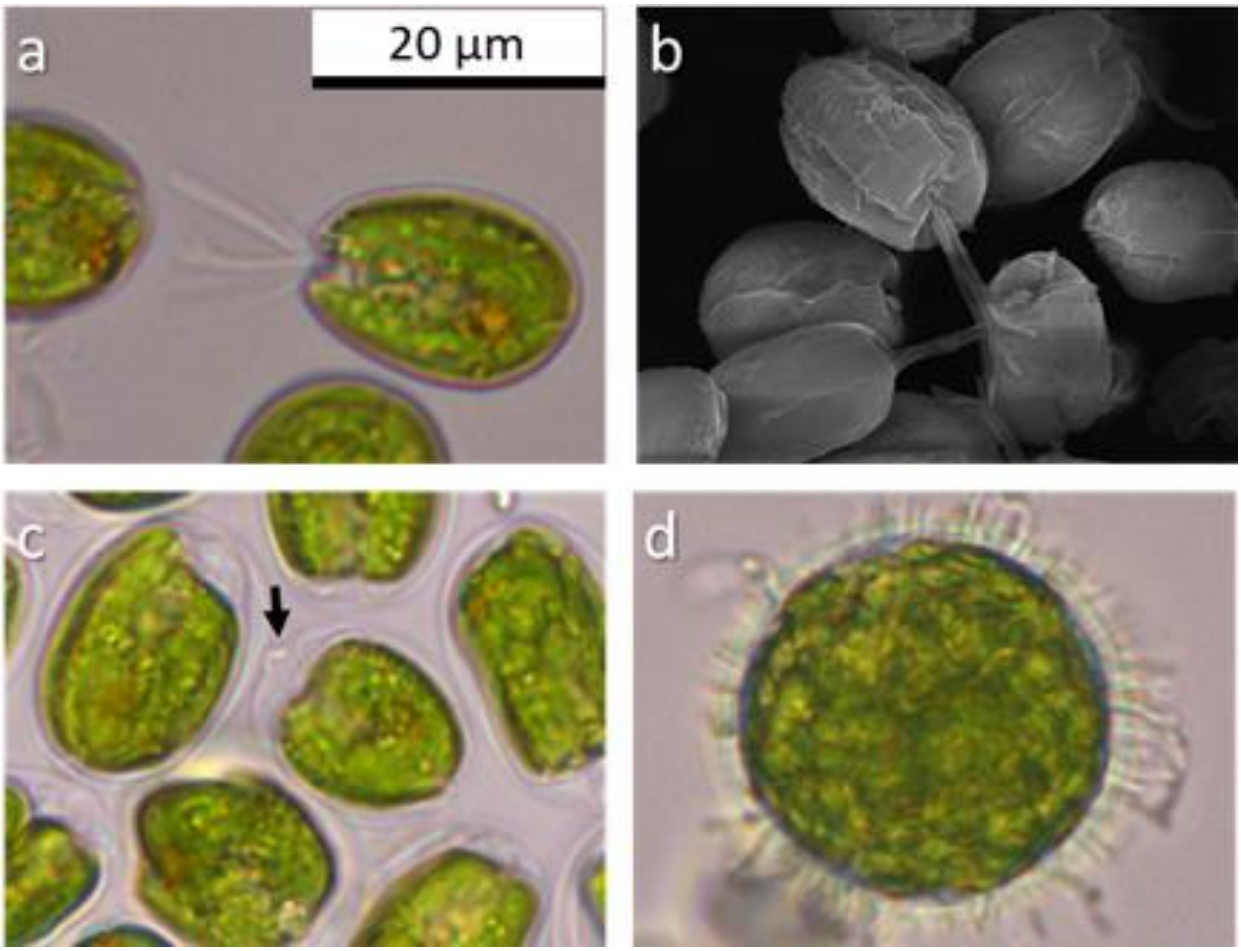

Figure 1. The cell structure of the investigated marine microorganism as observed by light (a, c, d) and scanning electron microscopy (b). a, b – note the presence of four flagella and the red-brown granules inside the cell (stigma); c – cells in the vegetative non-motile state: the arrow points to the teat or papilla. d – vegetative thick-walled cyst. The scale bar applies to all panels.

2. Sodium dodecyl sulphate and Triton X-100

Surface active agents act at surfaces and interfaces of different substances to lower the surface tension. Surfactants owe their characteristic to their amphipathic nature: the molecule consists of a polar, hydrophilic head group and a non-polar, hydrophobic tail (11, 12). Because of their excellent solubility and cleaning properties, they are used as detergents, emulsifiers, wetting and foaming agents, and many other sorts of cleaning products (6).

SDS is an anionic synthetic amphiphile that is commonly used for industrial purposes, but is also found in pharmaceuticals, household detergents, and cosmetic products like shower gels, toothpastes, and soaps. In terms of biological activity, SDS binds to various biomolecules such as starch, enzymes, proteins and nucleic acids, and may intercalate into various cellular fragments, e.g. phospholipid membranes (8, 13). It has the ability to solubilize cellular and nuclear membranes, denature proteins and disrupt their ultrastructure (9). In particular, SDS binding to proteins affects their surface charge, which may result in protein unfolding and loss of cell function (13).

TX100 is a non-ionic octylphenol polyethoxylate used as a polymer stabilizer, wetting and emulsifying agent (9). In biochemistry TX100 is used as a permeabilizing agent (e.g. for cell transfection, or intracellular labelling with antibodies), and as a lysing agent for extraction of cellular organelles, and certain proteins (14). At low concentrations, the permeabilization of membranes caused by TX100 is reversible (allowing for survival of cells). However, large quantities of TX100 that exceed the surfactant's critical micelle concentration (CMC) result in the disruption of cellular structure and over-permeabilization of cellular membrane, which can be lethal (14). The polar heads of TX100 molecules disrupt the hydrogen bonding within the lipid bilayer, leading to loss of integrity and membrane destruction (14). Non-ionic surfactants can also disrupt interactions of DNA and proteins, and of lipids and proteins, but are less effective with disrupting protein-protein interactions (9).

3. Materials and Methods

3.1 *Microorganism culture*

Xenic cultures of Tetraselmis chui CCAP CCAP 66/21b were cultivated in sterilized 50 mL tubes in sterile-filtered artificial marine water, which was prepared by dissolving sea salt mix (Reef Crystals, Aquarium Systems, France) in distilled water (22g/L) and was supplemented with Guillard's (F/2) Marine Water Enrichment Solution (ref.: G0154, Sigma Aldrich, USA). The culture was illuminated in 12h/12h (light/dark) cycle with the luminous intensity of 40-80 µmol m-2 s-1 in a bioreactor (incubated respirometer Echo, Echo, Slovenia). The culture status was routinely monitored by light microscopy and flow cytometry as described below. It was grown until the cell density reached approximately 1 × 109 cells/L. The microorganisms were

then harvested by 5-minute centrifugation at 300 g and re-suspended in marine water. Before the experiments, the biomass was washed twice in marine water (by the same centrifugation protocol), in order to eliminate the dead cells, accumulated discarded cell walls and debris, as well as a large portion of the bacteria. Finally, the culture was suspended in fresh f/2 marine water media for a final concentration of microorganisms in the range of 0.1 – 1 × 109 cells/L.

3.2 *Experimental setup*

Triton X 100 (Omnipour, ref.: 9410-OP, Calbiochem, USA) and SDS (ref.: L3771, Sigma Aldrich, USA) were prepared as 10% w/v solutions in marine water, and sterile filtered through 0.2-micron cellulose-acetate filters (Chromafil RC-20/25, ref.: 729030, Macherey-Nagel GmbH, Germany).

The experiments were performed in 96-microwell plates (TPP Tissue culture test plates, ref.: 92696, TPP Techno Plastic Products AG, Switzerland), which allowed for simple visual examination by light microscopy and FCM analysis. 100 µL of culture was added to the 100 µL of pre-prepared solution of surfactants in marine water, giving the total volume of 200 µL per well. The culture was exposed to five different concentrations of SDS or TX100 in the grown media: 1 g/L, 100 mg/L, 10 mg/L and 1 mg/L. These concentrations correspond to the following molar concentrations of surfactants: 3500, 350, 35, and 3.5 µM for SDS, and 1600, 160, 16, and 1.6 µM for TX100. An untreated sample (without added surfactant) was prepared as a negative control.

3.3 *Light microscopy*

Microplates with prepared samples were observed by an inverted microscope (Eclipse TE2000-S, Nikon, Tokio, Japan). The flagella detachment and loss of motility were the two primary and most evident effects of both surfactants on the investigated species. Therefore, the amount of surfactant causing immobilization of all cells was defined and expressed as minimal immobilization concentration (MIC):

$$\text{MIC [mol/number of cells]}=(c_{surfactant}[\text{mol/L}])/(c_{cells}[\text{number of cells/L}])$$

Where $c_{surfactant}$ is the molar concentration of the surfactant in the sample where no mobile cells were observed after 30 min exposure time at room temperature, and c_{cells} is the concentration of cells in that sample.

3.4 *Scanning electron microscopy*

Selected samples, incubated overnight at 22 °C and at 37 °C were prepared for analysis by scanning electron microscopy (SEM). The samples were incubated for two hours in 2% OsO4 and dehydrated in a graded series of ethanol (30-100 %) followed by a graded series of hexamethyldisilazane (mixed with absolute ethanol; 30 %, 50 % and 100 %), and finally air

dried. The dehydrated samples were coated with gold and palladium and examined by JSM-6500F Field Emission Scanning Electron Microscope (JEOL Ltd., Tokyo, Japan).

3.5 *Flow cytometry*

Flow cytometer (MACS QUANT, Miltenyi, Bergisch-Gladbach, Germany) was used for the quantification of cellular damage and the lytic effect of the surfactants. The measurements were performed after an overnight incubation of samples treated with 0.1 g/L of SDS and TX100, and untreated samples (at room temperature). The 0.1 g/L solution of surfactants in the marine water media resulted in a negligible event count in comparison to other samples, excluding the possible contribution of foaming to the FCM event count.

The concentration (event count), forward scattering signal (generally associated with particle size or volume), side scattering signal (influenced by cellular morphology, intracellular chemical composition and cell volume (15, 16)) and fluorescence (due to the chlorophyll contents) of particles were considered.

3. Results and discussion

For both SDS and TX100 treated cells, loss of motility was observed under the microscope by the evanescence of flagella and formation of a teat or papilla, which is a feature of non-motile cells (Figure 2). The flagellar remnants could not be observed, we therefore assume that flagella were dissolved by the surfactants. While no other visible signs of cellular damage were observed at MIC of SDS-treated samples, the pigment leakage was observed in a minority of cells at MIC of TX100 treated samples (Figure 2).
The cell density was 8 × 108 cells/L and 1 × 109 cells/L for TX100- and SDS- treated samples, respectively. Complete immobilization of cells after 30 minutes of exposure was observed in samples treated with 0.01g/L of TX100 (16 µM, MIC = 0.02 pmol/cell), and 0.1g/L of SDS (350 µM, MIC = 0.35 pmol/cell). As the critical micelle concentration (CMC) at 25°C in pure water is reported to be 12 mM for SDS and 0.26 mM for TX100 (10), the MICs are expected to be below the CMC for both surfactants. However, in saline water, CMCs of surfactants can be reduced due to the interactions of ions and surfactant's charged groups (10). In this study, the CMC values of SDS and TX100 in marine water were not determined and the effects of salinity are unknown.

Furthermore, we noted that all cells were not equally affected by the surfactant. The vegetative cysts that were present in all surfactant-treated samples (**Figure 2**) appeared intact even after three days of incubation at the highest concentrations of both surfactants. While the leakage of chlorophyll was observed, the cell wall itself appeared intact under the LM. Such preservation of cell wall integrity indicates that the wall itself cannot be completely degraded by the surfactant and/or that surfactants may enter the cell at a site which is not

protected by the cell wall. In the case of this species, such a site could be the opening in the cell wall where the flagella emerge.

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In images acquired by SEM (**Figures 1** and **3**) the cells in the untreated samples have preserved their flagella and have a smoother cell surface in comparison to surfactant-exposed cells. At the surfactant concentration of 0.1 g/L for TX100 and 1 g/L for SDS, the absence of flagella was noted in both cases. Besides the accumulated cellular debris and discarded cell walls, small vesicles, which are attached to cells or found in their surroundings have been observed (**Figure 3**). The small vesicles were a noticeable feature of both control and surfactant-treated samples. They accumulated on the cell surface and in its proximity (Figure 3). The quantity of these small vesicles was considerably larger in the surfactant-exposed samples and one possible explanation is that these are cell-derived extracellular vesicles (EVs). Presumably, the release of EVs could serve as a protective mechanism for cells to eliminate the surfactant and reduce its toxicity. However, vesiculation could also be a result of membrane adjustment to perturbations and concomitant changes in its curvature.

In contrast to TX100, 1 g/L SDS-treated cells have visibly shrivelled and wrinkled cell walls. These distinct features have probably emerged in the process of dehydration (needed for SEM analysis). Normally, the cell topology is preserved through the chemical fixation of intracellular components. Hence, the observation of cellular collapse seems to suggest loss of intercellular components, presumably due to their degradation and leakage induced by SDS. This assumption is further supported by the loss of green pigmentation as observed by LM. Additionally, the detachment of flagella is marked by an exposed teat in surfactant-treated cells and if examined closely, small tears or pores can also be spotted on the surface of wrinkled cell walls (**Figure 3**).

To test the impact of temperature, the samples of culture with and without surfactant were incubated overnight also at 37 °C (**Figure 4**). The increase in temperature alone resulted in a burst of submicron vesicles (**Figure 4a**), as also promoted the proliferation of various kinds of bacteria in the co-culture (**Figure 4c**). At 37 °C (**Figure 4b**) surfactant had even more pronounced effects than at 22°C, and in the most affected cells, some pores could have been observed in the remnants of the cell walls (**Figure 4d**).

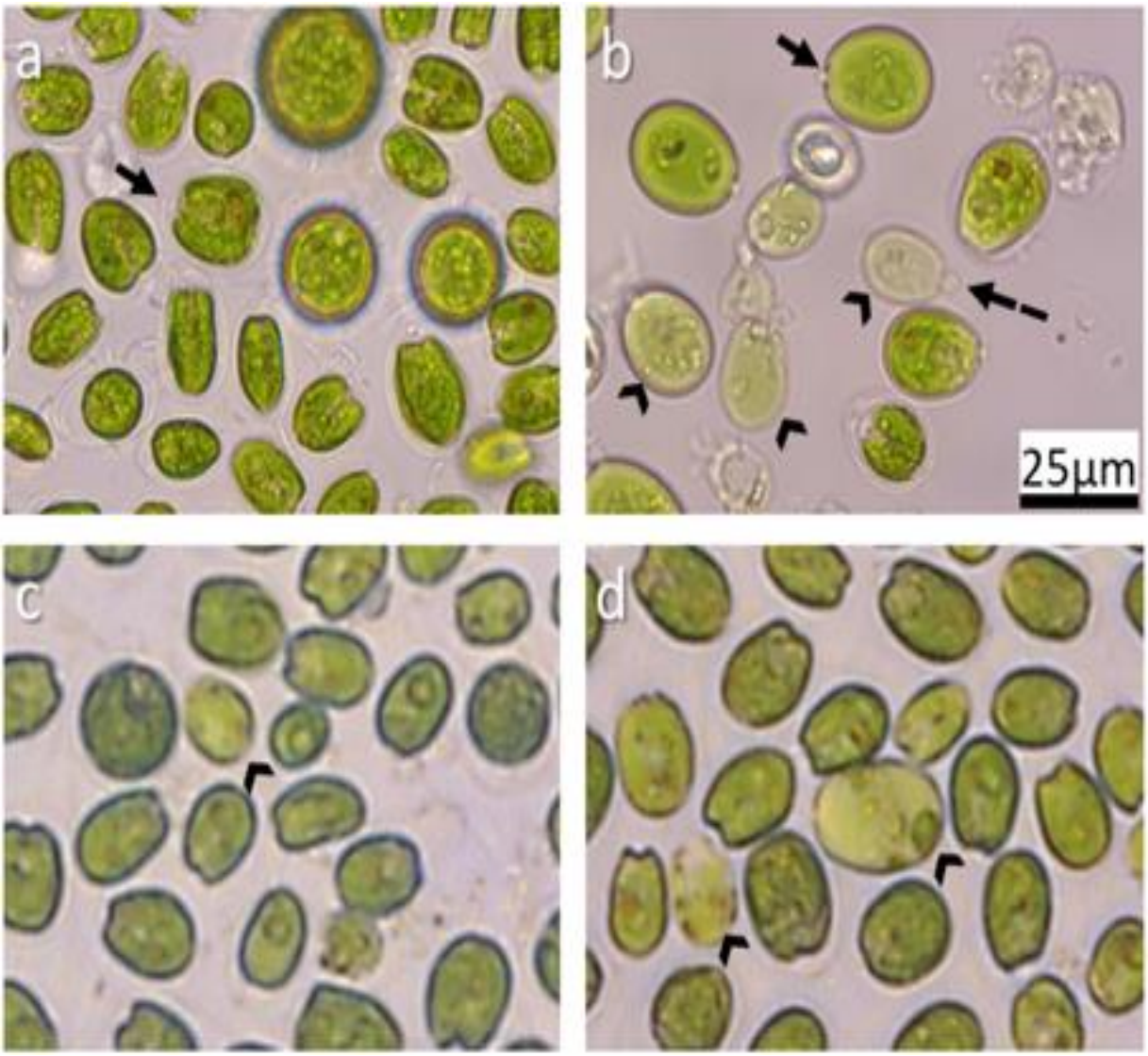

Figure 2. Light microscopy images of cells treated with SDS (a, b) and TX100 (c, d). Sample at a) MIC of SDS; b) 1 g/L SDS; and c) MIC of TX100 and d) 1 g/L TX100. Arrows point at the papilla, arrowheads point at leaking cells, and dashed arrow points at a vesicle forming at the flagellar pit of the cell.

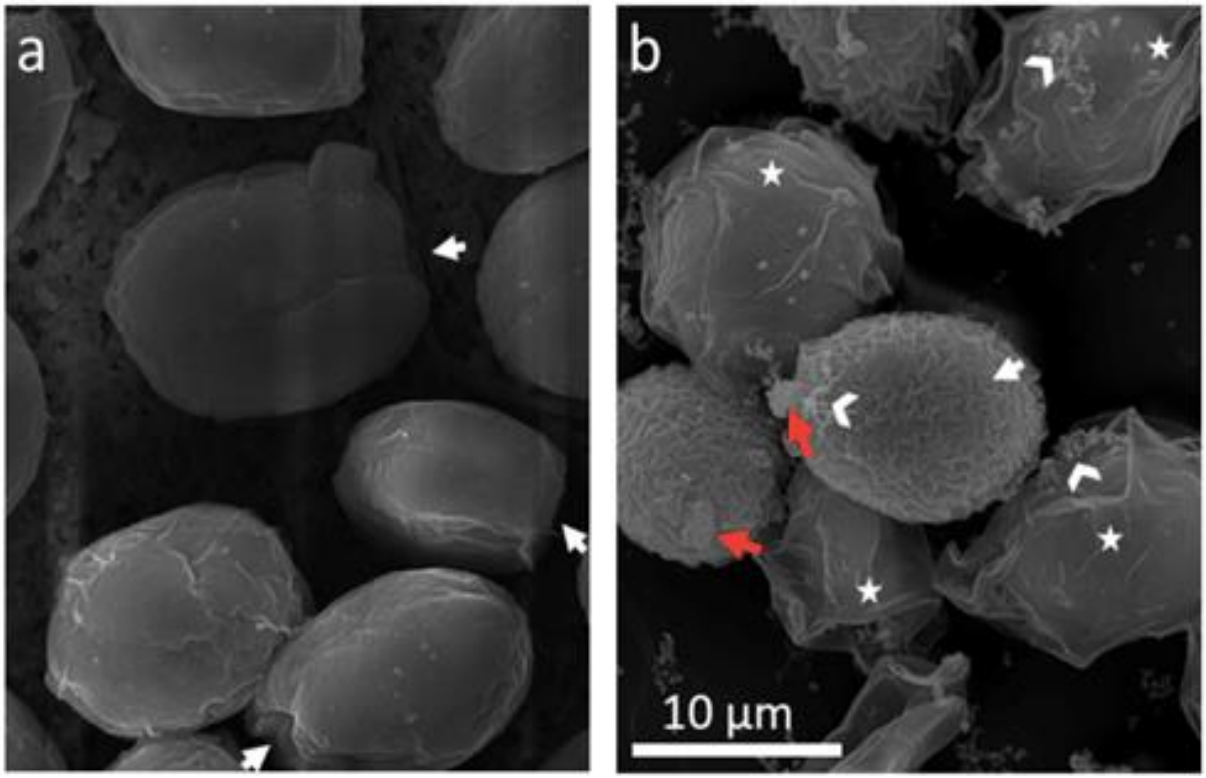

Figure 3. Scanning electron microscopy images of changes observed in cells due to the presence of surfactants. a: 0.1 g/L TX100, b: 1 g/L of SDS. The scale bar applies to both panels. White arrows point to cells that have lost flagella, red arrows point to the papilla, stars indicate the cells with collapsed cell walls, arrowheads point at small vesicles. Also, note the presence of cells with wrinkled cell surfaces in Panel b.

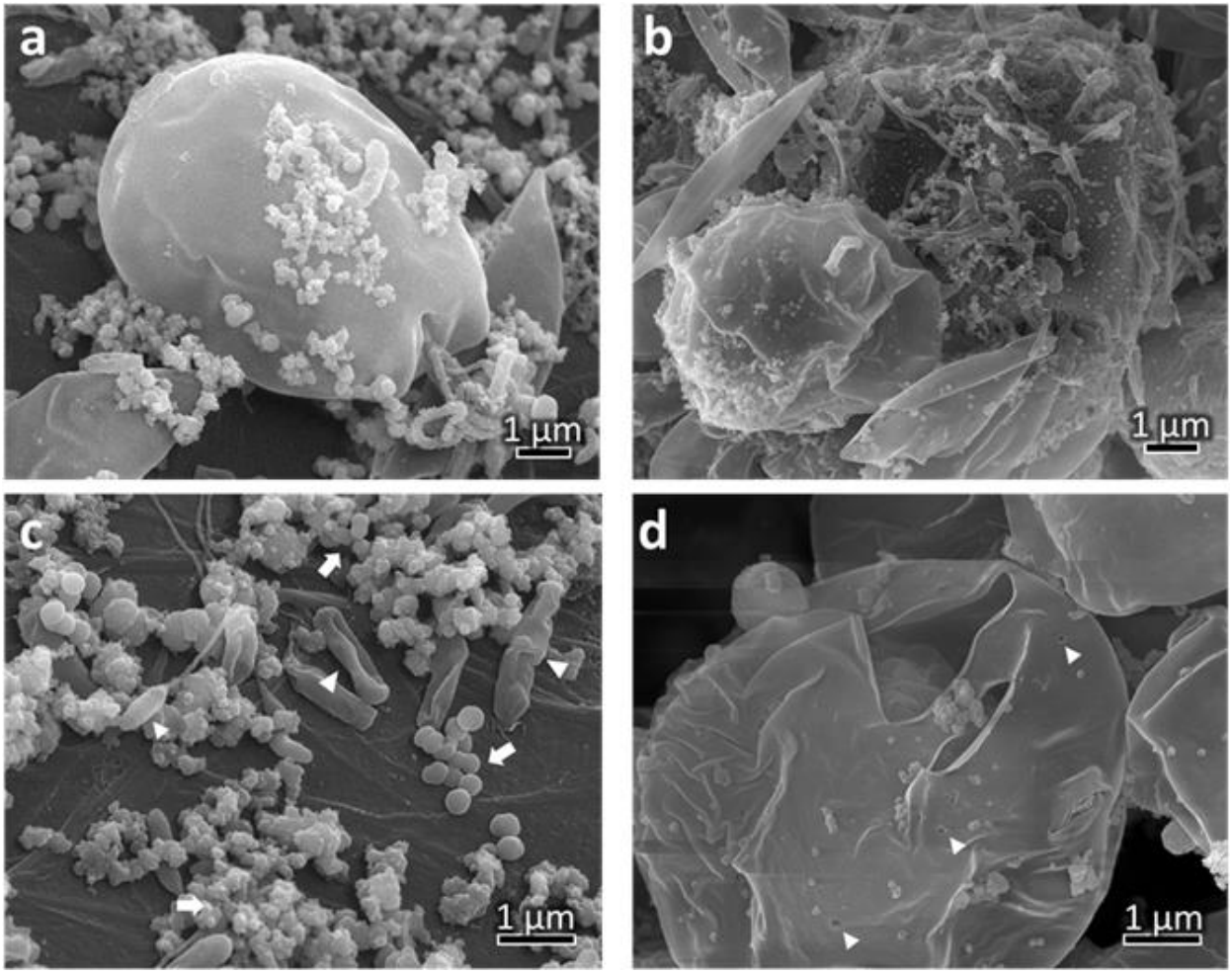

Figure 4. Scanning electron microscopy images of the microorganism incubated overnight at 37°C; in f/2 growth media (a, c) or in f/2 growth media supplemented with 0.1 % sodium dodecyl sulphate (b, d). Increased temperature as well as surfactant induce formation of nanoparticles of different topologies. Different bacteria (arrowheads) and abundance of small particles (arrows) can be observed in c. Pores in the cell wall (arrowheads) can be observed after the cell lysis by sodium dodecyl sulphate in d.

The scatter plots in **Figure 5** represent the FCM measurements of untreated and 0.1 g/L TX100 and SDS-treated samples (incubated at 22 °C). The gating was established based on the scatter plots obtained for the untreated sample of cells. The encircled population in untreated samples represents normally appearing intact cells, and the population in the square box represents smaller and more weakly scattering particles (cellular debris and bacteria).

In comparison to the untreated samples, the exposure of cells to 0.1 g/L of TX100 has resulted in a downward shift in the cell population. This decrease in the SSC signal indicates that cell density and volume had decreased, which could correspond to the observed leakage of intracellular contents in LM. Additionally, in both 0.1 g/L TX100- and SDS-treated samples, an increase in the event count and SSC signal amplification were detected in the boxed area

representing the smaller and more weakly scattering particles (**Figure 5**). Some of those particles were also fluorescent, indicating their chlorophyll content.

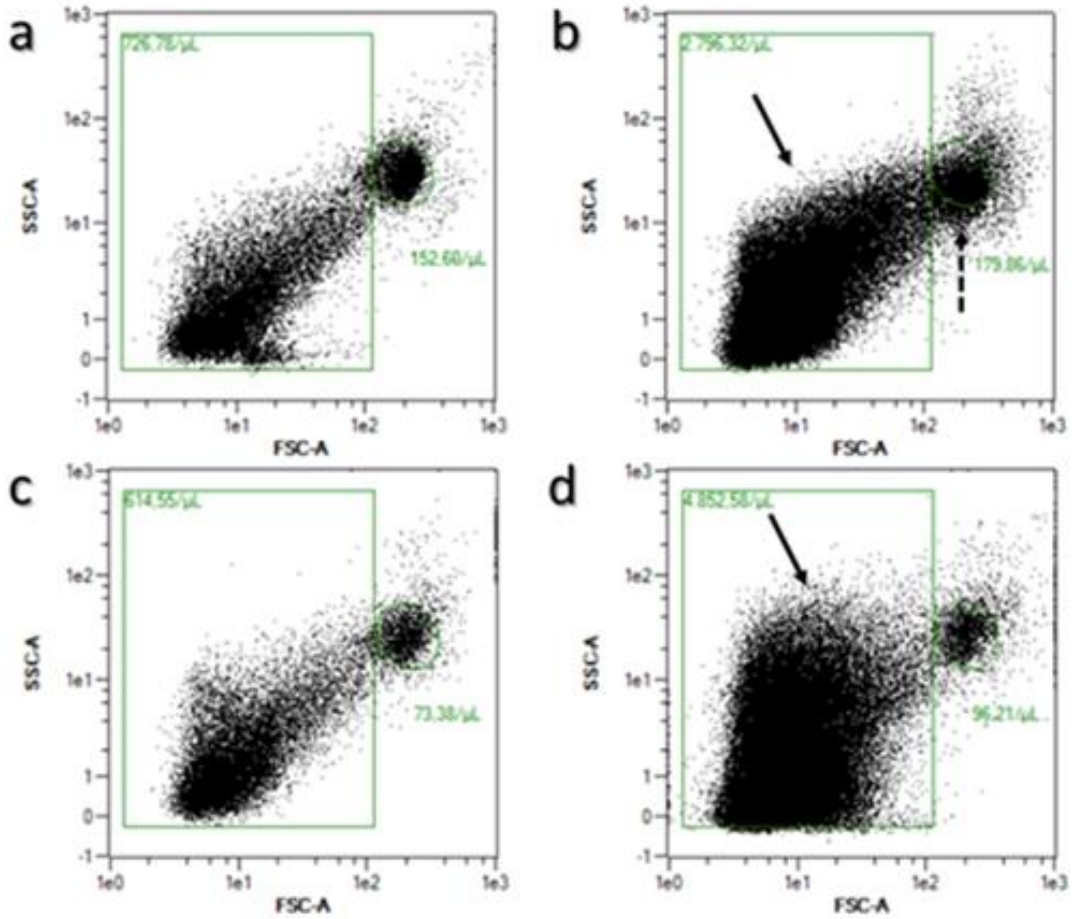

Figure 5. Flow cytometry measurements of cells incubated overnight in untreated (a, c) and 0.1 g/L surfactant-treated samples (b, d). Arrows point to increased number of events and SSC signal amplification in the area of small particles in 0.1 g/L surfactant-treated samples; the dashed arrow points to the downward shift of cell population in TX100- treated samples. The upper row (Panels a and b) represents the TX100-treated samples and the bottom row (Panels c and d) represents the SDS-treated samples.

Based on LM observations, we assume that this change is related to the surfactant-induced release of cellular components (e.g. parts of the flagella, discarded cell walls, intracellular proteins, lipids, etc.). In order to identify the source of signal amplification, fluorescent dyes could be applied. However, the use of dyes can produce artefacts and interfere with signal detection due to the presence of endogenous pigments in the cell, which can distort the quantitative signal emitted from the dye (16).

Conclusions

The results of this study highlight the importance of treating wastewaters from industrial and household settings to alleviate the potential burden of surfactants on aquatic ecosystems. The present study focused on the short-term effects of SDS and TX100 toxicity on a unicellular marine microorganism species. Loss of motility of microorganisms and leakage of the chlorophyll content occurred at concentrations of 0.02 pmol/cell and 0.35 pmol/cell for TX100 and SDS, respectively. In samples incubated with concentrations above the respective concentrations, visible signs of cellular damage were observed by light microscopy (loss of flagella and pigmentation) and electron scanning microscopy (loss of flagella and disruption of cellular integrity). The flow cytometry results showed a considerable increase in small particles count in surfactant-treated cells. According to scanning electron microscopy observations, some of those particles could be small vesicles that are released by the cells.

Prospective studies should further address the effect of sub-lethal, chronic surfactant exposure (e.g. on cell growth), and the reversibility of cellular damage. Furthermore, testing different microorganisms would provide a more comprehensive overview of surfactant toxicity in aquatic ecosystems. Surfactants can have a wide range of effects on living organisms and the exact mechanisms of their action are yet to be elucidated, which makes this subject an interesting one for future research.

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References

1. Dutka, BJ, and Bitton G, Toxicity testing using microorganisms. Vol. 2. CRC Press, 2019.
2. Arrigo KR. Marine microorganisms and global nutrient cycles. Nature 2005, 437.7057: 349-355.
3. Baharum SN, Beng EK, Mokhtar, MAA, Marine microorganisms: potential application and challenges. J Biol Sci 2010, 10.6: 555-564.
4. Lechuga M, Fernández-Serrano M, Jurado E, Núñez-Olea J., & Ríos F. Acute toxicity of anionic and non-ionic surfactants to aquatic organisms. Ecotoxicol Environ Saf 2016, 125: 1-8.

5. Invally K, Ju LK, Biolytic effect of rhamnolipid biosurfactant and dodecyl sulfate against Phagotrophic alga Ochromonas danica. J Surfactants Deterg 2017, 20.5: 1161-1171.

6. Ivanković T, Hrenović J, Surfactants in the environment. Arh Hig Rada Toksikol 2010, 61.1: 95-110.

7. Masakorala K, Turner A, Brown MT, Toxicity of synthetic surfactants to the marine macroalga, Ulva lactuca. Water Air Soil Pollut 2011, 218.1-4: 283-291.

8. Cserháti T, Forgács E, Oros G, Biological activity and environmental impact of anionic surfactants. Environ int 2002, 28.5: 337-348.

9. Boccafoschi F, Botta M, Fusaro L, Copes F, Ramella M, Cannas M, Decellularized biological matrices: an interesting approach for cardiovascular tissue repair and regeneration. J tissue eng regen m 2017, 11.5: 1648-1657

10. Ying GG, Fate, behavior and effects of surfactants and their degradation products in the environment. Environ int 2006, 32.3: 417-431.

11. Bajpai P, Biermann's Handbook of Pulp and Paper: Chapter 19: Colloid and Surface Chemistry. Third Edition, Elsevier, 2018

12. Moroi Y, Micelles: theoretical and applied aspects. Springer Science & Business Media, 1992

13. Sirisattha S, Momose Y, Kitagawa E, Iwahashi H, Toxicity of anionic detergents determined by Saccharomyces cerevisiae microarray analysis. Water Res 2004, 38.1: 61-70.

14. Koley D, Bard AJ, Triton X-100 concentration effects on membrane permeability of a single HeLa cell by scanning electrochemical microscopy (SECM). Proc Natl Acad Sci 2010, 107.39: 16783-16787.

15. Debelius B, Forja, JM, DelValls Á, Lubián LM, Toxicity and bioaccumulation of copper and lead in five marine microalgae. Ecotoxicol Environ Saf, 2009, 72.5: 1503-1513.

16. Hyka P, Lickova S, Přibyl P, Melzoch K, Kovar K, Flow cytometry for the development of biotechnological processes with microalgae. Biotechnol adv 2013, 31.1: 2-16

Research

PHYSICAL APPROACH TO THE CHARACTERISTICS OF LUMINOL CHEMILUMINISCENCE REACTION IN WATER

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Abstract

Chemiluminescence is the emission of light that is triggered by a chemical reaction. This kind of phenomenon occurs when an exothermic reaction produces molecules in an electronically excited state. When these molecules return to their ground states they release energy in the form of light. Here, the occurrence of light emission was observed in the oxidation of the model reagent of the aromatic carboxylic acid hydrazide family, luminol. It was found that amounts of oxides, catalyst and fluorescent dye play an important role in the occurrence of light emission. There is an optimum amount of each of these substances as regards the intensity of emitted light and activation of the reaction. At pH 9.00, the optimal system contained a 1.71 mM solution of copper(II) ions and 1.06 mM of fluorescein (as a disodium salt). The reaction was most successfully activated by a 0.050 % solution of hydrogen peroxide as the oxidant.

1. Introduction

There are many different types of energies in nature. Potential energy changes into kinetic energy as the apple falls from the tree. In the light bulb the energy in the form of heat is converted to light. We will focus on a process where the energy source is expressed without the heat source. It is known as luminescence or cold light (1).

In general, the luminescence type is defined according to the type of energy delivered. In nature we can frequently encounter bioluminescence, where an enzyme catalyzes oxidation of a substrate molecule (for example in fireflies; enzyme: luciferase, substrate molecule: luciferin) (2). The light can be used for defense or attack, communication, mating or simply just lighting the surrounding environment (3).

Two photoluminescence phenomena are known: fluorescence and phosphorescence. Fluorescence is a photoluminescent phenomenon that occurs with a constant inflow of energy (usually in the form of radiation), while phosphorescence can continue even if the energy supply is interrupted (4,5).

Chemiluminescence (CL) is the process of producing electromagnetic radiation in the form of light through a chemical reaction. Light may be ultraviolet, visible or infrared. It is a process in which the exothermic reaction results in the excitation of electrons in the molecules. When these electrons return to their ground state, they release photons (energy in the form of light). CL can be divided into direct or indirect chemical reaction. Direct CL can be represented by a general reaction scheme (6):

$$A + B \rightarrow [I]^* \rightarrow \text{products} + \text{light } (h\nu), \quad (1)$$

where A and B are reagents and [I]* are intermediat in the higher excited energy state.

In cases where the excited state of substance alone is not a sufficiently effective radiation asset, it can transfer energy to another type of asset (sensitizer, S), that later emits energy. In this case, we are talking about indirect type of CL. An example is the light emitted by activated glow sticks (7). Indirect CL can be represented by a general reaction scheme (6):

$$A + B \rightarrow [I]^* + S \rightarrow [S]^* \rightarrow S + \text{light } (h\nu). \quad (2)$$

CL's use can be found in chemistry, biochemistry, medicine, pharmacy, biology and biotechnology (8,9). There are many known CL compounds, which can emit energy in the form of light. The intensity of research in this field is gradually developing as scientists discover new reagents and their new uses in different fields (3), for example in labelling tumors in medicine (6,10).

2. Luminol chemiluminiscence reaction

Reaction between luminol (**Figure 1**) and hydrogen peroxide is an example of an direct CL reaction. Oxidation of luminol can take place in a protic (alcohol, water) or aprotic (DMSO, DMF) medium. Different oxidants are required for different solvent systems and different spectra of emitted light are obtained. In aprotic solvents, molecular oxygen and a strong base are needed for CL reaction while the maximum of the emission spectrum is at wavelength 485 nm. In protic solvents, strong base, molecular oxygen or hydrogen peroxide and a catalyzer are needed for oxidation. The maximum of the emission spectrum is at wavelength 425 nm. In both solvent types, the emission takes place at an excited 3-aminophthalate ion (4).

During oxidation, luminol emits turquoise blue light (its wavelength is in the range 425 - 485 nm) that is clearly visible in a dark room. The first reports of luminol CL were observed by the chemist Herbert Otto Albrecht in 1928, and since then CL has been intensively investigated.

To initiate the reaction in aqueous solutions, a superoxide radical anion ($O_2^{\bullet-}$) is formed upon the decomposition of hydrogen peroxide (H_2O_2). The precipitation leads to turbidity of the solution and consequently to a lower intensity of light. In analytical studies this information is considered to be important, as the turbidity of the solution (suspension) results in large variations in intensities. Luminol's optimum emission with regard to *pH* is observed in the *pH* range of 9, so it is important to maintain this value of *pH*. A carbonate buffer mixture is used to maintain the constant *pH* in such reactions (for example: $NaHCO_3 + Na_2CO_3 + (NH_4)_2CO_3$) (4).

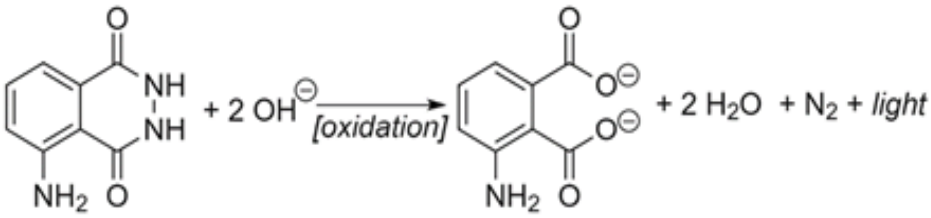

Figure 1: Reaction scheme of luminol oxidation.

Light emission duration depends on the oxidant (H_2O_2) concentration. The greater the concentration of oxidant the shorter is the duration of the reaction, therefore the light intensity is higher (and vice versa). Catalyst also plays an important role. The most commonly used catalysts are Cu(II) ions, but Co(II) and hexacyanoferrate(III) ions – $[Fe(CN)_6]^{3-}$ are useful too (4).

3. Objective, purpose and hypothesis

The aim of the research was to carry out CL reactions of luminol with various experimental variables such as different concentrations of Cu(II) ions, different weight percentages of hydrogen peroxide and the addition of fluorescent dye to the reaction system. By changing these parameters, we wanted to show the physical background of the reaction. We theoretically estimated the key parameters that are important in the description and characterization of light emission by using *Mathematica* software. We assume that each component in the reaction system (copper(II) ions, hydrogen peroxide and fluorescein) has a specific optimal concentration for light generation. Elevated concentrations of any component start to inhibit the light emission reaction.

4. Experimental methods

4.1 *Solutions*

The solution with reagent and catalyst in buffer (solution: SOL-1, adapted from (11)) 500 mL of deionized water was poured into a 1 L volumetric flask. 4.000 g of anhydrous sodium carbonate was quantitatively dissolved in water and 0.200 g of luminol was added. The contents were stirred on a magnetic stirrer until the reagent had completely dissolved. 24.000 g of sodium hydrogen carbonate and 0.500 g of ammonium carbonate monohydrate were added to the contents. Different masses of copper(II) sulphate pentahydrate catalyst and disodium fluorescein dye salt dye were added to the contents, corresponding to individual concentrations. After addition of all reagents, the contents in the volumetric flask were further diluted with deionized water and stirred on a magnetic stirrer until the mixture became completely homogeneous. The resulting solution was adjusted to *pH* 9.00. We used a Mettler Toledo *pH* meter, AG SG78 SevenGo Duo Pro, and performed a two-point calibration with a glass electrode. The *pH* was adjusted using a 1 M solution of NaOH or HCl.

Catalyst solutions, Cu(II)-ions: by dissolving copper(II) sulfate(VI) pentahydrate in a basic CL solution (SOL-1), catalyst solutions were prepared at the following concentrations: 1.71, 2.23 and 2.283 mM.

Fluorescence dye solution, fluorescein disodium salt: We prepared 3 different concentrations (0.27, 1.06 and 2.13 mM) of flourescein (we used flourescein in the form of disodium salt) in a catalyst in buffer solution (SOL-1).

Hydrogen peroxide as a solution of oxidant (solution: SOL-2, adapted from (3) and (11)): Diluted hydrogen peroxide solutions were prepared. First, a commercial, 30 % solution was standardized (i.e., determined by concentration) with potassium permanganate in an acidic medium, according to the literature procedure (12). 0.025, 0.050 and 0.10 % of the solution were then prepared from the commercial solution.

4.2 *Detection system*

To determine the intensity light of luminol oxidation, we assembled the system shown in **Figure 2** (adapted from (13)).

A Vernier LS-BTA type light sensor was immersed in a 100 mL beaker containing the basic CL solution (also with added fluorescein) with a catalyst as shown in **Figure 2**.

We connected the sensor by a computer interface to the computer system that processed data and plotted graphs using *Logger Pro 3* software. We adjusted the lux-sensor to the measurements. A sequence of measurements was performed automatically within 2 minute intervals with analysis rate 500 samples/minute. When the solution "SOL-2" was poured into solution "SOL-1", in complete darkness, we activated the computer system for plotting graphs.

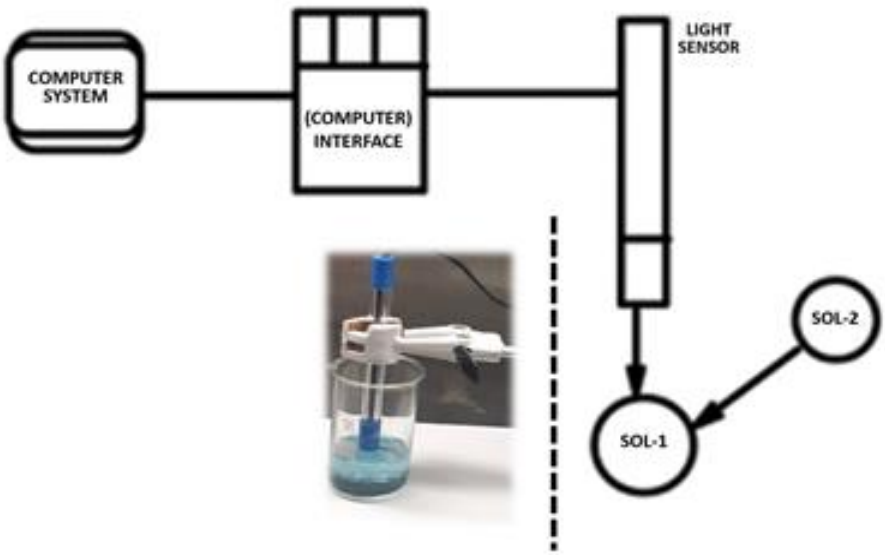

Figure 2: System for determination of the light intensity

4.3 *Physical approach to data editing*

The experimental data were converted to *Excel 2010* (Microsoft Office Professional, 2010), systematically edited and transferred to *Mathematica* (Wolfram Research, Inc., Mathematica, Version 9.0, Champaign, IL (2012)) where the data were fitted by the exponential relaxation. The parameters that yielded best fit to the experimental data were determined.

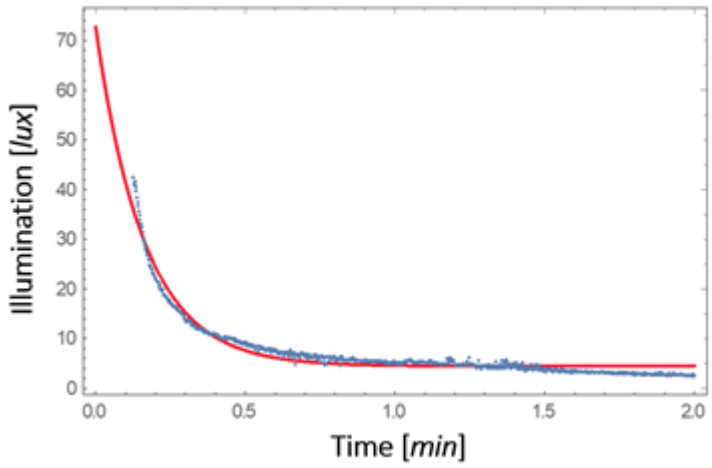

Figure 3: Intensity of emitted light due to luminol oxidation in dependence of time. Blue curve present experimental data, and red comparison of the experimental data and the curve obtained by fitting.

5. Results and discussion

5.1 *Influence of different concentrations of hydrogen peroxide on luminol oxidation*

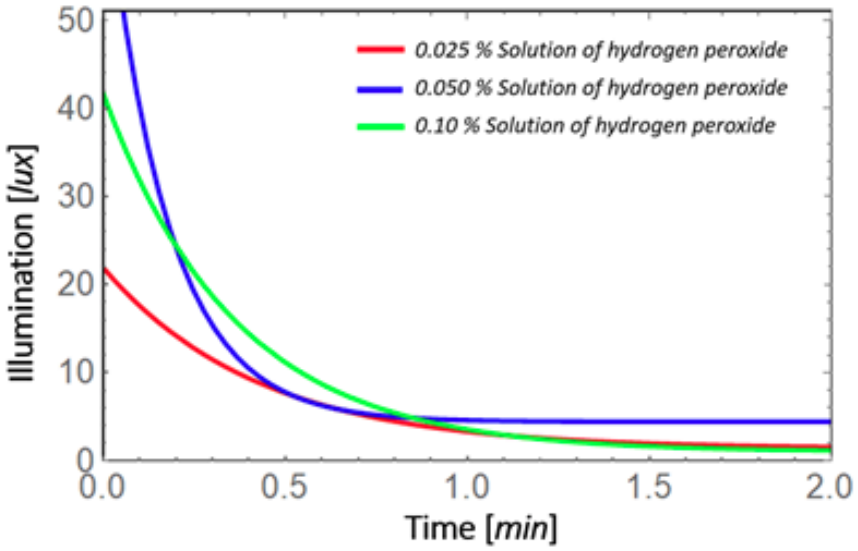

Figure 4: Intensity of emitted light due to luminol oxidation in dependence on time for different concentrations of hydrogen peroxide in the solution.

It can be seen in **Figure 4** that at hydrogen peroxide concentration 0.050 %, the blue curve has the highest initial value and falls most steeply. The green curve, which corresponds to

hydrogen peroxide concentration 0.050 %, has a lower initial value and falls less steeply. The blue curve in **Figure 4** corresponds to most emitted light, meaning the concentration of hydrogen peroxide there is optimal.

From the above we can conclude that the optimum hydrogen peroxide ratio in the reaction was exceeded for red and green curves of **Figure 4.**

5.2 *Influence of different concentrations of copper(II) ions on luminol oxidation*

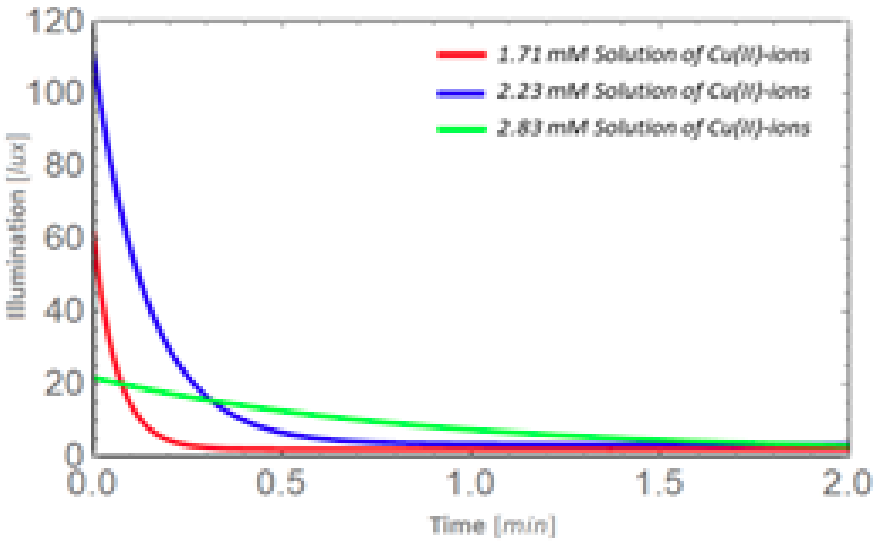

Figure 5: Monitoring the effect of different concentrations of Cu(II) ions on the luminol oxidation.

It can be seen in **Figure 5** that with increasing concentration of Cu(II) ions, the initial value and the slope of the curve increase. At the concentration 2.83 mM an excess concentration of the reagent inhibited the entire reaction (**Figure 5,** green curve). It can be observed that the highest concentration of Cu(II) ions (2.83 mM) yields almost 5 times lower initial value than the optimal one (2.23 mM)(**Figure 5,** green and blue curves, respectively).

5.3 *Influence of fluorescein concentrations on luminol oxidation*

Figure 6 shows that at constant catalyst and oxidant concentrations, fluorescein affects the course of the reaction. With increasing concentration, fluorescein induced activation of (catalytic amounts added) to the point where it began to act inhibitory; high amount of dye (2.13 mM) suppressed the entire reaction (**Figure 6**, green curve).

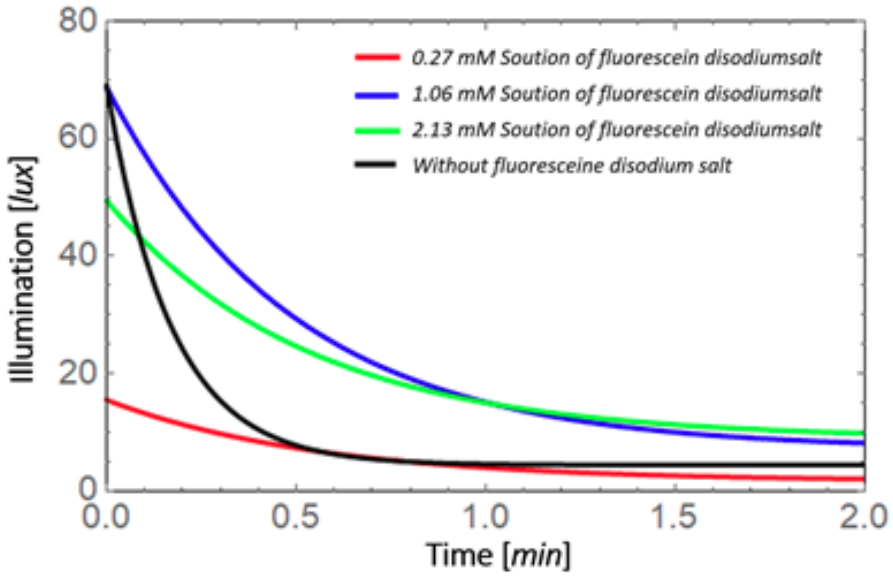

Figure 6: Intensity of emitted light due to luminol oxidation in dependence on time for different concentrations of fluorescein disodiumsalt in the solution.

5.4 *Quantitative determination of light emission*

The area under the best fit curve (dependence of illumination on time) is proportional to the emitted energy.

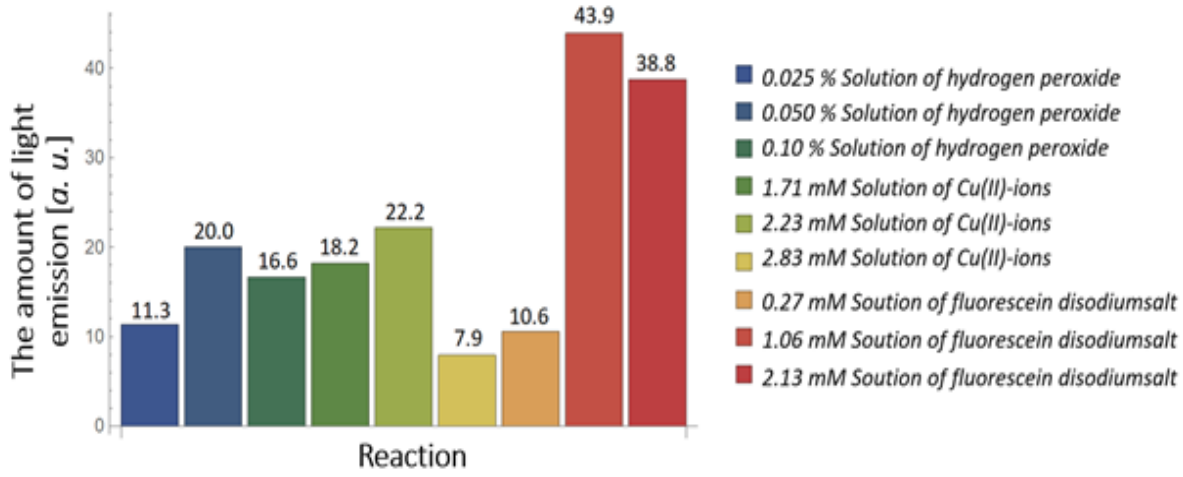

Figure 7: The amount of light emitted in luminol oxidation for different conditions in the solution and different activation modes, as indicated in the figure.

The hydrogen peroxide concentration with maximum yield (20.0 *a. u.*) was about 0.050 %. Copper(II) ions participate in the catalytic decomposition of hydrogen peroxide; the decomposition product is a superoxide radical anion that oxidizes luminol. The catalytic

decomposition was performed in a 0.050 % hydrogen peroxide solution at different copper(II) ion concentrations. We found that 2.23 mM concentration of copper(II) ions yielded maximal amount of emitted light (22.2 *a. u.*) while an increased concentration of copper(II) ions suppressed the emission of light. Fluorescein, a fluorescent dye in the form of a water-soluble disodium salt also affected the luminol oxidation reactions. Concentration of fluorescein 1.06 mM yielded an optimal amount of emitted light (43.9 *a. u.*).

Conclusions

We observed that changes in the concentrations of hydrogen peroxide solutions, copper(II) ions and fluorescein affected the luminol oxidation reaction. Our results showed that gradually increasing the concentrations of these substances increases emission of light emission. The maximum in the amount of emitted light is reached at an optimum concentration of the respective substance while further increase in the concentration of the added substances causes a decrease in the amount of the emitted light. In analytics practice, long-lasting CL reaction that produces a high amount of emitted light, is required.
The optimal system contained 1.71 mM of copper(II) ions and 1.06 mM of fluorescein (as disodium salt). The reaction was activated by a 0.050 % hydrogen peroxide solution. In this case, the light emission was highest.

Acknowledgements

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Rerefences

1. Arsov Z. Luminiscenca. Presek: List Za Mlade Matematike, Fizike, Astronome in Računalnikarje 2000;28:74–80. http://www.presek.si/28/1432-Arsov.pdf.
2. White EH, Steinmetz MG, Miano JD, Wildes PD, Morland R. Chemi- and bioluminescence of firefly luciferin. J Am Chem Soc 1980;102:3199–208. https://doi.org/10.1021/ja00529a051.
3. Jeran M, Drofenik I. Študij uporabe kemiluminiscenece luminola in organskih hidrazidov. Kemija v Šoli in Družbi 2010;22:11–4. https://www.kemija.net/clanek/1197.
4. Jeran M, Cvar S, Podgoršek Berke A. Uporaba fluorescenčnih barvil kot emisijskih občutljivcev pri kemiluminiscenčnih reakcijah v vodnem mediju. Kemija v Šoli in Družbi 2012;24:10–6. https://www.kemija.net/clanek/1346.

5. Munson CA, Gottfried JL, De Lucia FC, McNesby KL, Miziolek AW. Laser-based Detection Methods of Explosives. Counterterrorist Detection Techniques of Explosives, Elsevier; 2007, p. 279–321. https://doi.org/10.1016/B978-044452204-7/50029-8.

6. Jeran M. Odkrivanje in analiza biološkega gradiva s pomočjo svetlobnih reakcij. Proteus: Ilustriran Časopis Za Poljudno Prirodoznanstvo 2016;78:205–14. https://issuu.com/prirodoslovno.drustvo/docs/proteus_januar_2016_za_net.

7. Jeran M, Iskra J. Kemiluminiscenčna aktivnost diaril oksalatnih estrov. Kemija v Šoli in Družbi 2011;23:2–5. https://www.kemija.net/clanek/1273.

8. Li L, Arnold MA, Dordick JS. Mathematical model for the luminol chemiluminescence reaction catalyzed by peroxidase: peroxidase-catalyzed luminol chemiluminescence. Biotechnol Bioeng 1993;41:1112–20. https://doi.org/10.1002/bit.260411115.

9. Grofelnik G, Drobnič K. Uporabnost forenzičnega Bluestar Forensic testa pri latentnih krvnih sledeh. Revija Za Kriminalistiko in Kriminologijo 2008;59:166–73. https://www.policija.si/images/stories/Publikacije/RKK/PDF/2008/02/RKK2008-02_Grofelnik_Drobnic_BluestarForensicTest.pdf.

10. Iranifam M. Analytical applications of chemiluminescence methods for cancer detection and therapy. TrAC Trends in Analytical Chemistry 2014;59:156–83. https://doi.org/10.1016/j.trac.2014.03.010.

11. Shakhashiri BZ. Chemical demonstrations: a handbook for teachers of chemistry. Madison, Wis: University of Wisconsin Press; 1983.

12. Jeran M, Mohar B. O vodikovem peroksidu - kvantitativna določitev z uporabo titrimetrije. Kemija v Šoli in Družbi 2016:1–6. https://www.kemija.net/clanek/1564.

13. Jeran M, Nemec V, Drab M. Uporaba fizikalnih pristopov pri obravnavi emisijskih profilov reakcije kemiluminiscence organskega hidrazida v protičnem mediju. Kemija v Šoli in Družbi 2020;1:1 – 9. https://www.kemija.net/clanek/1714.

Review

POSSIBLE APPLICATIONS OF DIETHYLENETRIAMINE (DETA) IN CO_2 CAPTURING- A MINI - REVIEW

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Abstract

In the past decades, reduction of carbon dioxide (CO_2) emissions into the atmosphere has become a challenging goal. Capturing the CO_2 directly before storage is becoming a thriving alternative approach. Septavaux et al. (1) have proposed a CO_2 fixation method using diethylenetriamine (DETA) to produce a range of carbamation species that can be used for metal separation and recovery. They could show that lanthanum and nickel can be separated from the exhaust gases of vehicle engines by successive CO_2-induced selective precipitations. Individual metal components of La_2Ni_9Co alloys used to manufacture batteries for electric vehicles can also be separated. Here we suggest to use DETA as a mediator for an attractive interaction between like-charged macroions.

1. Introduction

Carbon dioxide (CO_2) emission into the atmosphere has increased at an alarming rate. In order to reduce CO_2 emissions, adequate measures for CO_2 capture and storage (CCS) or utilization (CCU) need to be taken (2). Since CCS is expensive therefore more attention is directed towards CCU because it has other economic advantages. CCU would significantly reduce the cost of storage due to recycling of CO_2 for further usage. In this context, Septavaux et al. (1) recently showed that the cost of CO_2 capturing with the industrial polyamine DETA can be reduced even further with another environmentally beneficial process (3). The authors proposed to selectively remove metal contaminants from water by adding DETA to aqueous solutions before bubbling CO_2 through the mixture. In this process, a series of ligand exchange reactions results in the formation of insoluble carbonates of the contaminating metals (4).

This contribution is a mini-review of DETA induced metal purification and recovery from waste streams using CO_2 fixation as proposed by Septavaux et al. (1). In addition, the possible application of DETA for mediating attractions in a solution of macroions is presented. Based on theoretical modelling, simulations and experiments with the chemically related molecule spermidine (5,6), we propose that DETA can induce attraction between like-charged macroions. Mobile DETA ions with spatially separated individual charges (**Figure 1**) can interact with like-charged macroins, as predicted by models using the mean-field level PB theory (5) and MC simulations (6). For instance, Gimsa et al. (6) studied the effect of spermidine, which is structurally similar to DETA (**Figure 1**), to induce attraction between borosilicate beads attached to AFM cantilevers and mica surfaces (both surfaces are negatively charged).

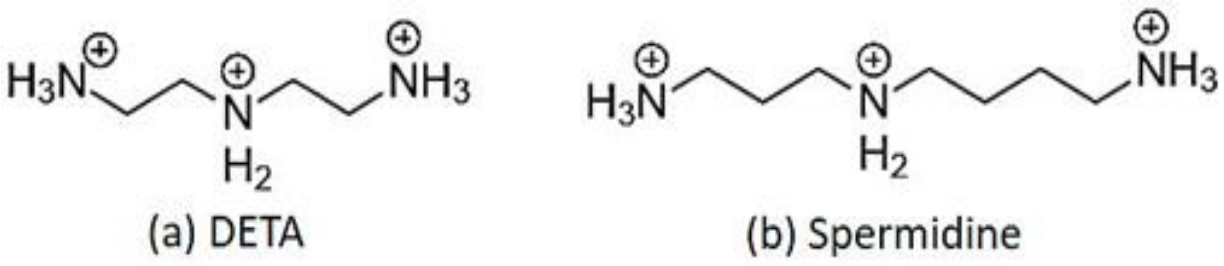

Figure 1. Structural representation of triprotonated (a) diethylenetriamine (DETA) and (b) spermidine molecule.

2. Cleaning of metal contaminants from waste streams using CO_2 capture and DETA

Septavaux et al. (1) demonstrated the efficiency of their technique by removing lanthanum as $La_2(CO_3)_3$ from a La–Ni mixture and separating the metal components of a La_2CoNi_9 alloy, as used in the electrodes of batteries, into the individual components with a purity of 97% or higher.

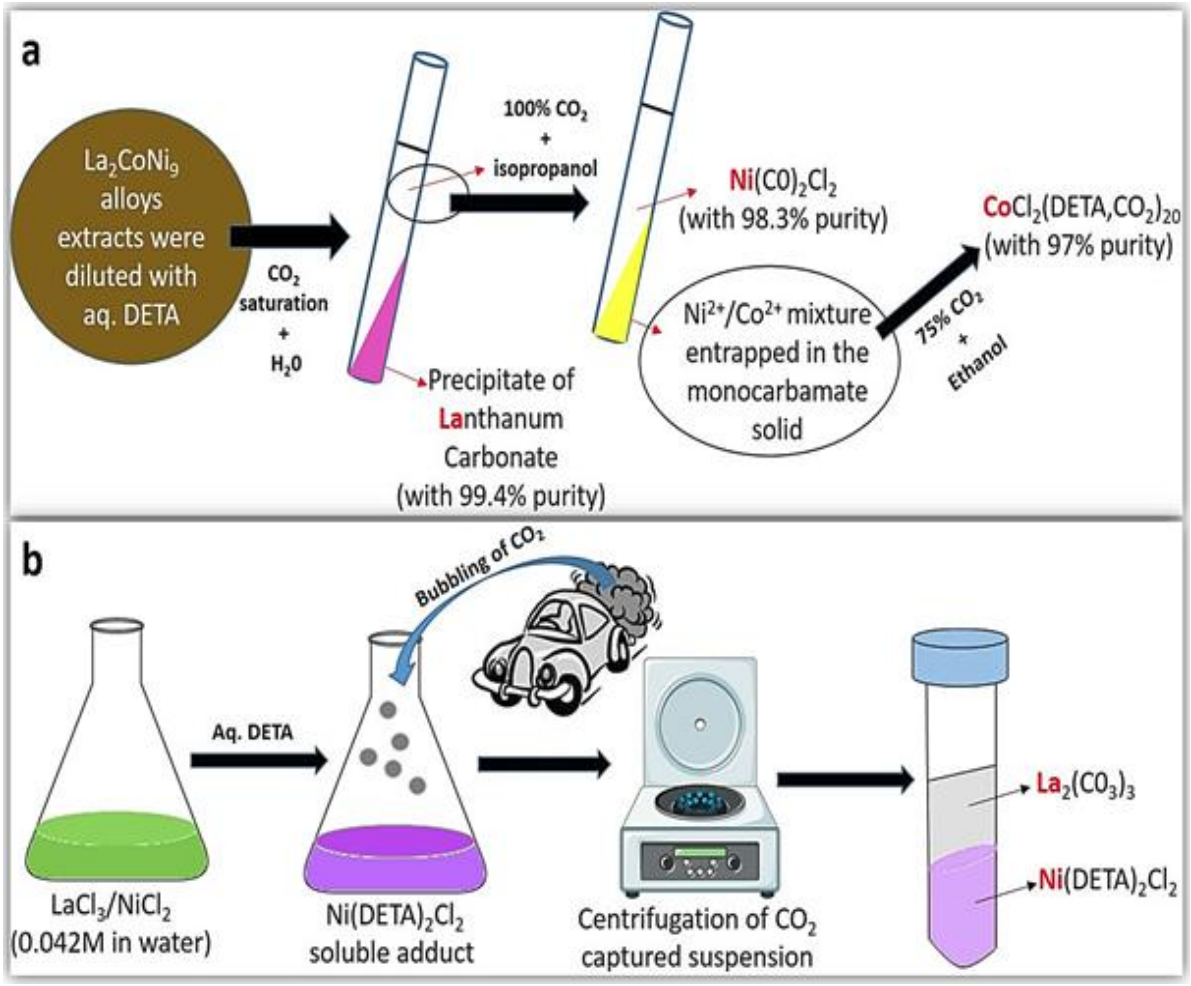

Figure 2. (a). Schematic of individual metal separation from La_2CoNi_9 alloys **(b)**. Schematic of bimetallic separation of LaNi liquid extract using gas from an internal combustion engine. Both mechanisms are suggested and described in details in (1).

In the *first case* (**Figure 2a**), the tri-metallic extract of the La_2CoNi_9 alloy was diluted with aqueous DETA after acid dissolution and saturated with CO_2. This yielded 99.4% of La, which was initially used in the form of carbonate. After dissolution of CO_2 and dilution with ethanol precipitates of two solids were sequentially obtained from the resultant solution. In the first

step, a solid precipitate was generated by loading DETA with CO_2 up to 100% and adding isopropanol.
Isopropanol decreased the polarity of the medium, in order to fully precipitate Co and to increase the Ni purity in the remaining solution (solution; $Ni(C0)_2Cl_2$). In the second step, precipitate was obtained by thermal stripping of CO_2 resulting in the release of Ni^{2+}/Co^{2+} mixture entrapped in the monocarbamate solid, followed by capture at CO_2 loading used for precipitation of Co in the first step (precipitate; $CoCl_2(DETA,CO_2)_{20}$).
In the *second case* (**Figure 2b**), Septavaux et al. (1) studied the possibility of direct metal separation from $LaCl_3$–$NiCl_2$, by capturing CO_2 from the exhaust pipe of internal combustion engine of vehicle using aqueous DETA. Lanthanum carbonate tetra hydrate was precipitated within minutes and was isolated from a $Ni(DETA)_2Cl_2$ complex (purple solution). After separation, both metals were recovered with 99% yield and 99% purity.

For a better understanding of the combinatorial chemistry (4) behind the conducted experiments, more insight into the mediating mechanisms is needed. DETA was chosen as a precursor for this system which acts as a complexing agent for metals because of the presence of three amine groups. These basic sites provide a backbone for mono-, di- and tri-carbamates (**Figure 3**), increasing the number of different complexes that can be obtained at the next level.

The first bond that DETA forms is a N–CO2 carbamate bond (Figure 3, highlighted), which generates a dynamic system of ligands based on carbamation process (dC) (7,8). Difference in the degree of protonation and carbamation provides a set of carbamation states from C0-C2 (where C denotes that species are generated in the carabamation system and the number indicates the number of carbamated sites on DETA). 13C NMR study of DETA, H2O and CO2 solution were conducted by Hartono et al (9). Their study revealed that at low CO2 loading carbamate species are produced, whereas dicarbamate species dominate at higher CO2 loading. Experiments revealed that bimolecular carbamation of C0 with intramolecular abstraction of a proton produced an assymetric monocarbamate (C1a) as the dominant species at low CO2 loading. X-ray analysis of C1aH indicated that it is a zwitterionic specie. On the other hand, intramolecular fixation of CO2 at the secondary nitrogen of DETA yielded C1sH. At high CO2 loading, fixation of second CO2 molecule on the triamine backbone can follow a termolecular pathway. That is, it can involve intermolecular abstraction of a proton from zwitterionic monocarbamate C1aH (11,12,1).

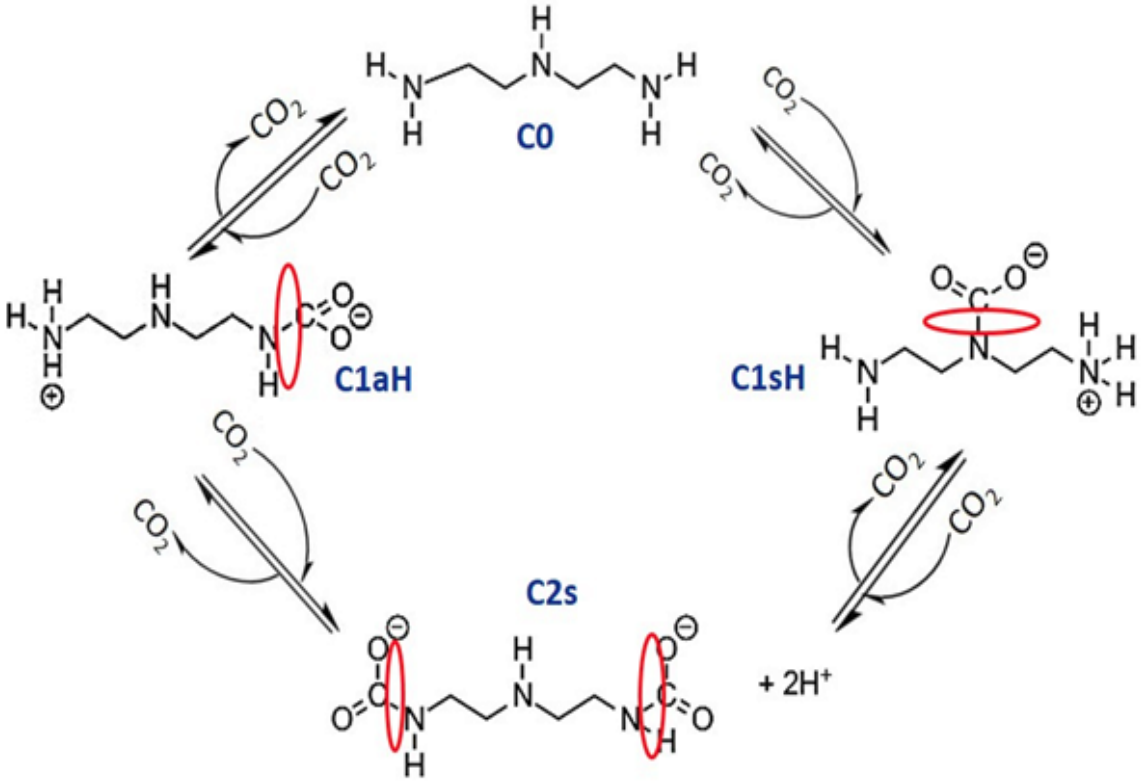

Figure 3. Network of carbamation species formed when DETA reacts with CO_2 (N-CO_2 bonds are marked with red circles), where C0 denotes carbamation, C1aH denotes asymmetric monocarbamate, C1sH denotes symmetric monocarbamate and C2s denotes symmetric bicarbamate species (described in more details in (1)).

Figure 3 illustrates how C0, C1a or C1s act as a base to generate C0H_2-C2s, C1aH_2-C2sH and C1sH_2-C2sH ion pairs, respectively. Products and reactants from the carbamation system combine with metal halides to form a second level library of complexes denoted by **dL** (Ligation process)(12). These metal chlorides (note that chlorides are strong cation binders) act as counter ions for cationic metal template forming a salt bridge with organic or organometallic cations. Members involved in first (dC) and second (dL) level systems usually contain hydrogen bonding sites (charged species) for further bonding. These charged species proceed to form ion pairing that encompasses the third level association denoted by **dIP** (ion pairing process). Finally, a combinatorial 3-level system is formed, denoted as **dCLIP** (4). In the dCLIP system, cationic metal complexes produced in dL system interact with carbamate oxyanions produced at the dC level through ion pairing. This leads to DETA induced complexation of metal particles (**Figure 4**). This process may later be used to balance protonated or metallated DETA-assisted amplification of complexes in solution.

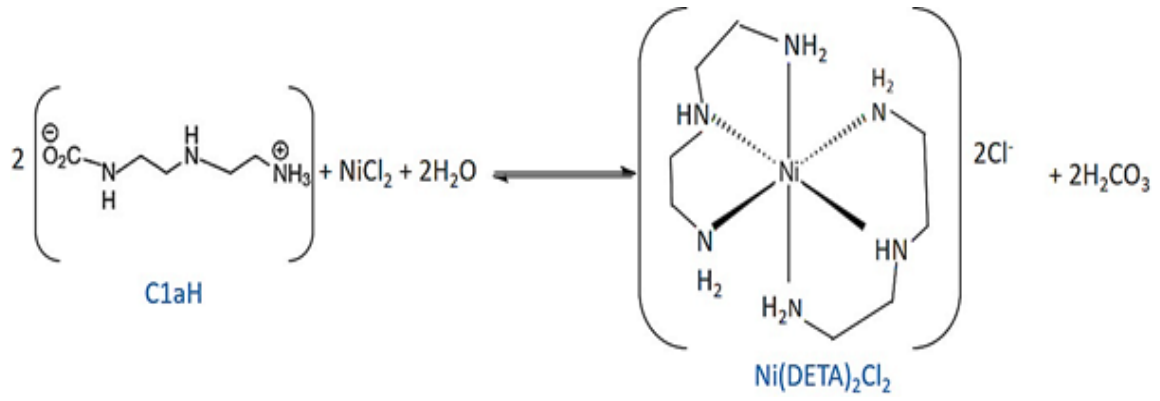

Figure 4. Reaction scheme of asymmetric monocarbamate (C1a) reacting with nickel chloride to yield $Ni(DETA)_2Cl_2$ complex.

Discussion

Electrostatic interaction between charged bodies is of fundamental importance in material science and biology (13). In the presence of multivalent ions, electrostatic interactions/ correlations render the nature of this interaction nontrivial (14). Superposition of electrostatic and van der Waals forces is explained forthrightly by the DLVO theory (13,15). Electrostatic double layer repulsion dominating at long distances may be overcome by van der Waals attraction at short distances (16). Meanwhile, some refinement by ion correlation and ion condensation have been introduced into the DLVO theory. Before employing Monte-Carlo simulations, Kirkwood and Shumaker experimentally studied ion correlation with polyvalent macroions (17). After studying charge fluctuations in the counterion cloud of polyions, Oosawa predicted the condensation of large ions (18) based on charge fluctuations, which develop attractive forces between induced dipoles of ion clouds of adjacent electric double layers (16). Also in the presence of multivalent counterions (CIs), like-charged surfaces can be mutually attracted (13,6,19-23).

Gimsa et al. (6) experimentally and theoretically (using Monte Carlo simulations) investigated the effects of spermidine (**Figure 1**) on the attractive forces between negatively like-charged surfaces. Experimentally, the effect of spermidine protonation on the attractive forces between borosilicate and mica surfaces was studied using AFM. For this borosilicate beads were attached to AFM cantilever tips. At pH 9.0 when spermidine is diprotonated, repulsion was detected above spermidine concentration of 0.02 mg/mL. This is mainly due to the fact that some spermidine molecules adhere parallel to the surface (see also **Figure 5**) and reduce the effective surface charge. And secondary factor is the energy barrier, which is generated by the repulsion of perpendicularly oriented positively charged headgroups of spermidine molecules, forming “brushes” at the surfaces. However, once the repulsive barrier forces are

overcome, a “bridging force mechanism” comes into effect generating maximum displacement forces. At pH 7.8, independent of spermidine concentration, no repulsion was observed due to the higher effective charges of the di- and triprotonated spermidine species. These species reduce the effective surface charge and reduce the free energy of the surfaces thus decreasing electrostatic repulsion (16). Monte Carlo simulations of the spatial distribution and orientational ordering of spermidine molecules showed that some of the rod-like spermidine molecules attach to the mica and

borosilicate surfaces in perpendicular orientation, while others are oriented parallel to the surface (16,6) (see also **Figure 5**). For small gap widths between the surfaces, this polyvalent cationic molecule mediates the so-called “bridging force mechanism” (6) that forms an electrostatic bridge-pull effect between the like-charged surfaces (20,21). This mechanism becomes more efficient at higher surface charges due to a quantitative increase in perpendicularly oriented molecules. Simulations also showed that the spermidine molecules condensed at the surface in parallel orientation. For large gaps, these molecules subside the effective surface charge densities reducing electrostatic repulsion (6,22). For narrow gaps, osmotic surface repulsion and counterion concentration is reduced due to lower effective surface charge densities (6).

In general, it was shown that that orientational ordering of quadrupolar counterions can induce attractive interactions between like-charged surfaces or macroions if the distances between the individual charges within the quadrupolar counterions are large enough (5,20,23). It was also shown that within Poisson-Boltzmann mean-field theory monovalent counterions cannot mediate attractive interaction between like-charged objects or surfaces (5,23).
However, if direct interactions are taken into account, also monovalent counterions can mediate attractive interaction between like-charged surfaces, but this transcends the mean-field approach (25). Spermidine-like biological molecules (24) (i.e. charged rod-like molecules) are an example of quadrupolar counterions, which have point-like charges located at well-defined finite distances (6).
Therefore, for finite separation distance between point-like charges within the individual molecules, intraionic positional correlations and orientational ordering are decisive for predicting short-range attractive bridging forces in the mean-field electrostatic models (5,6,21,22), as also confirmed by Monte Carlo simulations (6,23). From the theoretical studies (5,6), it can therefore be concluded that DETA-based polyvalent ions can function as mediators for attractive bridging forces between like-charged macroions (see **Figure 5**). Monte Carlo simulations (6,23) and theoretical models (5,21,23) are both successful in explaining the suggested bridging mechanism (5,6,20-23) for DETA-mediated attraction between like-charged macroions (**Figure 5**).

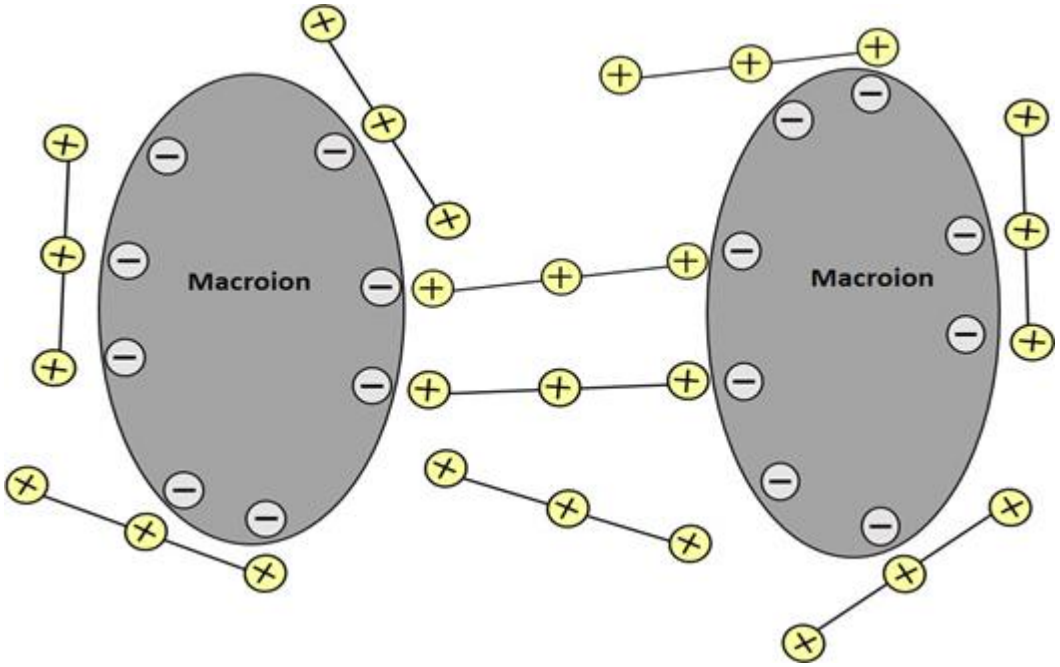

Figure 5. Schematic illustration of the proposed DETA-mediated attraction between like-charged macroions, assuming a triprotonated DETA state.

The DETA molecule, which is structurally similar to spermidine is a polycation with two primary and one secondary amine functionalities in the carbon chain (**Figure 1**). DETA can be in a tri-, di- or monoprotonated state. These properties increase the capacity of DETA to capture CO_2 at a high absorption rate (**Figure 3**). The kinetic rate constant for the primary amine group is higher than that of the secondary amine group or of water, i.e. the primary amine group is more reactive than the secondary amine group (10). Differences in the degrees of protonation, carbamation and CO_2 loading provide different carbamation sets for metal complexation. While some of the DETA molecules in a solution are free, others attach to the surface of macroions. At smaller distances the cationic DETA molecule would generate an electrostatic bridge-pull effect between like-charged macroions as shown in **Figure 5**.
To conclude, theoretical and experimental studies (5,6,20-23) have shown that orientational ordering and protonation of multivalent rod-like polyions like DETA, and the effective surface charge of macroions are important factors that determine the attraction between like-charged macroions mediated by rod-like polyions (**Figure 5**).
We hypothesise that spatially separated individual charges of DETA molecule cangenerate bridging forces that induce attraction between like-charged macroions. Our model helps in explaining the chemical interactions behind the DETA-induced metal complexation for the selective removal of metal contaminants from water.

Acknowledgement
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References

1. Septavaux J, Tosi C, Jame P, Nervi C, Gobetto R, Leclaire J. Simultaneous CO_2capture and metal purification from waste streams using triple-level dynamic combinatorial chemistry. Nature Chemistry 2020, 12: 202-212.

2. Mac Dowell N, Fennell PS, Shah N, Maitland GC. The role of CO_2 capture and utilization in mitigating climate change. Nature Climate Change 2017, 7: 243–249.

3. Rochelle GT. Amine scrubbing for CO_2 capture. Science 2009; 325: 1652–1654.

4. Corbett PT, Leclaire J, Vial L, West KR, Wietor J-L, Sanders JKM, et al. Dynamic combinatorial chemistry. Chem Rev 2006, 106: 3652–3711.

5. Bohinc K, Iglič A, May S. Interaction between macroions mediated by divalent rod-like ions. Europhys Lett 2004, 68: 494–500.

6. Gimsa J, Wysotzki P, Perutkova Š, Weihe T, Elter P, Marszałek P, et al. Spermidine-induced attraction of like-charged surfaces is correlated with the pH-dependent spermidine charge: Force Spectroscopy Characterization. Langmuir 2018, 34: 2725–2733.

7. Dell'Amico DB, Calderazzo F, Labella L, Marchetti F, Pampaloni G. Converting carbon dioxide into carbamato derivatives. Chem Rev 2003, 103: 3857–3898.

8. Yang Z-Z, He L-N, Gao J, Liu A-H, Yu B. Carbon dioxide utilization with C–N bond formation: carbon dioxide capture and subsequent conversion. Energy Environ Sci 2012, 5: 6602-6639.

9. Hartono A, da Silva EF, Grasdalen H, Svendsen HF. Qualitative determination of species in DETA–H_2O–CO_2 system using ^{13}C NMR spectra. Ind Eng Chem Res 2007, 46: 249-254.

10. Hartono A, Svendsen HF. Kinetics reaction of primary and secondary amine group in aqueous solution of diethylenetriamine (DETA) with carbon dioxide. Energy Procedia 2009, 1: 853–859.

11. Hartono A, Hoff KA, Mejdell T, Svendsen HF. Solubility of carbon dioxide in aqueous 2.5 M of diethylenetriamine (DETA) solution. Energy Procedia 2011, 4:179–86.

12. Grommet AB, Hoffman JB, Percástegui EG, Mosquera J, Howe DJ, Bolliger JL, et al. Anion exchange drives reversible phase transfer of coordination cages and their cargoes. J Am Chem Soc 2018, 140: 14770–14776.

13. Israelachvili JN. Intermolecular and surface forces. Third edition. Amsterdam: Elsevier, Academic Press, 2011.

14. Abrashkin A, Andelman D, Orland H. Dipolar Poisson-Boltzmann equation: ions and dipoles close to charge interfaces. Phys Rev Lett 2007, 99, 077801-04.

15. Cowley AC, Fuller NL, Rand RP, Parsegian VA. Measurement of repulsive forces between charged phospholipid bilayers. Biochemistry 1978, 17: 3163–3168.

16. Evans DF, Wennerström H. The colloidal domain: where physics, chemistry, biology, and technology meet. New York, NY: VCH Publishers, 1994.

17. Guldbrand L, Jönsson B, Wennerström H, Linse P. Electrical double layer forces. A Monte Carlo study. J Chem Phys 1984, 80: 2221–2228.

18. Oosawa F. Counterion fluctuation and dielectric dispersion in linear polyelectrolytes. Biopolymers 1970, 9: 677–688.

19. Besteman K, Zevenbergen MAG, Heering HA, Lemay SG. Direct observation of charge inversion by multivalent ions as a universal electrostatic phenomenon. Phys Rev Lett 2004, 93: 170802.

20. Urbanija J, Bohinc K, Bellen A, Maset S, Iglič A, Kralj-Iglič V, et al. Attraction between negatively charged surfaces mediated by spherical counterions with quadrupolar charge distribution. J Chem Phys 2008, 129: 105101-5.

21. May S, Iglič A, Reščič J, Maset S, Bohinc K. Bridging like-charged macroions through long divalent rodlike ions. J Phys Chem B 2008, 112: 1685–1692.

22. Perutková Š, Frank M, Bohinc K, Bobojevič G, Zelko J, Rozman B, et al. Interaction between equally charged membrane surfaces mediated by positively and negatively charged macro-ions. J Membrane Biol 2010, 236: 43–53.

23. Gongadze E, Velikonja A, Perutkova Š, et al. Ions and water molecules in an electrolyte solution in contact with charged and dipolar surfaces. Electrochimica Acta 2014, 126: 42–60.

24. Raspaud E, Durand D, Livolant F. Interhelical spacing in liquid crystalline spermine and spermidine-DNA precipitates. Biophysical Journal 2005, 88: 392–403.

25. Zelko J, Iglič A, Kralj-Iglič V, Kumar PBS. Effects of counterion size on the attraction between similarly charged surfaces. J Chem Phys 2010, 133: 204901-8.

Review

PHENOMENON OF LIGHT EMISSION IN INORGANIC MATERIALS: FLUORESCENCE ACTIVITY OF FLUORITE MINERAL

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Abstract

Fluorite is a mineral from halide group (in periodic table of elements) with the chemical formula CaF_2. Its name comes from Latin (lat. *Fluere* – to flow). Fluorite was firstly named by Georgius Agricola in the 16th as fluorspar, a jigsaw puzzle of the German and Latin words. Fluorite also gives the name to a special type of luminescence phenomenon – fluorescence. We present how important interdisciplinary approach is in research studies of fluorite mineral, chemical and physical parameters on fluorescence and fluorite's color.

1. About fluorite

Fluorite is a mineral with a chemical formula CaF_2. It crystallizes into an isometric or a cubic crystal system; it usually forms cubic or octahedral crystals and rarely dodecahedron crystals (1). Calcium fluoride is an ionic compound and its structure is well defined, however, perfect crystals do not occur in nature and on an atomic scale many defects in the crystal structure can be seen (2). Two most common defects in the fluorite's structure are called the Schottky defect and the Frenkel defect, respectively (2). The latter occurs when an ion (usually a cation) changes its position in the lattice (forms a vacancy and moves to another nearby location in the lattice where it becomes an interstitial ion) (2). Schottky defect refers to the occurrence of two oppositely charged ions leaving their lattice positions. Another relevant defect is *F*-center (also called color-center). It develops when one or more unpaired electrons occupy the vacancy in a lattice made by an anion. This defect is partially responsible for the purple color of fluorite (2–4).

Calcium fluoride in itself is a colorless substance, but many impurities and defects make it one of the most varied colored minerals. Vacancies, interstitial and all aforementioned defects importantly affect the color, as with new atoms in the lattice, absorption of light is also changed. Interstitial atoms of iron (Fe^{2+} and Fe^{3+}) tend to color the fluorite bluish, europium gives fluorite its yellow color, manganese (Mn^{2+} and Mn^{3+}) ions have an impact resulting in brown color and samarium is responsible for the green color of fluorite (2, 5). Also another important factor is the possibility of solid and liquid inclusions, fractures in crystals and colloid inclusions (2, 3, 6). Fluorite most commonly occurs in blue, green, purple and yellow color, somehow rarer are pink, dark purple, white and brown.

2. Introduction to light emission production

Light interacts with matter in many different ways. Most common processes include absorption, reflection, refraction, diffraction and scattering (7). Absorption of visible light is the process responsible for our perception of colors of objects. If a material is irradiated with broadband visible light and the material selectively absorbs some wavelengths of the light spectrum, the residual wavelength bands are perceived as the color of the material. The energy of absorbed light is equal to electronic energy gaps between ground state and excited states in the interacting molecules. Because these states of molecules have discrete energy levels, only light matching these levels is absorbed.

Most of the molecules subsequently undergo vibrational relaxation, where the energy is thermally dissipated and the molecule returns to its ground state. However, some of the molecules, where thermal dissipation is for example suppressed by rigid molecular structure, can undergo radiative emission of light. This process is happening in orders of nanoseconds and the original singlet multiplicity of the molecule is retained (8).

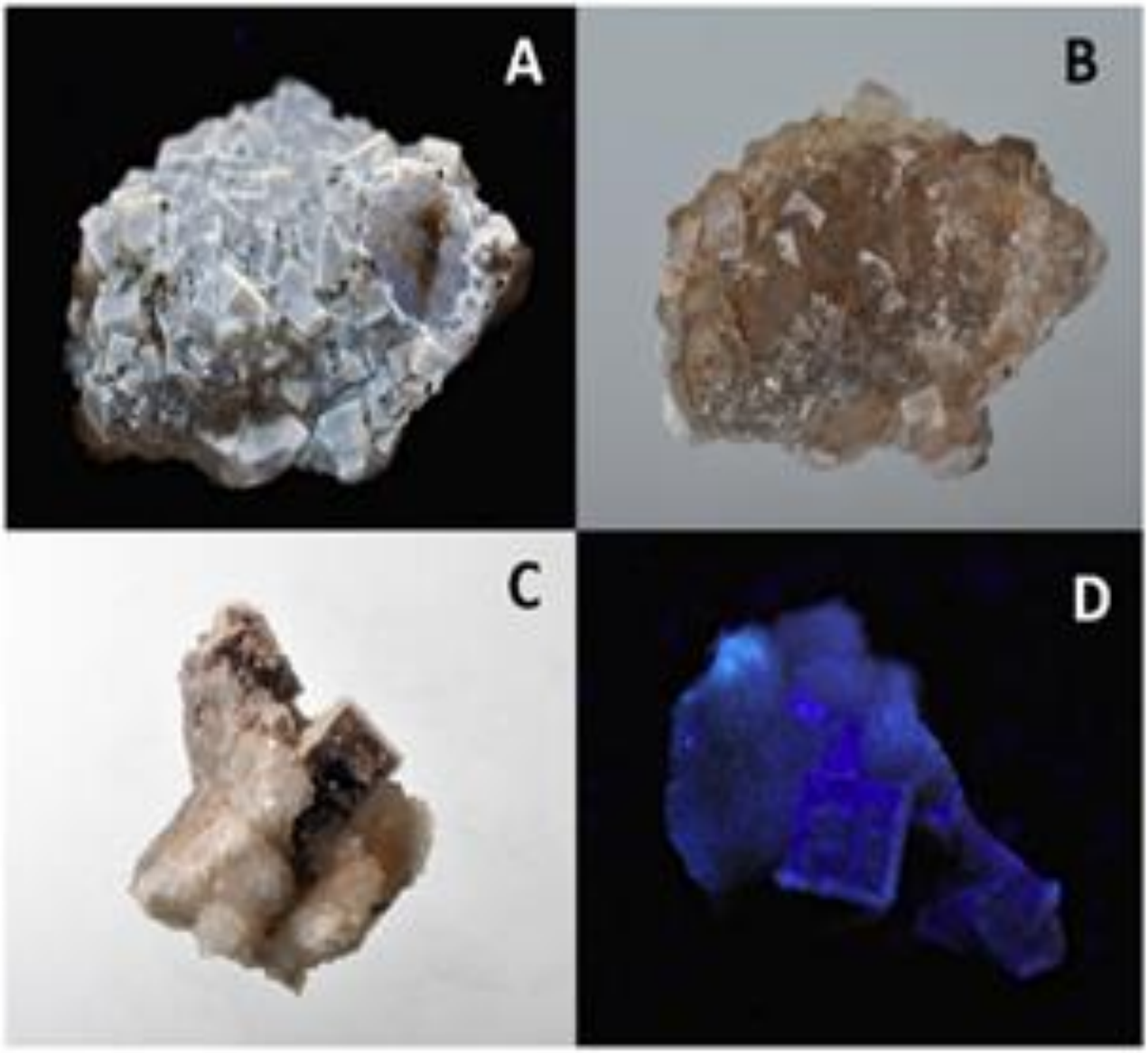

Figure 1. A: Small, well-crystallized fluorite sample exhibiting strong fluorescence under UV light. B: The same fluorite sample in visible light. C: The best fluorite specimens from Slovenia come from Blegoš valley. Fluorite crystals grow together with quartz. D: Fluorite from Blegoš, Slovenia under UV light. Fluorite and quartz can be easily distinguished. Courtesy of Nik Smerkolj and Miha Jeršek, with permission.

The energy of excitation is usually higher than that of emitted light because of the vibrational relaxation of a molecule in the excited state. However, in fluorite, a phenomenon where the emitted light has higher energy than the excitation energy, may take place. The phenomenon was first reported by J. Herschel in 1845 (9) and later described in detail by G. G. Stokes in 1852 (10). These works considered various materials and solutions, for example quinine solution and green fluorite mineral, where the specimens upon irradiation with light of lower wavelength emitted light with higher wavelength. This phenomenon was named by G.

G.Stokes “fluorescence”, similarly as the analogous term “opalescence” was derived from the opal mineral.Since then, experimental techniques employing fluorescence for detection, imaging and sensing became the essential tool in molecular biology, biochemistry, medicine and material science (11–13).

3. Origins of the fluorite mineral fluorescence

The majority of minerals do not exhibit fluorescence visible to human. Only approximately 15 % of minerals emit light visible to human (14). Emission of visible light occurs when minerals contain low concentrations of substances called activators. Activators are cations of metals (5,15), also they can be inherent defects in the crystal structure and organic compounds (2, 3).

Emission characteristics of fluorite fluorescence is determined by the environment where mineral has grown and by impurities that it contains (16). Impurities bind on different parts of crystal structure. Therefore minerals from the same locality can exhibit various emission bands with diverse intensities. Consequently, the color of fluorite fluorescence can vary even within the same specimen. The most common color of fluorite fluorescence excited by the short wavelength ultraviolet (UV) light (200–280 nm) and by the long wavelength UV light (315–400 nm) corresponds to the violet-blue part of the visible spectrum (~420 nm). It is caused by europium (Eu^{2+}), that substitutes calcium in the crystal structure (5). Less common are fluorite specimens that emit light in the green and red part of the visible spectrum. Also the combination of effects can appear as the white color of emitted light to a naked eye.

Green fluorescence occurs due to traces of erbium (Er^{3+}) (5), europium (Eu^{3+}) (17) and ytterbium (Yb^{3+}) (yellow-green fluorescence) (5). Rarer is a yellow fluorescence that occurs in samples containing dysprosium (Dy^{3+}), europium (Eu^{3+}) and ytterbium (Yb^{3+}) (yellow-green fluorescence) (5, 18). Samples exhibiting this fluorescence are found in New Mexico, Norway, Hunan province in China, Okayama prefecture in Japan and in Wisconsin, United States (19). Red fluorescence, which is equally rare as yellow fluorescence, occurs due to inclusions of europium (Eu^{3+}) (17) and samarium (19). Samples from Berber Asturias in Spain, Namur province in Belgium, Derbyshire in England and several locations in Mexico are reported to exhibit this fluorescence (19).

Fluorite is not the only mineral that exhibits fluorescence, some of more known are also barite, calcite, dolomite, hemimorphite, willemite and franklinite (2,6,20).

4. Other luminescence phenomenons and analytical methods

Besides fluorescence occurring in fluorites, also phosphorescence and thermoluminescence are types of luminescence (21–23). In phosphorescence emission of light lasts much longer than in fluorescence (even several days) (22). Duration of phosphorescence depends on the time the electron spends in the metastable energy state (24). Thermoluminescence occurs

due to electron displacements within the lattice caused by high-energy radiation (25). The longer a given object is exposed to the radiation, the greater energy is released in the form of thermoluminescence (26).

Fluorescence is widely popular in mineralogy and especially among collectors as it is a tool to identify minerals for which one only needs an UV flashlight – however, it can be used also to determine the concentration of an analyte (27). Fluorescence spectrometry is a method with which impurities at low concentrations can be detected in the sample (27). Also it gives information on the structure of the analyte by the frequency spectra of emitted light that may correspond to vibrational levels of the constituting atoms (28, 29). Fluorescence spectroscopy is widely used in geology, gemology, chemistry, biochemistry and medicine.

Conclusion

With the use of fluorescence, and the corresponding analytical methods and techniques mineralogy closely connects with fields of natural science. Combination of physical and chemical approaches provide insight into some key structural parameters at the level of elementary particles.

Acknowledgements

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References

1. Anthony JW, editor. Handbook of mineralogy. Tucson, Ariz: Mineral Data Pub; 1990.
2. Smerkolj N, Vene E, Koren J. Kemijski, biološki in fizikalni pristopi k raziskovanju fluoritnih mineralov. Ljubljana: National Institute of Chemistry and University of Ljubljana; 2018.
3. Smerkolj N. Vzroki za obarvanje in fluorescenco fluorita. Ljubljana: Primary school Vižmarje-Brod; 2015.
4. Byrne A, Hilbert DR. Color realism and color science. Behav Brain Sci 2003;26:3–21. https://doi.org/10.1017/S0140525X03000013.
5. Sidike A, Kusachi I, Yamashita N. Natural fluorite emitting yellow fluorescence under UV light. Physics and Chemistry of Minerals 2003;30:478–85. https://doi.org/10.1007/s00269-003-0341-3.
6. Smerkolj N, Vene E, Koren J, Jeran M. Kemijske in fizikalne lastnosti kot podpora pri raziskavi fluorita. Konkrecija 2019:108–12. http://www.dpmfs.si/konkrecija/konkrecija_2019.html.
7. Björn LO. The Nature of Light and Its Interaction with Matter. In: Björn LO, editor. Photobiology, New York, NY: Springer New York; 2008, p. 1–39. https://doi.org/10.1007/978-0-387-72655-7_1.
8. Kln P, Wirz J. Photochemistry of Organic Compounds. Chichester, UK: John Wiley & Sons,

Ltd; 2009. https://doi.org/10.1002/9781444300017.
9. Herschel JFW. IV. ’Αμόρφωϒa, no. I.— on a case of superficial colour presented by a homogeneous liquid internally colourless. Phil Trans R Soc 1845;135:143–5. https://doi.org/10.1098/rstl.1845.0004.
10. Stokes GG. XXX. On the change of refrangibility of light. Phil Trans R Soc 1852;142:463–562. https://doi.org/10.1098/rstl.1852.0022.
11. Hof M, Hutterer R, Fidler V, editors. Fluorescence spectroscopy in biology: advanced methods and their applications to membranes, proteins, DNA, and cells. Berlin ; New York: Springer; 2005.
12. Conn PM. Imaging and spectroscopic analysis of living cells. Amsterdam; Boston: Elsevier/Academic Press; 2012.
13. Wöll D, Flors C. Super-resolution Fluorescence Imaging for Materials Science. Small Methods 2017;1:1700191. https://doi.org/10.1002/smtd.201700191.
14. Verbeek ER, Hollister L, Duffy TS, Goodell L, Kasabach H. Fluorescence - The Sterling Hill Mining Museum. The Sterling Hill Mining Museum 2020. https://www.sterlinghillminingmuseum.org/fluorescence.
15. Mahdy NM, Shalaby MH, Helmy HM, Osman AF, El Sawey ESH, Zeid EKA. Trace and REE element geochemistry of fluorite and its relation to uranium mineralizations, Gabal Gattar Area, Northern Eastern Desert, Egypt. Arab J Geosci 2013. https://doi.org/10.1007/s12517-013-0933-2.
16. Dill HG, Weber B. Variation of color, structure and morphology of fluorite and the origin of the hydrothermal F-Ba deposits at Nabburg-Wölsendorf, SE Germany. N Jb Miner Abh 2010;187:113–32. https://doi.org/10.1127/0077-7757/2010/0169.
17. Dejneka M, Snitzer E, Riman RE. Blue, green and red fluorescence and energy transfer of Eu3+ in fluoride glasses. Journal of Luminescence 1995;65:227–45. https://doi.org/10.1016/0022-2313(95)00073-9.
18. Barmarin G. Luminescence, fluorescence and phosphorescence of minerals - Fluorite. Database of Luminescent Minerals 2009. http://www.fluomin.org/uk/fiche.php?id=29&name=FLUORITE&fbclid=IwAR2GcM_TT_mGzZo_ISNGFW_eU_Gicj2xU4qHtuA1gw-v6kwvmSURHPmoYw0.
19. Beadle R. The Colors of Fluorescence, Part I. Chicago Rocks & Minerals Society 2013. http://www.chicagorocks.org/article14_colors_fluorescence.htm?fbclid=IwAR1abIUGyOCvI7Jk-H-9-Ul5ehXoeDU5LqOv3_Zce5Z22KwfsPqrrZoF1nA.
20. Robbins M. The Collector’s Book of Fluorescent Minerals. 1983.
21. Hill JJ, Aron J. An Investigation of the Thermoluminescence of Fluorite Colored by X-Ray Irradiation. The Journal of Chemical Physics 1953;21:223–8. https://doi.org/10.1063/1.1698863.
22. Millson HE, Millson HE. Observations on Exceptional Duration of Mineral Phosphorescence. J Opt Soc Am 1950;40:430. https://doi.org/10.1364/JOSA.40.000430.
23. Meetei SD. Synthesis, Characterization and Photoluminescence of ZrO2 : Eu3+

Nanocrystals. Doctor of Philosophy in Physics. Manipur University, India, 2013.
24. The Editors of Encyclopaedia Britannica. Phosphorescence. Encyclopædia Britannica 2010. https://www.britannica.com/science/phosphorescence.
25. McKeever SWS. Thermoluminescence of solids. Cambridge [Cambridgeshire] ; New York: Cambridge University Press; 1985.
26. The Editors of Encyclopaedia Britannica. Thermoluminescence. Encyclopædia Britannica 2020. https://www.britannica.com/science/thermoluminescence.
27. Hooijschuur JH. Fluorescence spectrometry. Chromedia Analytical Sciences 2020. https://www.chromedia.org/chromedia?waxtrapp=mkqjtbEsHiemBpdmBlIEcCArB&subNav=cczbdbEsHiemBpdmBlIEcCArBP.
28. Schulman SG, Sharma A. Introduction to fluorescence spectroscopy. Microchemical Journal 2000;65:353. https://doi.org/10.1016/S0026-265X(00)00048-5.
29. Radboud University, Faculty of Science. Fluorescence Spectroscopy. Systems Chemistry 2020. https://www.ru.nl/systemschemistry/equipment/optical-spectroscopy/fluorescence/.

Review

MEMBRANE VESICLES AS UNIVERSAL ENVOYS

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Abstract

Membrane enclosed particles – vesicles – are generally found in the surroundings of living cells. From the second half of the past century onwards, the indications of their significant biological functionality have been increasingly attracting the attention of more and more researchers. Nowadays, vesicles are extensively investigated for their potential use as biological markers, therapeutics and drug delivery systems. In this article we recall a few basic properties of this peculiar communication media found in all domains of life.

1. Introduction

Membranes are one of the bases of living nature. They divide a system into “internal” (a cell) and “external” (the environment), and through the separation and localisation of chemical compounds (in organelles) enable simultaneous course of incompatible processes in a very small space. On the other hand, packaging of biologically valuable molecules into membrane-enclosed particles (vesicles) allows their exchange between distant systems in a form that protects them from modification and degradation upon many external factors. The vesicular extracellular transport is evolutionarily highly conserved and found in all domains of life (1, 2), which indicates its utility and efficiency. Studies have shown that extracellular vesicles participate in fundamental cell physiology such as cell growth, differentiation, development, metabolism, and apoptosis (3, 4). They are involved in stress response, intercellular competition, lateral gene transfers (via RNA or DNA), pathogenicity and detoxification.

2. Vesicle functionality

Vesicles carry active biological compounds such as proteins, nucleic acids, and small metabolites. If introduced into a cell in a certain amount, all of these have potential to modify its physiology. The observations suggest that vesicles – as vectors – may be able to cross the boundaries between different living beings. In this aspect one could find the vesicles having a lot in common with viruses. In a certain way membrane-possessing virions could be seen as a special type of vesicles. Like viruses, vesicles may carry genetic material that can directly influence the metabolism of a target cell. They may also exhibit some specificity regarding their uptake by different cells. However, vesicles in contrast to viruses lack the capacity of self-reproduction.

Despite the fundamental concept of heritable material being passed from generation to generation in vertical gene transfer, many species (mainly prokaryotes) are known to be capable of alternative mechanisms of gene exchange. This phenomenon is called horizontal gene transfer, and was found to occur even between different species. The probably most known example of this is bacterial exchange of genes for antibiotic resistance. Nonetheless, indications suggest that horizontal gene transfer probably impacts genomes of all species (5), even hundreds of human genes (6). As genetic material is precious, but (like many biological compounds) susceptible to degradation, vesicles in this aspect represent a convenient vector. Namely, they provide a long span with specific targets, and protection of genetic material from DNAses and RNAses which are abundantly present in the extracellular environment.

3. Vesicle »life« cycle

The key features related to the functionality of vesicles are their formation, stability and specificity. Vesicles can be considered as self-assembled structures. The power of a self-assembly principle is that multiple weak interactions between molecules bypass the need of high energetic input for setting up a fairly stable construction, and at the same time allow for

relatively easy rearrangement. The latter provides flexibility of form. Dynamics which is needed for its adaptability to changes in conditions is also crucial for its functionality (activity). Vesicle formation starts with blebbing. It is led by sorting and grouping of proteins and lipids (such as tetraspanins, BAR-domain proteins and ceramide) that modulate membrane curvature (7-9). Although promoted by the enzymes that regulate presence of membrane and perimembrane components (such as ESCRT and sphingomyelinases), blebbing can be seen as a spontaneous phenomenon. Meanwhile, the life of the vesicle is defined by two more complex, energy-requiring events – the fission of a bud from the membrane of origin and the fusion of the vesicle with the target one. Fission of a bleb with a thin neck can be triggered by mechanical forces, but in a more physiological sense, it is mediated by cytoskeleton remodelling (such as actin and myosin) and a series of kinase cascades (10).
The chance for fusion of the membranes of a vesicle and a target is conditioned by their direct contact, and is influenced by fluidity, curvature and extraluminal decoration of the two membranes. Membrane surface factors – including charge, exposure of ligands and glycosylation – may promote or repulse the nearing of the two membranes. Loosely assembled membranes with high dynamics of components (e.g. diffusion and translocation rate of molecules in bilayer) are presumed to have higher tendency to fuse with others than the rigid ones. In addition, curvature of the two proximal membranes delimits contact surface area between them, and at the same time sets their elastic tension. Relaxation of this elastic energy can be the driving force for the fusion (10).
Considering the specificity and following the mechanisms described in literature, the uptake of vesicles can be seen akin to the cell entry of enveloped viruses. It is proposed that interactions of specific ligands on a virus/vesicle with the corresponding receptors expressed by the recipient cell stabilize them in close contact, while at the same time they induce structural changes of the interacting membrane anchored proteins, causing bilayer perturbations and triggering membrane fusion (10), (11). In case of vesicles, one such factor is the SNARE complex, which compiling in a zippering manner brings the membranes of a vesicle and its target cell together, and strongly bends the linker regions (10). Between the membranes emerges a pore which quickly expands while the components of both membranes redistribute in accordance to the free energy minimum of the newly established system (10).

The longevity of vesicles in the extracellular space depends on the abundance and proximity of their potential recipients, the stability of components and robustness of their assembly in regard to environmental conditions. Besides the hydrophobic interactions of lipids and proteins in the membrane bilayer, the architecture of a vesicle can be supported internally by skeleton-like constructs (like nucleocapsid in case of enveloped viruses), and externally by glycosylation. The latter sustains a propitious microenvironment, providing the hydration layer, control over the pH, osmosis, and approaching of potentially harmful molecules. However, despite the idea of directed production and targeted uptake, we think that it should be borne in mind that vesicles are associates, of which formation, transformation, dissociation

and possible reformation is governed by physicochemical laws. The vesiculation can be stimulated by many factors. Indications such as the scanning electron microscopy images presented in the contribution of Štibler et al., 2020 (Figure 4, page XX in this proceedings, showing how increase in temperature or introduction of surfactants on cells may result in degradation of cells and production of abundance of submicron particles), suggest that internal (cell-directed) mechanisms can relatively easily be interfered by external factors. As all living beings share genetic inscription and basic chemistry, the productive cross-communications – intentional or coincidental – may not be that surprising, which makes vesicular traffic a believable factor in shaping phenotype and evolution of the organisms.

4. Balancing the ecosystems and promoting evolution

Vesicles of various sources can be found in significant quantities in aquatic environments (12), and they were confirmed to have various inter-special effects. Transport by extracellular vesicles is believed to prevent dilution of the active compounds and protect them from the lytic enzymes present in extracellular space, and thereby increasing the chance of their delivery into specific target cells in the effective amount (13). The extracellular vesicles were found related to processes like quorum sensing and biofilm formation, horizontal gene transfer, detoxification, and virus susceptibility.

In algae, for example, vesicles play a part in viral infections within positive and negative feedback loops. Viruses keep algal populations under the level of blooms, and in case of a persistent bloom they are key players in ending it (14, 15). Without efficient decomposition, the rapid multiplication of algae could result in depletion of nutrients, finally fatal for the algae. Constant lysis of algae, promoted by viruses, provides a nutrition source for the next generation of algae, and therefore enables their sustainable reproduction. Viral infectivity and viral reproduction is controlled through the lysis of algal cells, which cause the release of specific and nonspecific inhibitors – such as inefficiently or incompletely constructed viral particles and receptors anchored in degraded cell membranes (15). Algal vesicles can mislead, and capture the viruses, acting as inhibitors (13), or - on the contrary - enhance viral infectivity as shown in Emiliania huxleyi (14). The viruses, with the assistance of vesicles therefore maintain the dynamic balance, which enables survival of both.

The cell membrane of many unicellular organisms is covered with a more or less compact cell wall. How the vesicles cross the cell wall of bacteria, fungi, plants and algae is still not very well understood. The proposed mechanisms comprise existence of the pores, turgor pressure, and enzymatic cell-wall remodelling (16, 17). The pores in the cell wall can be observed when cells are lysed by detergents, as was demonstrated on the microorganism treated by sodium dodecyl sulphate (Figure 4d in Štibler U, Božič D, Hočevar M, et al., Toxicity of surfactants sodium dodecyl sulphate and triton x-100 to marine microorganisms, this proceedings). However, the observed pores often seem too small to allow the passage of the vesicles (Figure 4d in Štibler U, Božič D, Hočevar M, et al., Toxicity of surfactants

sodium dodecyl sulphate and triton x-100 to marine microorganisms, this proceedings, (17)). While vesicles might be compressed from the inside of the cell by the turgor pressure, the reverse transition seems to depend on some sort of active transport machinery. The underlying mechanisms still need further research in order to complete our understanding of vesicle action.

Despite the many unknowns, the evidence suggests that extracellular vesicular transport represents one of the core interspecies bridges involving uni- and multicellular organisms, influencing their existence, development and behaviour. Some studies show that exogenous regulative RNAs are present in the sera and tissues of various animals and that they can be acquired orally, through the food intake. They can cross into the bloodstream through the respiratory system or gastrointestinal tract without degradation, and – incorporated in vesicles – reach various organs, where they can alter gene expression (2, 18-21). It was reported that some plant-derived vesicles accumulate and act in the brain (20), meaning they can escape the host immune system, and cross the blood-brain barrier. The exogenous extracellular vesicles that can be taken up by specific human cells often contain immunomodulatory factors, reducing defence reactions through suppression of inflammatory signalling pathways (22) or exposure of targets for complement factors, reducing the effectiveness of host humoral defence (22, 23). In this way a pathogen can condition the host for its invasion. On the other side, exogenous molecules can escape the host-body systems for elimination by taking up(/incorporation into) the host's cell membranes (18).

5. Conclusion

In the modern era, the environment, alimentation, and microbiota are progressively gaining attention beyond nutrition and pathogenicity. Their acknowledged value is growing through better understanding of their roles in the ecosystems, human development and well-being, including behaviour and cognition. In this context, the research of vesicles has brought to light an unexpected information interflow, evincing once again how closely interlaced the organisms in this world are.

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References

1. Deatherage BL, Cookson BT, Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. Infect Immun 2012, 80: 1948-1957.

2. Woith E, Fuhrmann G, Melzig MF, Extracellular Vesicles-Connecting Kingdoms. Int J Mol Sci 2019, 20: 5695.

3. Latifkar A, Hur YH, Sanchez JC, Cerione RA, Antonyak MA, New insights into extracellular vesicle biogenesis and function. J Cell Sci 2019, 132: jcs222406.

4. Thery C, Zitvogel L, Amigorena S, Exosomes: composition, biogenesis and function. Nat Rev Immunol 2002, 2: 569-579.

5. Keeling PJ, Palmer JD, Horizontal gene transfer in eukaryotic evolution. Nat Rev Genet 2008, 9: 605-618.

6. Huang W, Tsai L, Li Y, Hua N, Sun C, Wei C, Widespread of horizontal gene transfer in the human genome. BMC genomics 2017, 18: 274.

7. Bassereau P, Jin R, Baumgart T, et al. The 2018 biomembrane curvature and remodeling roadmap. J Phys D Appl Phys 2018, 51: 343001.

8. Verderio C, Gabrielli M, Giussani P, Role of sphingolipids in the biogenesis and biological activity of extracellular vesicles. J Lipid Res 2018, 59: 1325-1340.

9. Akers J, Gonda D, Kim R, et al. Biogenesis of extracellular vesicles (EV): exosomes, micro-vesicles, retrovirus-like vesicles, and apoptotic bodies. J Neurooncol 2013, 113: 1-11.

10. Kozlov MM, McMahon HT, Chernomordik LV, Protein-driven membrane stresses in fusion and fission. Trends Biochem Sci 2010, 35: 699-706.

11. Plemper RK, Cell entry of enveloped viruses. Curr Opin Virol 2011, 1: 92-100.

12. Biller SJ, McDaniel LD, Breitbart M, Rogers E, Paul JH, Chisholm SW, Membrane vesicles in sea water: heterogeneous DNA content and implications for viral abundance estimates. ISME J 2017, 11: 394-404.

13. Schatz D, Vardi A, Extracellular vesicles - new players in cell-cell communication in aquatic environments. Curr Opin Microbiol 2018, 43: 148-154.

14. Schatz D, Rosenwasser S, Malitsky S, Wolf SG, Feldmesser E, Vardi A, Communication via extracellular vesicles enhances viral infection of a cosmopolitan alga. Nat Microbiol 2017, 2: 1485-1492.

15. Thyrhaug R, Larsen A, Thingstad TF, Bratbak G, Stable coexistence in marine algal host-virus systems. Mar Ecol Progr Ser 2003, 254: 27-35.

16. Brown L, Wolf JM, Prados-Rosales R, Casadevall A, Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. Nat Rev Microbiol 2015, 13: 620-630.

17. Casadevall A, Nosanchuk JD, Williamson P, Rodrigues ML, Vesicular transport across the fungal cell wall. Trends Microbiol 2009, 17: 158-62.

18. Zhang L, Hou D, Chen X, et al. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell res 2012, 22: 107-26.

19. Zhou Z, Li X, Liu J, et al. Honeysuckle-encoded atypical microRNA2911 directly targets influenza A viruses. Cell Res 2015, 25: 39-49.

20. Zhuang X, Teng Y, Samykutty A, et al. Grapefruit-derived Nanovectors Delivering Therapeutic miR17 Through an Intranasal Route Inhibit Brain Tumor Progression. Mol Ther 2016, 24: 96-105.

21. Baier SR, Nguyen C, Xie F, Wood JR, Zempleni J, MicroRNAs are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow milk and affect gene expression in peripheral blood mononuclear cells, HEK-293 kidney cell cultures, and mouse livers. J Nutr 2014, 144: 1495-500.

22. Buck AH, Coakley G, Simbari F, et al. Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. Nat Commun 2014, 5: 5488.

23. Codemo M, Muschiol S, Iovino F, et al. Immunomodulatory Effects of Pneumococcal Extracellular Vesicles on Cellular and Humoral Host Defenses. mBio 2018, 9: e00559-18.

Review

RISK OF VERTICAL TRANSMISSION OF SARS-CoV-2 INFECTION AND CLINICAL MANIFESTATION OF INFECTION IN PREGNANT WOMEN, FETUS AND NEONATES

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Abstract: Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported in Wuhan, Hubei Province in China, in December 2019. It became a global public health emergency, Manifesting with complications mostly in elderly and patients with underlying health issues. Pregnancy is a physiological state that predisposes women to viral infection, with concerns also relating to fetal and neonatal outcome. Therefore, pregnant women require special attention in prevention, diagnosis and management of COVID-19. Of major concern is whether there is a risk for vertical mother-to-fetus transmission and clinical manifestation of the SARS-CoV-2 infection in fetuses. Another concern is the development of serious clinical symptoms of COVID-19 in neonates and that infants born to mothers infected with SARS-CoV-2 are in greater risk to be infected. Existing literature reports mostly good maternal, fetal and neonatal outcomes of patients who were infected with COVID-19 in late pregnancy.

1. About COVID-19

In December 2019, an outbreak of the COVID-19 associated with pneumonia was reported in Wuhan, China The causative pathogen (identified as SARS-CoV-2) is a new strain of coronaviruses that are pathogenic to humans (1). Two other notable strains are Severe Acute Respiratory Syndrome CoronaVirus (SARS-CoV) and the Middle East Respiratory Syndrome CoronaVirus (MERS-CoV) (2). SARS-CoV-2 seems to have a considerably lower virulence than SARS-CoV and MERS-CoV, it was found to be much more transmissive by a close contact (3). Typically, respiratory viruses are most contagious when a patient is symptomatic, however in case of SARS-CoV-2, human-to-human transmission may also occur during the asymptomatic incubation period which was estimated between 2 and 14 days (4). Most COVID-19 patients have developed mild symptoms such as dry cough, sore throat, and fever, with spontaneous recovery in the majority of cases. Low percentage of infected have developed various fatal complications including organ failure, septic shock, pulmonary oedema, severe pneumonia, and acute respiratory distress syndrome (5). Novel coronavirus raised special concern for pregnant women, fetuses and neonates, since SARS-CoV and MERS-CoV have been found to cause severe complications in these vulnerable groups (6).

2. Occurrence and course of COVID-19 during pregnancy

During pregnancy physiological changes in women's immune and cardiopulmonary system occur, therefore there is greater risk for women to develop severe illness after being infected with respiratory virus (7). In 1912 influenza pandemic caused 37% mortality among pregnant women, despite the fact that overall population mortality rate of was 2-6% (8) and in 2009, pregnant women accounted for 1 % of patients infected with influenza A subtype H1N1 virus representing 5% of all H1N1-related deaths (9).

Before the appearance of SARS-CoV-2, also SARS-CoV and MERS-CoV were responsible for death and life-threatening complications during pregnancy, including renal failure and difficulty in breathing, which resulted in the need for endotracheal intubation and admission to an intensive care unit (10,11). The impact of COVID-19 on pregnant women seems to be less severe (7). Yet the risk for severe respiratory complications remains elevated in pregnant women with COVID-19 (12), in fetus and in newborn (13) with respect to uninfected subjects. Analysis of the medical records of the cohort of 41 pregnant women with COVID-19 showed an elevated risk of miscarriage, preterm birth, preeclampsia and Caesarian delivery, especially in the pregnant women with pneumonia. Mullins et al. (14) reported that 47% women affected by COVID-19 delivered preterm. Asymptomatic individuals and those with mild conditions had fever complications (13). Most pregnant patients have been treated with ribavirin, corticosteroids and antibiotics, however optimal treatment has not yet been established (15). According to Chen et al. (16) nine women in the third semester of pregnancy mainly had two symptoms – fever and cough, some of them also complained about muscle

pain, malaise, sore throat, shortness of breath and diarrhea. Possible symptoms of pregnant patients are also fatigue, headache and reduced fetal movement (17). Similar clinical manifestations were reported by Yu et al. (15) who monitored seven patients in the third trimester of pregnancy. Chen et al. (16) reported pregnancy complications in nine cases which appeared as fetal distress in two cases and as premature rupture of membranes in two cases. Laboratory tests data showed lymphopenia, increase in C-reactive protein level and multiple patchy ground-glass shadows in lungs detected by CT scans (16,15) (Figure 1). Infection was not fatal for any of the patients, also, none of them developed severe COVID-19 pneumonia.

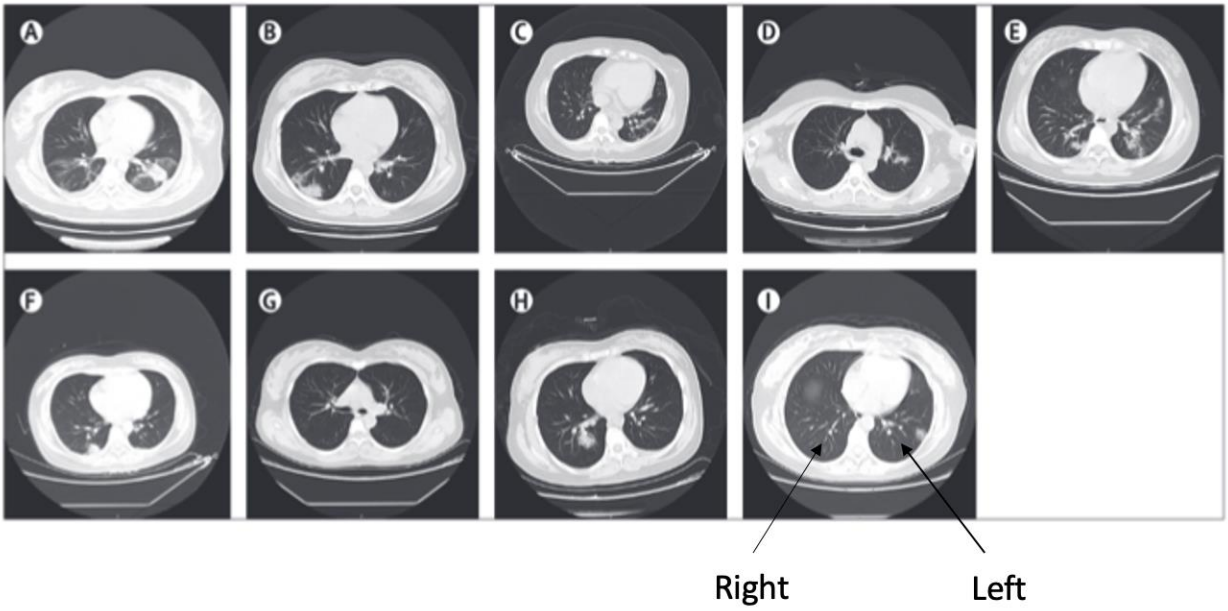

Figure 1: Chest CT scans of nine pregnant patients (transverse plane). (A) Patient 1: left-sided patchy consolidations and multiple bilateral ground-glass opacities. (B) Patient 2: subpleural patchy consolidation in the right lung and slightly infiltrated shadows around left bronchus. (C) Patient 3: bilateral multiple ground-glass opacities, prominent on the left. (D) Patient 4: left-sided patchy ground-glass opacity. (E) Patient 5: multiple ground-glass opacities bilaterally. (F) Patient 6: right-sided subpleural patchy consolidation. (G) Patient 7: bilateral clear lung fields with no obvious ground-glass opacities. (H) Patient 8: multiple bilateral ground-glass opacities, prominent on the right. (I) Patient 9: multiple bilateral ground-glass opacities. From (16).

3. Fetal outcome and possibility of vertical transmission of COVID-19 in pregnancy

Viral infection of a fetus can result in severe birth defects (18), pregnancy loss (18) or stillbirth (13), however, according to Chen et al. (16). Viruses rarely cross the placental barrier, yet some of the viruses can access decidua and placenta by passing from the lower reproductive tract or by hematogenous transmission, depending on gestational age, viral entry receptor expression, in-utero environment and maternal immune response to the virus (18). To our

best knowledge there has been no decisive evidence of in-utero transmission of SARS-CoV-2 in women who developed COVID-19 pneumonia in the third trimester of pregnancy, but there are still reasonable concerns that COVID-19 could be contracted in the womb (16). Immunoglobulin G (IgG) antibodies can be passively transferred across the placenta from mother to fetus from the end of the second trimester until birth, when they reach high levels (19). However, immunoglobulin M (IgM) antibodies' macromolecular structure is considered too large to be transmitted from mother to fetus (20). Zeng et al. (20) speculated that there is a possibility for the virus to cross the placental barrier, as they detected IgM antibodies in the serum of two infants that were born to infected mothers. They suggest that IgM could have been produced by the fetus after the virus had crossed the placenta. Similar conclusions were reported also by Dong et al. (21) who monitored a neonate with abnormal cytokine test results and elevated antibody levels two hours after birth. The laboratory results displaying inflammation and liver injury, beside the IgM antibodies, indirectly supported the possibility of vertical transmission. After the neonate tested positive for SARS-CoV-2, Wang et al. (22) performed nucleic acid tests for SARS-CoV-2 on the cord blood and placental specimens that we retained during the Cesarean delivery, and the results were negative.

Transmission during delivery and with breastfeeding

Because of the recent occurrence of the problem with SARS-CoV-2 infections, there is no data on preterm birth or perinatal complications if infection is acquired during the first or early second trimester of pregnancy. But delivery of women infected with SARS-CoV-2 in the third trimester was reported. Chen et al. (16) reported Cesarean delivery in all nine of monitored patients, to prevent infection of medical staff and the neonate. There were no cases of fetal death in this group. Four of the neonates were born before the term and none of these was considered to be directly infected with SARS-CoV-2. Samples of vaginal mucosa were not collected and therefore it couldn't be assessed whether transmission of SARS-CoV-2 infection took place during vaginal delivery. As reported by previous studies, there is no evidence of congenital infection with SARS-CoV-2 during fetal passage through the birth canal (23). Dong et al. (21) suspected that the infant was infected at delivery. Mother's vaginal secretions were negative for SARS-CoV-2. Infant's IgM antibodies were elevated two hours after the birth which indicates infection during pregnancy. It is usual for IgM antibodies to appear three to seven days after infection (21).

It was also suggested that there is no risk of vertical transmission of SARS-CoV-2 infection by breastfeeding, since the virus was not detected in the colostrum of infected pregnant women (15), (22). Fan et al. (24) concluded that it is possible that the mother produces sufficient neutralizing antibodies without developing serious conditions due to immune response to SARS-CoV-2 infection and these passive antibodies may have a protective effect on the infants via breastfeeding.

6. Clinical manifestation of COVID-19 infection in neonates

As reported by Di Mascio et al. (13) newborns of pregnant women infected with COVID-19 are at risk of neonatal death (one of 41 neonates died). Chen et al. (16) reported that after delivery, throat-swab samples of six newborns were negative for SARS-CoV-2. Also, the samples of respective amniotic fluids and cord blood were negative for SARS-CoV-2. It was found that Apgar score (a score for measuring health status of newborn children based on heart rate, respiration, color, muscle tone and reflex irritability) was ≥9 (maximum 10) 5min after the birth in all nine newborns. Studies, published in the first month after the outbreak of COVID-19 reported that in China, newborns are separated from their (infected) mother for at least first 14 days after the delivery or until mother tested negative for SARS-CoV-2 to prevent transmission of the virus with close contact. Zeng et al. (20) monitored six neonates, born to mothers with mild manifestation of COVID-19. None of these neonates tested positive for SARS-CoV-2, however antibodies were detected in serum of all six neonates. Using the serological test, concentrations of IgG to SARS-CoV-2 were elevated in five infants and IgM to SARS-CoV-2 were detected in two infants. Chen et al. (17) monitored four infants of infected mothers and tested three of them 72 hours after birth, using a throat swab. They all tested negative for SARS-CoV-2. Serious clinical symptoms such as fever, cough, or diarrhea were not observed in any of these neonates. Two of them had a rash, which disappeared spontaneously without treatment. One neonate had a mild dyspnea. It was considered that the dyspnea was caused by transient tachypnea. A non-invasive mechanical ventilation support was administered for three days. Yu et al. (15) reported that one of three tested neonates was tested positive for SARS-CoV-2 36 hours after birth, with mild shortness of breath symptoms but with no fever or cough. A neonate followed by Wand el al. (22) tested positive for SARS-CoV-2, but had no cough or vomiting. Chest CT showed thickened lung texture with no abnormalities in the heart or palate (Figure 2).

7. Conclusions:

Due to physiological changes during pregnancy, there is a greater risk for women to develop severe illness after being infected with respiratory virus. Before the appearance of SARS-CoV-2, SARS-CoV and MERS-CoV were responsible for death and life-threatening complications during pregnancy. H1N1 influenza A caused higher risk for severe complications and death among pregnant women. Hitherto collected data suggest that SARS-CoV-2 is not as severe for pregnant women as SARS-CoV, MERS-CoV and H1N1 influenza A. It manifests with mild or no complications when the maternal infection occurs in the third trimester. Also, there are no reports yet of severe defects of fetus, stillbirth or complications during and after the delivery. It is not yet clear whether SARS-CoV-2 can be transmitted vertically through placenta, during labor or with breastfeeding. Limited studies suspected that there is a possibility of the virus to cross the placental barrier, since IgM antibodies were detected in samples of the neonates a

few hours after birth. There was no data on mother's vaginal secretions and breast milk that tested positive for SARS-CoV-2.

Most of the reported cases resolved in total recovery for pregnant women infected with SARS-CoV-2 and neonates. Collection of clinical data and research is ongoing with the aim of answering questions about risk of vertical transmission during pregnancy, delivery and breastfeeding.

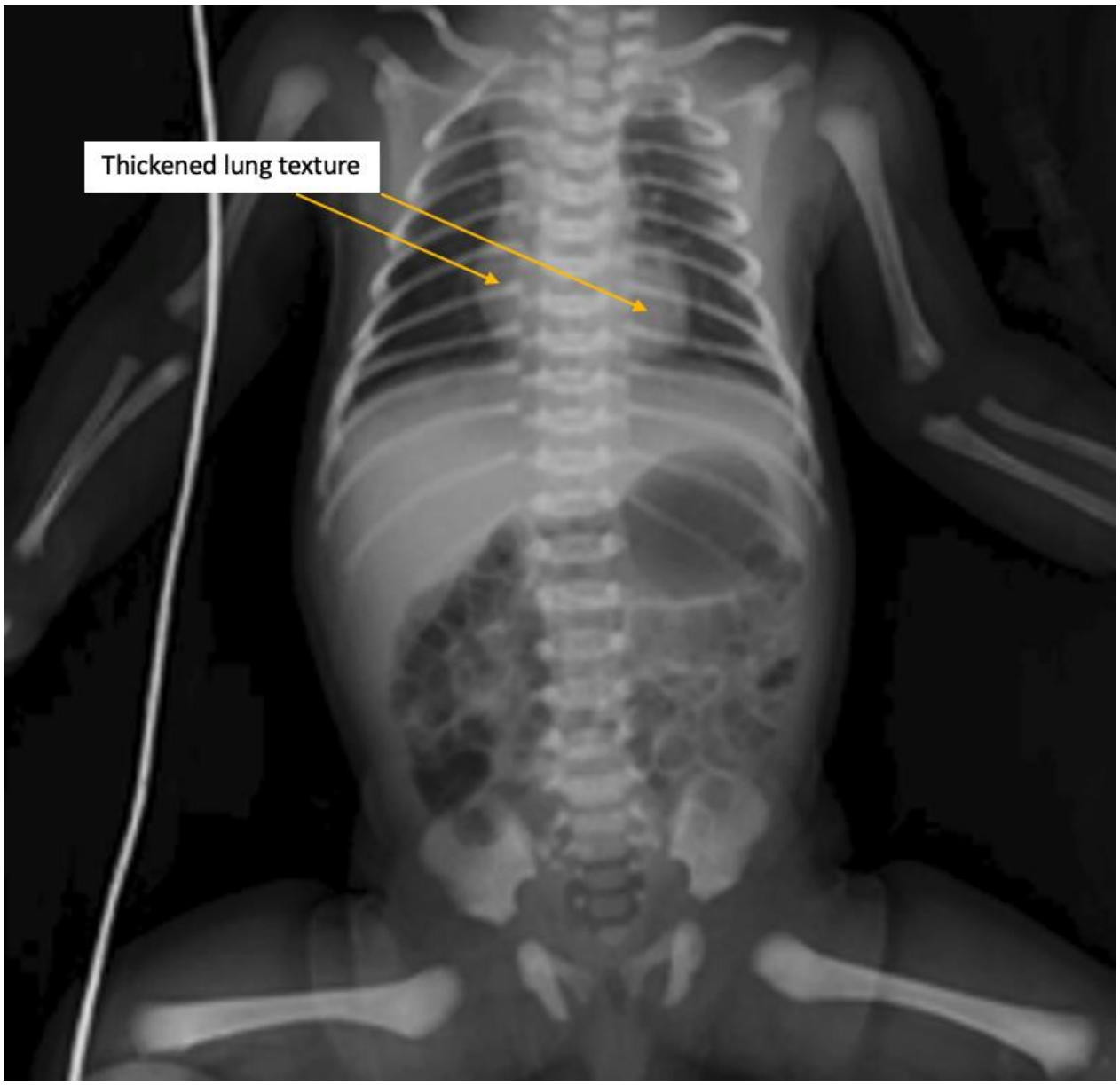

Figure 2: Chest radiograph of the newborn, showing thickened lung texture with no abnormalities in the heart or palate. From (22).

References

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med. 2020;382(8):727-33. Epub 2020/01/24. doi: 10.1056/NEJMoa2001017. PubMed PMID: 31978945; PubMed Central PMCID: PMCPMC7092803.

2. Qiao J. What are the risks of COVID-19 infection in pregnant women? Lancet. 2020;395(10226):760-2. Epub 2020/02/12. doi: 10.1016/S0140-6736(20)30365-2. PubMed PMID: 32151334.

3. Wu Z, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. JAMA. 2020. Epub 2020/02/24. doi: 10.1001/jama.2020.2648. PubMed PMID: 32091533.

4. Lauer SA, Grantz KH, Bi Q, Jones FK, Zheng Q, Meredith HR, et al. The Incubation Period of Coronavirus Disease 2019 (COVID-19) From Publicly Reported Confirmed Cases: Estimation and Application. Ann Intern Med. 2020. Epub 2020/03/10. doi: 10.7326/M20-0504. PubMed PMID: 32150748; PubMed Central PMCID: PMCPMC7081172.

5. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020;395(10223):507-13. Epub 2020/01/30. doi: 10.1016/S0140-6736(20)30211-7. PubMed PMID: 32007143; PubMed Central PMCID: PMCPMC7135076.

6. Favre G, Pomar L, Musso D, Baud D. 2019-nCoV epidemic: what about pregnancies? Lancet. 2020;395(10224):e40. Epub 2020/02/06. doi: 10.1016/S0140-6736(20)30311-1. PubMed PMID: 32035511; PubMed Central PMCID: PMCPMC7133555.

7. Yang H, Wang C, Poon LC. Novel coronavirus infection and pregnancy. Ultrasound Obstet Gynecol. 2020;55(4):435-7. Epub 2020/03/05. doi: 10.1002/uog.22006. PubMed PMID: 32134165.

8. Gottfredsson M. [The Spanish flu in Iceland 1918. Lessons in medicine and history]. Laeknabladid. 2008;94(11):737-45. PubMed PMID: 18974435.

9. Siston AM, Rasmussen SA, Honein MA, Fry AM, Seib K, Callaghan WM, et al. Pandemic 2009 influenza A(H1N1) virus illness among pregnant women in the United States. JAMA. 2010;303(15):1517-25. doi: 10.1001/jama.2010.479. PubMed PMID: 20407061; PubMed Central PMCID: PMCPMC5823273.

10. Wong SF, Chow KM, Leung TN, Ng WF, Ng TK, Shek CC, et al. Pregnancy and perinatal outcomes of women with severe acute respiratory syndrome. Am J Obstet Gynecol. 2004;191(1):292-7. doi: 10.1016/j.ajog.2003.11.019. PubMed PMID: 15295381.

11. Alfaraj SH, Al-Tawfiq JA, Memish ZA. Middle East Respiratory Syndrome Coronavirus (MERS-CoV) infection during pregnancy: Report of two cases & review of the literature. J Microbiol Immunol Infect. 2019;52(3):501-3. Epub 2018/06/02. doi: 10.1016/j.jmii.2018.04.005. PubMed PMID: 29907538.

12. Karimi-Zarchi M, Neamatzadeh H, Dastgheib SA, Abbasi H, Mirjalili SR, Behforouz A, et al. Vertical Transmission of Coronavirus Disease 19 (COVID-19) from Infected Pregnant Mothers to Neonates: A Review. Fetal Pediatr Pathol. 2020:1-5. Epub 2020/04/02. doi: 10.1080/15513815.2020.1747120. PubMed PMID: 32238084.

13. Di Mascio D, Khalil A, Saccone G, Rizzo G, Buca D, Liberati M, et al. Outcome of Coronavirus spectrum infections (SARS, MERS, COVID 1 -19) during pregnancy: a systematic review and meta-analysis. Am J Obstet Gynecol MFM; 2020.

14. Mullins E, Evans D, Viner RM, O'Brien P, Morris E. Coronavirus in pregnancy and delivery: rapid review. Ultrasound Obstet Gynecol. 2020. Epub 2020/03/17. doi: 10.1002/uog.22014. PubMed PMID: 32180292.

15. Yu N, Li W, Kang Q, Xiong Z, Wang S, Lin X, et al. Clinical features and obstetric and neonatal outcomes of pregnant patients with COVID-19 in Wuhan, China: a retrospective, single-centre, descriptive study. Lancet Infect Dis. 2020. Epub 2020/03/24. doi: 10.1016/S1473-3099(20)30176-6. PubMed PMID: 32220284.

16. Chen H, Guo J, Wang C, Luo F, Yu X, Zhang W, et al. Clinical characteristics and intrauterine vertical transmission potential of COVID-19 infection in nine pregnant women: a retrospective review of medical records. Lancet. 2020;395(10226):809-15. Epub 2020/02/12. doi: 10.1016/S0140-6736(20)30360-3. PubMed PMID: 32151335.

17. Chen Y, Peng H, Wang L, Zhao Y, Zeng L, Gao H, et al. Infants Born to Mothers With a New Coronavirus (COVID-19). Front Pediatr. 2020;8:104. Epub 2020/03/16. doi: 10.3389/fped.2020.00104. PubMed PMID: 32266184; PubMed Central PMCID: PMCPMC7098456.

18. Racicot K, Mor G. Risks associated with viral infections during pregnancy. J Clin Invest. 2017;127(5):1591-9. Epub 2017/05/01. doi: 10.1172/JCI87490. PubMed PMID: 28459427; PubMed Central PMCID: PMCPMC5409792.

19. Kohler PF, Farr RS. Elevation of cord over maternal IgG immunoglobulin: evidence for an active placental IgG transport. Nature. 1966;210(5040):1070-1. doi: 10.1038/2101070a0. PubMed PMID: 5950290.

20. Zeng H, Xu C, Fan J, Tang Y, Deng Q, Zhang W, et al. Antibodies in Infants Born to Mothers With COVID-19 Pneumonia. JAMA. 2020. Epub 2020/03/26. doi: 10.1001/jama.2020.4861. PubMed PMID: 32215589; PubMed Central PMCID: PMCPMC7099444.

21. Dong L, Tian J, He S, Zhu C, Wang J, Liu C, et al. Possible Vertical Transmission of SARS-CoV-2 From an Infected Mother to Her Newborn. JAMA. 2020. Epub 2020/03/26. doi: 10.1001/jama.2020.4621. PubMed PMID: 32215581; PubMed Central PMCID: PMCPMC7099527.

22. Wang S, Guo L, Chen L, Liu W, Cao Y, Zhang J, et al. A case report of neonatal COVID-19 infection in China. Clin Infect Dis. 2020. Epub 2020/03/12. doi: 10.1093/cid/ciaa225. PubMed PMID: 32161941; PubMed Central PMCID: PMCPMC7108144.

23. Shek CC, Ng PC, Fung GP, Cheng FW, Chan PK, Peiris MJ, et al. Infants born to mothers with severe acute respiratory syndrome. Pediatrics. 2003;112(4):e254. doi: 10.1542/peds.112.4.e254. PubMed PMID: 14523207.

24. Fan C, Lei D, Fang C, Li C, Wang M, Liu Y, et al. Perinatal Transmission of COVID-19 Associated SARS-CoV-2: Should We Worry? Clin Infect Dis. 2020. Epub 2020/03/17. doi: 10.1093/cid/ciaa226. PubMed PMID: 32182347.

Review

USE OF ELECTROPORATION FOR BACTERIAL INACTIVATION

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Abstract

Bacterial cell wall is the main barrier against the environment, and as such it is the main target for bacterial inactivation processes. One promising approach for inactivation is electroporation, which leads to increased membrane permeabilization and, if electric field is high enough, cell death. This makes electroporation useful method for gene transfection, extraction of biomolecules, preservation and sterilization of food, and wastewater treatment. In the food industry it is a useful non-thermal alternative to thermal treatments, because it preserves valued compounds such as vitamins and antioxidants, yet electroporation alone often induces insufficient inactivation to work as a standalone approach, motivating the investigation into synergistic treatments. Bacterial inactivation can be increased by combining electroporation with other physical methods or antimicrobial agents. This article describes what electroporation is, how it can be used to inactivate bacteria and potentiate the effect of antibiotics and presents some applications.

1. Electroporation

Electroporation (electropermeabilization) is a process of exposure of biological membranes to a sufficiently high electric field, so that the induced transmembrane voltage (TMV) exceeds a certain threshold value, which leads to a rapid and large increase of their electric conductivity and permeability. Nearly all cells maintain an electric potential difference between inner and outer side by ion pumps and channels in the membrane, termed resting transmembrane potential. If applied external field causes the transmembrane voltage to exceed the resting potential significantly, it causes permeabilization, which reflects formation of pores in the membrane, as well as chemical changes to the membrane lipids and proteins (1). Since this is a physical phenomenon, it occurs in the lipid bilayer membranes of both prokaryotic and eukaryotic cells (2).

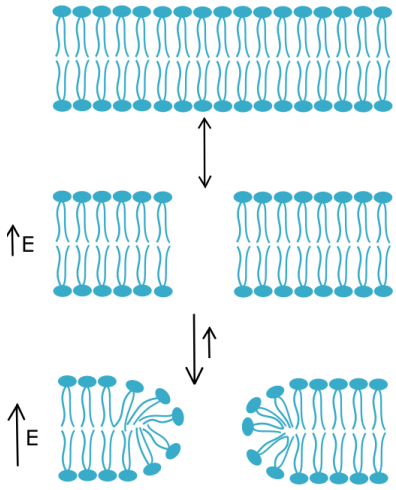

Figure 1: Schematic representation of pore formation. E=electric field.

Pore formation is initiated by water molecules orienting their dipoles along the local electric field created by the TMV and forming small clusters that extend into the hydrophobic core and merge to form a hydrophobic pore. Subsequently, the lipids around start to reorient their polar headgroups toward the water, thus stabilizing the pore into its hydrophilic state. Pore initiation time decreases exponentially with increasing electric field, but pore closure time is practically independent of the applied field and is completed within tens to hundreds of nanoseconds (1).

Threshold for a lipid bilayer electropermeabilization depends on its capacitance, dipole potential and on the nature of lipids' hydrophobic tails. Electric pulses also induce generation of reactive oxygen species that lead to lipid peroxidation which alters the composition and properties of lipid bilayers and enhance membrane susceptibility to pore formation (1). The build-up of TMV depends on the pulse duration and consists of two components. First is

dielectric response that results from reorientation of dipoles and is induced within picoseconds. This component is dominant for pulses that are much shorter than ~1 microsecond (3). Second component is redistribution of charges like ions in the surrounding solution that accumulate on both sides of the membrane and charge it as a capacitor. This component is slower, reaching plateau within microseconds, and is dominant for longer pulses (4).

Kinetics of electroporation can be divided into five stages: initiation (membrane conductivity and permeability start to increase; nanoseconds to microseconds), expansion (persistence and/or increase of conductivity and permeability as long as TMV is above the critical value; until the end of the pulse), partial recovery (membrane conductivity and permeability decrease rapidly but not fully after the TMV drops below the critical value; microseconds to milliseconds), resealing (if the cell is still viable, the membrane gradually recovers; seconds to minutes) and residual memory (stressors can lead to some residual alterations in cells physiological processes even after full membrane resealing; hours) (1).

Membrane electroporation can be either reversible or irreversible. Reversible electroporation occurs when the transmembrane potential is not sufficiently high, which leads to transient pores that can reseal after the electric field is removed. Irreversible electroporation on the other hand occurs when the voltage reaches a threshold for permanent pores, causing cell inactivation. This can happen also with reversible electroporation if resealing time is too long and leakage and/or uptake of toxic molecules takes place (5).

2. Electroporation of bacterial cells

In bacteria a difference in poration is observed between Gram-positive and Gram-negative bacteria, because of different membrane structure and composition. Gram-negative bacteria are more resistant to poration than Gram-positive bacteria, mainly because of the outer membrane, which functions as a permeability barrier, excluding certain drugs and antibiotics from penetrating the cell and because of lipopolysaccharides that have reduced mobility (1). Stronger electric fields that cause irreversible damage and cell death are already showing great promise as a safe and efficient method for inactivation of microorganisms in water and food (2,6). In contrast to electroporation, other physical and chemical methods of such inactivation are either less effective or produce toxic by-products (7). The intensity of electroporation can be precisely controlled by adjusting the pulse amplitude and duration, while the physical nature of the underlying phenomenon (formation of pores) makes it impossible for the microorganisms to develop resistance (8).

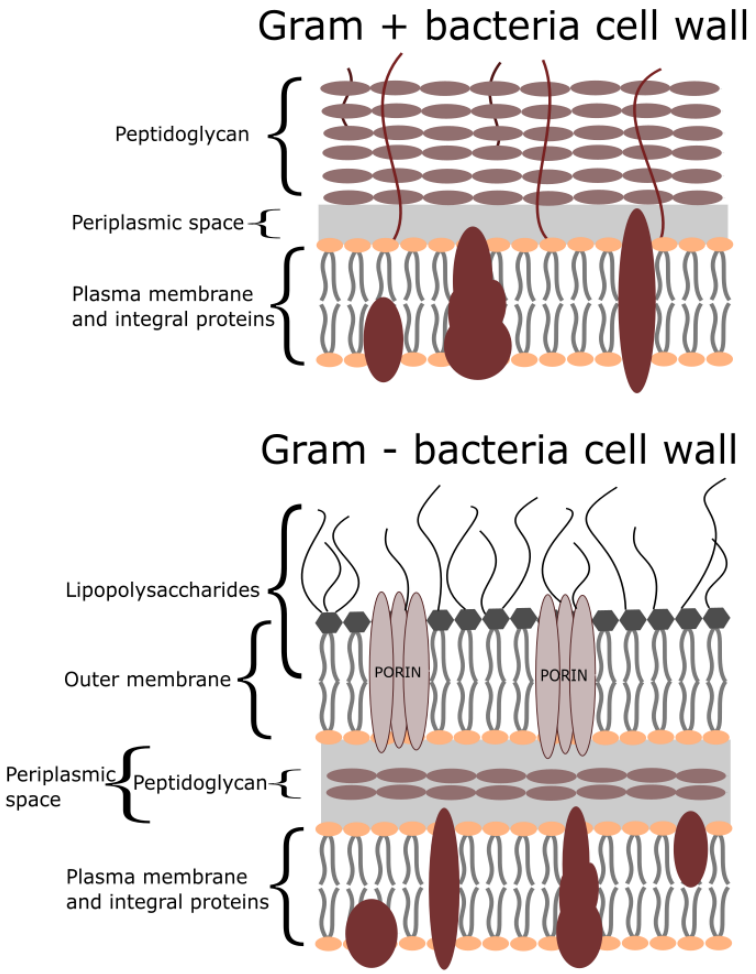

Figure 2: Cell wall composition of Gram-positive and Gram-negative bacteria.

3. Electroporation as a potentiator of antimicrobial efficacy

Antibiotics are used to prevent and treat bacterial infections and can be grouped based on their chemical structure, target, and mechanism of action. The main groups are: agents that inhibit cell wall synthesis, depolarize the cell membrane, inhibit protein synthesis, inhibit nucleic acid synthesis and inhibit metabolic pathways (9). Many bacterial species have become resistant to antibiotics, so that many of the available antibiotics are no longer effective against some infections. While electroporation has a demonstrated capability to inactivate microorganisms, it often induces insufficient inactivation to replace thermal treatments alone, motivating the investigation into synergistic treatments (other physical treatments or antimicrobials) (10,11).

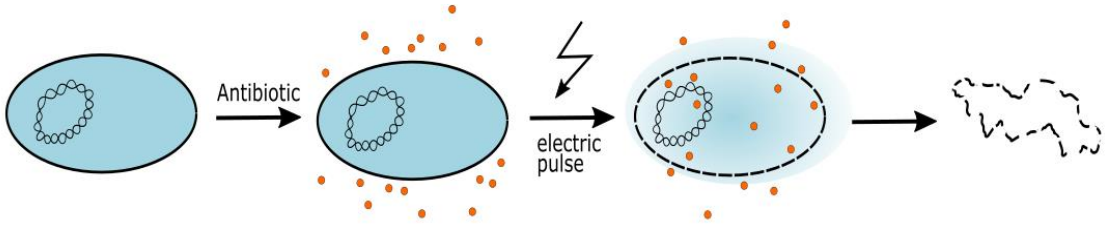

Figure 3: Schematic representation of uptake of antibiotic through pores, created by electroporation which leads to cell death.

In recent years, electroporation was also used to inactivate bacteria in combination with different antibiotics and other antimicrobial substances (10-14). These studies have generally found that such combined use is synergistic, with inactivation achievable at lower antibiotic concentrations and at reduced electric pulse amplitudes and/or durations, because antibiotics become more effective if the pathogens receive sublethal damage by electroporation. Electroporation enhances the microbial effect of antibiotics through pore formation, which leads to increased transport of molecules across the membrane. Antimicrobial compounds that are most studied in combination with electroporation are natural antimicrobials generally recognized as safe to humans, but also some clinical antibiotics (8,10,12-14).

Applications

Electroporation can be used in different areas, ranging from biomedicine (tissue ablation, electrochemotherapy, gene and drug delivery, cell fusion) and food processing (extraction, tissue dehydration, preservation and sterilization) to environmental applications (wastewater treatment, biofuel production) (15). Irreversible electroporation can inactivate both Gram-positive and Gram-negative bacteria, which can be used for food sterilization and chemical-free preservation (8), water decontamination (6,7) and potentially for treatment of infections (14).

3.1 *In food technology*

The purpose of food preservation is both to extend shelf-life and to preserve high content of vitamins, minerals, antioxidants, and other important nutrients, so there is a large market interest for non-thermal treatment methods. Pulsed electric field (PEF) is a suitable alternative because it inactivates microorganisms, preserves valued bioactive compounds due to low heat, and shortens total processing time. Use of electroporation for controlling the microbial contamination in the food industry can be either for temporary inactivation or complete sterilization, electric field strengths required for efficient inactivation of microbes in juices are within the range of 15-40 kV/cm (16). It is already used for inactivation of microorganisms in various drinks, liquid foods and solid food (8,10,15).

The efficiency of electroporation was found to depend on processing parameters, treatment chamber design, microbial properties and treatment medium characteristic (15). Limitations and challenges of PEF technology for food treatment are scaling-up difficulties, high initial equipment cost, possible air bubble formations and limited inactivation of some microbial species and particularly of bacterial spores (16). PEF is not capable of achieving commercial sterility, thus other preservation techniques or combinations will be required for sufficient quality and stability of the food during storage and distribution. To increase the efficiency of electroporation, synergistic approaches have also been described (combining electroporation with mild heating, other physical methods, or antimicrobials like bacteriocins, organic acids and essential oils) (8). Electroporation in food industry can also be used as pre-treatment to increase extraction or improve osmotic dehydration (16), to enhance peeling, for controlling enzyme activity and metabolism activity, organism growth stimulation, or as a stressing mechanism to promote production of metabolites (15).

3.2 *For wastewater treatment*

Wastewater is any water whose quality has been compromised by human activities. Hospital wastewaters that contain a variety of toxic substances (pharmaceuticals, antibiotics etc.) are among major reservoirs of pathogenic bacteria, especially those resistant to antibiotics, and are commonly discharged to the sewer systems, rivers, lakes, and seas without prior treatment. Therefore, effective water disinfection without causing carcinogenic by-products is becoming critical to our society. Irreversible electroporation is suited for bacterial decontamination and can also eradicate antibiotic-resistant strains and thus their spread into the environment (2). It was proven that with electroporation, no unwanted toxic by-products are released, no genotoxicity, phenotypic changes, adaptation of bacteria to repeated electroporation, or decrease in nuclease activity was observed (6,7). This would be an advantage of using PEF for decontamination of recycled waters, like those from hospital wastewaters that already contain antibiotics, swimming pools or industrial plants circulation flows .

3.3 *Potential clinical applications*

Most established use of electroporation in clinical medicine is electrochemotherapy, but the delivered energy is lower than what would be required for bacterial inactivation. Namely, bacterial cells are smaller than human cells, so stronger electric field is needed for electropermeabilization (17). Therefore, possible damage of host cells, which are generally larger, is among major concerns of using electroporation for bacterial inactivation in clinical medicine.

Some bacteria, especially human pathogens, can grow in the form of a biofilm, that consists of bacteria and extracellular matrix. These biofilms are hard to treat because antibiotics applied to the surface often cannot reach the bacteria inside. PEF can be used for inactivation of biofilm

bacteria (18) and it could also have a synergistic effect in combination with antibiotics, as it disrupts biofilms so that the antibiotics can reach bacteria inside.

Another potential use of electroporation is skin wound treatment. Before the wound is healed, it is often subjected to infections by bacteria, fungi, and viruses. The most problematic to treat are infections by multidrug resistant bacteria, so new methods are needed. In a study by Golberg et al. (2015) (17), they demonstrated an alternative burn wound disinfection with PEF in a murine model. This treatment reduced the bacterial load by more than four orders of magnitude, but remaining concerns are host cell damage and secondary infections due to incomplete disinfection or recontamination. Disinfection could be increased by combined treatment of existing antibiotic regimens and PEF, which would increase drug diffusion into biofilms and bacterial cells (17).

References

1. Kotnik T, Rems L, Tarek M, Miklavčič D. Membrane Electroporation and Electropermeabilization: Mechanisms and Models. Annu Rev Biophys 2019, 48: 63–91.

2. Kotnik T, Frey W, Sack M, Haberl Meglic S, Peterka M, Miklavcic D. Electroporation-based applications in biotechnology. Trends Biotechnol 2015, 33: 480–488.

3. Tarek, M. Membrane electroporation: A molecular dynamics simulation. Biophys J 2005, 88: 4045–4053.

4. Kotnik T, Miklavcic D. Theoretical evaluation of voltage inducement on internal membranes of biological cells exposed to electric fields. Biophys J 2006, 90: 480–491.

5. Wang T, Chen H, Yu C, Xie X. Rapid determination of the electroporation threshold for bacteria inactivation using a lab-on-a-chip platform. Environ Int 2019, 132, 105040.

6. Gusbeth C, Frey W, Volkmann H, Schwartz T, Bluhm H. Pulsed electric field treatment for bacteria reduction and its impact on hospital wastewater. Chemosphere 2009, 75: 228–233.

7. Rieder A, Schwartz T, Schön-Hölz K, et al. Molecular monitoring of inactivation efficiencies of bacteria during pulsed electric field treatment of clinical wastewater. J Appl Microbiol 2008, 105: 2035–2045.

8. Saldaña G, Álvarez I, Condón S, Raso J. Microbiological Aspects Related to the Feasibility of PEF Technology for Food Pasteurization. Crit Rev Food Sci Nutr 2014, 54: 1415–1426.

9. Reygaert CW. An overview of the antimicrobial resistance mechanisms of bacteria. AIMS Microbiol 2018, 4: 482–501.

10. Martín-Belloso O, Sobrino-López A. Combination of Pulsed Electric Fields with Other Preservation Techniques. Food Bioprocess Tech 2011, 4: 954–968.

11. Berdejo D, Pagán E, García-Gonzalo D, Pagán R. Exploiting the synergism among physical and chemical processes for improving food safety. Curr Opin Food Sci 2019, 30: 14–20.

12. Novickij V, Svediene J, Paskevicius A, et al. Induction of Different Sensitization Patterns of MRSA to Antibiotics Using Electroporation. 2018, 23, 1799.

13. Rubin AE, Usta OB, Schloss R, Yarmush M, Golberg A. Selective Inactivation of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* with Pulsed Electric Fields and Antibiotics. Adv Wound Care 2019, 8: 136–148.

14. Korem M, Goldberg NS, Cahan A, Cohen MJ, Nissenbaum I, Moses AE. Clinically applicable irreversible electroporation for eradication of micro-organisms. Lett Appl Microbiol 2018, 67: 15–21.

15. Mahnic-Kalamiza S, Vorobiev E, Miklavcic D. Electroporation in Food Processing and Biorefinery. J Membr Biol 2014, 247: 1279–1304.

16. Gabric D, Barba F, Roohinejad S, et al. Pulsed electric fields as an alternative to thermal processing for preservation of nutritive and physicochemical properties of beverages: A review. J Food Process Eng 2018, 41.

17. Golberg A, Broelsch GF, Vecchio D, et al. Pulsed electric fields for burn wound disinfection in a murine model. J Burn Care Res 2015, 36: 7–13.

18. Khan SI, Blumrosen G, Vecchio D, et al. Eradication of multidrug-resistant pseudomonas biofilm with pulsed electric fields. Biotechnol Bioeng 2016, 113: 643–650.

Review

DIABETES MELLITUS TYPE II AND URINARY EXTRACELLULAR VESICLES

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Abstract

Diabetes Mellitus type II (DM II) is a disease that mainly affects people over 40 years old with an unhealthy lifestyle. It develops due to insufficient insulin secretion and reduced sensitivity of target tissues (i.e., liver, muscles, adipose tissue) for insulin. DM II has acute and chronic complications. Diabetic kidney disease (DKD) is one of the chronic microvascular complications. In the beginning, the clinical picture is silent, so many researchers are aiming to find diagnostic biomarkers and identified urinary extracellular vesicles (EVs) as promising in diagnosis in DKD. EVs are membrane-bounded sub-micron particles deriving from all types of cells. They contain cell-specific origin molecules and act on neighboring, distant, and origin cells. Therefore, they play an important role in regulating physiological or pathological processes. It was found that urinary EVs can be shed by cells composing any part of the urinary tract or can be transfected to the urine from blood if the kidney function is impaired. However, it was suggested that most EVs originate from basal epithelial cells of collecting duct. Moreover, exosomes that are formed by renal tubular cells are secreted into the lumen of the nephron via the fusion of the multivesicular endosome with plasmalemma, therefore, they reflect cell metabolism and kidney function. For that reason, proteins from urinary exosomes could be the potential diagnostic biomarkers for DKD. Some of these exosomal proteins are e.g. MMP-2, MMP-9, ceruloplasmin, aquaporins 2 and 5, α-microglobulin/bikunin precursor (AMBP), histone-lysine N-methyltransferase (MLL3), and voltage-dependent anion-selective channel protein 1 (VDAC1), MLL3 and peptidase-IV (DPP IV).

1. Definition of Diabetes Mellitus type II (DM II)

The diagnosis of diabetes mellitus type II (DM II) based on the patient's clinical picture and the result of fasting plasma glucose level (independent of caloric intake). At least one of the following criteria must be fulfilled (1): typical symptoms of DM II (increased urinary excretion, increased thirst and unexplained weight loss) and plasma glucose level ≥ 11.1 mmol / L regardless of previous caloric intake and at any time during the day; fasting plasma glucose level ≥ 7 mmol/L, with no caloric intake for ≥ 8 hours; plasma glucose level ≥ 11.1 mmol/L after initiation of oral glucose tolerance test (2,3). The oral glucose tolerance test is performed when the fasting plasma level is 6.1 to 6.9 mmol /L. The test is positive if the glucose plasma level is more than 11.1 mmol/L two hours after the standardized glucose load. Standardized glucose load means that the subject drinks 75 g of glucose dissolved in 3 dL of water in approximately 5 minutes (2).

DM II mainly affects people over 40 years old with an unhealthy lifestyle. By the year 2030, it is expected that more than 435 million people will suffer from DM II. Therefore, DM II represents a major public health problem (4).

2. Mechanisms of DM II

It is considered that DM II develops due to insufficient insulin secretion because of degenerative processes of pancreatic beta cells and reduced sensitivity of target tissues (i.e., liver, muscles, adipose tissue) for insulin (2,3). Genetic factors and familial predisposition also influence the development of this disease (1,3). DM II affects kidney function and causes diabetic kidney disease (DKD).

3. Complications of diabetes mellitus II

In the beginning, the clinical picture of DM II is quite silent, so it is mostly diagnosed when complications are already present. According to the time of occurrence, complications can be divided into acute and chronic. Chronic complications of DM II are a result of damage of small or large blood vessels (microvascular and macrovascular complications, respectively) (5,6). Different organ systems can be affected; microvascular complications manifest as DKD, diabetic neuropathy, diabetic retinopathy, and diabetic foot (1,4,5,7). Macrovascular complications (i.e., coronary artery disease, peripheral arterial disease) are not only specific to DM II, but also for other diseases (6).

4. Diabetic kidney disease

DKD often leads to an end-stage renal disease requiring dialysis or kidney transplantation. It is also associated with a high risk of cardiovascular disease (1,4,5).

The earliest sign of DKD is albuminuria, which progresses to proteinuria and at the end stage to complete renal failure. If diabetic retinopathy and albuminuria or proteinuria are present, the probability of DKD is high (7).

DKD manifests clinically when renal function is severely compromised. The diagnosis is primarily clinical. However, it can be proven by histopathological examination of the kidney tissue obtained by biopsy. The latter is required only exceptionally because DKD can be diagnosed based on the combination of clinical, laboratory, and imaging data (4).

Due to the significant healthcare burden of DM II and DKD, new non-invasive diagnostic tools are being developed. Extracellular vesicles (EV) are considered as potential tools in the diagnosis of these diseases (8).

5. Formation and composition of EV

EVs are membrane-surrounded sub-micron particles deriving from all types of cells (8–10). They can be isolated from bodily fluids of humans, animals, plants and cell cultures including cultures of microorganisms (9–11).

EVs are classified into apoptotic bodies, microvesicles and exosomes according to their formation, size and composition (**Table 1**) (9) and are additionally described in Ramos Juarez et al. (2020) (12).

Table 1: Classification of EVs

	Apoptotic bodies	Exosomes	Microvesicles
Origin	Decaying apoptotic cells	Endosome	Plasma membrane
Size	50 nm - 5000 nm	40 - 100 nm	100 - 1000 nm
Mechanism	Apoptosis	Exocytosis	Ectocytosis

EVs – extracellular vesicles

EVs detected in isolates from bodily fluids or tissues can be formed also due to mechanical, chemical and thermal stress in the process of sampling, isolation and characterization (13,14).

EV membrane is composed of a phospholipid bilayer which includes membrane proteins, glycoproteins and lipids and reflects the contents of the mother cell (15,16). Besides,

EV proteins can be tissue-specific (17). Nucleic acids in the form of different types of RNA and DNA are also present in EVs (9,12,17).

6. The role of extracellular vesicles in diagnostics and treatment

EVs may originate from healthy or damaged cells. They contain cell-specific origin molecules and act on neighboring, distant, and mother cells. Therefore they play an important role in regulating physiological or pathological processes. Isolation and characterization of some types of EVs could be useful in the identification of neurodegenerative, infectious and autoimmune diseases and tumors. Some EVs could be also used for treatment as vaccines against tumors or infectious diseases, as regenerative or immunosuppressive therapy or as vectors for medications (8).

The application of EVs as diagnostic biomarkers has been widely researched in various types of cancer. It was found that EVs that are formed by tumor cells carry tumour antigens (8,18–29). In tumor diagnosis, EVs were isolated from various bodily fluids, most commonly from urine and blood. In the tumor treatment, EVs research is at the stage of clinical trials (8). Furthermore, the use of EVs is already established in clinical practice for regenerative purposes, since the platelet-rich plasma contains high levels of EV and has shown beneficial regenerative effects (30–34)

7. The role of urinary EV in diagnostics of disease

Urinary EVs were first found in the isolates from the urine of a rabbit suffering from nephrotoxic nephritis (35,36). Since urine is obtained rapidly and noninvasively, urinary EVs have become interesting as potential diagnostic biomarkers. The use of urinary EVs in the diagnosis of various urinary tract diseases is shown in **Table 2**.

It was found that urinary EVs can be shed by cells composing any part of the urinary tract or can be transported to the urine from blood if the kidney function is impaired. In normal kidney function, the exosomes are bigger than the glomerular pore size which is approximately 4,5 nm. Therefore they cannot pass the glomerular membrane (43,44). However, the study results of the filtration of EVs from the blood through the glomerular basement membrane into the urine are yet indecisive (15,45,46). Moreover, EVs can be formed in the nephron, which is the main functional unit of the kidney (45). However, it was suggested that most EVs originate from basal epithelial cells of collecting duct (47). It was suggested that exosomes that are formed by renal tubular cells are secreted into the lumen of the nephron via the fusion of the multivesicular endosome with plasmalemma; therefore they contain plasma membrane proteins (21,48) and reflect cell metabolism and kidney function (45).

Table 2: Overview of some urinary exosomal biomarkers which could be used in the diagnosis of urinary tract diseases.

Disease	Biomarker	Reference
Acute renal failure	ATF-3	(37)
Ischemic-reperfusion renal failure of the kidney after transplantation	AQP-1	(38,39)
Glomerular diseases		
Systemic lupus erythematosus	miRNA-26a, ADAM 10	(21,40)
Focal segmental glomerulosclerosis	WT-1, podocalyxin	(21,37)
IgA nephropathy	Aminopeptidase N, α1 antitrypsin, ceruloplasmin	(21,40,41)
Tubular diseases		
Autosomal dominant polycystic kidney disease	AQP-2, APO-A1, ANXA1 in ANXA2	(21,42)
Prostate cancer	catenin-d, integrin-a, integrin-b, PCA3	(18–20)
Renal cell carcinoma	ceruloplazmin, MMP-9, PODXL, carbonic anhydrase IX	(21,22)
Bladder cancer	LASS2, GALNT1, EDIL-3, MUC4, GTPase	(21,23–25)

Abbreviations: ATF-3: activating transcription factor 3; AQP-1: aquaporin; ADAM10: Disintegrin and metalloproteinase domain containing protein 10; WT-1: Wilms' tumour gene; AQP-2: aquaporin 2; APO-A1: apolipoprotein A1; ANXA1 and ANXA2: annexin A1 and A2; PCA3: prostate cancer antigen 3; MMP-9: metalloproteinase 9 matrix; PODXL: podocalyxin; LASS2: ceramide synthase 2; GALNT1: N-acetylgalactosaminyltransferase 1 polypeptide; EDIL-3: epidermal growth factor-like sequences and discoidin-like domain containing protein 3; MUC4: mucin 4

Due to the relationship between exosome size and glomerular basement membrane properties, it is believed that the majority of EVs found in isolates are exosomes (15,46). However, EVs detected in human urine do not originate only from the human; they can be shed from bacteria present in the genitourinary tract. Therefore, the applicability of urinary EV is promising also in the diagnosis of urinary tract infections (15).

8. Exosomal biomarkers in diabetic kidney disease

Proteins from urinary exosomes could serve as potential diagnostic biomarkers to detect DKD in early stages, to track DKD's progression and the success of treatment. However, many exosomal proteins are not DKD-specific (45). Besides, different exosome isolation methods can lead to different results; Gudehithlu et al. (2015) presented evidence in favor of the hypothesis that urinary exosomes in DKD represent changes in renal tissue and disease progression more accurately than other urinary markers. Isolation of urinary exosomes was performed by differential centrifugation, which also included ultracentrifugation. Gelatinase activity was measured with fluorescent gelatin and ceruloplasmin concentration by the enzyme-linked immunosorbent assay (ELISA). Decreased gelatinase activity (MMP-2 and MMP-9) and increased urinary exosomal ceruloplasmin activity were found in patients with DKD. Increased cerupoplasmin concentration was considered a sign of inflammatory response since oxidoreductase properties of ceruloplasmin are expected to protect tissues from oxidative stress (49). Elevated concentrations of ceruloplasmin-rich urinary exosomes may occur before microalbuminuria in DKD (45).

Glomerular and tubulointerstitial damage is noticed with the progression of the DKD also. That is why exosomes formed from tubular cells are a potential diagnostic biomarker in DKD (50). Rossi et al. (2017) found that concentrations of exosomal aquaporins (AQP2 and AQP5) are elevated in DKD compared to patients without DKD (50).

Zubiri et al. (2014) developed a protocol to determine the urinary EV proteome in patients with DKD (51). The goal was to detect an exosomal diagnostic biomarker. Despite high albumin concentration in the urinary samples, 25 DKD-specific proteins were found, such as α-microglobulin/bikunin precursor (AMBP), histone-lysine N-methyltransferase (MLL3), and voltage-dependent anion-selective channel protein 1 (VDAC1) (51). MLL3 has so far been detected only in the urinary exosomes of diabetic patients (45,51).

Sun et al. (2012) investigated association between dipeptidyl peptidase-IV (DPP IV) and DKD. They found that DPP IV in EVs isolated from urine positively correlated with proteinuria in DKD (52).

References

1. World Health Organization. Definition, diagnosis and classification of diabetes mellitus and its complications : report of a WHO consultation. Part 1, Diagnosis and classification of diabetes mellitus. 1999;(WHO/NCD/NCS/99.2). Available from: https://apps.who.int/iris/handle/10665/66040

2. Ribarič S. Mehanizmi nastanka in zapletov sladkorne bolezni. In: Temelji patološke fiziologije. 2nd ed. Medicinska fakulteta, Inštitut za fiziologijo, Ljubljana; 2011. p. 86–91.

3. Košnik M, Mrevlje F, Štajer D, Koželj M, Černelč P. Bolezni presnove. In: Interna medicina. 4th ed. Ljubljana: Slovensko medicinsko društvo; 2011. p. 769–805.

4. Košnik M, Štajer D. Interna medicina. 5th ed. Medicinska fakulteta, Slovensko zdravniško društvo; 2018. 861–946 p.

5. Forbes JM, Cooper ME. Mechanisms of Diabetic Complications. Physiol Rev. 2013 Jan 1;93(1):137–88.

6. Pongrac Barlovič D, Ravnik Oblak M, Urbančič Rovan V, Zaletel J, Simona F. Sladkorna bolezen. In: Košnik M, editor. Interna medicina. 5th ed. Ljubljana: Slovensko zdravniško društvo in Medicinska fakulteta Ljubljana; 2018. p. 868–87.

7. Lindič J. Sladkorna bolezen in ledvice. In: Bolezni ledvic. 3rd ed. Ljubljana: Slovensko zdravniško združtvo-Slovensko nefrološko društvo in Univerziteetni klinični center Ljubljana-Klinični oddelek za nefrologijo, Interna klinika; 2014. p. 285–90.

8. Fais S, O'Driscoll L, Borras FE, Buzas E, Camussi G, Cappello F, et al. Evidence-Based Clinical Use of Nanoscale Extracellular Vesicles in Nanomedicine. ACS Nano. 2016 26;10(4):3886–99.

9. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol. 2018 Jan 17;19(4):213–28.

10. Ogorevc E, Kralj-Iglič V, Veranič P. The role of extracellular vesicles in phenotypic cancer transformation. Radiol Oncol. 2013 Sep 1;47(3):197–205.

11. Doyle LM, Wang MZ. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. Cells. 2019 Jul 15;8(7):727.

12. Ramos Juarez AP, Ramila M, Giovambattista C, Trepiccione F, Pocsfalvi G. Extracellular vesicles in biomedicine: from biomarkers to tissue regeneration. In: Peer reviewed

proceedings. Ljubljana: Faculty of Health Sciences, University of Ljubljana, Slovenia; 2020.

13. Šuštar V, Bedina-Zavec A, Štukelj R, Frank M, Ogorevc E, Janša R, et al. Post - prandial rise of microvesicles in peripheral blood of healthy human donors. Lipids Health Dis. 2011 Mar 21;10:47.

14. Štukelj R, Ignaščenko IH, Peternelj S, Peruško M, Blažič T, Pajnič M, et al. Role of Blood Sampling in Assessment of Concentration of Extracellular Nanovesicles in Isolates from Peripheral Blood. In: Iglič A, Kulkarni CV, editors. Advances in Planar Lipid Bilayers and Liposomes [Internet]. Elsevier; 2014 [cited 2019 Jan 2]. p. 175–89. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780124186996000072

15. Barreiro K, Holthofer H. Urinary extracellular vesicles. A promising shortcut to novel biomarker discoveries. Cell Tissue Res. 2017;369(1):217–27.

16. Kim D-K, Lee J, Kim SR, Choi D-S, Yoon YJ, Kim JH, et al. EVpedia: a community web portal for extracellular vesicles research. Bioinformatics. 2015 Mar 15;31(6):933–9.

17. Driedonks TAP, Nolte-'t Hoen ENM. Circulating Y-RNAs in Extracellular Vesicles and Ribonucleoprotein Complexes; Implications for the Immune System. Front Immunol [Internet]. 2019 [cited 2019 Jun 16];9. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03164/full

18. Lu Q, Zhang J, Allison R, Gay H, Yang W-X, Bhowmick NA, et al. Identification of extracellular delta-catenin accumulation for prostate cancer detection. The Prostate. 2009 Mar 1;69(4):411–8.

19. Nawaz M, Camussi G, Valadi H, Nazarenko I, Ekström K, Wang X, et al. The emerging role of extracellular vesicles as biomarkers for urogenital cancers. Nat Rev Urol. 2014 Dec;11(12):688–701.

20. Bijnsdorp IV, Geldof AA, Lavaei M, Piersma SR, van Moorselaar RJA, Jimenez CR. Exosomal ITGA3 interferes with non-cancerous prostate cell functions and is increased in urine exosomes of metastatic prostate cancer patients. J Extracell Vesicles. 2013;2.

21. De Palma G, Sallustio F, Schena FP. Clinical Application of Human Urinary Extracellular Vesicles in Kidney and Urologic Diseases. Int J Mol Sci [Internet]. 2016 Jun 30 [cited 2019 Jun 16];17(7). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4964419/

22. Raimondo F, Morosi L, Corbetta S, Chinello C, Brambilla P, Della Mina P, et al. Differential protein profiling of renal cell carcinoma urinary exosomes. Mol Biosyst. 2013 Jun;9(6):1220–33.

23. Perez A, Loizaga A, Arceo R, Lacasa I, Rabade A, Zorroza K, et al. A Pilot Study on the Potential of RNA-Associated to Urinary Vesicles as a Suitable Non-Invasive Source for Diagnostic Purposes in Bladder Cancer. Cancers. 2014 Jan 22;6(1):179–92.

24. Beckham CJ, Olsen J, Yin P-N, Wu C-H, Ting H-J, Hagen FK, et al. Bladder cancer exosomes contain EDIL-3/Del1 and facilitate cancer progression. J Urol. 2014 Aug;192(2):583–92.

25. Smalley DM, Sheman NE, Nelson K, Theodorescu D. Isolation and identification of potential urinary microparticle biomarkers of bladder cancer. J Proteome Res. 2008 May;7(5):2088–96.

26. Skog J, Wurdinger T, van Rijn S, Meijer D, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and protein that promote tumor growth and provide diagnostic biomarkers. Nat Cell Biol. 2008 Dec;10(12):1470–6.

27. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol. 2008 Jul;110(1):13–21.

28. Ogata-Kawata H, Izumiya M, Kurioka D, Honma Y, Yamada Y, Furuta K, et al. Circulating exosomal microRNAs as biomarkers of colon cancer. PloS One. 2014;9(4):e92921.

29. Baran J, Baj-Krzyworzeka M, Weglarczyk K, Szatanek R, Zembala M, Barbasz J, et al. Circulating tumour-derived microvesicles in plasma of gastric cancer patients. Cancer Immunol Immunother CII. 2010 Jun;59(6):841–50.

30. Uršič B, Vozel D, Šuštar V, Kocjančič B, Dolinar D, Kralj-Iglič V. Extracellular Vesicles from Platelet-Rich Plasma as Conveyors of Regeneration Potential in Orthopedics. J Hematol Thromboembolic Dis [Internet]. 2014 [cited 2019 Jan 2];2(5). Available from: http://www.esciencecentral.org/journals/extracellular-vesicles-from-plateletrich-plasma-as-conveyors-of-regeneration-potential-in-orthopedics-2329-8790.1000163.php?aid=31838

31. Vozel D, Uršič B, Krek JL, Štukelj R, Kralj-Iglič V. Applicability of extracellular vesicles in clinical studies. Eur J Clin Invest. 2017 Apr;47(4):305–13.

32. Vozel D, Božič D, Jeran M, Jan Z, Pajnič M, Pađen L, et al. Treatment with platelet- and extracellular vesicle-rich plasma in otorhinolaryngology-a review and future perspectives. In: Pocsfalvi G, Bongiovanni A, Manno M, Kralj-Iglič V, editors. ABLSA 32: Biological Membrane Vesicles: Scientific, Biotechnological and Clinical Considerations. Elsevier; In press.

33. Tao S-C, Guo S-C, Zhang C-Q. Platelet-derived Extracellular Vesicles: An Emerging Therapeutic Approach. Int J Biol Sci. 2017;13(7):828–34.

34. Vozel D, Božič D, Jeran M, Jan Z, Pajnič M, Pađen L, et al. The role of platelet- and extracellular vesicle-rich plasma in the treatment of chronic postoperative temporal bone cavity inflammation: a randomized controlled clinical trial. In: Peer reviewed proceedings. Ljubljana: Faculty of Health Sciences, University of Ljubljana, Slovenia; 2020.

35. Musante L, Tataruch DE, Holthofer H. Use and Isolation of Urinary Exosomes as Biomarkers for Diabetic Nephropathy. Front Endocrinol [Internet]. 2014 Sep 26 [cited 2019 Jun 16];5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4176463/

36. Wiggins R, Glatfelter A, Kshirsagar B, Beals T. Lipid microvesicles and their association with procoagulant activity in urine and glomeruli of rabbits with nephrotoxic nephritis. Lab Investig J Tech Methods Pathol. 1987 Mar;56(3):264–72.

37. Zhou H, Kajiyama H, Tsuji T, Hu X, Leelahavanichkul A, Vento S, et al. Urinary exosomal Wilms' tumor-1 as a potential biomarker for podocyte injury. Am J Physiol Renal Physiol. 2013 Aug 15;305(4):F553-559.

38. Bedford JJ, Leader JP, Walker RJ. Aquaporin expression in normal human kidney and in renal disease. J Am Soc Nephrol JASN. 2003 Oct;14(10):2581–7.

39. Sonoda H, Yokota-Ikeda N, Oshikawa S, Kanno Y, Yoshinaga K, Uchida K, et al. Decreased abundance of urinary exosomal aquaporin-1 in renal ischemia-reperfusion injury. Am J Physiol Renal Physiol. 2009 Oct;297(4):F1006-1016.

40. Gutwein P, Schramme A, Abdel-Bakky MS, Doberstein K, Hauser IA, Ludwig A, et al. ADAM10 is expressed in human podocytes and found in urinary vesicles of patients with glomerular kidney diseases. J Biomed Sci. 2010 Jan 13;17(1):3.

41. Moon P-G, Lee J-E, You S, Kim T-K, Cho J-H, Kim I-S, et al. Proteomic analysis of urinary exosomes from patients of early IgA nephropathy and thin basement membrane nephropathy. Proteomics. 2011 Jun;11(12):2459–75.

42. Pocsfalvi G, Raj DAA, Fiume I, Vilasi A, Trepiccione F, Capasso G. Urinary extracellular vesicles as reservoirs of altered proteins during the pathogenesis of polycystic kidney disease. Proteomics Clin Appl. 2015 Jun;9(5–6):552–67.

43. Cheng Y, Wang X, Yang J, Duan X, Yao Y, Shi X, et al. A translational study of urine miRNAs in acute myocardial infarction. J Mol Cell Cardiol. 2012 Nov;53(5):668–76.

44. Wiwanitkit V. Glomerular Pore Size Corresponding to Albumin Molecular Size, an Explanation for Underlying Structural Pathology Leading to Albuminuria at Nanolevel. Ren Fail. 2006 Jan 1;28(1):101–101.

45. Xu W-C, Qian G, Liu A-Q, Li Y-Q, Zou H-Q. Urinary Extracellular Vesicle: A Potential Source of Early Diagnostic and Therapeutic Biomarker in Diabetic Kidney Disease. Chin Med J (Engl). 2018 Jun 5;131(11):1357–64.

46. Pisitkun T, Shen R-F, Knepper MA. Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci U S A. 2004 Sep 7;101(36):13368–73.

47. Fraser KB, Moehle MS, Daher JPL, Webber PJ, Williams JY, Stewart CA, et al. LRRK2 secretion in exosomes is regulated by 14-3-3. Hum Mol Genet. 2013 Dec 15;22(24):4988–5000.

48. Chen C-L, Lai Y-F, Tang P, Chien K-Y, Yu J-S, Tsai C-H, et al. Comparative and targeted proteomic analyses of urinary microparticles from bladder cancer and hernia patients. J Proteome Res. 2012 Dec 7;11(12):5611–29.

49. Gudehithlu KP, Garcia-Gomez I, Vernik J, Brecklin C, Kraus M, Cimbaluk DJ, et al. In Diabetic Kidney Disease Urinary Exosomes Better Represent Kidney Specific Protein Alterations Than Whole Urine. Am J Nephrol. 2015;42(6):418–24.

50. Rossi L, Nicoletti MC, Carmosino M, Mastrofrancesco L, Di Franco A, Indrio F, et al. Urinary Excretion of Kidney Aquaporins as Possible Diagnostic Biomarker of Diabetic Nephropathy [Internet]. Journal of Diabetes Research. 2017 [cited 2019 Jun 14]. Available from: https://www.hindawi.com/journals/jdr/2017/4360357/

51. Zubiri I, Posada-Ayala M, Sanz-Maroto A, Calvo E, Martin-Lorenzo M, Gonzalez-Calero L, et al. Diabetic nephropathy induces changes in the proteome of human urinary exosomes as revealed by label-free comparative analysis. J Proteomics. 2014 Jan 16;96:92–102.

52. Sun A, Deng J, Guan G, Chen S, Liu Y, Cheng J, et al. Dipeptidyl peptidase-IV is a potential molecular biomarker in diabetic kidney disease. Diab Vasc Dis Res. 2012 Oct;9(4):301–8.

Review

PHYSICAL THERAPY AND ERGONOMIC ASSESSMENT OF WORK-RELATED DISORDERS

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Abstract

For at least 70 years, records testify about ergonomics and human factors. During the World War II, there was a need to deal with the fatigue of the employees and soldiers, stress, injury, poor performance due to mismatch between manpower and technology, unusual work schedules, and demanding and threatening work conditions. Over this period, ergonomics researchers and practiotioners addressed a broad diversity of the problems. Focused solutions and proper managemnet of specific problems (microergonomics) were sought and the limitations of the entire work progress (macroergonomics) was dealt with. Microergonomics factors deal with designing the proper characteristics of the tasks, tools, technology, environmental conditions, and capacity or knowledge of each individual or of groups of employees, to accomplish work effectively and safely. Microergonomics adresses the specific ergonomics risk factors in a particular task, job, or operation. Macroergonomics factors deal with broader issues such as the organization of the work pocess, the coordination of tasks and activities among employees and groups, the supervision of processes and employees and integration of manpower, technology, tasks, and environmental features. Improvements in ergonomics and the corresponding solutions must account for all levels in order for their effects to be successful (1). Ergonomic assessment is usually evaluated by analysing body posture, movement, recurring and forceful activities and maximum force, or by an increase of the muscle load over time (2). Specifically, in physical therapy, the pain and movement limitations are frequently related with numerous musculoskeletal problems or work-related musculoskeletal disorders (MSDs).

1. Environment - human interaction

The main importance of prevention in health maintenance is the capability to recognize various environmental and personal factors that interact and produce situations leading to injury risk. To indentify the interaction between human and environmental risk factors to ergonomic injuries, holistic and integrated approach is needed. Researchers presented the »balance model« as a holistic model of workplace and human interaction (3-7). Each element of this model can produce demands or loads on the workers while the combination of different effects can produce even greater demands and consequently induce stress or strain potentially leading to injury. Additionally, each of the exposed elements of the »balance model« such as task, technology, environment, work organization, load, personal capacity, strain and disorder, can be improved to reduce the risk of injury (6).

2. Psychological, physiological and biomechanical strain

The environment comprise the physical and social attitudinal environment in which people live and function. Environmental factors include closer personal factors such as the physical neighborhood in which the person lives, attitudes of coworkers, physical structure of the neighborhood and broader societal attitudes towards people with differing abilities. The role of psychosocial work stress was already demonstrated as a potential risk factor for numerous chronic diseases (8). A wide range of individual attributes that can affect injury risk include perceptual/motor abilities, physical capabilities, capacity such as strength and endurance, body mass index, current health status, susceptibilities to disease, personality, intelligence, heredity, and behavior (8). There are numerous environmental characteristics including technology and material used by the individuals that could influence the injury risk in every working or relaxing environment. The demands for a work activity and the way in wich tasks are conducted can influence strain in individuals and subsequent injury. Work task demands can be separated into physical requirements, mental requirements, and psychological considerations. Furthermore, the environment exposes individual to climate changes, different materials and chemical, and physical agents that can cause harm or injury if the exposure exceeds safe limits (7).

Work-related upper limb and neck musculoskeletal disorders (MSDs) are one of the most common occupational disorders worldwide affecting associated direct and indirect costs (9). Work-Related musculoskeletal disorders (MSDs) are among the most widely spread occupational problems in both, developed and developing countries, in industries and services (6). They cause increasing expenses of salary compensation and health costs, declining productivity and lower quality of life. Ergonomic assessment of MSDs involves the evaluation of risk for developing a range of disorders to muscles, nerves and joints, primarily of the upper limb and low back, associated with occupational tasks (2).

3. Physical therapy and ergonomic assessment of work-related MSDs

The Federation of State Boards of Physical Therapy defines physical therapy practice as examining, evaluating and testing the individuals with mechanical, physiological, and developmental impairments, activity limitations and participation restrictions or other health and movement-related conditions (10). Physical therapy aims to determine diagnosis, prognosis, and plan of treatment and intevention and to assess the on-going effects of intervention. Furthermore, it is directed towards alleviating activity limitations, reducing the risk of injury, reducing impairment, reducing activity limitations, and allowing participation. It includes promotion and maintenance of fitness, health, and wellness in populations of all ages and at all levels of work. It is implemented as a practice, an administration, consultations, education and research. Physical therapists examine, evaluate, diagnose, and intervene at the level of activity limitation. Furthermore, we agree that physical therapists can use the their knowledge and clinical skills to prevent, reduce, or eliminate impairments and injuries and to enable the individuals to achive the most optimal quality of life possible. Impairments are problems in body function or structures that are deviations from generally accepted population standards, defined as a loss or deficiency, or other abnormality.

Physiological impairment is an alteration in any physiological function such as aerobic capacity, muscle performance (strength, power, endurance), joint mobility (hypomobility/hypermobility), balance, posture, motor function or mental function. Physical therapist intervention practice can most significantly modify physiological impairments (10). Structural impairment is an abnormality or loss of structure such as a hip anteversion, structural subtalar varus, structural genu varum, or congenital or traumatic loss of a limb. However, environmental factors impacting functioning and disability include products and technology, natural environment and human-made changes to environment, support and relationship, attitudes, services, systems, and policies (10). We support the idea that these factors can help the person to be resilent. However, they can also create psychological, physiological and biomechanical barriers.

Physical therapists dealing with pain and movement limitations are familiar with numerous musculoskeletal problems. The first evaluation procedure in physical therapy includes observation of the individual. Then, the anamnesis is recorded and physical testing of the capabilities and performance are made. The physical therapy evaluation procesure includes static and functional postural analysis. Physical load of work is usually evaluated by analyzing body posture, movement, recurring and forceful activities and maximum force, or increasing muscle load over time. Observational and instrument-based techniques are proposed in research to provide a quantitative measure for the degree of discomfort and postural strain caused by different body positions. Rapid Entire Body Assessment (REBA) is one of the observational postural analysis – based ergonomic assessment tools (11).

The observational techniques may include Ovako Working Posture Assessment System (OWAS), Posture, Activity, Tools and Handling (PATH), Quick Exposure Check (QEC), Rapid Upper Limb Assessment (RULA), American Conference of Governmental Industrial Hygienists Threshold Limit Value (ACGHI TLV), Strain Index (SI), Occupational Repetitive Actions (OCRA), NIOSH Lifting Equation. REBA provides a quick and easy measure to assess a variety of working postures for risk of work-related MSDs. It divides the body into sections to be coded independently, according to movement planes. It offers a scoring system for muscle activity throughout the entire body. The presented REBA tool (**Figure 1**) also gives an action level with a sign of importance and requires minor equipment such as a »pen and paper method« (11).

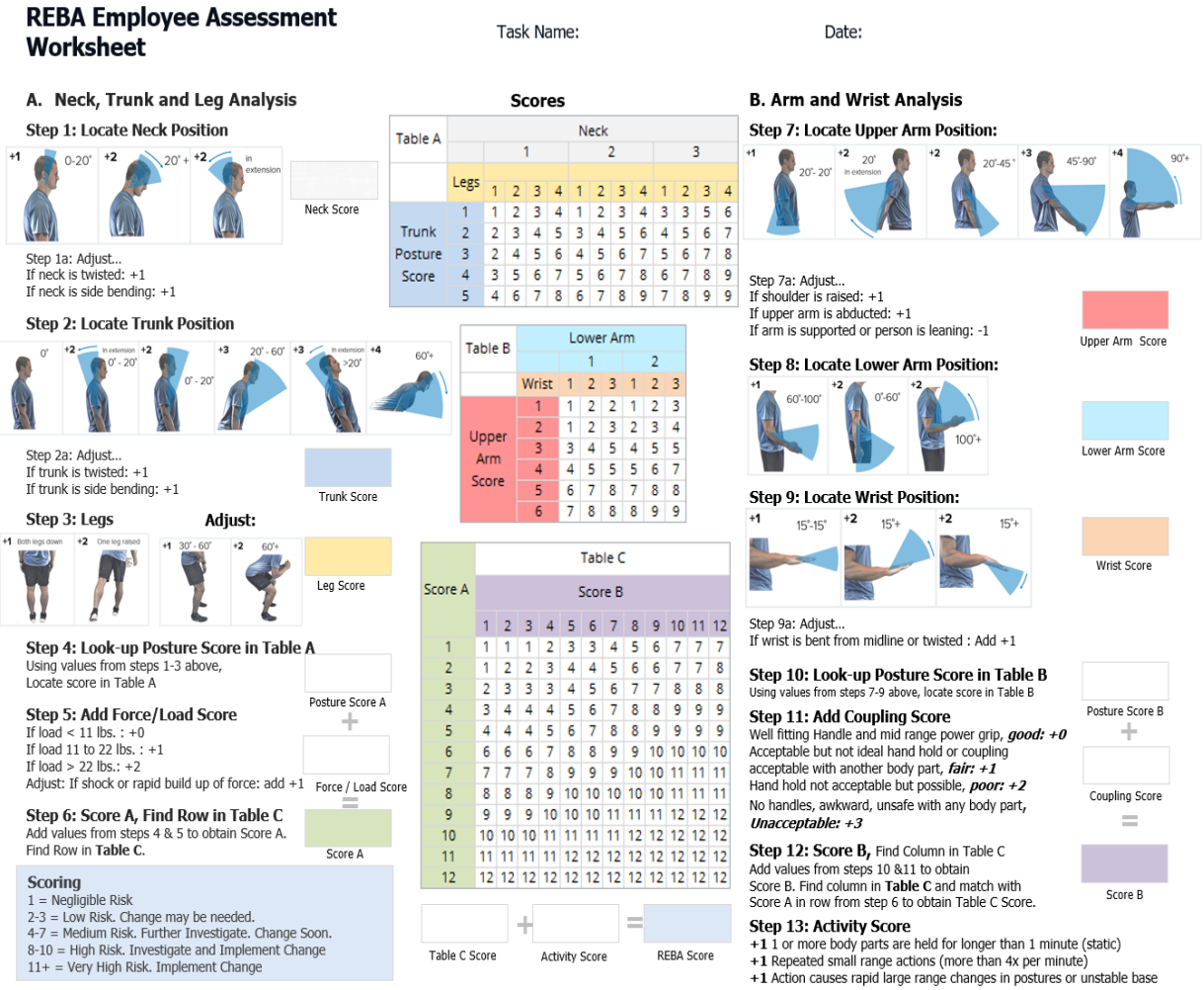

Figure 1. An ergonomic assessment tool Rapid Entire Body Assessment (REBA) to observe postural analysis (from (11) Hignett and McAtamney, 2000, original worksheet Developed by Dr. Alan Hedge).

4. General approach to an ergonomic program

The observational techniques of ergonomic assessment used in industry for their noninterference with the work performed should follow simplicity of use and low price (12, 13). The pressented assessment tool such as REBA is one of the widely used observational ergonomic assessment tools in various industries and services. Based on a review of different observational techniques, it is shown that the purpose of their development are various uses and therefore they are applied in a multiple workplace circumstances. Each technique has its own posture classification application, which is different from other techniques, so different positional load rates can be assigned for a given posture, based on the technique used.

On the other hand, there are lots of studies that evaluate many techniques with regards to their performance and dependability (12). We strongly agree with (2) that additional work is needed to support the REBA as a tool to assess work related musculoskeletal disorders risks. Furthermore, we suggest that the future programs of risk prevention and reduction should based on detailed physiological and ergonomical evaluations. The specific indivuduals capabilities and abilities as well as the exposure of environmental factors may identify important risk factors of specific work or repeated task. Namely, physical therapists usually treat pain and movement restrain as a result of musculoskeletan problems, which may also be associated with working overload. Additionaly, physical therapists as a health workers contribute to the assessment of an individual's physical abilities and workload therefore there is a specific need for accurate knowledge of ergonomic assessment of work-related disorders.

Conclusions

To conclude, through the the view of physical therapy and environmental physiology, we suggest the evaluation of absolute and relative ergonomic load of the individuals in specific living, working and relaxing environment.

References

1. Smith J M, Human factors and ergonomics in health care and patient safety. Edited by Pascale Carayon. 2nd ed. 2017, 597-610.

2. Madani AD, Dababneh A, Rapid Entire Body Assessment: Literature review. American Journal of Engineering and Applied Sciences 2016, 9 (1): 107-118.

3. Smith MJ, Sainfort PC, A balance theory of job design for stress reduction. International Journal of industrial Ergonomics 1989, 4, 67-79.

4. Smith and Carayon, New technology, automation and work organization: Stress problems and improved technology implementation strategies. International Journal of Human Factors in Manucaturing 1995: 5, 99-116.

5. Carayon P, Smith MJ, Haims M, Psychosocial aspects of work-related musculoskeletal disorders. Human factors 1999, 41 (6), 644-663.

6. Smith MJ, Karsh BT, Moro FB, A review of research on interventions to control musculoskeletal disorders. In Work-Related Musculoskeletal Disorders. Washington, DC: National Research Council 1999, pp.200-229.

7. Carayon P, Smith MJ, Work organization and ergonomics. Applied Ergonomics 2000, 31, 649-662.

8. Kivimäki M, Kawachi I, Work Stress as a Risk Factor for Cardiovascular Disease. Curr Cardiol Rep 2015, 17(9): 630.

9. Hoe CW V, Urquhart M D, Kelsall L H, Zamri N E, Malcolm R S. Ergonomic Interventions for Preventing Work-Related Musculoskeletal Disorders of the Upper Limb and Neck Among Office Workers. Cochrane Database of Systematic Reviews 2018, 23;10: CD008570.

10. Brody LT, and Hall CM, Therapeutic Exercise. Moving toward function 2018, pp.: 2-37.

11. Hignett and McAtamney, Applied Ergonomics, 2000, 31:201-205.

12. Kee D, Karwowski W, A comparison of three observational techniques for assessing postural loads in industry. International journal of occupational safety and ergonomics 2007, 13:1, 3-14.

13. Janowitz I, Gillen M, Ryan G, Rempel D, Trupin L et al. Measuring the physical demands of work in hospital settings: Design and implementation of an ergonomics assessment. Applied Ergonomics 2006, 37: 641-658.

Review

TECHNIQUES OF TYMPANOPLASTY IN OTORHINOLARYNGOLOGY

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Abstract

Tympanoplasty is an operation on the tympanic membrane and in the middle ear. By removing the diseased tissue, restoring the ossicles and closing the tympanic membrane perforation it is aimed at establishing a functioning middle ear that ensures good transmission and adequate amplification of sound. Here we describe the historical development of tympanoplasty, its surgical technique and classifications and categorizations of tympanoplasties.

1. Introduction

The function of the outer ear is to transmit sound to the middle ear. This is achieved by anatomy of the external, middle and internal ear (**Figure 1**). The normal transmission of sound can be worsened by an altered anatomy, which may be congenital or the result of injury, tumours or severe inflammation. The term "-plasty" in the medical sense describes the surgery which is aimed at restoring the original functional state of the organ. In the case of tympanoplasty, the relevant organs are the outer and the middle ear.

Besides transmittance of sound, the roles of the middle ear are also sound amplification and inner ear protection. Tympanic membrane perforations or damaged ossicles cannot provide sound amplification, and cause conductive hearing loss, named chronic otitis media. In the treatment of conductive hearing loss, a functioning middle ear that ensures good transmission and adequate sound amplification needs to be established. With different types of tympanoplasties, the diseased tissues are removed, the ossicles are restored and the perforation in the eardrum is closed to enable the function of the middle ear (2).

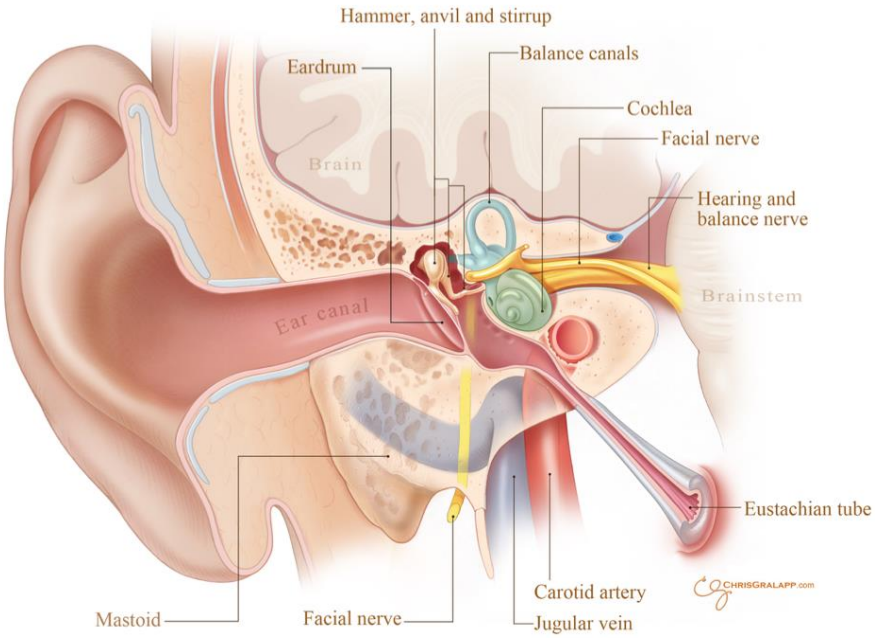

Figure 1. Anatomy of the ear: external ear (auricle, external ear canal), middle ear (tympanic cavity, tympanic membrane, ossicles) and inner ear (balance canals, cochlea). Ossicles: Hammer (lat. Malleus), Anvil (Incus) and Stirrup (Stapes). From (1).

Myringoplasty is a surgical procedure which is confined to the eardrum alone, without manipulation of the ossicles or middle ear. When manipulation of the tympanic membrane and eradication of disease from the middle ear is done, then we call this procedure a tympanoplasty. If it is done in conjunction with manipulation of the ossicles it is called ossiculoplasty. Surgery that includes manipulation of mastoid is known as mastoidectomy (3).

2. History of tympanoplasties

The development of tympanoplasty is one of the most interesting of all surgical procedures in otology . The first reported attempt to repair a perforated tympanic membrane was described in 1640, when M. Banzer used pig's bladder which he stretched across an ivory tube and placed in the ear and obtained temporary hearing improvement (4).

The first true tympanoplasty was reported in 1878, by E. Berthold (3). With emergence of antimicrobials, incorporation of operating microscopes and the newly designed microscopic instruments in the 1950s, the revolutionary change of tympanoplasty began. The article "Tympanoplasty as an operation to improve hearing in chronic otitis media and its results" by H. Wullstein (5) described the operation with a goal to improve hearing and protect the middle ear from the outside environment.

In the beginning, the overlay technique was used in all tympanoplasty surgeries. Split thickness and full thickness skin autologous grafts were used, however, poor long-term outcomes with adverse effects like graft eczema and desquamation often resulted. Thus surgeons searched for alternative grafting materials (6). In 1959, the use of vein graft showed satisfactory repair however, graft atrophy and re-perforation over a period of few months appeared (7). In 1961, the use of fascia in grafting was discovered which solved most of the problems faced with free skin grafts (8). Removing all components of the skin as an intact unit is defined as a full-thickness skin graft, and a piece of skin cut at a thickness varying between 0.196 mm and 0.441 mm is considered to be a partial- or a split-thickness skin graft (9).

In 1964, the first tympanoplasty using homograft tympanic membrane was reported in the USA and promising results were obtained by many authors (10). Sterilization and acquisition of homografts was a disadvantage of this technique. Different homografts have been used including loose areolar tissue, perichondrium, cartilage fat, periosteum and fascia. The internal structure of mucopolysaccharides and collagen most probably accounts for its high success rate of fascia: collagen provides the tensile strength to the wound area and the complex mucopolysaccharides play a role in the healing process by attracting fibroblasts into the wound area by chemotaxis (11).

The tympanoplasty surgical procedure has improved dramatically over the years. Various techniques have been attempted in order to achieve better results with improved hearing (3).

The first ossiculoplasty was reported by A. Hall and C. Ryztner in 1957; they used autograft ossicles (12). W. House et al. in 1966 introduced ossicular repair with homograft ossicles by sculpting the ossicles and fixing them (13). In the late 1970s, a high-density polyethylene sponge (HDPS) that had nonreactive properties was developed. In 1972, R. Wehrs used homograft ossicles (14). In order to reduce preparation time and obviate concern about the disease transmission later, he designed hydroxyapatite prosthesis in 1989 (14). He concluded that this prosthesis performed well and fulfilled its function of emulating the homograft ossicles (13).

3. Surgical technique and classification

Depending on the extent of the middle ear damage, several types of tympanoplasty can be distinguished. Lower numerical mark means less extensive surgical procedure, and better functional outcome and higher expectation that postoperative hearing can be achieved.
The types of tympanoplasty are depicted in Figure 2: Type I – only tympanic membrane closure without operating on the rest of the middle ear; Type II – tympanic membrane closure with restoration of the partially damaged ossicles which do not require surgical correction; Type III – tympanic membrane closure and restoration of the broken ossicular chain; Type IV and V – the difference in exposure to sound waves between the oval and round window is attempted and at the same time the middle ear is closed (15).

For type III, IV and V tympanoplasties re-shaped patient's auditory bones (homografts) or artificial implant prostheses, which replace the ossicles can be used (15). Artificial prostheses are made of different materials - ceramic, fluoroplastic, plastic, cement, and from titanium (15). In recent decades, the use of auditory ossicles of dead donors (i.e. allografts) has been abandoned (16).

Over the years, many classifications and categorizations of the middle ear and mastoid surgeries have been proposed. For instance, the one presented by H. Wullstein in 1956 on tympanoplasty is well known and still used today (15). With the goal to unify diverse classification of otologic surgeries, International Otology Outcome Group created a new international classification system which is expected to facilitate international collaboration that will lead to the development of high level evidence in otologic surgery (17).

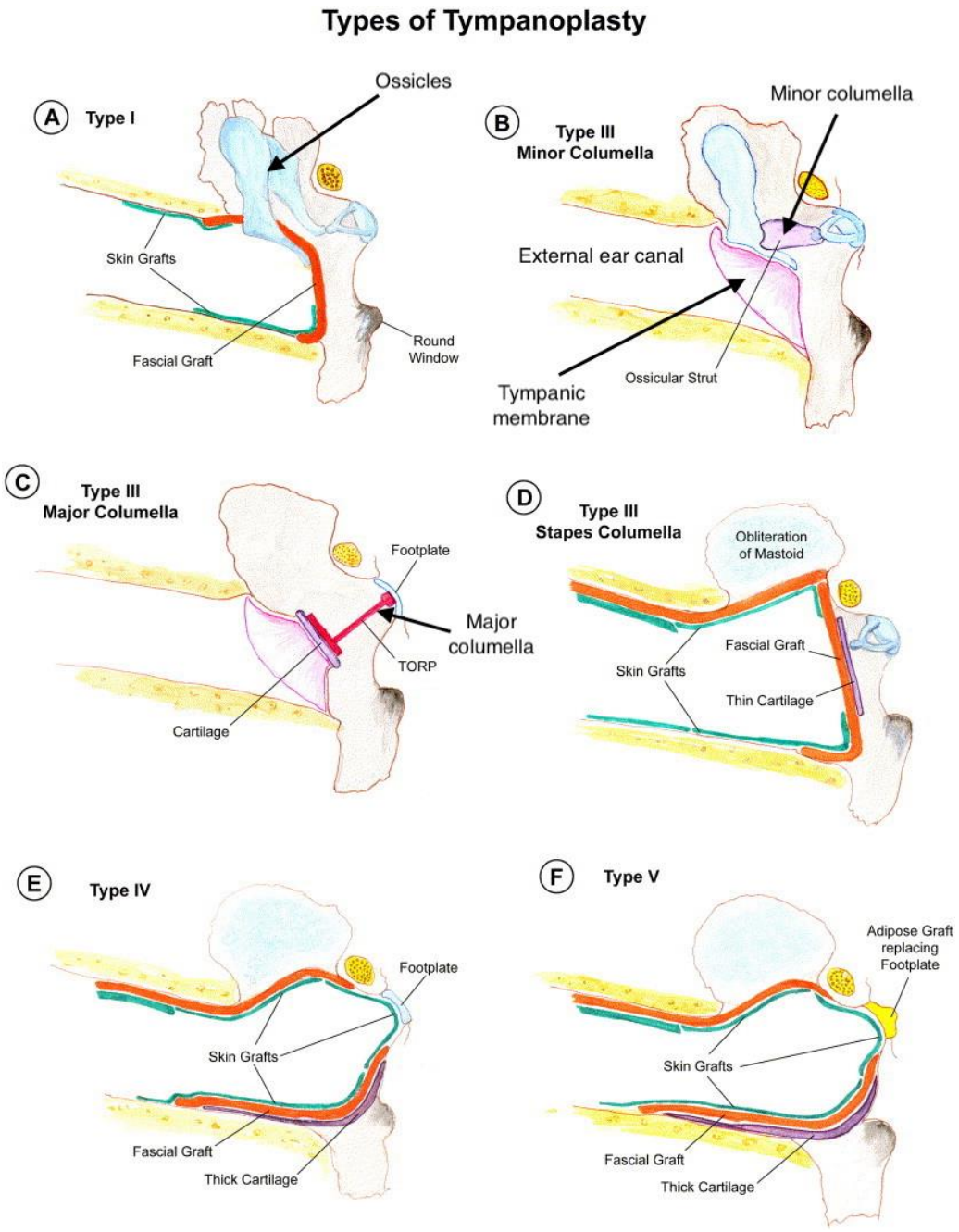

Figure 2. Wullstein classification of tympanoplasties. B and C are combined with ossiculoplasty. Orange-fascial graft, blue-ossicles, green-skin graft, yellow-bone. Minor columella-graft between malleus and stapes suprastructure, major columella-graft between tympanic membrane and stapes footplate. From (15).

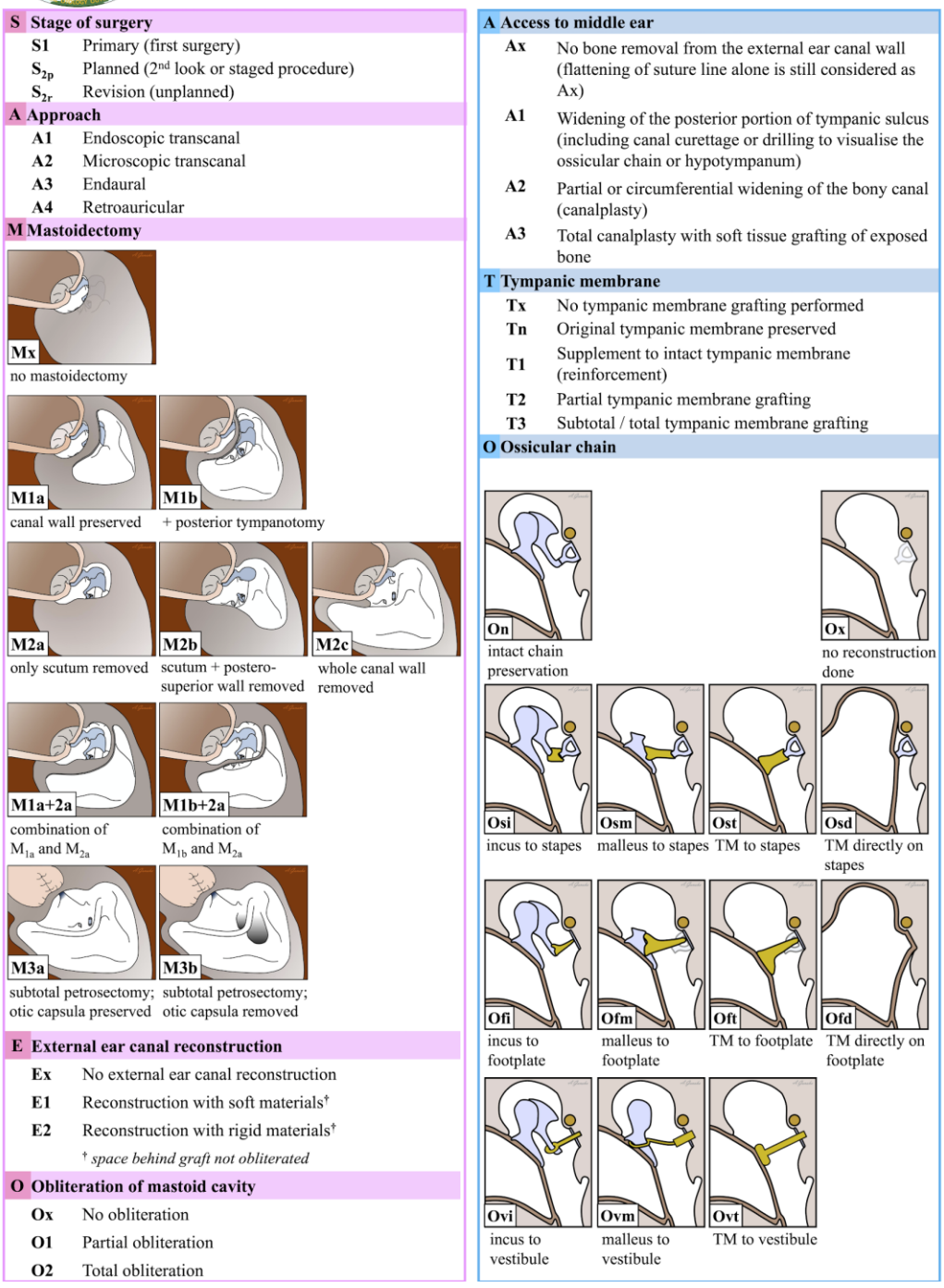

Figure 3. International classification of otologic surgeries . From (17).

Anaesthesia

Tympanoplasty can be performed either under local or general anesthesia. General anesthesia is preferred in children and adults who can't tolerate local anesthesia. In either case, the ear canal skin is injected with local anesthetic in combination with epinephrine for vasoconstriction (18).

Approaches and incisions

The approach should provide complete visualization of the perforation. It depends on the perforation size, the anatomy of the external auditory canal, and the surgeon's preference (18). Three main approaches are used in tympanoplasty: Transcanal (through intact ear canal), Endaural (skin is cut anterosuperior to the external auditory canal, between the tragus and crus of helix), Postauricular (behind the ear).

Graft placement

Graft can be placed medially or laterally to the tympanic membrane remnant (18). Lateral graft technique (overlay): the graft is placed laterally to the fibrous layer of the tympanic membrane remnant but medially to the malleus handle. Prior to graft placement, complete removal of the squamous epithelium from the lateral surface of the tympanic membrane remnant should be done to avoid iatrogenic cholesteatoma formation. It also requires a bony canaloplasty for complete anterior visualization and proper graft placement (18). Medial graft technique (underlay): the graft is placed medially to the tympanic membrane remnant (18).

Numerous different types of grafts can be used for tympanoplasty: Autologous grafts: temporalis fascia, fascia lata, periosteum, perichondrium, cartilage with and without perichondrium, veins, fatty tissue, and skin (19); allografts: dura mater, pericardium, temporalis fascia, amniotic membrane, skin, cornea, peritoneum, veins, and aortic valve (20); alloplastic grafts: paper, absorbable gelatin sponge, acellular dermal matrix (21).

Ossiculoplasty

Persistent infection can cause ossicular chain fixation or discontinuity. Ossicular fixation from tympanosclerosis should be suspected as the cause of conductive hearing loss when a history of chronic infections is present, or when tympanosclerosis is seen on the tympanic membrane. Tympanosclerosis can hinder motion of the eardrum and the ossicles. In severe cases, it can totally immobilize the stapes, malleus, and incus. It is the end product of chronic infection and appears as a chalky white mass that involves the tympanic membrane and the middle ear.

Tympanosclerosis most commonly involves the tympanic membrane (myringosclerosis), but it usually does not cause profound hearing loss (18). Significant conductive hearing loss is usually a result of the fixation of the ossicles in the middle ear or epitympanum (18). Chronic otitis media in almost any form can result in the disruption of the continuity of the ossicular chain. Cholesteatoma is the most common cause. Even without active infection, chronic eustachian tube insufficiency and tympanic membrane retraction that results in prolonged contact of the tympanic membrane with the tip of the incus and stapes can cause ossicular necrosis (18).

Reconstruction of the ossicular chain is done using autografts, homografts, and allografts. Autografts are either bone or cartilage, removed from the patient and sculpted to serve as an interposition graft (18). Homograft ossicles and en-bloc tympanic membranes with attached ossicles may be available through regional tissue banks, and graft rejection is extremely rare; however, their usage has decreased considerably, largely as a result of the fear of potential transmission of human immunodeficiency virus, hepatitis, and Creutzfeldt-Jakob disease (20). Alloplastic grafts come in two basic configurations. A partial ossicular replacement prosthesis is used when the stapes superstructure is present, and a total ossicular replacement prosthesis is used when the superstructure is absent. Polymers, ceramics, and metals are three most frequently used materials (18).

Tympanoplasty using platelet rich plasma

Throughout history many different approaches for tympanic membrane repair have been established. Tympanoplasty using platelet rich plasma is a tympanoplasty procedure with conjunction of autologous platelet rich plasma as an adjunct to promote tympanic membrane healing. Recent studies have shown positive effects of platelet rich plasma on surgical outcome (22, 23). A possible role of extracellular vesicles in the healing process has been indicated (23). However, to our best knowledge only around dozen studies have been published on this topic, hence further studies are needed to determine the efficacy of platelet rich plasma on tympanic membrane healing.

Presently, different preparation procedures are being considered and different nomenclature is being used. It is therefore necessary to better understand mechanisms of postoperative wound healing in order to produce an optimal preparation for individual patient (24, 25).

4. Conclusion

From using swine intestines in the 17th century, to implementing synthetic prostheses for ossicular reconstruction in the 20th century, the tympanoplasty evolved greatly. The goal of tympanoplasty is to eradicate underlying disease and provide the best possible functional hearing for the patient. Evolution of tympanic membrane repair has gone in the direction of

giving way to maximum postoperative hearing using minimal instrumentation. Learning from published treatment methods, otology specialists are still developing newer strategies and techniques for performing this operation. To give best long-term results, platelet rich plasma presents great promise for effective postoperative wound healing.

References

1. Patient's Guide to the Normal Ear. Otolaryngology — Head & Neck Surgery (OHNS). https://med.stanford.edu/ohns/OHNS-healthcare/earinstitute/education/patients-guide-to-the-normal-ear-and-hearing.html
2. Battelino S. Motnje sluha: vrste, odkrivanje, zdravljenje in (re)habilitacija. In: Rehabilitation. 2012. p. p12-17. Supplement 1; vol. 11.
3. Sarkar S. A Review on the History of Tympanoplasty. Indian J Otolaryngol Head Neck Surg. 2013 Dec. 65 (Suppl 3):455–60. doi:10.1007/s12070-012-0534-5
4. Banzer M, Acidalius G, Wittenberg U. Disputatio Medica Inauguralis De Auditione Laesa. Röhnerus; 1640
5. Wullstein H. The Restoration of the Function of the Middle Ear, in Chronic Otitis Media. Ann Otol Rhinol Laryngol. 1956. Vol.65. https://doi.org/10.1177/000348945606500416
6. Sooy FA. A Method of Repairing a Large Marginal Tympanic Perforation. Ann Otol Rhinol Laryngol, 1956. 65(4):911-4. doi: 10.1177/000348945606500402.
7. SHEA JJ. Vein graft closure of eardrum perforations. J Laryngol Otol, 1960. 358-362. doi: 10.1017/s002221510005670x
8. Patterson ME, Lockwood RW, Sheehy JL. Temporalis Fascia in Tympanic Membrane Grafting. Tissue Culture and Animal Studies, 1967. Arch Otolaryngol. 85(3):287-291. doi:10.1001/archotol.1967.00760040289010
9. Herndon DN. Total Burn Care: Expert Consult - Online. Elsevier Health Sciences; 2012. 976 p. ISBN 9781437727869
10. Austin DF. Transcanal tympanoplasty. Otolaryngol Clin North Am. 1972, 5(1):127–43.
11. Pohlman ME. The Artificial Ear Drum.Ann Otol Rhinol Laryngol, 1951. https://doi.org/10.1177/000348945106000109
12. Hall A, Rytzner C. Stapedectomy and autotransplantation of ossicles. Acta Otolaryngol. 1957, 47(4):318–24. https://doi.org/10.3109/00016485709130348
13. Mudhol RS, Naragund AI, Shruthi VS. Ossiculoplasty: Revisited. Indian J Otolaryngol Head Neck Surg. 2013, 451–454. doi: 10.1007/s12070-011-0472-7.
14. Wehrs RE. Incus replacement prosthesis of hydroxyapatite in middle ear reconstruction. Am J Otol. 1989, 10 :181–2.
15. Zupancic J, Battelino Saba. Današnja vloga alograftov v ušesni kirurgiji = The present role of allografts in otosurgery. Med Razgledi Suppl. 1996, (35):37–42.

16. Merchant SN, Rosowski JJ, McKenna MJ. Tympanoplasty. Oper Tech Otolaryngol-Head Neck Surg. 2003, 14:224–236. doi: 10.1053/S1043-1810(03)00092-7
17. Yung M, James A, Merkus P, Philips J, Black B, Tono T, et al. International Otology Outcome Group and the International Consensus on the Categorization of Tympanomastoid Surgery. J Int Adv Otol. 2018, 14:216–26. doi: 10.5152/iao.2018.5553
18. Richardson M, Flint PW, Haughey B, Lund V, Niparko JK, et al. Cummings Otolaryngology - Head and Neck Surgery. Elsevier Health Sciences, 2014. 4198 p.
19. Mohamad SH, Khan I, Hussain SSM. Is cartilage tympanoplasty more effective than fascia tympanoplasty? A systematic review. Otol Neurotol. 2012 ,33: 699–705. doi: 10.1097/MAO.0b013e318254fbc2
20. Van Rompaey V, Farr MRB, Hamans E, Mudry A, Van de Heyning PH. History of otology allograft tympanoplasty: A historical perspective. Otol Neurotol. 2012, 34:180-188.
21. Haynes DS, Vos JD, Labadie RF. Acellular allograft dermal matrix for tympanoplasty. Curr Opin Otolaryngol Head Neck Surg. 2005, 13:283–6. doi: 10.1097/01.moo.0000172820.97322.8d.
22. Gökçe Kütük S, Özdaş T. Impact of platelet-rich fibrin therapy in tympanoplasty type 1 surgery on graft survival and frequency-specific hearing outcomes: a retrospective analysis in patients with tympanic membrane perforation due to chronic otitis media. J Laryngol Otol. 2019 , 133:1068– 1073. doi: 10.1017/S0022215119002391
23. Vozel D, Božič D, Jeran M, Jan Z, Pajnič M, Pađen L, et al. Platelet-and extracellular vesicle-rich plasma as a treatment modality for chronic bone cavity inflammation: a randomized controlled trial. Socrat Lect 3 Int Minisymp. 2020 Apr 17;
24. Vozel D, Jeran M, Božič D, Pađen L, Pajnič M, Uršič B, et al. Applicability of platelet- and extracellular vesicle-rich plasma in medicine. Socratic Lectures. 2019.
25. Vozel D, Božič D, Jeran M, et al. Treatment with platelet- and extracellular vesicle-rich plasma in otorhinolaryngology-a review and future perspectives. In: Pocsfalvi G, Bongiovanni A, Mauro M, Kralj-Iglič V, editors. ABLSA 32: Biological Membrane Vesicles: Scientific, Biotechnological and Clinical Considerations. Amsterdam, The Netherlands, Elsevier, in press.

Review

HYPOTHESES ON ORIGIN AND FORMATION OF VIRUSES

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Abstract

There are quite a few different hypotheses of how viruses emerged, yet none of them has been affirmed. In this contribution we present state of the art hypotheses of virus origin. Also we describe the mechanism of virus replication and discuss possible origins of the SARS-CoV-2 virus.

1. The emergence of viruses

The origin of viruses is hard to trace due to the fact that they do not leave fossils (1). It was suggested that viral proteins date before the divergence of life and have infected the common ancestor of all living organisms, LUCA (2). Furthermore it was indicated that they evolved separately throughout the Earth's history (2).

The question of the viruses' origin is still a topic of debate, with different theories within the scientific community and there is still not a widely accepted answer. Currently we have three main hypotheses amongst virologist (2): progressive hypothesis, regressive hypothesis and virus-first theory.

Progressive hypothesis

Progressive hypothesis predicts that viruses have emerged from genetic material of more complex organisms (1). They evolved from chromosome or plasmid DNA or mRNA that were a parts of the cell genome, and attained capability of escaping the cell. Through progressive process these particles of DNA or RNA gained the ability to move between cells (1,3) and enter another cell, thus infecting it (3). They developed mechanisms to replicate their genome in another cell (3).

Regressive hypothesis

The regressive hypothesis suggests that viruses might have been free organisms that became obligatory intracellular parasites (4). For example, the Smallpox virus and Mimivirus are much more complex and bigger than others, which makes them less dependent on host cells (5). Based on the evidence, it has been suggested that smaller cells have developed a symbiotic relationship with more complex cells, but with time they became dependent on the host cells (4,6). With dependence they lost some of their genes, which were previously essential (4,6).

Virus- first hypothesis

The virus first hypothesis states that viruses existed first (7). This hypothesis claims that viruses developed from proteins and nucleic acids before the emergence of cells and became more complex with time (1).. Eventually, enzymes for membrane synthesis evolved and the first cells appeared. This hypothesis' critics argue that some genes that are crucial for the replication of viruses do not exist in the cell genome (8-10) suggesting that viruses couldn't have emerged before cells but rather they co-existed and co-evolved, which is what the co-evolution hypothesis claims (9,10).

1. Evolution of viruses

Studies of different viruses have indicated that viruses did not evolve from a single virus but most likely have multiple independent origins in different time periods (11,12). It seems that there is no clear line between living and non-living entities, instead, life followed the evolutionary continuum (12).

Researching viral evolution is in many ways different from researching evolution of other species, viruses are smaller than colloidal particles forming sedimentary rocks that fossilize plants and animals (13) – this means that viruses cannot leave behind physical fossils. However, their DNA sequences can be found in the genome of many of today living organisms (9). These DNA sequences, called endogenous viral elements, give valuable information about evolutionary history of viruses (13). Also, important information comes from measuring mutation rates of viruses. The latter allows application of a molecular clock – technique that uses mutation rates of biomolecules to deduce the time in prehistory when two or more life forms diverged (12). Viral polymerases are error prone which means that RNA or DNA mutations are relatively frequent. Also, reproduction in host cells makes them more susceptible to mutations (12). As with other species, natural selection occurs – those viruses whose phenotype is best adapted to the environment will ultimately survive (2).

The way viruses reproduce in their host cells makes them susceptible to the genetic changes that help to drive their evolution. The RNA viruses are especially prone to mutations (14). Mechanisms for correcting mistakes do not work for RNA and when an RNA virus replicates in its host cell, changes in its genes are occasionally introduced, some of which are lethal (14). One virus particle can produce millions of progeny viruses in just one cycle of replication; therefore, the production of a few unfunctional viruses does not considerably endanger the continuation of the virus identity (14). Most mutations of viral genome are "silent" and do not result in any obvious changes to the progeny viruses (14). Some confer advantages that increase the fitness of the viruses in the environment (14).

These could be changes to the virus particles that disguise them, so they are not identified by the cells of the immune system, or changes that make antiviral drugs less effective (14). Many viruses (for example, influenza A virus) can "shuffle" their genes with other viruses when two similar strains infect the same cell (14).

This phenomenon is called genetic shift and can be a cause of the appearance of new and more virulent strains (11). Other viruses change slowly as mutations in their genes gradually accumulate over time - a process known as antigenic drift (18). Through these mechanisms new viruses are constantly emerging and present a continuing challenge in attempts to control the diseases they cause (14).

1. Biological implications of mutation, recombination and reassortment

Viruses can be divided in those with DNA and those with RNA genetic material. Four replicative schemes are relevant with regard to the occurrence of mutations during viral genome replication (14):

1. DNA → DNA → DNA

2. DNA → RNA → DNA

3. RNA → RNA → RNA

4. RNA → DNA → RNA

The nucleic acid written in the first and third place in each of the four schemes is the one found in the parental and progeny viral particles. The nucleic acid in the middle position indicates the type of nucleic acid that acts as a replicative intermediate (not the transcripts involved in gene expression that participate in the infectious cycle) (15). The presence or not of RNA in the scheme determines the potential of genetic variation of the virus. RNA viruses and DNA viruses that have RNA as replicative intermediate (that is, viruses that follow replicative schemes 2, 3 and 4) display elevated mutation rates that have been estimated in 10-5 to 10-3 per nucleotide copied (15). The molecular basis for high mutation rates is the absence of a proofreading-repair activity in the viral RNA-dependent RNA polymerases (RdRp) and RNA-dependent DNA polymerases (RdDp) (also termed reverse transcriptases (RT)), as evidenced by functional and structural studies (15).

High mutation rates of RNA viruses and some DNA viruses result in dynamic distributions of mutants called viral quasispecies (14). Viral quasispecies constitute reservoirs of genetic and phenotypic variants, with several biological implications for viral evolution and pathogenesis, some of which have become apparent over the last few years (15).

2. Origin of coronaviridae

Corona in Latin means crown. The name refers to their characteristic appearance under the electronic microscope - bulbous surface projections reminiscent of a crown. This structure is created by viral spike proteins (16).

Coronaviruses are mainly pathogens of birds and mammals (16). They are RNA viruses and are currently classified into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. Alphacoronaviruses and Betacoronaviruses are present exclusively to mammals, whereas gammacoronaviruses and deltacoronaviruses primarily infect birds. The greatest diversity of coronaviruses was found in bats and avian species – suggesting these species as their natural reservoirs (16).

Molecular clock dating analysis of coronaviruses suggests that the most recent common ancestor of SARS-CoV-2 existed around 10,000 years ago (16). But this estimation could be wrong for coronaviruses that have a unique proofreading mechanism (error-correcting process that corrects mutations that occur during the replication) which would lower their mutation rate far below other RNA viruses. Therefore, there could be a substantial underestimation of the length of the natural evolutionary history of coronaviruses (16).

Evolution of SARS-COV-2

SARS-CoV-2 virus has four structural proteins: Proteins S (spike), E (envelope), M (membrane) and N (nucleocapsid) (17). Genetic sequencing showed that its S proteins enable high affinity binding to angiotensin converting enzyme (ACE-2) of humans, ferrets, cats and other organisms with high receptor homology (18).

SARS-CoV-2 is the seventh coronavirus known to infect humans; SARS-CoV, MERS-CoV and SARS-CoV-2 can cause severe disease and death, whereas HKU1, NL63, OC43 and 229E only cause mild symptoms. The part of the genome that codes receptor-binding domain (RBD) in the spike protein is the most variable part of the coronavirus genome.

Understanding the origin of the virus is very important for future prospects: if SARS-CoV-2 pre-adapted in animals, then it is very likely that it can happen again. This means that a virus with the right set of mutations exists in an animal so all that is needed for a pandemic to start is for a human to come in contact with the infected animal) (18). On the other hand if the virus adapted in humans, the same set of mutations would have to occur in order to start a new pandemic (in this case humans must first get infected with a virus from an animal and later a set of specific mutations must happen in order for the virus to start a pandemic (17).

Also, the virus is mutating and changing throughout the pandemic. Currently, eight SARS-CoV-2 strains with slightly different genome have been evidenced throughout the world (19).

2. The mechanism of viral replication

Viruses have to enter a living host cell in order to replicate and continue spreading. They carry a genetic code for proteins, while the host cell provides the necessary energy and environment for the virus to replicate (20).

Viral attachment

The process starts by the attachment of protein ligands on specific cell wall receptors. Glycoproteins S (spike proteins) in the shape of spikes are sticking out of the viral capsid of the human coronavirus giving it the distinct shape of a crown (9). Protein S is the main determinant of the viral entry into a host (21) and has two main domains (S1 and S2) (22). It was found that the coronavirus binds to the ACE2 receptor of the host with its receptor domain on the C-end of

the S1 protein subunit (22). This is followed by the formation of an endosome and is the first crucial determinant for the entry of the virion into the cell (22).

Viral entry

If the attachment is successful, viral entry follows. Non-enveloped viruses enter with translocation or endocytosis, while enveloped ones use membrane fusion or endocytosis (23). It was indicated that the key part of viral entry is the proteolytic cleavage of protein S; enzyme protease first cleaves the protein into two main domains (S1 and S2) and then further cleaves the cleavage domain within the S2 subunit (S2') (22). This exposes the fusion protein (23), which causes fusion of the cell and viral membrane (20). The exact mechanism of the fusion is still not completely understood. There are indications that the process involves cleaving of protein S by Cathepsin L protease (21,23) These processes were found to depend on pH (22,25,26). Evidence ACE2 receptor localization in the early endosome suggests that a virus can also enter by endocytosis (26). However, other, pH-independent routes of entry have been reported. Possible entries by endocytosis involve chlatrin (26,27) and curved lipid rafts (caveolae) (28).

Lipid rafts are cholesterol, sphingolipid and caveolin-enriched membrane microdomains within which the interactions between membrane proteins are facilitated. Furthermore, it was found that ACE2 receptors are located within the lipid rafts (26). It was suggested that interactions between receptors lead to the activation of the signal pathway and viral entry into the cell (28).

Viral replication

After entry, viral capsid shell is removed by the proteolytic enzymes in the cell (24). This is followed by directing cell metabolism into forming new virions. DNA viruses are replicated in cell nucleus by cell transcriptase whereas RNA viruses are replicated in cytoplasm by their own enzymes (22).

Coronaviruses contain a single-strand positive sense RNA which acts as a mRNA (24). mRNA is capped on its 5' end and polyadenylated on its 3' end (24). The replicase gene takes two thirds of the 5' end of the genome and has two open reading frames (ORF) - 1a and 1b (25). 16 non-structural proteins are coded, some of which also have enzymatic function (e.g. RNA primase, helicase and N7 methyltransferase) (25). The latter has the ability to recognize and correct mistakes during the process of replication. The remaining third of the genome on the 3' end codes structural proteins: protein S, envelope protein E, membrane protein M and nucleocapsid N (25). Newly synthesized protein N forms a nucleocapsid whereas newly formed proteins M, S and hemagglutinin esterase are incorporated in the endoplasmic reticulum, where last modifications occur (glycosylation, phosphorylation…) take place. These new viral proteins and nucleocapsid join into a new virus particle, which is transported by the Golgi vesicles to cell membrane where exocytosis takes place to release the virions (25).

Conclusions

Viral infections have been causing diseases since the dawn of time and new pathogens are going to continue to emerge and spread in the future. Further research on different viruses helps us define the propensity to jump between species, mutate and find significant reservoirs. Understanding their entry, proteins and replication significantly improves our ability to find a cure and design preventive measures.

References

1. Emerman M and Malik HS (2010) Paleovirology: Modern Consequences of Ancient Viruses. PLoS Biol 8(2).

2. Wessner DR (2010). The Origins of Viruses. Nature Education 3(9):37. doi: 10.1016/j.jmb.2005.08.060.

3. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J and Devon K (2001). Initial sequencing and analysis of the human genome. Nature 409, 860–921.

4. Andersson SGE, Zomorodipour A, Andersson JO, Thomas Sicheritz-Pontén T (1998). The genome sequence of Rickettsia prowazekii and the origin of mitochondria. Nature 396, 133–143.

5. La Scola B, Desnues C, Pagnier I, Robert C (2003). A giant virus in Amoebae. Science 299.

6. Drummond A, Pybus OG, Rambaunt A (2003). ADV Parasitology 54, 331-358

7. Koonin EV, Martin W (2005). On the origin of genomes and cells within inorganic compartments. Trends in Genetics 21, 647–654.

8. Villarreal LP. Viruses and the Evolution of Life. American society for microbiology. 2005. DOI: 10.1128/9781555817626

9. Viral evolution. Wikipedia. Retrieved April 2020, from https://en.wikipedia.org/wiki/Viral_evolution

10. Moelling K, Broecker F. Viruses and Evolution – Viruses First? A Personal Perspective. Front. Microbiol. 2019; 10:523. doi: 10.3389/fmicb.2019.00523

11. Sonali S, Joshi. Origin of life: The panspermia theory. Helix. 2008

12. Emerman M, Malik HS (2010). Paleovirology--modern consequences of ancient viruses. PLoS Biol; 8(2):e1000301. doi: 10.1371/journal.pbio.1000301

13. Connor RJ, Kawaoka Y, Webster RG, Paulson JC (1994). Receptor specificity in human, avian and equine H2 and H3 Influenza virus isolates. Virology 205:17–23.

14. Sussman M, Topley WWC, Wilson GK, Collier LH, Balows A. Topley & Wilson's microbiology and microbial infections. Arnold. 1998. 11-12. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC501099/pdf/jclinpath00276-0077e.pdf

15. Domingo E. Mechanisms of viral emergence. Vet Res. 2010;41(6):38. doi:10.1051/vetres/2010010

16. Coronavirus. In Wikipedia, The Free Encyclopedia. Retrieved 10:09, July 29, 2020, from https://en.wikipedia.org/w/index.php?title=Coronavirus&oldid=969992211

17. Wertheim JO, Chu DK, Peiris JS, Kosakovsky Pond SL, Poon LL. A case for the ancient origin of coronaviruses. J Virol. 2013;87(12):7039–7045. doi:10.1128/JVI.03273-12

18. Andersen KG, Rambaut A, Lipkin WI et al. The proximal origin of SARS-CoV-2. Nat Med. 2020; 26, 450-452. doi: 10.1038/s41591-020-0820-9

19. Dr. Liji Thomas, M., 2020. Eight Strains Of Coronavirus Afflicting The World. [online] News-Medical.net. Available at: <https://www.news-medical.net/news/20200331/Eight-strains-of-coronavirus-afflicting-the-world.aspx> [Accessed 29 July 2020].

20. Koonin EV, Senkevich TG, Dolja, VV. The ancient Virus World and evolution of cells. Biol Direct. 2006; 1,29. doi: 10.1186/1745-6150-1-29

21. Banejee A, Mossman K, Misra Vikram. Viruses can cause global pandemics, but where did the first virus come from? The conversation. 2018. https://theconversation.com/viruses-can-cause-global-pandemics-but-where-did-the-first-virus-come-from-94551

22. Belouzard, S., C. Chu, V. in Whittaker, G.R., Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites, Proceedings of the National Academy of Sciences (2009), 106 (14) 5871 – 5876; https://www.pnas.org/content/106/14/5871

23. Domingo E. Mechanisms of viral emergence. Vet Res. 2010;41(6):38. doi:10.1051/vetres/2010010

24. Avšič Županc, T., Drinovec, B., Koren, S., Marin, J. in Poljak, M. (2011). Splošna medicinska virologija. V S. Koren in J. Marin (ur), Razmnoževanje virusov (str. 23 – 36). Ljubljana: Medicinski razgledi

25. Petrovec, M. in Poljak, M. (2011). Medicinska virologija. Ljubljana: Medicinski razgledi

26. Wang, H., Yang, P., Liu, K. et al. SARS coronavirus entry into host cells through a novel clathrin- and caveolae-independent endocytic pathway. Cell Res 18, 290–301 (2008). https://doi.org/10.1038/cr.2008.15

27. Wang, S., Gui, F. Et al. Endocytosis of the receptor binding domain of SARS-CoV spike protein together with virus receptor ACE2. Virus Research 136: 8-15, 2008. https://doi.org/10.1016/j.virusres.2008.03.004

28. Jezernik, K., Sterle, M. in Veranič, P. (2015), Celična biologija, učbenik za študente Medicinske fakultete. Ljubljana: DZS

Review

ROLE OF ESCRT COMPLEX IN VIRAL PARTICLE FORMATION

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Abstract

The endosomal sorting complexes required for transportation (ESCRT) is believed to be involved in many important mechanisms in the cell such as vesicular pathway, cytokinesis and also virus budding. ESCRT pathway was shown to be crucial in membrane fission events. Enveloped viruses are released from infected cells by budding of the host cell membranes. Some viruses, such as orthomyxovirus, togavirus and coronavirus are thought to be ESCRT – independent while ESCRT pathway is likely to be the crucial mechanism of budding of the viruses such as HIV, HTLV and Ebola virus. In this work we describe a view on some important elements of the ESCRT pathway.

1. Introduction

The endosomal sorting complexes required for transportation (ESCRT) are thought to be a crucial apparatus of the mechanism, which enables the formed vesicles to separate from the cytoplasm (1). The ESCRT complex is believed to be formed from cytosolic protein complexes ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III. It is supposed to be an evolutionarily highly preserved complex that contributes to many important mechanisms in the vesicular pathway including budding and vesiculation of the virus (1,2).

For viral replication to complete, it is essential for the enveloped viruses to emerge from the plasma membrane. This process enables them to gain their lipid bilayers, which is needed for their complete formation. Much attention was dedicated to cellular ESCRT complex and its role in virus budding. It is believed to be of great importance in membrane fission events and is therefore used by lots of different viruses in order to make their replication possible. It is likely to be the crucial mechanism of budding of HIV, HTLV and Ebola viruses (1).

2. ESCRT pathway

The pathway of budding is supposed to be analogous to the two abscission stages of cytokinesis; 1) membrane deformation – the host cell membrane is wrapped around the budding virion and 2) membrane fission, when the neck of the bud is severed (3). The ESCRT complex is believed to enable the final step in budding – membrane fission and the release of the virion (3).

Three classes of factors oprating in specific order were considered in ESCRT complex function, 1) adaptor proteins define the ESCRT binding site, 2) early acting factors initiate the ESCRT assembly and 3) late acting factors mediate the fission (3). The adaptor proteins localize specific membrane domains and enable the function of early-acting ESCRT factors (3). Early-acting ESCRT factors consist of two components; Bro1 and ESCRT-I/II complexes. They have the possibility to bind late-acting ESCRT-III factors that recruit VPS4 ATPases. These, in turn, mediate membrane constriction and fission (3).

3. ESCRT-mediated resease of HIV-1 from infected cells

ESCRT proteins of the host cell are used by human immunodeficiency virus type 1 in order for HIV-1 to release infectious viral particles from the cell (4). Recruitment of the ESCRT complex is almost essential for production of infectious virus although the main structural protein of HIV-1, Gag, is capable of assembling and budding from membranes on its own (4).

The HIV-1, which is an enveloped virus, pinches off from the infected cells to complete its replication cycle in a process driven by the viral Gag protein (4). There are four domains in this protein – matrix, capsid, nucleocapsid and p6 –, connected by linker peptides (4). Matrix binds to the plasma membrane lipid phosphatidylinositol bisphosphate and thereby targets Gag to the plasma membrane (4). Next, capside organizes the shape of the membrane bud whereas function of nucleocapsid is to pack the RNA genome. P6 is a polypeptide at the C-terminus of the Gag and it recruits cellular proteins TSG101 and ALIX to initiate budding of the virus from

the plasma membrane (4.) Additionally, the insertion of Gag protein into the inner leaflet of the host plasma membrane is promoted by the N-terminal myristoylation (4).

ESCRT subunits polymerize and recruit other proteins (VPS4, the ESCRT-Related ATPase) at the neck where membrane abscission occurs. ESCRTs are later recycled back to the cytosolic pool. (4)

4. Importance of ubiquitin in ESCRT pathway and virus budding

Role of the ESCRT complex in viral replication was extensively studied in the case of the HIV-1 virus. Retroviral HIV virions concentrate ubiquitin which appears to play a role in the budding process (3). It was found that the lack of ubiquitin inhibits virus budding from host cell's membrane (3). Furthermore, it was proven that ubiquitin can sometimes function as a late assembly domain in the budding process if fused with retroviral Gag proteins and that the known early-acting mammalian ESCRT proteins ALIX, ESCRT-I and ESCRT-II all include ubiquitin-binding domains (3). In most cases, however, the key ubiquitin receptors have not been identified. Also, the functional target of enzymes that bind ubiquitin (process: ubiquitylation) has not yet been completely identified. However, viral structure proteins are most likely the targets of ubiquitylation and their budding efficiency correlates with the formation of K63 (a protein which enhances HIV-1 budding) linked poly-ubiqutuin chains on HIV-1 glycosaminoglycans (3). Consequently, ESCRT recruitment by viral structural proteins may be analogous to ESCRT recruitment by mono- and K63-linked poly-ubiquitylated membrane proteins that are being directed into multivesicular bodies (3). In this and perhaps other cases, ubiquitylation of the ESCRT itself may play a key functional role (3).

Conclusions

ESCRT has a function as a protein sorting and membrane sculpting and scission machinery. Its action has been studied in the process of cytokinesis, multivesicular body pathway and HIV budding (1). Viruses must cross cellular membranes efficiently to spread the infection. Complex interactions between host and viral factors are required in both entry and budding. However, while some enveloped viruses encode their own membrane fusion proteins to mediate entry, some other viruses instead harness the cellular ESCRT machinery to effect membrane fission during egress (3). Further study is required to reveal ESCRT function in cellular vesiculation and abscission, in molecular mechanisms underlying membrane deformation as well as in multivesicular body pathway (1,3).

References

1. Schmidt O, Teis D. The ESCRT machinery. Curr Biol. 2012, 22(4):R116–R120. doi:10.1016/j.cub.2012.01.028

2. Morita E, Sandrin V, McCullough J, Katsuyama A, Baci Hamilton I, Sundquist WI. "ESCRT-III protein requirements for HIV-1 budding". Cell Host Microbe. 2011, 9 (3): 235–42. doi:10.1016/j.chom.2011.02.004

3. Votteler J, Sundquist WI. Virus budding and the ESCRT pathway. Cell Host Microbe. 2013, 14(3):232–241. doi:10.1016/j.chom.2013.08.012

4. Hurley JH, Cada AK. Inside job: how the ESCRTs release HIV-1 from infected cells. Biochem Soc Trans. 2018, 46(5):1029–1036. doi:10.1042/BST20180019

Review

DOUBLE MEMBRANE BOUND VESICLES

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Abstract

The proposed theme of the discussion is to review how and in which systems double membrane vesicles are formed and how the double membrane vesicles are connected with different kind of viruses, in particular with the betacoronavirus (SARS, MERS). It is acknowledged that viruses depend on host cell machinery for the replication of their genome and the generation of progeny virus particles. Moreover, many viruses were found to be formed in the sub cellular microenvironments or mini-organelles known as “virus factories” or viroplasm. These viral factories are thought to create a platform bringing together the replicase proteins, virus genomes and host proteins required for replication. Depending on the family and genus to which the virus belongs, the rearranged cellular membranes may be derived from various organelles including the endoplasmic reticulum, late endosomes/lysosomes or the mitochondrial outer membrane. Plus-strand RNA viruses induce the formation of two types of vesicles: single-membrane vesicles formed by negatively curved membranes and initiated by invaginations of pre-existing membrane bilayer, giving rise to spherules or vacuoles toward the lumen of targeted cell organelle and double-membrane vesicles formed by positively curved membranes. Viruses from Togaviridae, Bromoviridae and Nodaviridae families induce small invaginations called spherules or larger spherule-lined cytopathic vacuoles, whereas those from the Arteriviridae, Picornaviridae and Coronaviridae families generate a more extensive network of different membrane structures during the temporal and spatial dynamics of virus replication. These membrane changes include not only convoluted membranes and single-membrane vesicles, but also a massive network of double membrane vesicles.

1. Introduction

Upon infection, positive strand RNA viruses (+RNA viruses) rearrange the membranes of the host to create replication organelles. For a few virus types it has been proven that replication organelles are the sites of viral RNA synthesis (1). Compartmentalizing of viral genome is thought to bring viruses some advantages, like generating a more suited micro environment for replication (concentrated viral and host proteins and intermediates necessary for replication), preventing degradation of viral RNA by host enzymes and protection against host's cellular intrinsic antiviral signalling pathways (1,2). Depending on the family and genus to which the virus belongs, the rearranged cellular membranes may be derived from various organelles including the endoplasmic reticulum (ER), late endosomes/lysosomes or the mitochondrial outer membrane (3).

In the last two decades, with the increasing use of electron tomography, RNA+ viruses are documented to induce one of two basic morphotypes of membrane modifications: invaginations or double membrane vesicles (DMVs) (2), the latter being the more prominent one (4).

Within *the inner part of* invaginations (where the membrane curvature is negative), single-membrane vesicles are formed from organelle membrane bilayer, giving rise to spherules toward the lumen of the targeted cell organelle (3).

Virus-induced invaginations are better understood as their structure is simple as helical and icosahedral forms and they are thought to have a common topography among different virus groups (2). In contrast, DMVs architecture and connectivity, to other organelles or between each other, differ among different virus families, in some cases even within the same genus (2).

2. Differences and similarities in DMVs (different families and genus of viruses)

Common traits between different families

As the name suggests, DMVs are vesicles with double lipid bilayer, of about 60-400 nm (sizes differ in different types of viruses), usually connected to or derived from the membrane of endoplasmatic reticulum (ER). DMV-inducing viruses that are known today, are: *flavoviridae, picornaviridae, nidovirales* (2,5). *Some viruses* can induce other nanostructures that interact with DMVs and form a larger network. For example, in SARS-CoV 1 (coronaviruses, *nidovirales*), they interact with *reticular network of double membranes*, also called convoluted membranes (2,8) (**Figure 1**). These convoluted membranes appear to be the major accumulation sites of the viral replicase subunits.

They can interact with modified endoplasmatic reticulum membranes to form a large reticulovesicular network (2). Zippered endoplasmatic reticulum is another type of structure that can be connected or in the proximity of DMVs (*Nidovirales*). It is also important to distinguish between DMVs and double membrane spherules (DMS). DMVs can be connected

to other membrane modifications by a neck like structure, where both membranes are opened, forming a passage from the cytosol to the inside of the vesicle, or the inner membrane is closed. It is unclear if the closed membrane contains any smaller channels which could allow the intermediates or proteins to be exchanged with the cytosol. However, researchers found a significant correlation between DMVs with the spherules, suggesting that these two are spatially and temporally linked (1,2).

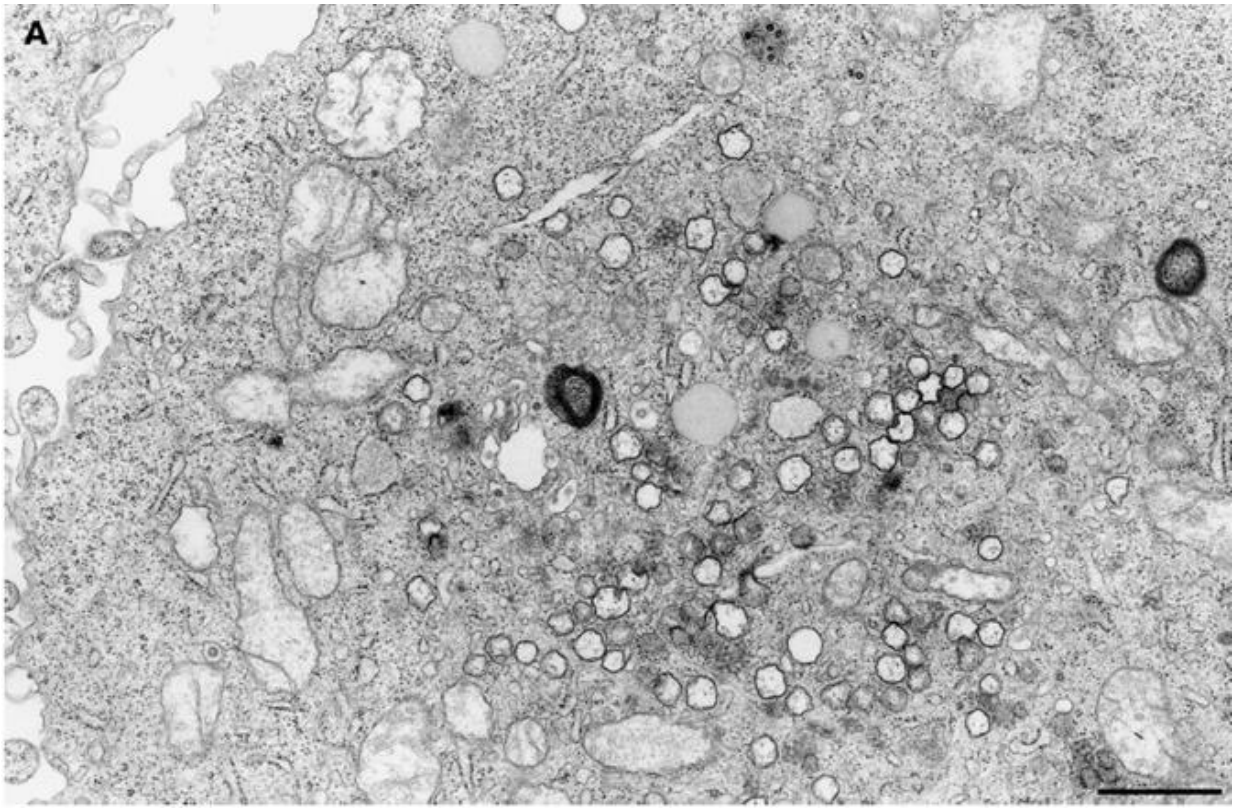

Figure 1: Transmission electron micrograph of virions in Mouse hepatitis virus-infected cells. Bar length is 2 micrometers. From (6).

2.1 *Flaviviridae family*

Hepatitis C virus (HCV) is the most prominent in *Hepaciviruses* genus, where DMVs (approximately 150nm in diameter) are accumulated and embedded in a membranous matrix, so called membranous web (5). These DMVs (**Figure 2**) contain non-structural proteins (nsps) and double-stranded RNA, which implies that this is the site of RNA replication (2,5). Other structures induced by Hepatitis C virus are clustered vesicles, single membrane vesicles, continuous vesicles or multi-membrane vesicles. Most of the DMVs were reported to be built as closed, only about 10% has a small pore, connecting the cytosol to the interior of the vesicle. It is still unclear if the replication complex is inside the vesicle or on the membrane (5).

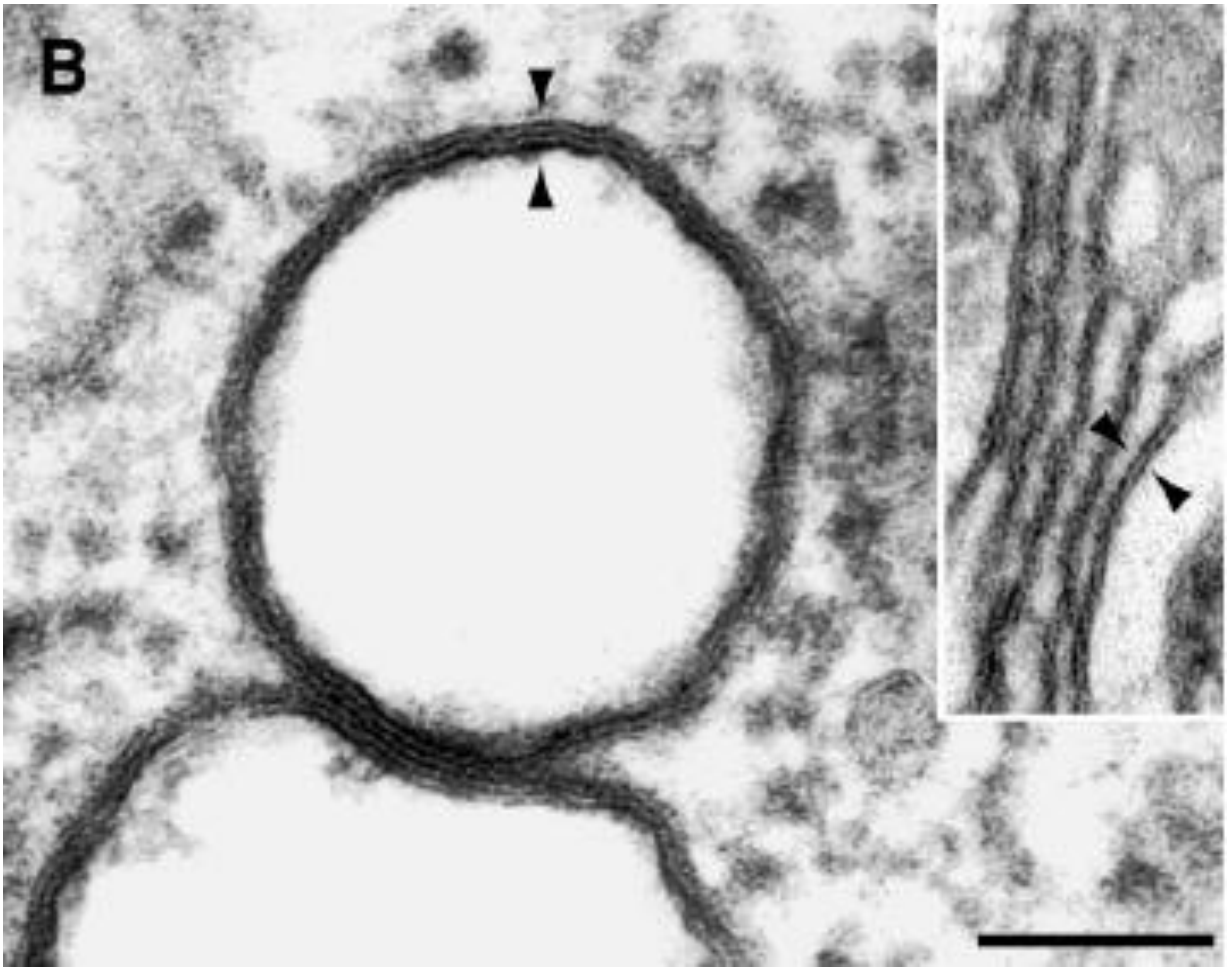

Figure 2: Double membrane vesicle in a Mouse hepatitis virus-infected cell. Inset: … Bar length is 100 nm. From (6).

2.2 *Picornaviridae family*

Only a few representatives from this family have been analysed, most from genus Enterovirus. Poliovirus is one of the best studied viruses so far. It induces single membrane vesicles first and DMVs later. Because of the disappearance of single membrane vesicles when DMVs were recorded, it is suggested that single membrane vesicles are converted to DMVs. DMVs are about 100-300nm in diameter. Because of the absence of replicase proteins in DMVs they are suspected to have a different function as the DMVs induced by other viruses. This different function is still not clear. It has been suggested that DMVs play a role in viral maturation as DMVs could act like a scaffold for the assembly of viral replication complexes by providing an organization facilitating viral replication (5). The same is thought also for picornavirus coxsackievirus B3 (CVB3), also from genus Enterovirus (2), although here the DMVs were also part of multilaminar vesicles (DMVs enwrapped by other membranes, always remaining opened to the cytoplasm) later in infection. *(5)* Membrane modifications induced by Enterovirus seem to *include* isolated compartments with no membranous connections to cellular organelles (2). In this they are very different from *Nidovirales* family where vesicles

remain connected to the organelle membranes or other vesicles, even 4 hours post infection (5).

2.3 *Nidovirales family*

This family is one of the most researched ones from DMVs aspect. This is important because coronaviruses and arteriviruses are part of this family. The first ones infecting a wide range of species, from pets, swines, humans, and thus causing important animal and human diseases (1). It was indicated that DMVs formation is a common trait in *Nidovirales* family, since they have been proven in every nidovirales infection documented thus far (from genus Arteriviridae and coronavirus) (1,2). Both researched genuses induce similar formation of double membranes as well as ER derived DMVs located mostly in the perinuclear region (5). Although diameters of DMVs are documented to be very diverse also in the same genus (2), in general DMVs induced from coronaviruses are on average twice the size of the Arteriviridae. Diameters of coronaviruses-induced DMVs span from 140 to 350nm. These are the largest DMVs observed to date. Both in SARS-CoV1 (betacoronavirus) and Equine arteritis virus (EAV) large reticulo-vesicular networks have been detected. Only the *Gammacoronavirus*-infectious- bronchitis- virus-induced DMVs were isolated, with no DMV-DMV connections and only a small number of DMVs were reported to be connected to the ER. There is also lacked presence of free ribosomes on the outer membrane. Researchers suggest that to determine if this is a specific gammacoronavirus trait or it can also be applied to other nidovirales (sub)groups further analysis has to be done (2).

2.4 *Arteriviridae genus*

A few virus types from this genus have been analysed, the most researched one being equine viral arteritis (EVA). The DMVs from equine arteritis virus infections are about 100 nm in diameter and they appear in the perinuclear region of the cell and then proliferate in number, giving rise to cytoplasmic clusters that often also show an increased presence of free ribosomes (2). Neck-like structures connecting DMVs' outer membrane with ER have also been observed. Researches have suggested that this and the fact that the outer DMVs' membrane is decorated with free ribosomes, is an indication that ER is the most likely membrane donor (2). Majority of DMVs are connected to other modified ER membranes, forming a large reticulovesicular network.

Coronaviruses

Coronaviruses have the largest RNA genome and subgenomic template RNA (4,5). Replication organelles for alpha-, beta-, gamma- and deltacoronaviruses genera have been characterised (1). A common characteristic of coronavirus-infected cells, which are absent in arteriviridae, are convoluted membranes, firstly documented in Mouse hepatitis virus (2,6) and later also in alpha- and betacoronaviruses, while gamma- and deltacoronaviruses induced the formation of zippered endoplasmatic reticulum with tethered double-membrane spherules (1). In has been proposed that DMVs precede CM formation, since the latter has been observed predominantly at later time points after infection (2,4), when viral proteins or polyprotein

fragments accumulate. That is why it has also been proposed that CM are a type of cubic membrane (4). Although these double-membrane vesicles have been well characterized, the mechanism behind their formation remains unclear, including which viral proteins are responsible. Nsp3 has membrane disordering and proliferation ability, both in its full-length form and in a C-terminal-truncated form. nsp3 and nsp4 working together have the ability to pair membranes. nsp6 has membrane proliferation ability as well, inducing perinuclear vesicles localized around the microtubule organizing center. Together, nsp3, nsp4, and nsp6 have the ability to induce double-membrane vesicles that are similar to those observed in SARS coronavirus-infected cells (7).

In the same genera the type and size of membrane structures are likely to be a fundamental requirement for virus replication machinery, but it is not related to the pathogenicity of the genera of viruses. DMVs are induced in both pathogenic and apathogenic virus infected cells. Alterations in efficiency of induction of membrane rearrangements or variations in the type of structures induced by the virus are not a clear indicator for viral pathogenesis. This indication is especially important for drug and vaccine development (8).

3. Mechanisms of DMV formation

Both viral and host cellular factors are thought to be important for the biogenesis of +RNA viral replication organelles. Some of the nsps, encoded in viral RNA, have been proven or predicted to have special transmembrane domains which have been shown to modify the host membrane and form DMVs (*Nidovirales*) (1,2,4,9) Some of the host factors implicated in the membrane modifications include proteins involved in lipid metabolism. It is suspected that this way viruses get enough energy for their replication. (2)

In the context of *nidovirales*-induced DMV formation, two alternatives have been proposed: enwrapping (also known as protrusion and detachment) and double budding (2,4,9). Mechanisms have similar important steps (membrane pairing, fission and induction), with different orders in which these events occur. The enwrapping model begins with membrane pairing of two lipid bilayers in the membrane organelle of the donor cell (in *Nidovirales*, the membrane organelle is most likely endoplasmatic reticulum). The paired membrane then curves in two ways, the outer membrane positively, the inner negatively. This way a double membrane vesicle is sticking out of the lumen of the organelle but is still connected to it. As the curvatures get larger, the opening would get narrower and eventually could be sealed with fission. The inner membrane would be the first to seal, making an inner compartment containing cytosolic material and the outer membrane continuous with the endoplasmatic reticulum (2,4,9).

The second mechanism starts with a negative curvature, giving rise to a single membrane vesicle oriented towards the lumen of the organelle. A second budding event, this time toward the cytosol, would result in DMV oriented toward the cytosol, its outer membrane still connected to the original membrane (2,4,9).

Historically many of the researchers favour the wrapping mechanism although they are not contrary exclusive, mostly because of the presence of membrane pairings and its curvature in electron microscope at earlier hours of infection (1,2,4) In a research conducted in 2016 (on equine arteritis virus) the presence of intermediates of double budding mechanism (SMV in ER lumen, close to the membrane) was confirmed and interpreted as structures that may be waiting to begin the second wrapping (2)

The formation of DMVs is possible because of two fundamental cellular mechanisms: membrane bending and membrane pairing. Regarding curvature, several processes can drive the bending of cellular membranes. This can happen for example by generating asymmetry in the bilayer by differential insertion of proteins or irregularly shaped lipids, oligomerization of proteins that can be integrated into or associated with cellular membranes (2,9,10). It is currently unknown how *Arteriviruses* and coronaviruses accomplish membrane bending but it has been proved that some specific nsps or more importantly their specific formation helps with this (1,2,4,9). These specific nsps have transmembrane-spanning features. They span the membrane multiple times which allows them to have luminal loops (one or more). Some of those luminal loops contain sites for N-linked glycosylation and these are proven to be critical for viral replication. It is currently unknown how their membrane insertion is accomplished. nsps luminal loops are also thought to make the membrane pairing possible (2). In a Nature review article from 2008 (9) it is stated that although studies confirmed that individual viral proteins can promote the formation of membrane modifications in nidovirales and picornavirus families, it is likely that there are also cellular membrane factors involved (9). For example, the components of COPII complex (ER-Golgi transport) were found to colocalize with virus-induced vesicles in the case of PV (*Picornaviridae*) and it was therefore proposed that ER-Golgi transport intermediates might initiate formation of the PV-modified membranes that are involved in RNA replication (9). It is important to state that this has been proven for *Picornaviruses*, and not *Nidovirales*. I

4. Importance of DMVs for viruses

As said before, +RNA viruses are thought to use replication organelles as a form of protection from host's antiviral response and to facilitate new viral RNA replication (2). Replication complexes are part of replication organelles and they are formed from nsps. It has been proven that Mouse hepatitis virus nsps are integral transmembrane proteins of DMVs (6). Because of this, and also considering that most of newly-synthesized viral RNA has been detected near the inner DMV membrane, it is indicated that the replication complex is membrane-bound in DMVs and that DMVs are the sites of viral RNA synthesis (6). One of the first *Nidoviruses* that this was confirmed in was Mouse hepatitis virus (6).

Conclusions

To fully determine if RNA synthesis happens in DMVs, first the function of double stranded dsRNA has to be better understood. Improved microscopic techniques such as electronic microscopy and cryo-microscopy could reveal important facts about the virions. The involvement of the host cell membrane and host metabolism in the formation of newly-

formed virions should be given more attention. A crucial aspect for better understanding virus-inducted replication organelles and possibly for the design of antiviral strategies, is to uncover the mechanisms leading to the formation of their replication organelles.

References

1. Doyle N, Hawes PC, Simpson J, Adam LH, Maier HJ, The Porcine Deltacoronavirus replication organelle comprises double-membrane vesicles and zippered endoplasmic reticulum with double-membrane spherules. Viruses. 2019;11(11):1030. Doi:10.3390/v11111030

2. Van der Hoeven B, Oudshoorn D, Koster AJ, Snijder EJ, Kikkert M, Bárcena M, Biogenisis and arhitecture of arterivirus replication organelles. Virus Res. 2016;220:70-90.doi: 10.1016/j.virusres.2016.04.001

3. Blanchard E, Roingeard P, Virus-induced double-membrane vescicles, Cell Microbiol. 2015 Jan 1; 17 (1): 45-50. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5640787/

4. Oudshoorn D, Rijs K, Limpens RWAL, et al. Expression and cleavage of middle east respiratory syndrome coronavirus nsp3-4 polyprotein induce the formation of double-membrane vesicles that mimic those associated with coronaviral RNA replication. mBio. 2017;8(6):e01658-17. Doi:10.1128/mBio.01658-17

5. Harak C, Lohmann V, Ultrastructure of the replication sites of positive-strand RNA viruses. Virulogy. 2015; 479-480:418-433. Doi: 10.1016/j.virol.2015.02.029

6. Gosert R, Kanjanahaluethai A, Egger D, Bienz K, Baker SC, RNA replication of mouse hepatitis virus takes place at double-membrane vesicles. J Virol. 2002; 76(8):3697-3708. Doi: 10.1128/jvi.76.8.3697-3708.2002

7. Angelini M, Severe Acute Respiratory Syndrome Coronavirus Nonstructural Proteins 3,4 and 6 Induce Double-Membrane Vescicles, mBio, e:00524-13, doi:10.1128/mBio.00524-13

8. Maier HJ, Neuman BW, Bickerton E, Keep SM, Alrashedi H, Hall R, et al, Extensive coronavirus-induced membrane rearrangements are not a determinant of pathogenicity. Scientific Reports. 2016; 6(1). Doi: 10.1038/srep27126

9. Miller S, Krijnse-Locker J. Modification of intracellular membrane structures for virus replication. Nar Rev Microbiol. 2008;6(5):363-374. Doi: 10.1038/nrmicro1890

10. Iglic A, Kralj-Iglic V, Effect of anisotropic properties of membrane constituents on stable shape of membrane bilayer structure. In: Tien HT, Ottova-Leitmannova A, editors. Planar Lipid Bilayers (BLMs) and their Applications. Amsterdam, The Netherlands, Elsevier, 2003, pp. 143–172

Review

MEMBRANE STRUCTURE OF CORONAVIRUSES

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Abstract

Coronaviruses, from the order of *Nidovirales*, are enveloped and spherical viruses that have been noted for having the longest positive-sense RNA genome. Coronaviruses are known to infect birds and mammals, in which they primarily target epithelial cells and are generally associated with gastrointestinal and respiratory infections. Due to the current COVID-19 pandemic in the human population of severe pulmonary disease, caused by a coronavirus, the objective of this article was to present the membrane structure of Coronaviruses and to try to link it to their size and shape in order to better understand their biological function.

1. Introduction

Coronaviruses belong to the Nidovirales order. The most significant differences between the viruses belonging to Nidovirales order are thought to be in the number, type, and sizes of the structural proteins (2). All coronaviruses are pleomorphic RNA viruses which contain characteristically crown-shaped peplomers with 80-160 nm in size and RNA that consists of 27-32 kilobase (kb) pairs with positive polarity (RNA can serve both as genome and messenger RNA, which can be directly translated into proteins) (1). The spikes on the surface of the virion are a defining feature which gives the virion the appearance of solar corona, thus the name coronavirus. Within the lipid envelope of the virion is a helically symmetric nucleocapsid which is uncommon among positive sense RNA viruses (2). As a consequence of different structural proteins there are differences in the structure and morphology of virions (2). It is important to understand the main structural proteins of coronaviruses so we can study the differences between the specific viruses belonging to the same order and hopefully find pan-coronaviruses targets and also specific targets for potential cure or vaccine. In the following review we will focus on the structural proteins that form the virus membrane.

However, before we can discuss membrane proteins of coronaviruses, we must first understand their genome. About two-thirds of the viral RNA encodes viral polymerase (RdRp), RNA synthesis material and two large nonstructural polyproteins (they are not involved in host response modulation, ORF1a-ORF1b) (2). The remaining third of their genome encodes four structural proteins: spike (S), membrane (M), envelope (E), nucleocapsid (N), and other helper proteins (2). The length of the CoV genome indicates high variability for ORF1a/ORF1b and for four structural proteins.

2. Membrane proteins of coronaviruses

The Spike protein (S)

The S protein is a homotrimer and forms the distinctive club-shaped spike structures on the surface of the virus which give the coronaviruses their name. Due to its characteristic structural conformation the protein S belongs to the class I fusion proteins. Its function is to mediate attachment to the host receptor and to enable virus entry into the target cells (2). In most coronaviruses, host cell protease (members of cathepsin family, transmembrane protease serine 2) cleaves the S protein at the S1/S2 and the S2' site forming polypeptide S1 and S2 (1,3). S1 polypeptide becomes the receptor-binding domain and S2 the membrane-fusion subunit through which viral and host membrane fuse.

The S-protein/host receptor interaction determines tissue tropism and range of potential hosts. Some of host receptors for coronaviruses (S proteins) are peptidases (e.g. aminopeptidase N), angiotensin-converting enzyme 2, CEACAM1 (Carcinoembryonic antigen-related cell adhesion molecule 1) and DPP4 (dipeptidyl-peptidase 4). In the infected host cells S proteins that don't

get assembled into new virions mediate cell-cell fusion between infected and adjacent uninfected cells thereby forming multinucleated cells safe from host immune response. The host antibody response is mainly directed towards the S1 domain, whereas S2 domain is generally immunologically silent (2).

The membrane protein (M)

The M protein is the most abundant structural protein of coronaviruses and is thought to give the virion its shape (2). It is suggested that M protein is a dimer with two conformations, *long* and *compact* (M LONG and M COMPACT), inducing membrane curvature and binding to the nucleocapsid (1). Although the M protein is not sufficient for virion formation (for that M and E proteins are needed), it is responsible for the majority of protein-protein interactions required for assembly of coronaviruses (2).The M protein can also bind to the nucleocapsid, and with this interaction promotes the completion of virion assembly (2).

The envelope protein (E)

E proteins are highly divergent among coronaviruses but have a common shape. It was suggested that the E protein has ion channel activity - a viroporin function which facilitates assembly and release of the virus by altering the host secretory pathways (1). A role as an ion channel could be the basis for trafficking of virions in the secretory pathways and for membrane permeability that are essential for virus growth. There are also some indications for a role of the E protein in inducing membrane curvature and preventing M protein aggregation (2). Two topologies – hairpin and transmembrane, have been demonstrated, their relative proportion is thought to affect membrane curvature (1).

The nucleocapsid protein (N)

N protein is the only known protein in the nucleocapsid (2). Two separate domains compose the N protein, a N-terminal domain (NTD) and a C-terminal domain (CTD) (2). In vitro experiments have shown that both these domains can bind to viral RNA by different mechanisms. Optimal binding of the N protein to RNA requires contributions from both domains of the protein - NTD and CTD (2). N protein was found to be heavily phosphorylated (2), which has been suggested to trigger structural changes enhancing the affinity for viral versus non viral RNA (2).

Also N protein binds to nspr3 (key component of the replicase complex) and to M protein (2). These protein interactions are likely helping the viral genome in binding to the replicase-transcriptase complex and subsequently in packaging the encapsidated genome into viral particles (2).

Hemmagglutinin-Esterase (HE)

HE is present in a subset of β-coronaviruses. The protein exhibits hemagglutinin function, binds sialic acid on surface glycoproteins and has acetyl-esterase activity (2). It is presumed that with these activities HE enhances S-protein-mediated cell entry and virus spreading through therespiratory mucosa (2).

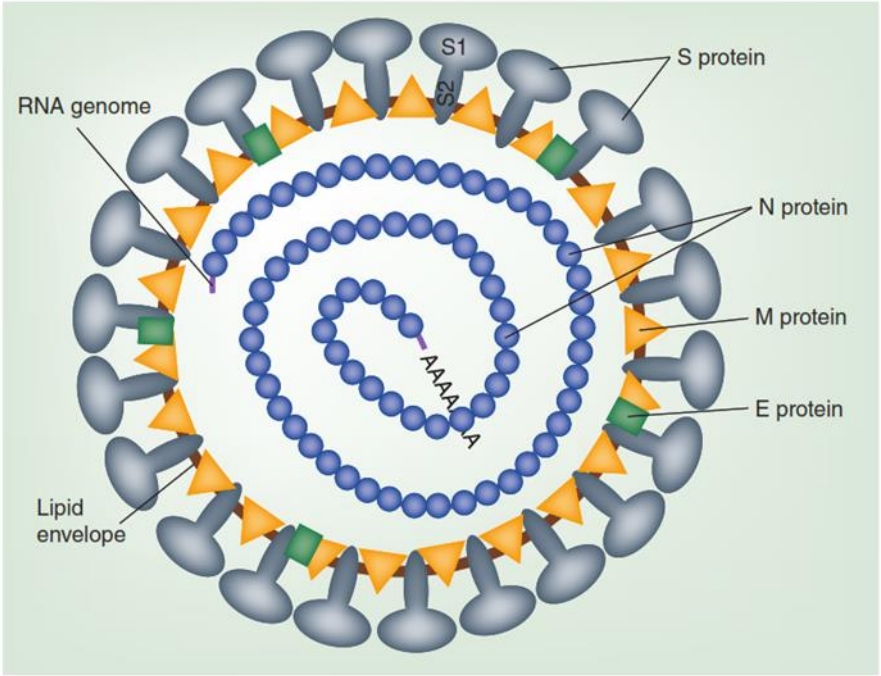

Figure 1. Schematic representation of a coronavirus particle. From (1)

3. SARS-CoV-2 membrane

It is suggested that SARS-CoV-2 is phylogenetically closely related to SARS-CoV (3). Phylogenetic analysis has shown that there is only 12.8% of difference in gene sequences between SARS-CoV-2 and SARS-CoV in S protein and 83.9% similarity in minimal receptor-binding domain (4). Although antigenic analysis proposes great antigenic differences between most of the CTL epitopes of SARS-CoV-2 and SARS-CoV, some of the S protein epitopes were found to be similar. S protein in SARS-CoV-2 (1273 amino acids) was found to be longer for 18 amino acids more than in SARS-CoV (1255 amino acids) (4).
SARS-CoV and SARS-CoV-2 S spike proteins are known to interact with angiotensin-converting enzyme 2 (ACE2). In the S1 subunit (the receptor-binding domain of the SARS-CoV-2 spike (S) protein), changes were observed compared to SARS-CoV that had a significant effect on SARS-CoV-2 spike/ACE2 interaction. The binding energy was reduced. A higher affinity of the SARS-

CoV-2 spike protein to the human ACE2 receptor was suggested which could be the reason for the rapid viral spread in humans (5).
In the receptor-binding domain of the SARS-CoV-2 spike protein (S1) several amino acid substitutions were detected in comparison to SARS-CoV but no significant difference in their structures was noted. The mutations are thought to switch co-receptor use and increase the viral pathogenesis by improving the ability of interaction with the human ACE2 receptor and also the receptor recognition. The reason behind improved ability of interaction could be the presence of two loops around the receptor-binding domain of SARS-CoV-2 (5).
Glycosylation sites present on viral S glycoprotein are thought to affect the internalization of the virus (3). There is evidence that SARS-CoV-2 uses various viral novel glycosylation sites compared to SARS-CoV (4). It was suggested that mutations in the spike protein could have an effect on the tropism of a virus, including new hosts (4).
SARS-CoV-2 E protein has some modifications with respect to homologous CoV E protein:a substitution at position 69, a positively charged Arg replaces negatively charged Glu or Gln or Asp; a deletion in position 70; and a modification at positions 55-56, where Ser-Phe replaces Thr-Val. These changes are considered to affect the protein conformation and protein-protein interactions (6).
Mutation occurring at the N-terminus region, in the ectodomain of M glycoprotein is thought to play a role in the interaction with the host cell (6).

4. Structural proteins and coronavirus virions shape and size relationship

Coronaviruses have characteristically club-shaped spikes, resembling solar coronas, that project from the cell membrane, and the virion shape is spherical, with an average size of 125 nm (2). Studies that considered the shape of the virion to be determined by opposing forces (expanding and collapsing force) indicated that the ground energy state of virions is spherical (7). If we look at virions as a protein-decorated vesicles, we can therefore expect that their shape should be similar to the shape of vesicles unless modified by the effects of viral protein interactions. N protein and genomic RNA were found to affect the virion size and shape variation presumably through interactions with the M protein (2). Coronavirus structural proteins such as E,M, N were found to increase the bending constant of the vesicle membrane and can therefore make vesicles more rigid (7). There are two types of M proteins, long and compact which can differently affect virions characteristics (7). Elongated M protein is associated with rigidity, multiple clusters of spikes and a relatively high range of membrane curvature (7). In contrast, compact M protein is associated with flexibility, low spike density, larger particles size and low curvature (7). Some studies show the importance of spike proteins; spikeless virions were significantly larger suggesting that spike incorporation was linked to the virion size (7).
Researchers have also found that SARS-CoV particles appeared to have clusters of spikes at one or two spots on the viral envelope, where M-long is common, suggesting that M-long mediates

spike incorporation (7). This could also be the reason why compact M is associated with low spike density, due to inefficient S protein incorporation into virus membrane (7). These spike clusters were significantly associated with more curved ends and therefore shaping spheric virions into more of an ellipsoidal structure (i.e. closed surface of which all plane cross sections are either ellipses or circles) (7). Therefore there is some relationship of structural proteins with virions shape and size, however further research is required.

Conclusions

Hitherto research indicates that the prominent proteins of corona viruses are spike (S), membrane (M), envelope (E) and nucleocapsid (N) proteins. Membrane proteins determine the size and shape of the virions, as well as the rigidity of the virion membrane. The structure of the coronavirus membrane is connected to its biological function: structural protein S takes part in internalization of the virus in the host cell and mutations in its shape could affect tropism of the virus, causing its spread to new hosts. However, these conclusions must be validated with additional research.

References

1. Entedar A. J. Alsaadi, Jones I.M., Membrane binding proteins of coronaviruses, Future virology. Vol.14. No.4, Published Online: 29 Apr 2019. doi: 10.2217/fvl-2018-0144

2. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. Methods Mol Biol. 2015;1282:1–23. doi: 10.1007/978-1-4939-2438-7_1

3. Li F. Structure, Function, and Evolution of Coronavirus Spike Proteins. Annu Rev Virol. 2016;3(1):237–261. doi: 10.1146/annurev-virology-110615-042301

4. Kumar, S., Maurya, V.K., Prasad, A.K. et al. Structural, glycosylation and antigenic variation between 2019 novel coronavirus (2019-nCoV) and SARS coronavirus (SARS-CoV). Virus Dis. 31, 13–21 (2020). doi: 10.1007/s13337-020-00571-5

5. Ortega JT, Serrano ML, Pujol FH, Rangel HR. Role of changes in SARS-CoV-2 spike protein in the interaction with the human ACE2 receptor: An in silico analysis. EXCLI J. 2020;19:410–417. Published 2020 Mar 18. doi: 10.17179/excli2020-1167

6. Bianchi, M.; Benvenuto, D.; Giovanetti, M.; Angeletti, S.; Ciccozzi, M.; Pascarella, S. Sars-CoV-2 Envelope and Membrane Proteins: Differences from Closely Related Proteins Linked to Cross-species Transmission? Preprints 2020. Published Online: 7 April 2020. doi: 10.20944/preprints202004.0089.v1

Review

Extracellular vesicles in rheumatoid arthritis

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Abstract

Rheumatoid osteoarthritis (RA) is an inflammatory chronic and systemic disease mostly associated with joints, especially of the hand, although it often affects other body systems such as the skin, its complications can severely damage the lungs and heart. The exact pathogenesis of rheumatoid arthritis is still fairly unknown. Extracellular vesicles (EVs) are submicron cellular fragments which are mobile with body fluids and mediate cell-to-cell communication. EVs carry different substances like proteins, and different RNAs. The scope of this contribution in the potential role of EVs in rheumatoid arthritis.

Introduction

Rheumatoid osteoarthritis is an inflammatory chronic and systemic disease mostly associated with joints, especially of the hand, although it often affects other body systems such as the skin, its complications can severely damage the lungs and heart (1). According to a 2010 study (2), the global prevalence of RA is estimated to be 0,24%, with it being about 2-3 times higher in the US and northern Europe (2). RA is a debilitating disease and presents a heavy burden for individuals as well as a large financial burden for the society (3). Consequently, it is important to understand its pathophysiological properties and enable us to treat it with new, more efficient methods.

Despite acknowledging the role of immune system, in particular T-cells, the exact pathogenesis of RA is still fairly unknown. Recently, increasing attention has been devoted to the role of membrane-enclosed cellular fragments in fundamental physiological and pathophysiological processes. A possibility should therefore be considered that these fragments (called extracellular vesicles (EVs)) can play a role also in development of RA.

EVs were hitherto found to play a role in a cell-to-cell communication including cell proliferation (4). EVs carry different substances such as proteins and different types of RNAs. Pathological processes in the body may affect the EV cargo. There are multiple types of EVs (5). The acknowledged type classification consists mainly of three subgroups according to their size, biogenesis and the cell of origin: microvesicles, apoptotic bodies and exosomes (5).

1. The importance of EVs in the pathogenesis of rheumatoid arthritis

1.1 *Role of EVs in the immune system - antigen presentation and formation of immune complexes*

The cells of the immune response are macrophages, dendritic cells and B lymphocytes (4). Antigen-presenting particles (APPs) and antigen-presenting cells (CPPs) carry molecules that lymphocytes recognize and develop an immune response against them (4). Autoantigens are antigens originating from the organism itself. EVs, in particular those originating from platelets may function as APPs (4). As a response, anti-APP antibodies form immune complexes that stimulate production of leukotrienes by neutrophils (4). These molecules dispose in joints and induce inflammation (4). Moreover, presenting autoantigens by EVs induces immune response against the body's own cells. In the case of rheumatoid arthritis, anti-CPP and anti-APP antibodies against these EV-carrying antigens in the serum can also be useful for diagnostic purposes.

1.2 *Inflammation*

Fibroblast-like synoviocytes are cells that are found in the connective tissue of the joints and are important for the development of RA (4). EVs may function as a cytokine (especially TNF-α) – carriers and cause the inflammation also indirectly (1). TNF-α activates fibroblast-like synoviocytes, more specifficaly, their nuclear transcription factor NF-κB, that promotes an inflammatory response by synthesis of inflammatory mediators, most commonly interleukins IL-6 and IL-8 and activation of IL-1(4). EVs cause the inflammation and hypercoagulability also directly, by stimulating the synthesis of pro-inflammatory mediators like TNF-α and thrombin (4).

An important class of receptors, associated with inflammation in RA, are toll-like receptors (TLR), especially TLR-2 and TLR-4. It was found that activation of TLR-4 by EVs leads to a synthesis of cytokines that promote inflammation (4) and that TLR are activated by EVs of patients with RA to a much larger extent than by EVs of healthy individuals (4). However, EVs also contain anti-inflammatory proteins such as ANXA1 (1).

1.3 *EVs as miRNA carriers*

It was indicated that micro RNA (miRNA) molecules play an important role in the pathogenesis of RA (1). miRNA molecules serve as intercellular and intracellular messengers; their primary role is post-transcriptional gene silencing (1). The roles of several miRNA molecules in RA pathogenesis have been found (1). However, only a small percentage of extracellular miRNAs are packed into EVs (1). miRNAs interact with Argonaut 2 protein (1) - a protein which after binding to a miRNA molecule either inhibits translation or cleaves the target mRNA (6). The two most well-known EV-derived miRNAs involved in RA pathophysiology are miR-155 and miR-146a (4). In patients with RA, both these miRNAs have been found to be up-regulated (4). However, their roles in the process of inflammation are controversial; miR-155 is pro-inflammatory, as it was found to increase the production of TNF-α, as well as of other inflammatory molecules while miR-146a suppresses inflammation by downregulating TNF-α and other inflammatory mediators (4).

4. EV use in RA clinical practice

4.1 *Biomarkers*

Although a few markers (rheumatoid factors, anti-citrullinated peptide antibodies), that can be detected before the onset of symptoms of RA, are already known and used, these clinical markers lack the needed sensitivity and specificity for assessing RA activity. EVs were suggested be very promising biological markers for RA and might become the primary markers in the future (4).

Levels of EVs in isolates from sera were found elevated in RA patients comparing to the healthy controls while the protein and miRNA contents in the respective samples differed (4).

Additionally, some of the RA-specific miRNAs seem to be present in only in specific disease phases (4) and as such could prove very useful in detecting RA earlier and more accurately.

4.2 *EVs as drug delivery vehicles*

EVs are showing some properties which could be used in the treatment of RA: drugs could be encapsulated into EVs, which protect therapeutic agents from enzyme degradation and the immune response. EVs also have the ability to cross biological membranes, including synovial membranes, thus they can deliver the drugs directly into the synovial fluid (7). The current treatment options for RA are systemic and lead to systemic side effects, such as immunosuppression. By delivering drugs directly to the synovial joints, many of drug side effects could be avoided and the efficiency of the current treatment methods could be improved. Additionally, direct application provides an opportunity for new pharmacological agents to be used. As for now, no EV-packed drugs for RA treatment have to our best knowledge been reported, but EV-encapsulated drugs have been used in treatment of other diseases (7). However, other forms of lipid-like nano-cages, such as liposomes, have been used in the treatment of RA and have shown promising results (7). This indicates that EVs could prove useful in treating RA in the future, for which further research is needed.

Conclusions

It seems that EVs play an important role in RA pathology. Firstly, EVs play an important role in the formation of immune complexes: in inflammation they can function as antigen-presenting cells. Secondly, EVs serve as carriers for different molecules, such as cytokines and miRNAs. Their ability of transporting different molecules could also be used for a new way of delivering drugs directly to the joint, thus reducing side effects. Additionally, EVs in patients with RA were found to be different from those found in healthy individuals (1), leading to a belief, that EVs can be used as biomarkers for discovering RA in its earlier stages. It seems that using EVs is a promising approach for improving our understanding of RA, however this remains to be proved.

References:

1. Fu H, Hu D, Zhang L, Tang P. Role of extracellular vesicles in rheumatoid arthritis, Mol Immunol, 2018; 93:125-132; DOI: https://doi.org/10.1016/j.molimm.2017.11.016
2. England BR, Mikuls TR, Epidemiology of, risk factors for, and possible causes of rheumatoid arthritis. (https://www.uptodate.com/contents/epidemiology-of-risk-factors-for-and-possible-causes-of-rheumatoid-arthritis#H1554584259)
3. Cross M, Smith E, Hoy D, Carmona L, Wolfe F, Vos T, et al., The global burden of rheumatoid arthritis: estimates from the global burden of disease 2010 study. BMJ, July 2014 – Volume 73-7 https://ard.bmj.com/content/73/7/1316.long

4. Withrow J, Murphy C, Liu Y, Hunter M, Fulzele S, Hamrick MW. Extracellular vesicles in the pathogenesis of rheumatoid arthritis and osteoarthritis. Arthritis Research & Therapy 2016, 18:286. doi: 10.1186/s13075-016-1178-8
5. Doyle LM, Wang ZM, Overview of extracellular vesicles. Their origin, composition, purpose and methods for exosome isolation and analysis. Cells 2019; 8: 727, doi:10.3390/cells8070727
6. Ender C, Meister G; Argonaute proteins at a glance. J Cell Sci 2010; 123:1819-1823; doi: 10.1242/jcs.055210
7. Sokolov AV, Kostin NN, Ovchinnikova LA, Lomakin YA, Kudriaeva AA. Targeted Drug Delivery in Lipid-like Nanocages and Extracellular Vesicles. Acta Naturae, 2019, 11:28-41 DOI: 10.32607/20758251-2019-11-2-28-41

Reflection

REFLECTIONS ON SCIENCE, TRUTH AND COMMUNICATION DURING COVID-19 EMERGENCY IN ITALY

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Abstract

I have reflected on the aspect of the COVID-19 emergency in Italy as an Italian presently living abroad and observing the Italian media broadcasting from my native country. In particular, the relationship between science, its presentation by the media and limitations posed upon citizens by the state instruments is considered.

1. Public debate has an important role in COVID-19 crisis development and its solution

Due to recent COVID-19 outburst and the concomitant crisis, the relevant issues such as the spread of the virus, and attempts at finding the optimal cure, economic issues generated by the pandemics, various topics and issues on human behavior as well as the public reactions, hopes and prospects , are daily discussed on Italian national TV RAI and Mediaset networks. It's obvious that the most important topic is how to defend against the virus and therefore the TV broadcasted scientific programs in which virologists, epidemiologists, scientists and philosophers discussed various aspects of the pandemics, comparing different ideas and different points of view. However, as observed, the debates at the Italian national TV did not always seem to contribute to clarification of the problems for those waiting to learn how to defend themselves from the disease. It happened that we witnessed the clash of ideas up to verbal attacks and denunciations to the scientific and judicial authorities. For example, Dr. Stefano Montanari, an expert in nanopathologies and scientific popularizer of no-vax movements, was denounced by the official scientific representatives of the association 'Patto Trasversale della Scienza' (1) for having expressed doubts about the spread of the virus and a possible vaccine (2). In addition, the Authority for Guarantees in Communication (AGCOM) required the social networks to obscure all the contents that disseminate scientific information not related to accredited sources (3). Questions can be posed why a discourse that is necessary in scientific approa+ch has been banned, and why the public debates, both political and civil, have been restricted by the authorities that were supposed to assure the freedom of expression granted by the Italian Constitution in art. 21? (4)

Moreover, deliberate release of misleading news (called also 'fake news') or disinformation ('hoax') could occur since the dissemination of information today takes place also through the variegated and poorly controlled world of social media. Fake news and hoax have become fashionable as they offer a certain degree of comfort to those who listen to them. When in fact someone denounces a news as false, 'hoax' or 'fake news', it creates a doubt, that could in turn somehow lead us to the truth. And the truth is comforting, sheltering us from ambiguous interests, from the slippery ground of lies and bad faith. The truth is the truth and there is no question.

But, for an observer, it is difficult to differentiate false informations from true ones if even the true ones are poorly defined. In order to recognize that some informations are false, the true ones should be given being convinced that something is the case (i.e. high certainity). This certainty could be achieved by the results of science supported by ample evidence and explanation.

Scientific results and transmission of their interpretations to the public are great allies within official communication. They delimit the territory of the discursive possibilities in general. It is of interest to observe how these two allies have been performing in the COVID-19 issues.

Many people frequent daily televison encounters where journalists, politicians, authors, directors, opinion-formers, celebrities and scientific communicators express themselves and disseminate information. The public is expected to trust the released information based on the credibility of the broadcasters (journalists, politicians, traditionally accredited broadcasters, etc). These, in turn, often substantiate their own words on the current position of science. The World Health Organization at international level and its Italian counterpart, the Italian Institute of Health, have become important sources of scientific information. It may seem that these organizations have taken the position of indisputable authority and untouchable guarantee of credibility as regards the non-scientists.

An Italian theologian Vito Mancuso has presented an interesting view upon the science and medicine: 'If there were no science, we would know nothing about this disease, nor would we have any idea how to fight it. If anything, it is the idol of science that holds us captive, deluding us of being in the condition to dominate all the forces of nature. Even death.' (5)

However, it seems that the science has not yet acknowledged a relevant explanation of the mechanisms of the disease attributed to the SARS-CoV-2 virus. Evidently, many are searching the solution, and the ways they are undertaking are different. There are differences in opinion regarding scientific hypotheses as well as regarding treatment of the diseased.

2. Search for truth

The history of science is full of reversals, revolutions, clashes, conflicts, debates, differences, abuses. Italians play an important role in this history, because some of the founding fathers of modern science have been protagonists and victims of such conflicts. In 1600, for example, Giordano Bruno, an Italian Dominican friar, philosopher, mathematician, poet, cosmological theorist, was considered by the Church an anti-Christian thinker. He reiterated the infinity of the universe, the multiplicity of worlds, the motion of the Earth. (6) He was brought before the Holy Office where he tenaciously defended his ideas and, after refusing to renounce, was declared an Eritrean and condemned to be burned at the stake.

In the same century Galileo Galilei, a scientist, writer and a poet was a supporter of the heliocentric system enunciated by Copernicus placing the Sun at the center of the universe and planets revolving around it. This disagreed with what was affirmed by the Holy Scriptures and the Gospels. Unlike Giordano Bruno, Galileo Galilei retracted and saved his life. In short: the truths for which some time ago one could be silenced by condemnation to death, are today recognized as completely founded (7).

Figura1. : Giordano Bruno. https://www.museosandomenicomaggiore.it/Il-museo-di-san-domenico-maggiore-ricorda-giordano-bruno/

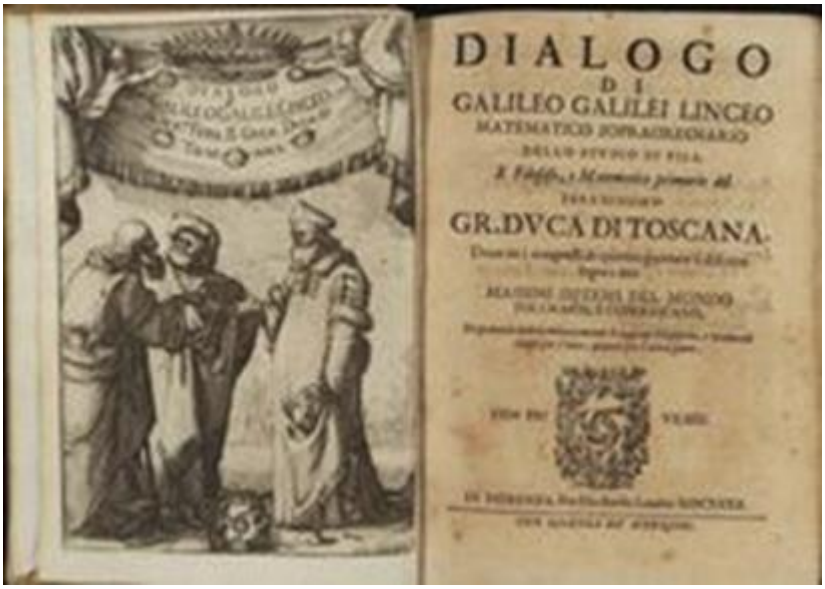

Figure 2. A page from the book of Galileo Galilei: 'Dialogo sopra i due massimi sistemi del mondo' (7).

Are we sure that we know so well how science works? What are the characteristics of scientific ' truth' as stated by a scientist? How can scientific data be taken into account? How should scientific results be interpreted? These are important questions since the answers of certain scientists seem to be dictating the guidelines for government action.

The demand for science to discover an objective truth - a truth residing in objects independent of the observer, a true truth in any case in any time - seems to surpass the possibilities. Accordingly, it was suggested by a philosopher K. Popper (8) that acknowledgement of a scientific theory is temporary. It can be interpreted that the scientific theories are conjectures or provisional hypotheses which will eventually be either recognized by a more general or upgraded theories – or rejected on the basis of new data. He suggested that a theory is not scientific unless it admits the possibility of being rejected, and sooner or later, overcome by some other more convincing theory.

Thomas Khun, a historian of science and epistemologist, argued that science proceeds by stages of evolution and paradigms (set of theories and models providing explanations of reality) that have nothing to do with the unchangeability of an indisputable truth (9). He asserted that scientific paradigms can also be surpassed by others because of the strength of conviction that scientists have, not just scientific theories. Even a theory can surpass another theory because the supporters of the old one have aged and the supporters of the new one, that are younger and more vigorous, are able to assert their arguments in the rhetorical field more effectively.

Werner Heisenberg, for example, one of the founders of quantum mechanics, introduced the principle of uncertainty according to which reality is knowable only up to extent that it is modified by the observer herself/himself in the act of observation. The scientist, therefore, cannot consider an unchangeable and definitive truth because from the moment she/he approaches the object of her/his study, just by approaching it, she/he may modify it (10).

The history of science could also be read as the history of motives and strategies - the history of arguments with which that particular species of human – scientists - with all their imperfections, have succeeded from time to time in persuading others of the usefulness of their research and results.

So much is enough to say that science is not a field in which once a truth has been discovered it can be considered indisputable, but par excellence the place of the debate in which the truth not only can be, but must be questioned, because scientific progress is based on a development of the explanatory models.

If the truth of today were to be considered indisputable we would have to renounce to the search - the same search that has led so many centuries ago to the description that the earth revolves around the sun. I think that science should be a place where no one can tell others that some road is forbidden, but rather the place where all roads can be practicable at least in principle within the ethical constraints.

The line of principle does not always coincide with the line of facts. Scientific research costs a lot, it needs tools with increasingly complex technologies that only large institutions can afford.

So in addition to the logic within scientific discoveries and the history of their alternation (which should already question the idea of 'truth' as definitive and indisputable) there is an equally important problem, that of research funding. Within the funding of scientific research, public or private, there is always someone who decides to allocate it to one or other research laboratory. Now, who decides what research and researchers are to be funded? Political and economic institutions are involved in these processes. More precisely, those institutions that are wealthy enough to afford such funding and that presumably expect a return on investment.

It is reasonable to think, then, that researchers would be encouraged to take the avenues of research more interesting to the funders and not decide to take the roads that would be unproductive - or worse - damaging to the funders. Their work and livelihood are at stake. Thus it is very difficult to speak of "free" research or "pure" research because research is driven by interests and pressures of another nature. Indeed, it is literally made possible thanks to resources that are external to scientific logic in the strict sense. There are only few scientists who can carry out their research in a completely free way, such as for example theoreticians that do not need expensive equipment for their research. Others are hindered either because they lack resources or mechanisms for dissemination of their work.

3. The realibility of the data

Another question concerns the data and their evidence. One could conclude based on the notions of the philosophers (as given above) that certainty is not possible. An abuse can occur by equalizing the inherent uncertainty in scientific discourse with hoaxes and thereby generating chaos. This is at least in the times of health uncertainty the main foundation of difficulty in discourse.

In the specific case of coronavirus, the data that are disseminated are being reported in a time that does not allow the analysis and interpretation including different aspects within the care they deserve; Ilaria Capua, Director of One Health Center - University of Florida has outlined that certain time is needed to envisage the predictive power of the tests (11). Also, the swab tests have limited sensitivity and specificity.

4. The scientific discourse is superior to dogma as it gives more possibilities to find the solution

As after 2 months since the outbreak of the coronavirus disease in Italy a superior scientific hypothesis on the mechanism of the disease has not been acknowledged, it seems reasonable to welcome any evidence – based attempt to resolve the medical and public crisis. It is therefore not favorable to hinder the scientific discourse by authorities for not agreeing with their view on the problem. A "masonic" conception where truth seems to be within the reach,

but to only a few elected (on the basis not always connected to the science), and all others must limit themselves to accepting it as a verb from above, would prevent development of methods that could enable or accelerate solving the problem. However, some elements of such approach could have been detected in public presentations in Italian television while Italy has been the number 1 country in the world as regards the number of deaths assigned to COVID-19 crisis in March 2020.

There exists a possibility of development of science-related rhetoric which is subjected to television salons, where effort is invested to give the impression that it is there that scientific truth is formed. Thus, while the scientific journals will examine the theories which are given - as true or false - and while the courts will examine the legal notices and complaints, the television rhetoric could lead the citizens to perceive as true or false things that have not yet been verified or refuted, in the name of the claims that in the time of a “war”, such rhetoric is necessary.

To unite the forces, in war time, it is convenient to renounce freedoms and prevent spreading of dangerous opinions. Measures have been taken restricting movement and behavior of citizens in many countries all over the world. Our freedom as citizens is being at stake. One may feel as standing on a shaky bridge with water flowing under and can be asking whether there is something which regulates the current flow as well as the citizens. In order to keep us safe, this body accepts the full burden of decisions by taking away the freedom. Perhaps one may feel safer within these measures because that water flows too vigorously and shows corpses floating in the current.

I felt that in Italy the tension and fear by the citizens is very high. Watching on television the scene of the military tanks carrying nightly from the Bergamo city cemetery to the cremation centers the dead due to COVID-19, is an image of anguish and impotence (12).

The description of the plague of 1630 in the book of the ‘I Promessi Sposi’ written in 1847 by Alessandro Manzoni, an Italian writer, poet and novelist, immediately comes to mind. Many analogies described in the novel 'I Promessi Sposi' can be found between the plague epidemic and COVID-19: the same territory of Lombardy-Veneto up to Tuscany; another similarity between the two epidemics lies in the approach taken at the beginning, at a time when the pandemic was not yet mentioned, underestimating the seriousness of the first cases of the plague. The quarantine, which prohibited gatherings, was issued when epidemy was already widespread. The passage of people between cities and regions was blocked, exactly as it was decided recently (13).

Over the centuries, epidemics have been curbed by the same methods, in particular through quarantine and government restrictions, which are correct and necessary to minimise as much as possible the spread of the epidemic.

Unfortunately, we will have this kind of situation, this sword of Damocles, always on us because viruses and bacteria are indebellable. So we need to learn to live with that.

Scientific research has an important role to play in the search for an antidote. All roads are viable for research provided that the ethical code of conduct is respected. I think that the principle of the freedom of research must coexist with that of a "science with conscience" - an inseparable pair when it comes to health.

References

1. Patto Trasversale per la Scienza: https://www.pattoperlascienza.it
2. Interview to Stefano Montanarihttps://www.youtube.com/watch?v=Q5TznJkiGDA
3. AGCOM – Autorità per le garanzie nella comunicazione. Letter for video and social media sharing platforms related to Coronavirus. 21.03.2020 https://www.agcom.it/documentazione
4. Costituzione Italiana. Parte prima diritti e doveri dei cittadini. http://www.governo.it/it/costituzione-italiana/principi-fondamentali/2839
5. Mancuso Vito. Stiamo già cambiando. 12.04.2020. Interview https://www.vitomancuso.it/2020/04/12/stiamo-gia-cambiando/
6. Giordano Bruno. De Immenso et innumerabilibus. Argomento del terzo dialogo.
7. Galileo Galilei. Dialogo sopra i due massimi sistemi del mondo.
8. Popper KR. Il pensiero essenziale. Brani scelti dall'autore come testamento spirituale. Il problema dell'induzione. Armando, Roma, 1998 p.123
9. Kuhn, Thomas S. (1970). The Structure of Scientific Revolutions. Enlarged (2nd ed.). University of Chicago Press. pp. 210. ISBN 978-0-226-45803-8. LCCN 70107472.
10. Heisenberg W. Über den anschaulichen Inhalt der quantentheoretischen Kinematik und Mechanik [On the intuitive content of kinematics and mechanics in quantum theory], in Zeitschrift für Physik, vol. 43, 1927, pp. 172–198, DOI:10.1007/BF01397280
11. Ilaria Capua. Coronavirus: https://www.la7.it/dimartedi/video/coronavirus-la-proposta-della-virologa-ilaria-capua-per-la-ripartenza-21-04-2020-320687
12. LA 7 – 22 marzo 2020 . https://www.youtube.com/watch?v=8sx0zRbHAbM
13. Alessandro Manzoni. I Promessi Sposi. Capitolo XXXI e XXXII . pp 583-624

Reflection

AN EMPOWERMENT PERSPECTIVE ON COVID-19 PANDEMIC

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Abstract

Due to its fascinating complexity, empowerment has gained since its inception a place of honour in a wide variety of contexts, proving to be highly innovative and fruitful, both from the point of view of theoretical evolution and the development of valid application tools. In the 1970s the concept of empowerment revolutionised the theory and practice of health promotion and care as well as the relation between patients and health professionals and managers. Patient empowerment has emerged as a process through which patients acquire competence, in terms of knowledge, attitudes and skills to gain more control on their life and health and to be fully responsible of their recovery process. Last December, the emerging Coronavirus disease 2019 or COVID-19 outbreak caused an unpredictable global health crisis, which has turned in a human, economic and social crisis, strongly affecting people's lives. The pandemic has caused a strong disempowerment at both individual and community level, undermining self-esteem, sense of control and power, and faith in the future. Moreover, the crisis impacted the most vulnerable, the hardest, enhancing existing social and economic disparities. The massive scale and sheer unpredictability of the outbreak has made it extremely challenging for healthcare managers, politicians and governments to respond. Empowerment programs are needed in different contexts to take action and respond to the crisis, starting with the promotion of a culture of prevention, health care and well-being of the entire community. Here we give a brief overview first of the empowerment theory and then of some key aspects of the global crisis caused by the COVID-19 outbreak, highlighting the desirable characteristics that aid programmes should have in an empowerment perspective and focusing on empowerment in health care.

1. The theory of Empowerment

The concept of empowerment refers to the emergence of latent resources and the conscious development of the potential of the individual or community, with the consequent increase in the possibility of having an impact on the social, political and economic context of belonging. The concept developed between the 1950s and 1960s in the United States, within the political language of movements for the emancipation and conquest of the civil rights of women and ethnic minorities. Empowerment represented giving voice and power to those who did not have it, offering a possibility of emancipation from a situation of weakness, passivity and marginalization to become active citizenship, engaged in the social and political context. Since then, empowerment has been widely accepted and used by many psychological, social and scientific disciplines, including medicine and health care. The novelty of empowerment is all played on the positive meaning of the term power. Power is no longer seen as the dominant and manipulative power of the strongest over the weakest, which in turn struggles to upset the balance, but as the creative power to build reality. Therefore, it is no longer a limiting resource for which to compete, but a potential that everyone possesses within and that in some cases has to be activated in order to fully express itself (1).
The development of the theory of empowerment is due to Julian Rappaport and Mark Zimmermann in the context of community psychology. Rappaport was the first to define empowerment as an "intentional process... centred on the local community, involving mutual respect, critical processing, caring and participation in the group" (2). Later, he himself better defined empowerment as a potential for skills that everyone possesses, but that need to be released. This implies that each individual has within him or herself the skills needed to take control of his/her life and improve his/her status, but these skills are not always used to the full (3). Empowerment, therefore, is the process as well as the result of discovery and expression of its own power.

Subsequently, Zimmermann had the great merit of proposing a systematization of the theory of empowerment framing its growing complexity. At the basis of the model there are four fundamental assumptions. First of all, (i) empowerment is a continuous variable, which can be present at different levels; (ii) it is an evolutionary construction, which varies not linearly over time; moreover it depends (iii) on the context, the situation and the particular aspect that is taken into consideration; and (iv) on the characteristics of the population (age, gender, social group, culture) (4,5). In other words, empowerment is neither a stable personality trait, nor a permanent result that once acquired can no longer be lost, nor a dichotomous variable, present or absent, but an extremely dynamic construct. The process of empowerment has been compared to an adventurous and eventful journey, made of commitment, arrests, retreats and shortcuts, but always aimed at increasing the degree of empowerment, whose maximum level can be considered an ideal to strive for and to be inspired by.

Secondly, Zimmermann highlighted three fundamental concepts, that become true milestones of empowerment, that are the control, i.e. the perceived and/or real ability to influence decision-making processes, the critical awareness, that is the knowing and understanding of the socio-political context and the functioning of power structures and, last but not least, the participation, as the tendency to take action to achieve the desired results and to develop strategies for social changes. However, the most significant aspect of its model was the definition of three different levels of empowerment. The first one is the individual or psychological level, which focuses on the individual intrapersonal, interpersonal and behavioural variables, and is accompanied by self-esteem, sense of control on its own life and active participation to social life. The second one is the organizational level, which deals with the mobilization of resources and opportunities for members to participate in groups and organizations. Finally, a third level focuses on social or community level, which addresses the activation of citizens with respect to socio-political structures and the development of their ability to influence social change (5). In this way, Zimmermann made explicit the multidimensional and multilevel nature of empowerment, in which different constructs, both purely psychological and social, converge synergistically.

It is pretty intuitive that individual, organization and community empowerment are interlinked concepts that are distributed along a continuum. As people become empowered, they can work together in groups to create positive changes and to challenge the system, increasing the empowerment of the entire community. Empowered communities for their part are more competent to offer to their members the resources and opportunities to express their potential and to increase their control of their own life and consequently their welfare.

2. Empowerment in health care

In the 1970s the concept of empowerment appeared in the medical and psychotherapeutic literature as a solution to increase the effectiveness and efficiency of clinical and rehabilitative interventions, to successfully manage the consequences of stress due to the pathology both in patients and caregivers, and more generally to promote self-diagnosis, prevention and health promotion. Since the World Health Organization (WHO) published the Alma Ata Declaration in 1978 and subsequently the Ottawa Charter for Health Promotion in 1986, the concept of empowerment has become a central element in the theory and practice of health promotion and care. Until then, healthcare professionals' long tradition of making decisions for the patients was based on a belief that they knew what was best for patients. According to the empowerment theory, instead, patients are, besides professionals, experts on their own bodies, symptoms and situation and, thus, should be treated as a partner in healthcare with both rights and responsibilities. Patient knowledge and active participation is necessary to succeed in treatment (6).

Within the field of healthcare, the concept of empowerment has been used on two levels. First, it has been used more in general to describe a relationship between health and power, based on the assumption that individuals who are empowered are healthier than those who are not. Empirical evidence has generally supported the existence of an inverse relationship between social stress and health, with income and social status indicators positively associated with health status, whereas chronic stressors including poverty and racism are associated with increased morbidity and mortality. In short, those who are empowered are healthier than those who are not: powerlessness is a broad-based risk factor for disease (7).

Second, at level of patients, empowerment has been referred to a process by which they come to recognize their dignity and their abilities, to have confidence in themselves and in the future, over which they feel to have an influence, and are therefore engaged in the cure and maintenance of their health. Through personal empowerment, patients learn how to leave the passive attitude of dependence towards the professional reference figures (e.g. the doctor, the psychotherapist, the nurse), to whom decisions and responsibility have been delegated, in order to become co-responsible of their own healing process. A successful empowerment process can occur when patients come to terms with their threatened sense of security and identity and develop a renewed and valuable sense of the self, together with a sense of mastery and control on its own life and responsibility regarding its own health. Moreover, active patients would manage self-care better, thereby easing the economic constraints on the healthcare sector and having an impact not just on their own health but on the overall society. Besides the individual component, there is also a collective patient empowerment as a process that gives groups the power to express their needs and take action to meet those needs and improve their quality of life (8). According to the deeper empowerment philosophy to be healthy, people must be able to bring about changes, not only in their personal behaviour, but also in their social situations and the organisations that influence their lives (6).

Health care providers can support the process by putting their professional competence at the service of patients as well as acting as health coaches helping patients to take in charge their healing process, and, if necessary, to develop changes in their habits and life-style. Healthcare providers can give detailed and clear information to patients regarding their disease, the impact on their life, the therapeutic approaches available, and the benefits and disadvantages of each one. They can also give important information about prevention and healthy habits. Moreover, through a caring and trustful relationship, healthcare professionals have the delicate role to sustain, without substituting to, patients to develop competence, not only in terms of knowledge, but also of skills and attitudes apt to gain greater control over decisions and actions affecting their health and overall well-being. Through interactions with health professionals and also with their peers, patients can develop new perspectives by reframing and reinterpreting their illness, which in turn leads to better adjustment to their long-term condition (8).

Finally, healthcare managers, politicians and governments should allocate resources and services and take action to enhance patient empowerment at different levels, starting with the promotion of a culture of prevention, health care and well-being at level of the entire community. Approaches to improve equity of services, reduce institutional barriers, enhance participation in local government, strengthen civil society associations and create healthy public policies can also concur to improve community health (9). For the development of effective empowerment promoting interventions, adequate study designs, methods, and indicators for assessing empowerment are essential. Besides indicators reflecting individual and psychological constructs, indicators for the organizational and political components of empowerment should also be considered (10). Standard and guidelines for high-quality evaluations of empowerment will be fundamental to measure adequately this complex construct in the different contexts of application and progressively identify the best roadmap to follow.

3. The COVID-19 pandemic and its disempowering implications

In December 2019, a new infectious respiratory disease emerged in Wuhan, Hubei province, China and rapidly diffused overall the world. A new class of Coronavirus, known as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has been found to be responsible for occurrence of this disease, named by WHO as Coronavirus disease 2019 or COVID-19. Since the first human coronavirus detected in the 1960s, SARS-CoV-2 is the seventh coronavirus that is known to infect humans; four of them cause mild illness, while the other three, SARS-CoV, MERS-CoV and SARS-CoV2 can lead to extremely serious disease(11,12).

As far as the history of human civilization is concerned, there are instances of severe outbreaks of diseases caused by a number of viruses, but the COVID-19, which has been characterized as a pandemic by WHO, has the hallmarks of a “landscape scale” crisis. An unexpected event or sequence of events of enormous scale and overwhelming speed, that caused an unprecedent global health crisis, that was managed in a very unprecedent way, that most if not all of us would think impossible. COVID-19 patients and their parents have been experienced a really heavy situation, in which rules on isolation and physical distance to limit contagion has made impossible relations and has not allowed to mitigate disease and loss with love and tenderness. The death at the time of COVID-19 has been so crude. Moreover, hospital staff has been exposed to a strong stress, not just for the risk of contagion, but also for the great commitment in curing COVID-19 patients. Above all, health professionals experienced a great sense of impotence towards an unknown and potential lethal disease and the consciousness to be the only ones that could give solace to isolated and lonely patients.

Now the situation in most countries seems better, in terms of both contagiousness and severity of the disease, an evidence that may contribute for some aspects to disorient us even more, if we compare it with all that we have just lived. What we are now living is much more than a

health crisis, and it is attacking societies at their core, causing a human, economic and social crisis, that is spreading human suffering and upending people's lives.

Several countries have been taking a phased response to managing the coronavirus outbreak, with each phase having implications for actions that healthcare professionals and members of the public are expected to take. The initial phase focused on containing the outbreak. In order to contain the virus, public health teams were committed in identifying, tracing and monitoring those who were known to have come into close contact with anyone who has tested positive for coronavirus, or who had been in an affected area. Part of this process has included asking those who have travelled back from affected areas to undergo supported isolation at specialist facilities or to self-isolate at home for 14 days.

However, as the scale of the outbreak grew, the response moved from the contain phase to a delay phase, in which many countries decided for a more or less complete lock-down of most activities, including educational, industrial and commercial ones. Governments asked to the entire population to stay at home, limiting movements only to the essential activities and to local sites, almost completely avoiding social relations. With lockdowns, schools and social services were suspended, and more people working from home, home became the workplace and classroom for several family members at the same time. In many cases this has placed an extra care burden on households, usually women, that were coping with extraordinary circumstances, competing priorities and increased pressures and might jeopardize focus, effectiveness and productivity for all (13). During such a crisis, people in most countries of the world experienced loss of control on their own life and reality, loss of their freedom to live, work and have social relationships, in summary loss of the possibility to make decisions about their health and life. Moreover, self-isolation may result in considerable stress, especially if the duration is long and uncertain, there are pre-existing mental health conditions, low autonomy, need of clinical treatments, poor access to basic supplies (e.g. food, water, home space), financial loss, and stigma around the disease. To complicate the scenario, the lack of clear, firm and supporting information, and the climate of panic created by mass media. In the following phase, people were allowed to go out and restart slowly normal activities, always wearing masks and in the respect of the social distancing and hygiene rules. In this delicate restart phase of coexistence with the virus and the fear, increasingly divergent positions of political leaders as well as scientific committees and scientific experts has created great uncertainty, elevated stress and anxiety, and prompted tunnel vision, with poor faith in the future.

In a word, people, even the ones that always perceived themselves pretty powerful and influent, have been feeling pretty disempowered, insecure and at the mercy of something unknown and potentially lethal and in some cases of leaders unprepared to face the complex situation and indicate the right direction.

The disempowerment has been even stronger for those individuals who belong to social groups in the most vulnerable situations, including people living in poverty, older persons, persons with disabilities, chronical pathologies, mental health conditions or psychological problems. Early evidence indicates that the health and economic impacts of the virus are being borne disproportionately by poor people, refugees, migrants, or displaced persons (14). Moreover, even though emerging numbers indicate that the COVID-19 may be more lethal for men, women are those taking the bigger socio-economic hit from the global pandemic. Among the reasons for this, there is the overrepresentation of women in precarious employment, including the informal sector, where their benefits and protection are inadequate or lacking. In addition, women make up the majority of front-line workers in health care and other professions that are based on personal interaction, exposing them to higher risks of contagion (13,15). Increasing evidence is pointing out to several indirect effects on COVID-19 pandemic outbreak in a plethora of different contexts. Among them the increment of death caused to other diseases, especially heart failure, due to limited access to hospitals, increase of children domestics accidents and heightened risk of domestic violence and sexual abuse (13,15). In conclusion, the societal and economic consequences are hitting all populations around the world, giving a strong and overall shaking to the perception of power and control that human beings have on their own life. Moreover, the crisis has impacted the most vulnerable, the hardest, exacerbating existing social and economic inequalities.

4. An empowerment perspective to efficiently deal with the crisis

The pandemic has caused a strong disempowerment at both an individual and a community level. At an individual level, COVID-19 pandemic has affected self-esteem, sense of control and power, and faith in personal abilities and possibilities to have an impact on the reality and the future. At a level of community, pandemic has made evident that human beings, despite their scientific, social, cultural and technological evolution, might be extremely vulnerable to natural events.

It has greatly impacted on the ability to obtain data, interpret the reality and know the problem as well as on the power, both perceived and actual, to find the better solution and take the necessary actions to solve the issues. The massive scale of the outbreak and its sheer unpredictability has made it challenging for politicians, leaders, and health managers to respond. Empowerment programs are needed at different levels to respond to the crisis and desirably construct a new equilibrium better than the previous one, more sustainable and equitable.

The novelty of empowerment interventions upset traditional practices of aid and support to third parties. Developing projects from an empowerment perspective means no longer focusing on the problem, the disease, the lack, but on the resources, strengths and at the same time the desires of people in disadvantaged conditions, such as bad health or economic troubles.

Focusing on the potential instead of the difficulty of the subject, like a Copernican revolution, determines a reversal of theoretical perspective and development of approaches. The objectives of the intervention change, no longer consists in curing, restoring, healing the negative aspects, but rather in strengthening the positive ones, promoting well-being and ultimately creating something new, more in line with the identity, characteristics and aspirations of the subject (individuals, groups and communities) (1). As a consequence, the role of the figures, such as doctors, nurses, operators, volunteers and in general caregivers involved in the aid intervention changes profoundly. There are no longer those who have power and those who do not, those who offer knowledge, support, resources, solutions and those who passively receive them, but the relation between disempowered subjects and operators is more equilibrate and symmetric. In such relation the dialogue is fundamental. For the empowerment of people facing a problem, information and communication are the golden key and the basis for an active participation to the solution of the problem. First of all, the information that is exchanged has to be meaningful, understandable and possibly individually adapted

Different forms of information can help listeners to stay safe, cope mentally, and connect to a deeper sense of purpose and stability. Formal structures and processes, such as guidelines, similarly need to be developed and institutionalized to provide ongoing, systematic opportunities for disempowered subjects to have information and participate in decision making (7). However, communication should lead not just to a simple, transference but to the co-creation of knowledge in which empowering people are strongly involved.

In the field of healthcare, as example, communication between health professionals and patients should favour subsequent decision making, helping patients in being aware of the importance of their role in self-management of their own health and overall quality of life. On the other way, health professionals would take great advantage from the dialogue with patients, becoming more competent in deeply understanding their needs and difficulties, finding with them the better therapeutic options, developing personalised and more effective solutions and, finally, increasing their experience and competence in the treatment of such type of patients.

According to the theory of double empowerment, to reach the objectives of an intervention, operators involved in promoting the empowerment of others, need to have empowerment opportunities themselves. Piccardo and Martini, thanks to the transdisciplinary analysis of various bibliographic sources and to the critical analysis of different experiences, highlighted how caring for others can be effective and last only "as long as one takes care of oneself and

receives in turn care from those close to one another"(16). The two authors also underlined the risk inherent in care-giving interventions, in which the operators could respond in a more or less unconscious way to their need to feel indispensable, irreplaceable and therefore capable, ending up reinforcing the sense of passivity and loose of control in the disempowered subjects.

As a consequence, the development and/or reinforcing of bonds of dependence may end up harming the recipients of the intervention as well as the operators themselves, resulting, paradoxically, in the establishment of a chronical disempowered attitude in both actors and recipients of the aid. On the contrary, in order to genuinely help the other to rediscover its own potential, the caregiver should first of all be engaged in a process of self-development and self-empowerment, taking advantage of the opportunities given by the context and the organization (16).

First, as previously discussed, empowerment opportunities for operators come directly from the recipients of the intervention, establishing a horizontal, symmetrical and reciprocal relationship in which all the actors are mutually involved in a supporting relationship and receive benefits from it. Second, these opportunities can come from the organization or community of which they are part, through enlightened leadership at the service of their members, training activities, exchanges of views, sharing of responsibilities and cooperation among peers. In health care, the education and support for patients and also health professional are crucial to a successful participation process as both groups need to have the right skills, knowledge and attitudes. A facilitating management and supportive care environment should be put in place and sufficient time and financial resources should be allocated. Double empowerment is not only an expression of the coherence between the goals of the organization or community and the path to pursue them, but it is crucial to the real achievement of those goals. For example, several additional benefits have been described for hospitals that promote nursing power. Hospitals that allowed their staff autonomy over their own practice and active participation in decision making about patient care issues were the most successful in recruiting and retaining nurses. Patient satisfaction has also been shown to improve when there was more organizational control by staff nurses. Empowerment for nurses may consist of different components, including a workplace that has the requisite structures to promote empowerment, the acknowledgement that there is power in the relationships and caring that nurses provide and, above all, a psychological belief in one's ability to be empowered. A more thorough understanding of these three components may help nurses to become empowered and use their power for better patient care (17).

Conclusion

The relationship theorised by double empowerment may resemble also the socratic method, in which cooperative argumentative dialogue between individuals stimulates critical thinking, drawing out and comparison of ideas thorough hypothesis elimination and logical tests, with the final aim to help a person or group to discover their own beliefs about a specific issues.

In the case of empowerment, the final outcome of the process is the discovery and the expression of power, as a person, a group and/or a community. With the advent of Covid-19 pandemic, all of us have been experiencing to be strongly interconnected and that, to

efficiently address global crisis, a global intervention is needed. As the strength of the chain is given by the strength of the weaker link, in the same way the strength, the power and the level of maturity of a society is measured by the level of strength and power of the most vulnerable subjects. Even though in a short range of time powerful subjects or countries will be less hit from the crisis, at a longer term the increasing disempowerment of the weaker subjects, communities, and countries will impact the overall society. So it is not just a sign of solidarity, but also of intelligence to support the empowerment of the more disadvantageous categories to enhance the level of empowerment of the entire society, and in a more general way of all human beings, as well as its resilience and its capabilities to overpass crisis, develop new potentials, new scenarios and evolve.

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References

1. G.L. Liguori, Le molte facce dell'empowerment: esperienze di vita, missione, contrasti e libertà nel mondo del no profit [The many facets of empowerment: experiences of life, mission, contrasts and freedom in the world of non-profit], 2019. Thesis in Psychological Science and Techniques, University Suor Orsola Benincasa, Naples.

2. J. Rappaport, In praise of paradox: A social policy of empowerment over prevention, Am. J. Community Psychol. (1981). https://doi.org/10.1007/BF00896357.

3. J. Rappaport, Terms of empowerment/exemplars of prevention: Toward a theory for community psychology, Am. J. Community Psychol. (1987). https://doi.org/10.1007/BF00919275.

4. D.D. Perkins, M.A. Zimmerman, Empowerment theory, research, and application, Am. J. Community Psychol. (1995). https://doi.org/10.1007/BF02506982.

5. M.A. Zimmerman, Empowerment Theory, in: Handb. Community Psychol., 2000. https://doi.org/10.1007/978-1-4615-4193-6_2.

6. I. Holmström, M. Röing, The relation between patient-centeredness and patient empowerment: A discussion on concepts, Patient Educ. Couns. 79 (2010) 167–172. https://doi.org/10.1016/j.pec.2009.08.008.

7. K.J. Roberts, Patient empowerment in the United States: a critical commentary, n.d.

8. E.M. Castro, T. Van Regenmortel, K. Vanhaecht, W. Sermeus, A. Van Hecke, Patient empowerment, patient participation and patient-centeredness in hospital care: A concept analysis based on a literature review, Patient Educ. Couns. 99 (2016) 1923–1939. https://doi.org/10.1016/j.pec.2016.07.026.

9. D. Wallerstein, What is the evidence on effectiveness of empowerment to improve health (Health Evidence Network report);, (2006). http://www.euro.who.int/Document/E88086.pdf.

10. V. Lindacher, J. Curbach, B. Warrelmann, S. Brandstetter, J. Loss, Evaluation of Empowerment in Health Promotion Interventions: A Systematic Review, Eval. Heal. Prof. 41 (2018) 351–392. https://doi.org/10.1177/0163278716688065.

11. J. Cui, F. Li, Z.L. Shi, Origin and evolution of pathogenic coronaviruses, Nat. Rev. Microbiol. (2019). https://doi.org/10.1038/s41579-018-0118-9.

12. Y.R. Guo, Q.D. Cao, Z.S. Hong, Y.Y. Tan, S.D. Chen, H.J. Jin, K. Sen Tan, D.Y. Wang, Y. Yan, The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak - an update on the status, Mil. Med. Res. 7 (2020) 11. https://doi.org/10.1186/s40779-020-00240-0.

13. UNWOMEN, Women as a force for accelerated and inclusive economic recovery post COVID-19 in Asia and the Pacific., 1–6.

14. Committee for the Coordination of Statistical Activities (CCSA), How COVID-19 is changing the world : a statistical perspective, (2020) 1--90. https://unstats.un.org/unsd/ccsa/%0Ahttps://unstats.un.org/unsd/ccsa/documents/covid19-report-ccsa.pdf.

15. C. Wenham, J. Smith, R. Morgan, COVID-19: the gendered impacts of the outbreak, Lancet. (2020). https://doi.org/10.1016/S0140-6736(20)30526-2.

16. C. Piccado, M. Martini, Il doppio empowerment. Il lavoro di cura delle donne nelle imprese di servizio e il pieno sviluppo delle potenzialità di chi è destinatario della cura e di chi la eroga [The double empowerment. The work of caring for women in service enterprises and the f, Svilupp. Organ. 205 (2004) 19–39.

17. M. Manojlovich, Power and Empowerment in Nursing, (2007). https://doi.org/10.3912/OJIN.Vol12No01Man01.

Reflection

COPING WITH STRESS DURING COVID-19

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Abstract

Stress is the body's reaction to stimuli from the environment. It is a state of tension of the organism in which the defense is triggered, while the organism is faced with a threatening circumstance. This is followed by a non-specific reaction of the organism, which leads to “fight or flight” reaction. The latest threat to global health is the ongoing outbreak of the respiratory disease that was given the name Coronavirus Disease 2019 (COVID-19) which was recognized in December 2019 and quickly became characterized as a global pandemic by March of 2020. Coronavirus-19 (COVID-19) is transforming every aspect of our lives. Altough large numbers of people troughout the world will show resilence to the profound loss, stress, and fear associated with Covid-19, the virus will likely exacerbate existing mental health disorders and contribute to the onset of new stress-related disorders for many.

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1. Introduction

Stress is a physiological, psychological and behavioural response of an individual who tries to adapt and get used to internal and external pressures, i.e. stressors. A stressor is an event, situation, person or object that an individual experiences as a stress element and results in stress. The stressor temporarily upsets the balance of the individual (1).

Stress is the body's response when the balance is temporarily disturbed, both physically and mentally, due to internal or external influences called stressors. Stress is a normal and expected response. Negative and positive stress is a daily and completely normal challenge for an individual in the developed world, both in the adult and younger population (1).

A certain amount of stress is necessary for a normal life. Problems arise when there are too many stressors, they are too frequent, too strong or last too long, and when an individual does not have good coping strategies. In this case, stress can even lead to illness, including depression and anxiety (1).

Stress and stressors are not equally experienced by all people or equally difficult to solve, because they are determined by the individual's personality, his experience, energy equipment, the circumstances in which they occur and the wider and narrower environment in which the person lives. The life orientation of the individual as well as the strength and quality of interpersonal relationships with the people around us are also important. This means that a certain event can be a stressor for some, while for others not that much. When we encounter a stressful situation, a distress signal comes into the hypothalamus, which is the part of the brain that functions as the main controlling organ of our body. From there signals are sent forward to the body and internal organs as a warning to prepare for “fight or flight” (1).

2. Bio-psychological basis of a stress response

The amygdala, hippocampus, and ventromedial region of the prefrontal cortex are part of the neural connections in the brain that regulate stress responses. The signals travel along a stress axis, namely the hypothalamus-pituitary-adrenal gland, and activate the autonomic nervous system, which regulates the body’s response to stressful situations (2).

The amygdala is also called the survival center because it triggers a “fight or flight” reaction by quickly assessing the gravity of the situation, alerting the brainstem, and activating the stress axis and sympathetic nervous system that prepares the body to respond to danger (2).

The hippocampus begins to function only between the second and third year of life, although connections between it and the amygdala are already being built from birth. Its task is to regulate information about external stimuli and, according to the circumstances, regulate the uncontrolled responses of the amygdala so that it does not unnecessarily trigger a stress

response. When the hippocampus begins to function, memories also begin to be stored with its help, so that their contents are accessible to recall, so that events can be consciously remembered and also expressed, which we call explicit memory. Thus, the hippocampus allows us to shape experiences and recognize different contexts. It makes sure that we respond to each danger differently, according to the circumstances. For example, the response calms down when the prefrontal cortex recognizes that there is a rope in front of us and not a snake (excluded from the proverb: "Whoever was bitten by a snake is afraid of a twisted rope."). Once it becomes clear that the stimulus is harmless, the parasympathetic nervous system is involved, which inhibits the vagus nerve, allowing the body's responses to be calmed (2).

The prefrontal cortex gives meaning to experiences and organizes our interaction with the world. It is the organ of memory and foresight, while also the seat of cognitive, rational control of experience and behavior. By recognizing the differences between a "rope" and a "snake," it directs the response of the lower centers. The ventromedial part of the prefrontal cortex controls the functions of the amygdala and thus regulates the emotional response, while the dorsolateral part is associated with the hippocampus. The latter allows for conscious thinking about the experience and directs attention to perceptions, memories, or individual ideas. The orbitofrontal cortex allows the recognition of emotional signals. In contrast to the amygdala, it also interprets them, distinguishing between "rope" and "snake," thus modulating amygdala responses (2).

The stress axis or hypothalamic-pituitary-adrenal axis regulates the body's stress response. The stress axis activates the amygdala in response to danger. The sympathetic part of the autonomic nervous system is immediately activated, which responds to the danger by stimulating physical arousal and by secreting cortisol (2).

3. The "fight or flight" reaction

The "fight or flight" reaction is a signal that we are in danger. A quick reaction that involves the functioning of the sympathetic nervous system and the inhibition of the parasympathetic nervous system. Emotional, chemical and physiological changes occur in the body, manifested in rapid heartbeat, rapid, shallow and irregular breathing, tense muscles, cold palms and feet, stomach problems, feelings of fear and threat (1). The effects of the sympathetic nervous system are immediate, affect the whole body and are long lasting. We also call it the short-term survival system (2). People are different and so not all have the same symptoms. Symptoms of a stress response develop within minutes of a stressful event and last from a few hours to a few days (1).

The parasympathetic nervous system, on the other hand, works in the opposite way, reducing the heart rate, dilating blood vessels and speeding up digestive processes (1). The parasympathetic nervous system is associated with a low level of activation, namely it

promotes rest and regeneration. We also call it the long-term survival system. The parasympathetic nervous system is activated more slowly than the sympathetic nervous system, and its effects are not as intense (2). These two nervous systems are usually in balance, but when we find ourselves in a stressful situation, this balance is disrupted and the sympathetic system prevails (1).

In extreme circumstances, when a person feels that he is in great danger, in a hopeless situation or even in a life-threatening situation, acute activation of the parasympathetic nervous system can lead to the whole body blocking (2).

Table 1: Symptoms and signs of stress. From (1).

BODY SYMPTOMS	BEHAVIOR	MOOD CHANGES	CHANGES IN THINKING	THOUGHT CONTENT
accelerated heartbeat headache sweaty hands indigestion fast, shallow and inconsistent breathing cold hands and feet irritated stomach sickness diarrhea muscle cramps pain	lack of will disorganization weeping insomnia or too much sleep	lack of determination no sense of humor tension nervousness depression irritability impatience anger aggressiveness	forgetfulness decreased concentration poor judgement mathemathical mistakes difficulty thinking fantasy life	I can't do this! something terrible is happening to me! it's verry hard! I don't feel good! I'm going insane! it's too much! poor self-esteem

In a stressful situation, we respond with our body, different thoughts, emotions and behavior, as it is shown in Table 1. Relaxation techniques are very helpful in overcoming stress and can be very effective if performed regularly, as they work similarly to regular physical exercise, and as long as we perform them, the effects are visible. Otherwise, the effects disappear (1).

As soon as we assess that the situation is no longer dangerous, we experience the “relaxation reaction” that includes slower breathing and a calm heartbeat, as well as a feeling of comfort. People who develop a “relaxation reaction” and other ways of managing stress early do not feel so helpless and have more choices while responding to stress (1).

4. Stress during the COVID-19 pandemic

Research and clinical observations suggest that during times of pandemic many people exhibit stress related responses. In the research study by Steven Taylor et al. (3) they developed the 36-item COVID Stress Scales (CSS) to measure these features, as they pertain to COVID-19. The CSS were developed to understand and assess COVID-19 related distress. They were developed and initially validated in population-representative samples from Canada and the United states. A stable 5-factor solution was identified, corresponding to scales assessing COVID-related stress symptoms: 1. Danger and contamination fears, 2. Fears about economic consequences, 3. Xenophobia, 4. Compulsive checking and reassurance seeking, 5. Traumatic stress symptoms about COVID-19.

The scales performed well on various indices of reliability and validity and they were intercorrelated, providing evidence of COVID Stress Syndrome. The scales offer promise as tools for better understanding the distress associated with COVID-19 and for identifying people in need of mental health services (3). The results from the research study by Steven Taylor et al. (3) indicate that 28% of general population sample had elevated anxiety and 22% were experiencing clinicali significant depressive symptoms. Fot the total PHQ-4 scale (depression and anxiety), the proportions were as follows, as based on cutoffs reported by Kroenke et al.: normal (54%), mild symptoms (23%), moderate symptoms (13%) and severe symptoms (10%). These findings are consistent with studies of responses to trauma (e.g., earthquakes, fires, floods), which show that most people are resilient to stress, altough a significant minority are prone to experinece stress-related psichopathology (11). These findings are also consistent with the emerging findings from China, which suggest that more than 25% of the general population experienced moderate to severe levels of stress-related symptoms in response to COVID-19 (4, 5). They are also similar to those reported during the SARS outbreak (6) and in the 2009 H1N1 pandemic (7, 8).

The CSS can also be used in studies to predict which people are most likely to engage in safety behaviors, like hygiene behaviors, social distancing, and the uptake of a vaccine, when one becomes available. (3)

Studies of previous epidemics and pandemics show that anxiety, or the lack thereof, is an important driver of behaviour (9). People with too litle anxiety about a viral outbreak are less likely to engage in hygiene behaviours (e.g. handwashing), less likely to adhere to physical distancing mandates and are less likely to get vaccinated if a vaccine is available (9). On the

other hand, people with excessive anxiety are more likely to engage in socially disruptive behaviours, such as panic buying and surging unnecessarily into hospitals and clinics when they misinterpret their minor aliments as signs of serious infection. (10)

Given the role that anxiety plays in shaping behavioral responses to viral outbreaks, both behaviors that can mitigate as well as those that can facilitate the spread of infection, it is critical that public health decision-makers, health officials, and health care providers understand the nature and degree of adverse psychological responses to the current COVID-19 crisis.

Research and clinical observations (9) suggest that during times of pandemic many people exhibit fear and anxiety-related distress responses that include the following: fear of becoming infected, fear of coming into contact with possibly contaminated objects or surfaces, fear of foreigners who might be carrying infection, fear of the socio-economic consequences of the pandemic, compulsive checking and reassurance-seeking regarding possible pandemic-related threats, and traumatic stress symptoms about the pandemic (e.g. nightmares, intrusive thoughts).

It is anticipated that when this pandemic passes, significant mental health needs will emerge in the public. These predictions are based on prior pandemics, where anxiety, depression, and traumatic reactions were observed (such as following quarantine due to SARS). (9, 12) Accordingly, the development of a pandemic-specific measure such as the CSS can serve to aid in identifying individuals at risk for adverse emotional reactions both during and post-pandemic. (3)

5. How to cope with stress

One of the important strategies for coping with stress, which helps us to achieve greater control over events and thus greater resilience to stress, is also planning our response. Some stressful situations can be predicted in advance and so we can prepare for our possible responses. (1)

A balanced diet, regular physical exercise and sleep quality also have a major impact on an individual's resistance to stress. Regular physical exercise has a beneficial effect on our well-being, as during exercise the “happiness hormones” are produced, which strengthens our resistance to stress. (1)

In addition, it is necessary to cultivate positive thoughts about oneself and regularly use positive encouragement. It is important to remember the qualities and abilities that an individual has. Life planning also works preventively. It is especially important not to forget about stimulating activities. It is important to maintain a balance between the things that need

to be done and between those things that we want and want to do. The harmonious distribution of stress and relaxation in life is important. (1)

Psychotherapy is also effective in overcoming stress. Research done by magnetic resonance imaging has confirmed that in long-term (process) therapies, the structure of the brain actually changes. This permanently increases the ability to calm down, alleviate anxiety and helps to better regulate emotions as a whole, as well as changing the way you look at others and at yourself. (2)

Medications are also important in controlling stress. They help by providing substances called neurotransmitters that affect the connections between brain cells and thus alter sensation and experience. They only work as long as we take them, but they do not contribute to the formation of new connections, as psychotherapy does. This is why their effects, unlike long-term psychotherapy, are not permanent. (2)

6. Conclusion

For today, strong and short-term stress is not as dangerous as the stress we are exposed to every day, about which we are sometimes not even aware of. It's important to know that a pinch of stress doesn't hurt, but it can instead spice up life. Of course, if there isn't too much of it and if we can control it. A certain amount of stress helps us cope with life and it is also an integral part of our lives, which we cannot escape. We need to learn to overcome and master it.

References

1. Selye, H. (1950). Stress. Montreal: Acta, 1955.

2. Lambert, M. J. (1979). Psychotherapy outcome research.

3. Taylor, S., Landry, C., Paluszek, M., Fergus, T. A., McKay, D., & Asmundson, G. J. (2020). Development and initial validation of the COVID Stress Scales. Journal of Anxiety Disorders, 102232

4. Qiu, J., Shen, B., Zhao, M., Wang, Z., Xie, B., & Xu, Y. (2020). A nationwide survey of psychological distress among Chinese people in the COVID-19 epidemic: implications and policy recommendations. General psychiatry, 33(2).

5. Wang, C.; Pan, R.; Wan, X.; Tan, Y.; Xu, L.; Ho, C.S.; Ho, R.C. Immediate Psychological Responses and Associated Factors during the Initial Stage of the 2019 Coronavirus Disease (COVID-19) Epidemic among the General Population in China. Int. J. Environ. Res. Public Health 2020, 17, 1729.

6. Cheng, S. K. W., Wong, C. W., Tsang, J., & Wong, K. C. (2004). Psychological distress and negative appraisals in survivors of severe acute respiratory syndrome (SARS). Psychological Medicine, 34, 1187-1195.

7. Rubin, G. J., Amlôt, R., Page, L., & Wessely, S. (2009). Public perceptions, anxiety, and behaviour change in relation to the swine flu outbreak: cross sectional telephone survey. Bmj, 339, b2651.

8. Wheaton, M. G., Abramowitz, J. S., Berman, N. C., Fabricant, L. E., & Olatunji, B. O. (2012). Psychological predictors of anxiety in response to the H1N1 (swine flu) pandemic. Cognitive Therapy and Research, 36(3), 210-218.

9. Taylor, S. (2019). The psychology of pandemics: Preparing for the next global outbreak of infectious disease. Cambridge Scholars Publishing.

10. Asmundson, G. J., & Taylor, S. (2020). Coronaphobia: Fear and the 2019-nCoV outbreak. Journal of anxiety disorders, 70, 102196.

11. Galatzer-Levy, I. R., Huang, S. H., & Bonanno, G. A. (2018). Trajectories of resilience and dysfunction following potential trauma: A review and statistical evaluation. Clinical Psychology Review, 63, 41-55.

12. Hawryluck, L., Gold, W. L., Robinson, S., Pogorski, S., Galea, S., & Styra, R. (2004). SARS control and psychological effects of quarantine, Toronto, Canada. Emerging infectious diseases, 10(7), 1206.

Reflection

REFLECTIONS ON MULTIETHNOLECTS

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Abstract

Standard or the “right” language is sometimes regarded as a superior way of expressing while dialects are regarded as a part of history and multiethnolects are regarded as 'cultural aliens'. Consequently, contemporary speakers of dialects and multiethnolects may be undervalued in society. Here we consider a possibility to acknowledge the value of dialects and multiethnolects and discuss the consequences of their usage. We argue that the application of multiethnolects can influence a language change and it can increasingly impact the society, as addressed by modern literature and sociolinguists.

1. Introduction

If language should be defined as a tool, it is of interest to consider the findings of linguists of the 20th century and continue to develop their theories (1). In the 19th century, the field of linguistics was concerned primarily with historical development and change of languages, with the aim to reconstruct their older versions (2, 3), while the modern languages came into the focus in the 20th century (1). Researchers of the modern language include Karl Bühler (4) (**Figure 1**) and Roman Jakobson (5) - the linguists who performed pioneering work on the role of the language. In 1934, German psychologist and linguist Karl Bühler, in his book Theory of Language (4), defined language as a tool: "*It is an ancient insight that tools and language are among the most human things about human beings* […]. *Language is related to the tool; it, too, is one of the implements used in life, it is an organon.*" Along these lines, he considered language not only a complex phenomenon, but also as a tool that serves for different purposes and can be used to perform various tasks.

Bühler's theory had a significant impact on the Russian-American linguist Jakobson (5), who up-graded the findings of his German counterpart. Jakobson (5) proposed six functions of language that were necessary for communication to take place.: 1) referential, 2) poetic, 3) emotional, 4) phatic, 5) conative, and 6) metalingual (6,7). As Jakobson (5) explains: "1) the referential function is oriented toward the context (the dominant function in a message like 'Water boils at 100 degrees'); 2) the poetic function (e.g., 'Smurf') puts the focus on the message for its own sake; 3) the emotive function is oriented toward the addressers in the interjections 'Bah!' and 'Oh!'; 4) the phatic function serves to establish, prolong or discontinue communication or confirm whether the contact is still there as in 'Hello?'; 5) the conative function is oriented toward the addressee (imperatives and apostrophes); (1) the metalingual function is used to establish mutual agreement on the code (e.g., a definition);"

However, in the last few years, the question of the seventh function of language has arisen reflecting the power of a language to constitute the reality (7), especially in contemporary literature (9). The novel function includes, for example, questions about the manipulative ability of language. An example of contemporary work that replays the idea of whether language can be an unprecedented and manipulative tool is the novel The Seventh Function of Language by the French writer Laurent Binet (9). In the novel, a possibility is considered that one who knows the seventh function of language can convince anyone of anything, and consequently acquire power (9).

Figure 1. Karl Bühler, German psychologist and linguist. From (6).

2. Multiethnolect vs. standard language ideology

In Europe, that has seen immigration on a large scale, new forms and uses of language are constantly emerging (10). Sociolinguistics in the study of language is therefore increasingly turning its attention to areas of emerging new multilingual urban speech taking place in certain neighbourhoods or communities formed on the ethnic basis. These ethnic dialects began to develop mainly in larger cities with at least 500,000 inhabitants in multilingual immigration countries, such as Germany (11). Ethnic dialects emerge in particular among groups of adolescent speakers. They are born in the informal spontaneous talk of multiethnic peer groups and usually used by monolingual young people from non-immigrant backgrounds as well as by their bilingual peers. These linguistic forms and styles are named multiethnolects (10). Typically, such multiethnolects (called also broken speech or broken language) are characterized by simplification of grammar and vocabulary of the language and negligence of its rules. In Germany, such multiethnolect is called “Kiezdeutsch”, in the Netherlands it is called “Straattaal” and in Sweden, it is called “Rinkeby-Svenska” (10). Mulitethnolects are considered as a threat to the good functioning of society (12). This fear is based on the assumption that speakers have no choice: they are unable to use other forms of the national language (10).

Despite the fact that these variations are criticized and marked as inappropriate it could not be denied that they contain important elements of creativity, as new words and new sentence structures are created whereas novelties are introduced also in the rhythm and sonority of the language (8).

The problem of multiethnolects, however, arises when we talk about linguistic value systems (11). There is a general agreement that the standard language (13) is regarded as the only 'right' or 'proper' language and is a base for 'the right usage'. The standard language (13) represents high-culture or the upper-class language use, because complexity requires effort and proper education. It also stands for national unity therefore it is often idealised as it was described by Heike Wiese, a German sociolinguist, who has stated (11): "*Standard language ideology seems to be particularly powerful in Germany, with strongly restrictive and puristic tendencies.*" The conflict occurs because the linguistic value systems interact with patterns of inclusion and exclusion, which shape language ideologies and power relations (11). Standard language ideologists want to preserve the 'old' language considering also the benefit of mutual understanding but they do not always show understanding or even interest towards ethnical dialects.

Dialects are regarded as a part of history, reflecting geographical position and represent the folk culture (14) but multiethnolects on the other hand are sometimes regarded as 'cultural aliens' (11). The impact of the ethnolects on the standard language (13) is considered as a linguistic threat (12), primitive (12) as subjected to uneducated people of the under-class (15). In addition to that, it was suggested that those who are speaking the 'babble' of a migrant out-group are more likely to fail school, are not able to build a successful career and are not capable of complex thought (11). Many among youngsters speak those ethnolects due to their 'immigrant background' and/or due to their social background – friends, school, neighbourhood (11) or as it seems to them trendy to speak ghetto talk that they describe as "cool" (16). An example of the media and sources of street language is music, especially rap.
The rappers began to incorporate into their lyrics the language they knew, which was close to them, which marked where they came from, with which they could express their feelings and react critically to current political developments and inequality in society. Ghetto talk connected young people, often from poorer suburbs, into a continuous whole and created a community in which they felt strong (17). In addition, ethnic dialects expressed seem to be convincing (18) in songs that have therefore the so-called street credibility (17) and consequently can embrace a larger group of listeners.

In principle no-one should be considered as a linguistic outsider; instead, ethnic dialects should be regarded as a source of a potential enrichment of the language in its on-going development, as language is a living substance and is subjected to change.

3. The duality of multiethnolects

Although politicians in Germany disagree, there are indications that multiethnolects have been present since post-war (2nd World War) immigration waves to Western Europe and have been denied over 50 years (19). Multiethnolects or ghetto talk has been therefore still considered as the language of the oppressed and stigmatized (10).

Therefore, the question arises: where can we find the value, greatness and power of multiethnolects? One possible answer to this question is given by the novel *Broken German* (20) by the Israeli writer Tomer Gardi **(Figure 2**), which has gained a lot of attention in Germany. Not only does the text deal, among other things, with language as a tool of power, but it is also written entirely in 'broken' German, the colloquial language of immigrants; sociolinguists would define it as multiethnolect. The author did not opt for this version of the German language because he would have intentionally curtailed or harmed the German language (21). He chose it because he heard it on the streets of Berlin and felt it as a 'pure real' language, a language that lacks nothing, that is no less valuable than others, and that has absolutely no less influence or power (20).

The point is that the adaptation of German language in his novel does not seem to be a consequence of linguistic poverty or some form of language decay. Instead, the gaps and incisions in his German language stem from German history and current political situation, partly due to the lack of transparency in the German language (e.g., a passive form), and partly due to the urgent need for a new language that will reflect current status in the European society and the world (21). The power of the literalized ethnic dialect is also reflected in the fact that Eurocentrism and all its internalized norms and values are called into question. In addition to that, it is an answer to the fear of foreign infiltration (20). So, we could come to the realization that actually one does not talk only about the language of immigrants, but also about immigration of language.

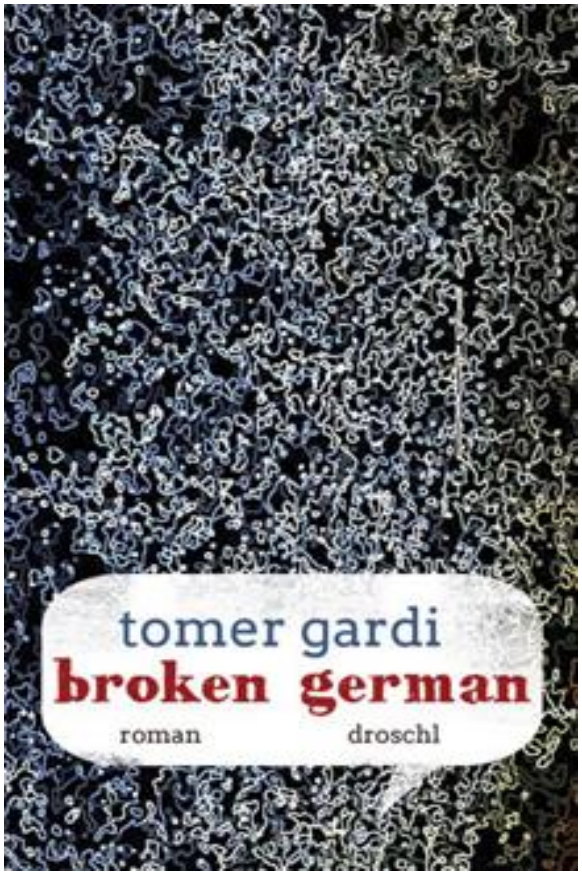

Figure 2. The front page of the novel *Broken German* (20).

Multiethnolects are linked to multiethnic rather than monoethnic neighbourhoods and cross ethnic boundaries, including speakers with non-migrant background. At the same time, using multiethnolects reflects a choice, a self-positioning of its speaker within a complex multi-ethnic urban setting. It signals that the speaker belongs to a certain group, emphasising its status as a youth language (22). Furthermore, multiethnolect is characterised by a high linguistic diversity with rich language contact opportunities, making e.g. »Kiezdeutsch« something like a pioneer dialect and an integral part of the linguistic landscape of German (23).

Conclusions

Changing the language seems inevitable, even though some would wish to conserve it and think that they could preserve and protect it through glorification. However, the alternative we are left with is to consider the interruptions, fractures, incisions, folds, anomalies, and gaps in multiethnolects for its development. In the end, the question which language is more accepted may not be the most important one, but how to overcome problems of inequality in society and thus preserve the dignity of every human being.

References

1. Elspass S. (2007), A two-fold view 'from below': New perspectives on language histories and language historiographies, Germanic Language Histories “from Below” (1700-2000), De Gruyter, 3-5.

2. Lehmann, W. P. (1991), The importance of models in historical linguistics, Language Typology 1988: Typological Models in Reconstruction (Ed. Hewitt H.-J. J., Lehmann, W. P.), John Benjamins Publishing Company, p. 7.

3. Nerlich, B. (1989), The evolution of the concept of "linguistic evolution" in the 19th and 20th century, Lingua 77 (2), 101-112.

4. Bühler K. (2011), Theory of language, the representational function of language, John Benjamins Publishing Company, Amsterdam / Philadelphia (in German: Sprachtheorie (1934): Die Darstellungsfunktion der Sprache. Jena: Gustav Fischer, Stuttgart), 434 p.

5. Jakobson, R. (1984), Russian and Slavic grammar, Studies 1931-1981 (Ed. Waugh l. R., Halle M.), Walter de Gruyter & Co., Berlin, 151 p.

6. Krevs Birk U. (2016), Einführung in die germanistische Linguistik, Znanstvena založba Filozofske Fakultete Univerze v Ljubljani, Ljubljana, p. 65.

7. Powers E. (2017), Word games, Books, Arts & Manners, National Review Inc., 40-41.

8. Biesler J., (2012), „Kiezdeutsch ist sehr komplex“ Sprachwissenschaftlerin erforscht Jugendslang (“Kiezdeutsch is very complex”), Deutschlandfunk, Campus & Karriere https://www.deutschlandfunk.de/kiezdeutsch-ist-sehr-komplex.680.de.html?dram:article_id=39255

9. Binet L. (2015), The 7th function of language, Grasset et Fasquelle, France, 359 p.

10. Cheshire J., Nortier J., Adger D. (2015) Emerging multiethnolects in Europe, Queen Mary’s Occasional Papers Advancing Linguistics, vol 33, 1-27.

11. Wiese H. (2015), This migrants’ babble is not a German dialect!”: The interaction of standard language ideology and ‘us’/‘them’ dichotomies in the public discourse on a multiethnolect, Language in Society 44, 341–368.

12. Neuland E., Schlobinski P. (2018), Handbuch Sprache in sozialen Gruppen (Handbook of Language in social Groups), Walter de Gruyter GmbH & Co, Germany, p. 223.

13. Smakman D. (2012) The definition of the standard language, A survey in seven countries, International Journal of the Sociology of Language, 218, 25-58.

14. Vincent N. (2014), Similarity and diversity in the evolution of Italo-Romance morphosyntax, Diachrony and Dialects (Ed. Benincà P., Ledgeway A., Vincent N.), Oxford University Press, 1-19.

15. Siegel J. (2010), Second Dialect Acqusition, Cambridge University Press, Cambridge, p. 146.

16. Von Leszczynski U. (2014), Kiezdeutsch ist bei allen Jugendlichen beliebt (Kiezdeutsch is popular among all adolescents), Märkische Allgemeine, Kultur, Jugendsprache des Deutschen https://www.maz-online.de/Nachrichten/Kultur/Kiezdeutsch-ist-bei-allen-Jugendlichen-beliebt

17. Gültekin U. (2017), How migrants shaped rap - A conversation with hip hop researcher Hannes Loh (in German: Wie Migranten Rap geformt haben – Ein Gespräch mit HipHop-Forscher Hannes Loh, Vice, interviews https://www.vice.com/de/article/pggb5y/wie-migranten-rap-geformt-haben-ein-gesprach-mit-hiphop-forscher-hannes-loh

18. Witzeck E. (2019), Rapper In Den Charts, Man spricht deutsch, Frankfurter Allgemeine Zeitung (Rapper In The Charts, One Speaks German) https://www.faz.net/aktuell/feuilleton/pop/warum-deutschsprachige-musik-ploetzlich-so-erfolgreich-ist-16126000.html

19. Heunlich D. W. K. (2016), The roots of 'multiethnolects': effects of migration on the lexicon and speech of german-speaking school children, Dissertation, The University of Texas at Austin, USA, 24-26.

20. Gardi T. (2016). Broken German, Literaturverlag Droschl GmbH, 144 p. https://www.droschl.com/book/broken-german/

21. Kastberger K. (2016), Wir schaffen das! Migration und Literatur, Zeit online, Kultur https://www.zeit.de/kultur/literatur/2016-08/literatur-migration-tomer-gardi-broken-german

22. Freywald U., Mayr K., Özcelik T., Wiese H. (2011) Kiezdeutsch as a multiethnolect (Ethnic Styles of Speaking in European Metropolitan Areas, (Ed. Friederike Kern, Margret Selting),

23. Bunk O., Pohle M. (2019) »Unter Freunden redet man anders«: The register awareness of Kiezdeutsch speakers, The Sociolinguistic Economy of Berlin, Cosmopolitan Perspectives on language, Diversity and Social Space (Ed. Heyd T., von Mengden F., Schneider B.), Walter de Gruyter, Berlin, 97-124.

Reflection

CLINICAL EXERCISE THERAPY AND CLINICAL ART THERAPY FOR OLDER ADULTS

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Abstract

Health, by its comprehensive definition, is also an expansive reflection of time and space. Health can be affected by where we play, learn, live, work and create. The priority task of most health professionals is to restore, maintain and enhance the stable health status of the individual. The purpose of this article is to apply physical therapy by a comprehensive interdisciplinary clinical exercise approach. The promotion of health prevention must be perfectly structured as part of a broader treatment of multidisciplinary dimensions and targeted at the health of the individual. In many cases, preventive activity through the promotion of physical activity is an exceptional opportunity to develop a holistic approach to programs for well-being of an individual and of society.

1. Introduction

Physiotherapists as health professionals emphasize the importance of physical activity in the clinical practice of different health specificities. Clinical exercise therapy as a part of the protocol of physical therapy of older adults should be strategically incorporated in the daily routine of an individual.

The promotion of physical activity has undeniable health benefits, specifically in maintaining the physical independence of older adults (1). A comprehensive treatment approach may represent the pooling of multiple individual abilities. For example, clinical art therapy as one of the holistic aspects of individual and community based complementary programs, can bring physical activity closer as an additional incentive. We suggest further studies of the comprehensive approach based on biological, behavioural, and environmental factors as a challenging and potentially holistic exercise/activity therapy.

The following topics are discussed in presented paper: (1) the biological and psycho-social changes related to age; (2) the proposal to establish a comprehensive approach to investigate the connections across individual biological, behavioural, and environmental factors of physical therapy; (3) the potential connections of clinical art therapy as a challenging method to stimulate the desire for further physical activity of the elderly; (4) the application of theory into practice and support for training motivation.

2. Biological and psychosocial changes related to age

The extensive biological and psychosocial components of the aging process were discussed in the literature (1,2). The biological factors are expressed in reduced physical fitness and abilities with aging. Deterioration of physical capacity was associated with psycho-social vulnerability of older adults (3). Poorer social functioning is associated with weaker health outcomes and has been identified as a priority for intervention of health prevention. There is also good evidence to suggest that integrated physical and mental health interventions are effective in improving social functioning of older adults (3).

Extensive biological, psychosocial and environmental factors make the physiotherapy recommendations more complex in the population of older adults. The decisions should be open for examining the effectiveness of an extended comprehensive health care approach including psychosocial interventions, for example clinical art interventions, on associated physical therapy outcomes in the later stages of life.

3. Biological, behavioural, and environmental factors of physical therapy

Biological impairments of physical capacity of older adults contribute to limited autonomy and associated behavioural changes. A possible consequence of both is usually also in the

environmental constraint or environmental change of the individuals (for example, changing the home living environment for community living). Physiotherapy can contribute to maintaining and enhancing an individual's physical independence.

The importance of regular participation (i.e., 30 minutes/day on most days of the week) in activities of moderate intensity (such as walking, climbing stairs, biking, or gardening), which increase the accumulated daily energy expenditure and maintain muscular strength should be encouraged in older adults (4).

4. Physical therapy and clinical exercise therapy of older adults

The frailty syndrome is frequent in older adults that include a lowered activity level, poor exercise tolerance, and loss of lean body and muscle mass. Poor exercise tolerance is related to the aerobic endurance of older adults. An exercise program that includes aerobic exercise can significantly improve peak oxygen consumption by ~10-15% (2). Furthermore, clinical exercise programs of physical therapy should include resistance training to increase muscle strength and mass. Although the increase in muscle mass in response to resistance training may be attenuated in frail older adults, resistance training can significantly improve muscle strength, particularly in institutionalized patients, by ~110% (2). The combination of both aerobic and resistance training target specific components of frailty is recommended for older adults.

5. Clinical art therapy as a comprehensive approach of motivation and further treatment participation

The physical therapy treatment of older adults is sometimes questionable from the point of view of general motivation or motivation for physical activity alone. Maintaining the diversity of activities may be one of the approaches that improves motivation. Short intervention of clinical art therapy (5)(as for example 10-15 minutes of free drawing (**Figure 1**)) before the physical therapy session may serve as a stimulus to general well-being and as a motivation to participate actively in further physical activity/exercise therapy intervention.

Health experts and public policy should focus on ways of increasing dimensions of lifestyle activities in older adults, as well as on increasing the availability and accessibility of senior and community centre programs for promoting physical activity throughout the life span.

Figure 1. A drawing of a 85 years old expresses a youthful and optimistic view on life.

6. Discussion

The ability to exercise is an essential determinant of health and of the quality of life. The population of older adults represents the population where a sedentary lifestyle is frequent (1). A comprehensive approach of clinical exercise therapy as physical therapy should be part of the continuity of efforts for the elderly. Physical activity extends longevity and reduces the risk of physical disability (2). The researchers demonstrated on the molecular level, that exercise reduces frailty by decreasing muscle inflammation, increasing anabolism, and increasing muscle protein synthesis. They concluded that more studies are needed to determine which exercises are best suited, most effective, and safe for this population. Based on the available studies, an individualized multicomponent exercise program that includes aerobic activity, strength exercises, and flexibility is recommended to treat frailty (2). Inconsistent physical activity or physical inactivity is associated with a decrease in exercise capacity that predisposes to frailty and health decline of older adults. Numerous alternatives are investigated contemporary to prevent muscle loss and improve functional fitness in frail older populations.

Furthermore, Lopes et al. (2019)(6) investigated the effects of strength training performed with blood flow restriction on strength, muscle mass, Insulin-like Growth Factor (IGF-1), endothelial function, microcirculation, inflammatory biomarkers, and oxidative stress. They suggested strength training with blood flow restriction as a potential novel therapeutic approach for older adults with sarcopenia. The researchers concluded that the key factors in improving health are exercising at a moderate-to-vigorous level for at least 5 days per week and including both aerobic and strengthening exercises.

Therefore, the challenge for health professionals is to increase physical activity and exercise participation in older adults. Taylor (2013) reported some success when physicians have given specific, detailed and localised information to their patients (7). Furthermore, Taylor (2013) suggested that additional high-quality research is needed to continue to address the issue of non-participation in physical activity and exercise of a high enough level to ensure health benefits (7).

Conclusions

Physical therapists should be actively encouraged to engage individual older adults in training programs. Clinical art therapy as one of the holistic therapeutic approaches integrated in a physical activity program could serve as a potential strategy related to health benefits. We strongly agree with researchers (8) who emphasize the development of transdisciplinary theories and the study of environmental and biological factors in order to address the connections between individual and group behaviour as a part of physical activity promotion.

Additionally, we propose further study of a comprehensive approach incorporated with additional alternative methods of encouragement and motivation of older adults. Clinical art therapy may serve as one of the potential methods incorporated in physical therapy.

Also we suggest further study of the comprehensive factors based on biological, behavioural, and environmental factors as a challenging and potential holistic therapeutic approach to individualized multicomponent exercise programs for older adults.

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The first author is indebted to her grandmother Iva for Figure 1.

References

1. Ricci NA, Cunha AIL, Physical Exercise for Frailty and Cardiovascular Diseases. Adv Exp Med Biol. 2020;1216:115-129. doi: 10.1007/978-3-030-33330-0_12. Review.

2. Aguirre LE, Villareal DT (2015), Physical Exercise as Therapy for Frailty. Nestle Nutr Inst Workshop Ser. 83:83-92.

3. Smart EL, Brown L, Palmier-Claus J, et al. (2020), A systematic review of the effects of psychosocial interventions on social functioning for middle-aged and older-aged adults with severe mental illness. Int J Geriatr Psychiatry. May;35(5):449-462.

4. DiPietro L. (2001). Physical activity in aging: changes in patterns and their relationship to health and function. Review. J Gerontol A Biol Sci Med Sci. Oct;56 Spec No 2:13-22.

5. Malchiod CA, (2003). Handbook of Art Therapy, The Guilford Press. New York-. Available from https://www.academia.edu/8449655/Handbook_of_art_therapy

6. Lopes KG, Bottino DA, Farinatti P, et al., (2019). Strength training with blood flow restriction - a novel therapeutic approach for older adults with sarcopenia? A case report. Clin Interv Aging. Aug 14;14:1461-1469.

7. Taylor D, (2014). Physical activity is medicine for older adults. Postgrad Med J. Jan;90(1059):26-32.

8. Satariano W, McAuley E, (2003). Promoting physical activity among older adults: From ecology to the individual. American Journal of Preventive Medicine Volume 25, Issue 3, Supplement 2, October, Pages 184-192.

Reflection

MUSICAL PROGRAMME ACCOMPANYING THE NAPLES SYMPOSIUM AND 2020 SOCRATES LECTURES

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Abstract

The article describes the musical programme accompanying two scientific symposia held on 11 February and 17 April 2020. Owing to social changes attributable to the coronavirus pandemic, the events in April took place using the latest technologies via the social networks. The present paper highlights the diversity of these events and the on-line adaptation of musical activity to the new situation.

1. Introduction

The purpose of this article is to present two completely different concerts, organized for a second time by Veronika Kralj-Iglič, Aleš Iglič and Anna Romolo. The February cultural evening in Naples, devoted to music, science and friendship, took the classic form of a concert in a hall with a sizeable audience, while the second was the musical accompaniment for the 2020 Socrates lectures, which in order to meet the challenge of the pandemic had recourse to a completely new kind of concert. At both international events, scientists also joined some professional musicians in making musical contributions.

In particular, it was interesting to see that a new concept of music presentation took place subject to adaptation to isolation due to pandemic. The significance of the changes the world has seen in recent decades recalls the major events of a hundred years ago, which the ethnomusicologist Roberto Leydi has described as „an unusual thirty years between the nineteenth and twentieth centuries, the years of the rise of the bourgeoisie, crisis and the decline of yesterday's world: the formation of our modernity. These are the years when the invention of the automobile, the telegraph and the aeroplane changed the human sense of time and distance“ (…). Photography, film, the phonograph and the gramophone change the notion of the past (…) (1). All those changes are reflected in art. With reference to music, it was as early as 1913 that Claude Debussy remarked: “The century of the aeroplane must have different music“ (2). A parallel can be drawn between the current rise of the development of artificial intelligence, the indispensability of the Internet in all areas of life and, finally, the emergence of a pandemic with the new virus, art becoming available to audiences in new forms.

2. Concert in Naples

In the very centre of Naples, next to the famous church and monastery of St Chiara, a concert entitled »Pulcinella e il Dragone« took place on 11 February 2020 in the cultural centre »Domus Ars«. Pulcinella, a Neapolitan mask from the Commedia dell'arte, and the Ljubljana dragon symbolized friendship and cooperation between the two cities (**Figure 1**).

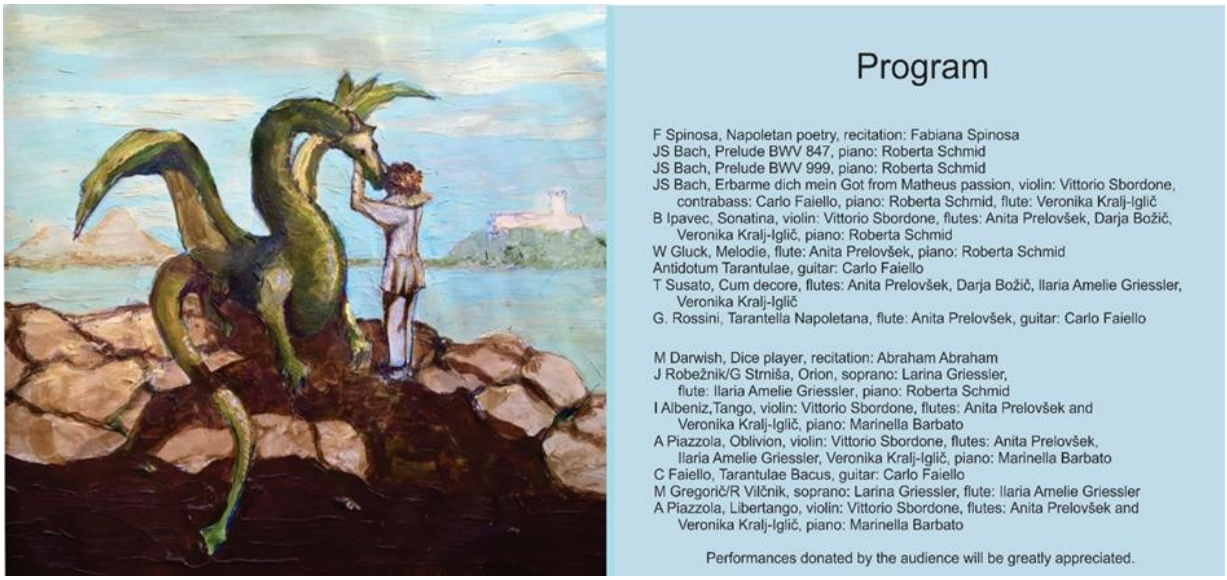

Figure 1. Invitation to the event dedicated to music, science and friendship on February 11, 2020 at Domus Ars, Via Santa Chiara, Napoli.

Figure 2. The concert sale in Domus Ars. Photo: Marco Sammarco.

»Domus Ars« is a concert hall in what used to be the church of San Francesco delle Monache, dating from the beginning of the fourteenth century (**Figure 2**). The stage of the hall is located

in the former church presbytery and is aligned on each side by the remains of tombstones. The concert, which began with a recital of poetry by Pablo Neruda , “Ode to the ocean algae” (by Anna Romolo accompanied by guitarist Carlo Faiello)(**Figure 3**), featured performers from Slovenia, Italy and Palestine. Among them there was a scientist from Palestine, who recited verses in Arabic language (**Figure 4**). The programme had been carefully chosen by the organizer and included Slovenian and Italian music and works from the European treasury of classical music.

In the light of the church background, the music of J. S. Bach was a very good choice for the Neapolitan pianist Roberta Schmid (**Figure 4**), while an aria from St Matthew's Passion Erbarme Dich Mein Got was performed by Veronika Kralj-Iglič on the flute, the Italian violinist Vittorio Sbordone and Roberta Schmid (**Figure 5**). Vittorio Sbordone played also at a similar event a year ago (3). Bach's music, despite the fact that he travelled very little in his life, is an example of global music that has its place in any historical time and in any part of the world.

Figure 3. Anna Romolo reciting Ode to the ocean algae by Pablo Neruda with improvised accompaniment by Carlo Faiello on the guitar. Photo: Marco Sammarco.

Figure 4. Roberta Schmid performing preludes by J.S.Bach and Abraham Abraham reciting poetry of M. Darwish. Photo: Marco Sammarco.

Figure 5. Erbarme Dich Mein Got by J.S. Bach was performed by (from the right side) Vittorio Sbordone, Veronika Kralj-Iglič, and Roberta Schmid with the assistence of Anna Romolo. Photo: Marco Sammarco.

This year, also for the second time in Naples, the guests included the flautist Anita Prelovšek, who with piano accompaniment of Roberta Schmid played the famous Melody by C. Gluck, an original and frequently performed flute solo from the opera »Orpheus and Eurydice« (**Figure 6**).

Figure 6. Anita Prelovšek and Roberta Schmid performing Gluck's Melody.
Photo: Marco Sammarco.

A special feature was a performance of the first movement of Benjamin Ipavec's Sonatina, otherwise known as the Serenade for Strings, but there is also an original unorchestrated version for four-hand piano. This composition was played by an ensemble of piano, solo violin and a flute quartet made up of Anita Prelovšek, Veronika Kralj-Iglič, Ilaria Giessler and Darja Božič (**Figure 7**), all from Ljubljana while Maurizio Chiurazzi joined them on the flute to play a transcribed "Cum decore" by T. Susato (**Figure 8**). The flute seems to be a popular instrument, being played by many scientists, as Filippo Staiano, a professor of flute at the Naples Conservatory, was pleased to say after the concert. The choice of music composed by Ipavec, who was a medical doctor by profession, also symbolically connected music, medicine and science.

Figure 7. Sonatina by Benjamin Ipavec as performed by (from the right side) Vittorio Sbordone, Anita Prelovšek, Iparia Griessler, Darja Božič, Veronika Kralj-Iglič and Roberta Schmid. Photo: Marco Sammarco.

Figure 8. The ensemble performing "Cum decore" by T. Susato. Photo: Marco Sammarco.

Mention should also be made of a Robežnik's song, »Orion«, performed by Larina Griessler, with accompaniment of Roberta Schmid (**Figure 9**) which enabled the Slovenian language to be heard in the hall of »Domus Ars«. The audience was also very enthusiastic about the Neapolitan tarantellas, both old and traditional but also modern ones by composer Carlo Faiello, who accompanied the instrumentalists Anita Prelovšek, Vittorio Sbordone and Veronika Kralj-Iglič on the guitar (**Figure 10**).

By embodying all of these features, the concert, which was followed by a social gathering with a dinner for the audience and the participants, fully justified its dedication to music, science and friendship (**Figure 11**).

Figure 9. Larina Griessler and Roberta Schmid performing Robežnik's In thousand years when we'll no longer be. Photo: Marco Sammarco.

Figure 10. Performance of Napoletan Tarantellas. Photo: Marco Sammarco.

Figure 11. Buffett by Francesca Schiavo. Photo: Marco Sammarco.

3. Musical performances during quarantine

Throughout the time when no concerts and other cultural events were possible, many artists decided to adapt to the situation by putting on alternative performances. The need to express themselves and to share their emotions with the audience is inherent to the nature of all artists and also manifests itself in circumstances that are not conducive to art or favourable to its practitioners. During the First World War, for example, the pianist Arthur Rubinstein immediately seized the opportunity to put on an improvised concert. In an overcrowded bar in Montparnasse he came across compatriots from Poland and one of them offered him a seat: »When we got to know each other better, I discovered that my neighbour is a doctor of medicine and loves music just like most people with this profession.« The doctor invited him to his hospital, where they even had a piano in the canteen. »I couldn't resist as I hadn't seen a piano since I left London. (…) I timidly saw the piano in the corner of the room, approached it, opened the cover and struck a few notes. It was terribly out of tune, two or three keys were mute. But I sat down and started playing. I played Beethoven's Pathetic Sonata.

I've never played it like that before. It wasn't about how it sounded - it was about how I felt. I almost cried, just like everyone else« (4).

Another example is that of the French composer Olivier Messiaen, who wrote his Quartet for the end of time (Quatuor pour la fin du Temps) in an internment camp in 1940, performing

(with some other musicians), in front of an audience of four hundred people imprisoned in the camp (5).

The Indian conductor Zubin Mehta also mentions making music in wartime several times in his memoirs (6). Among other such events, he describes how during the Gulf War in 1991 he performed as a pianist with some of his musical colleagues in the mornings in »unimaginable spaces«, but with music he hoped to »take away fear from people and give them some joy« (6). Three years after these events, he performed in bombed-out Sarajevo with the Sarajevo Symphony Orchestra. Although the string instruments were missing some strings and the clarinets were without reeds, all the players tried to do their best (6).

As the examples quoted above illustrate, even the most difficult wartime conditions have not discouraged musicians from performing. Today, during the coronavirus pandemic, musicians have been resourceful in finding new forms of performing. Some musicians, both amateur and professional, for instance, have decided to perform from the balconies of apartments or to play outdoors in front of retirement homes. Others, including many leading names in various genres, have opted for what have come to be called »couch concerts« from their homes, which have been broadcast live on-line. They have often used a smartphone to record their performances and posted the results on YouTube. Others have tried to make music on-line together with musical colleagues from some distance away, which is also made possible using a special Internet app. The world has been brought together by recordings of chamber, choral or even symphonic compositions. Cases in point are the Orchestre national de France, which played a piece from Ravel's Bolero with each member recording his or her part from home (7). Many top opera houses and other musical institutions that organize concerts have offered free recordings of past performances on-line. Some musicians have taken part in concerts broadcast live from empty halls or places without an audience, such as the performance by the famous pianist Denis Matsuev organized by the Moscow Philharmonic with the support of the Russian Ministry of Culture in an empty Tchaikovsky Hall in Moscow. In May, the Slovenian Philharmonic offered a free on-line series of chamber concerts performed live by members of the orchestra from the Slovenian Philharmonic Hall.

The sound quality of on-line concerts is not always of the best, but this is only the first attempt to perform on the Internet or in the form of »couch concerts«. Even the first recordings of music, made possible by Edison's invention of the phonograph in 1877, namely, a device for recording and also playing music (1), were of poor quality compared with today, of course, but for all that revolutionary. We can expect that the sound and image quality of on-line concerts will continue to improve significantly in the future.

4. Music at the 2020 Socrates lectures

Although the symposium was held on-line via Zoom, as an intermezzo there were some musical performances. The organizer of the symposium spent quite some time investigating the possibilities of playing live music on-line. The musicians who were invited to participate doubted whether a performance on the Internet could provide real aesthetic pleasure, but the organizer convinced them that such a »concert« would be acceptable to those attending. She has already had some experience of this from a similar event she recently ran on-line. During the preparations for the symposium, she tested the sound together with the musicians on-line in order to find the best one. Each performer was asked to prepare a solo performance, as the idea of two remote musicians playing together at the same time on-line did not produce satisfactory results.

Musical performances during the symposium were divided into three sets and took place during the breaks between lectures. The final result exceeded expectations regarding the sound, which was better than during the test performances. Instead of being on stage, the performers played or sang in their apartments and were not dressed in concert attire. Listeners were able to make comments and congratulate the musicians by writing on »Chat«, while those who had a microphone and a camera turned on could even applaud.

The first to perform was flautist Anita Prelovšek, who played without accompaniment the Siciliano from Bach's Sonata in E-flat major for flute and piano or harpsichord. The second break was attended by a participant from India, who sang an Indian song, while the third part was provided by Elena Starceva Somun, a pianist and piano teacher from Russia who has been living in Slovenia for fifteen years, and her son Emil, who started learning the trombone this school year. The young musician played three short songs with CD accompaniment and was well received by the audience. His mother played music from the film „Titanic“ which could be said to have a certain relevance to the words of Rajko Muršič, the ethnologist and cultural anthropologist: „This virus could be a warning that we are all on the same ship, which has common problems“ (8).

Conclusion

Concerts are always a welcome accompaniment to scientific symposia, being an opportunity for relaxation, artistic enjoyment and personal contacts. Many scientists are lovers of classical music and even perform it. Both at the concert in Naples as well as in the programme of music in the Internet symposium, some scientists actively participated as performers, which gave them even more satisfaction. The brief programme of music accompanying the on-line symposium enriched the event with several quite different musical performances.

Thanks to modern technologies, computers, Internet and smartphones, it is possible for musicians to maintain contact with an audience even during quarantine. However, on-line

performances or just listening to recordings cannot replace concerts in concert halls, where there is a live link between performers and listeners. Or, to paraphrase Kosovel, „This is the essence of art: the living contact between man and man, man and his surroundings, between man and the universe" (9).

References

1. Leydi R, Druga godba: etnomuzikologija, Ljubljana, ŠKUC, Znanstveni inštitut Filozofske fakultete, 1995, pp 17.

2. Griffiths P, Brève histoire de la musique moderne de Debussy à Boulez, Paris, Fayard, 1992, pp 86.

3. Prelovšek A, Art and science – two halves of the whole, Socratic lectures, Proceedings of the 2.nd international symposium 2019, V. Kralj-Iglič ed., 2019: 102-109.

4. Rubinstein A, My many years, Ljubljana, DZS, 1983, pp 439.

5. Rischin R, For the end of time: the story of the Messiaen quartet, Ithaca, N.Y., Cornell University Press, 2003.

6. Mehta Z, La partitura della mia vita, Excelsior, Milano, 2007, pp. 203-294.

7. https://www.youtube.com/watch?v=Sj4pE_bgRQI

8. Bandur S, Nič ne more nadomestiti pristnega stika med ljudmi, Delo, 23.4.2020, pp. 12.

9. Kosovel S, Selected poems, Mladinska knjiga, Ljubljana, 2004.

Reflection

ON THE ORIGIN OF THE CULTURAL AND NATIONAL IDENTITY OF ALPINE SLOVENES

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Abstract

In the article, we describe how Slovenes were a part of the European cultural space already in the early medieval period. In the initial phase of this process, the local Roman and Romanized population and Christian missionaries from the Eastern Roman Empire (Byzantium) and Ireland played an important role. We indicate that Sclavinia can be equated with Slovenia, i.e. the region of the Slovenes, already in the medieval period. Furthermore, we point out that the Slovene princes of Carantania, which can be regarded as part of Sclavinia, had, besides the privileged and free armed social group of the kosezi, a particularity known only to Slovenes, an accompanying high nobility already prior to the establishment of the Frankish social/feudal system. Accordingly, the castles of early Slovene high nobility also differed significantly from early feudal Frankish towers. In the article, we furthermore highlight that many Slovene scholars and clerics, such as the 15th century first residential bishop of Vienna George Slatkonja (Slatkonia) from Ljubljana and the humanist, bishop, university chancellor, educator of the future Emperor Maximilian I. and later the prince of the Holy Roman Empire Thomas Prelokar from Celje, or the scholar John Popovič (Popowitsch) from Celje, the pioneer of the standard Austrian variety of the German language from the 18th century, and many others, laid the intellectual/spiritual foundations of the Habsburg state, while the territory originally settled by Slovene ancestors (Sclavinia), which was much larger than the present-day Slovene independent state, formed the core of the Habsburg state in central Europe and also the core of present-day Austria. And finally, the Slovene principality of Carantania, with Slovene as its official language, can be considered as a legal basis of the later Habsburg state.

Some historians believe that Slovenes were drawn into the European cultural circle by (Bavarian) feudalism, although it is this very same feudalism that also prevented them access to the resources needed for their social and cultural development – it is precisely this fact, they suggest, that presented one of the most significant obstacles for the further development of the Slovene nation. We cannot agree with this viewpoint. Rather, Slovenes owe their inclusion in the medieval European civilisation, which originates from European antique traditions stemming from ancient Greece, primarily to Roman and other indigenous peoples, as well as to Christian missionaries from the non-Germanic parts of Europe (1), i.e. Ireland and the Eastern Roman (Byzantine) Empire, who spread Christianity and the antique culture in the region of the Diocese of Salzburg, from where missionaries came to Carantania, Carniola and Lower Pannonia (**Figure 1**). The first Apostle of Carantania Modestus, for example, was sent to Carantania on the orders of the Irish monk Vergilius of Salzburg (2). Overall, Irish monks contributed greatly to the preservation of the European civilisation in the Early Middle Ages also in other parts of Europe (3).

By passing on the preserved antique knowledge, for example literacy, onto the barbarian peoples, Byzantine missionaries from Greece and Aquileia (4,5) as well as Irish missionaries (monks) preserved the European civilisation, which is based on the accomplishments of ancient Greece in the fields of philosophy, science, art, and political organisation (democracy). European Christian theology also developed following the tradition of ancient Greek, Hellenist, and Roman philosophy. One may consider the Balkans and, more broadly speaking, Southern Europe as the cradle of European civilisation and, up to the beginning of the decline of the Byzantine Empire in the 14th century, as the region with the most advanced civilisation and continuous tradition since Greek antiquity. In these areas, the level of general literacy was much higher than across the royal courts of Western Europe, where people were mostly illiterate (6). Thus, since even Charlemagne was illiterate, it was not feudalism that caused the ancestors of modern-day Slovenes to become part of European culture and civilisation – they rather joined this circle with the conversion to Christianity, which was accompanied by cultural progress.

Similarly, it was not the Arab translations and comments of classical Greek and Roman works that were the main cause of the renaissance in Italy. The *spiritus movens* of start of the renaissance start in Italy was the great number of Byzantine Greek scholars and other emigrants who escaped to Italy after the fall of Constantinople in 1453 (7), who brought to Italy the knowledge of their (Greek and Roman) civilization, which had mostly survived the medieval dark ages in the rest of Europe in the Byzantine East Roman Empire. The process partially started in Italy already in the time of crusaders' conquest of Constantinople in 1204.

Even before the conquest of Constantinople by the crusaders, the close contacts between Kiev Russia and the Byzantine empire gave rise to intense cultural development in 11th and 12th century Kiev Russia, where the works of Homer, Democritus, Epictetus, Aristotle, Plato, and

classical works from old Greek and Roman times ranging from philosophy to history, law, etc., were widely known within the educated Russian class and were in many cases also completely or partially translated into the Old Church Slavic, as for example the Josephus's History of Judaic War (8). The remarkable literary achievements of Kiev Russia from the 11th to 12th centuries were written by the Russian secular and cleric elites. The high cultural level at the princely court of Russian Kiev can be very difficult to find in the same period at similar courts in the Western and Central Europe, i.e. outside the Byzantine Empire. It should be also be stressed that many Russian works from this period were written in the vernacular, an achievement which was repeated in most nations in the west and in central Europe much later, as they did not start to write vernacular prose before the 13th century, most of them only in the 14th century (8)(Dvornik, 1956). There were but few princes (or none) from the west and central Europe of that period who could write such remarkable literary documents as *Poučenie,* written by the Kievan prince Vladimir Monomach, who died in 1125 (8). Since Kiev was destroyed during the Mongol and Tatar invasions of the 13th century, the Kiev transfer of Greek and Hellenistic culture to the neighbouring central Europe (for example to Poland) was unfortunately stopped (8). The Mongol and Tatar invasions were Russia's greatest disaster, because there was not enough time to complete the transfer of cultural and spiritual inheritance of Byzantium. The division of Kiev Russia into smaller principalities did not stop the cultural development of Russia. However, it was geographically more limited, for example to principalities like Novgorod, which had an unusually high level of literacy for that time period and developed further into a republic. The Republic of Novgorod preserved certain cultural, social, and political traditions from the time of Kiev Russia and even improved some of them (8), but this was not enough since the rest of Russia was mainly cut off from this development, which ended in the 15th century with the annexation of the Republic of Novgorod by the Grand Duchy of Moscow.

Going back to Slovene history, in the lands of Alpine Slovenes the role of Roman and other indigenous peoples must not be overlooked when considering the process of conversion of Alpine Slovenes to Christianity and the transfer of the culture of antiquity, especially in the case of mixed marriages, which were likely no rarity at the time (9). Not all indigenous peoples migrated or were eradicated in the course of the conversion to Christianity, which means that Slovene culture did not emerge completely anew. Indeed, a burned-down antique settlement discovered by archaeologists does not automatically signify that it was violently demolished by newcomers – fires were and still are a part of everyday life (9).

For example, some Slovene historians assumed that the ancient Roman town of Celeia, today's city of Celje, formerly also known as the Celtic Keleia, died down along with its Christianity (diocese) at the end of the 6th century, which they then associated with one of the colonisation waves of the ancestors of modern-day Slovenes (10).

As it turned out, however, the Bishop of Celeia Andrew is still mentioned in written records as late as in 680 (9). Therefore, it is hardly surprising that the old Celtic name for the city of Celje, probably in its Latin version, was passed down to our ancestors. Moreover, bishops could be banished, even by the church community itself. Something similar could be said for many other old Slovene settlements and towns, for example Ptuj, which also preserved its antique name. All this testifies to the fact that the ancestors of present-day Slovenes did not populate an uninhabited area. Along with the Czechs, the Slovenes are the westernmost settled Slavic people in Europe. In addition, the Slovenes are the Slavic people located closest to the centre of the old Roman Empire, since the western part of today's Slovenia was a part of Italy. Slovenes were able to progress so quickly precisely due to establishing settlements in provincial areas belonging to antique culture and intermixing with its inhabitants.

What should also be taken into account is the influence of the neighbouring peoples, who at that time already partially converted to Christianity. The Slovene Carantanian Princes Gorazd (749–751) and Hotimir (752–769), both inaugurated on the Prince's Stone (**Figure 2**), were christened on the island of Herrenchiemsee at the Bavarian Lake Chiemsee (11), where they lived in an Irish monastery school. The beginnings of converting Slovenes to Christianity go further back than those of other Slavic nations, such as the Croats and the Poles. Prince Mieszko, the founder of the Polish Piast dynasty, converted to Christianity just before the year 1000, 250 years later than the Carantanian Princes Gorazd and Hotimir. Many other non-Slavic European nations also converted to Christianity much later than the ancestors of Slovenes. Norway converted to Christianity in the period between the 10th and the 11th century, Finland in the second half of the 12th century, after it had been conquered by Sweden. The Lithuanians converted to Christianity in 1386, under the rule of Jogaila (Jagiełło), after they formed a common state with the Poles, which means that they accepted Christianity as their religion approximately 600 years after the christening of the Slovene Carantanian Princes Gorazd and Hotimir. The oldest known preserved text in any Slavic language, written in the Latin alphabet, is of Slovene origin – the Freising Manuscripts (Slovene: Brižinski spomeniki), dating from the 10th century (**Figure 3**), which may even constitute the oldest preserved Slavic text in the world (12). Historian Igor Grdina believes that the Carantanian Church used the Slovene language of the highest social classes of Slovene society at the time, and thereby elevated it above being merely a dialect (12,9).

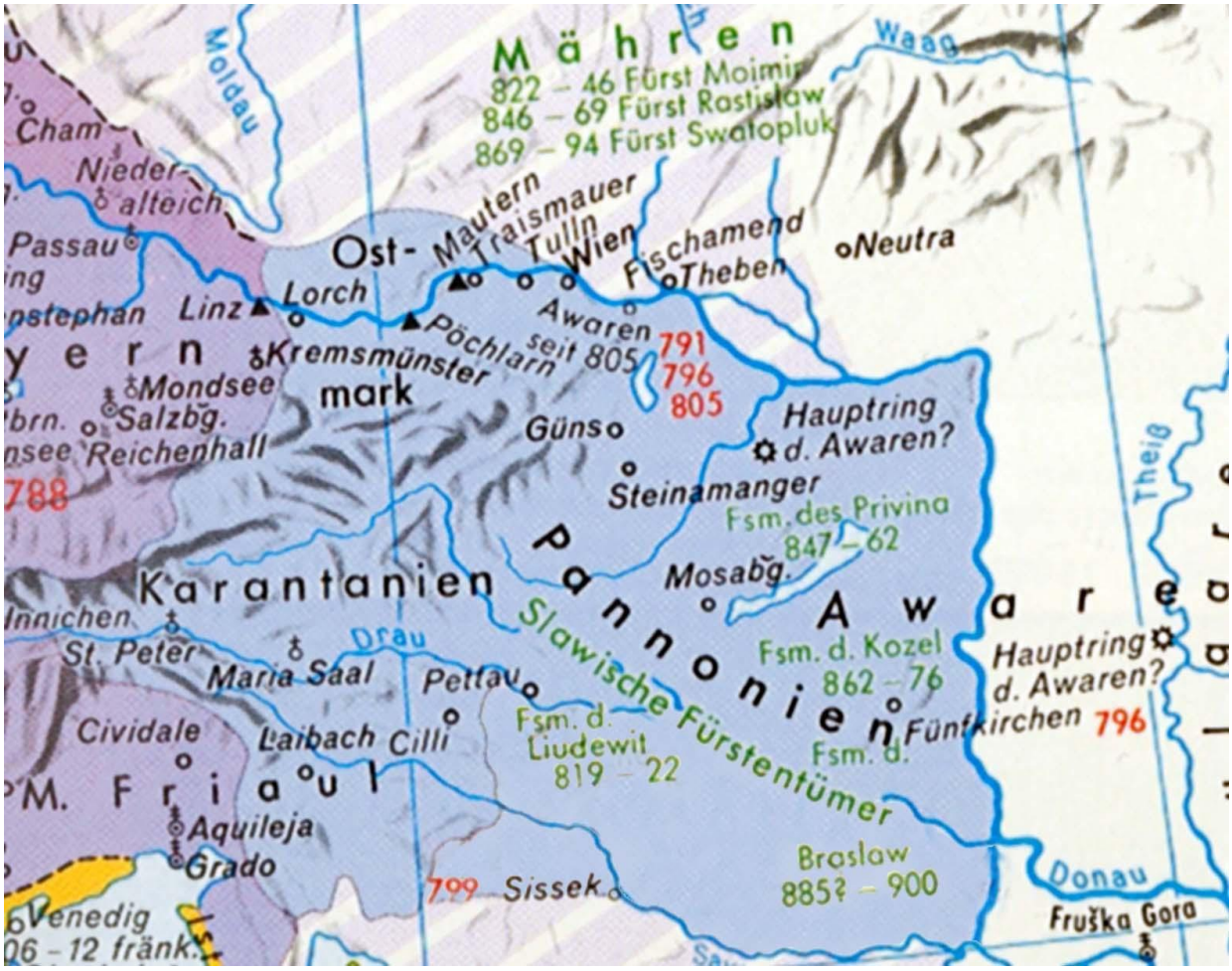

Figure 1. The region of adjacent Alpine Slovene princedoms Carantania and Lower Pannonia with their border provinces at the time of Charlemagne (742–814) and later (adapted from (13)). The region inhabited and ruled by Alpine Slovenes is denoted in the blue colour. Carantanian princes in the 8th and 9th century were Borut (740–749), Gorazd (749–752), Hotimir (752–769) and then from the year 769 onwards Valtunk, Pribislav, Semika, Stojmir, and Etgar, who was prince of Carantania until 828. In the figure are written also the names of some Slovene princes of the border provinces Pannonia and Carniola. In the 9th-century manuscript on the conversion of Bavarians and Carantanians to Christianity (*Conversio Bagoariorum et Carantanorum*), Carantania and Lower Pannonia north of the Drava River are designated as Sclavinia, i.e. Slovenia, which is borders on the Moravians to the north (see this figure). This indicates that the terms Sclavs and Sclavinia (**Figure 5**) in *Conversio* do not relate to the Slavic peoples in general, but rather to a specific Slavic people and region (9,12), i.e. to Sclavs (Slovenes) and Sclavinia (Slovenia).

Up until the last 130 to 140 years of the Habsburg Monarchy, i.e. until the beginning of the formation of the German nation in the modern sense as we know it today (at the end of the 18th and the beginning of the 19th century), which exerted strong pressure on other peoples to undergo germanisation, the ancestors of present-day Slovenes were not so dramatically

hindered in their social and cultural development as was believed until recently (14,15). This will be better illustrated in the following paragraphs with a variety of examples.

Figure 2. The Prince's Stone was originally set within the stronghold of the Krn Castle (Curtis Carantana, Civitas Carantana, Karnburg Castle), which was the seat of the Carantanian Prince (**Figure 6**). The Prince's Stone was used to inaugurate Slovene Carantanian princes between the 6th century and the year 820, approximately. After that, it was used to inaugurate Carantanian dukes and, finally, Carinthian dukes. Up until the year 1414, the inauguration was performed in the Slovene language. The ceremony itself is the oldest known such ritual in the Slavic world. The Prince's Stone is composed of the lower part of an ancient Ionic column from the period of the Roman Empire.

When the 6th century settlement of the Alpine Slovenes (Sclavorum gens) in the regions of today's Slovenia, Austria, Italy, Croatia, and western Hungary was at its end, the intermixing of newcomers (the majority) and the indigenous people (the minority) resulted in the formation of two major Slovene tribal regions – *Carantania* and *Carniola* (16), both of which the Lombard writer Paul the Deacon wrote about in the 8th century (17). One of the records of Oton II's deeds of donation from 973 states that "Krajina" or "Kranjska" (Chreina Marcha) is a local (Slovene) name for Carniola ("Karniola").

The main historical source of knowledge about the conditions in Carantania in the Early Middle Ages is the *Conversio Bagoariorum et Carantanorum*, a manuscript on the conversion of Bavarians and Slovene Carantanians to Christianity written in 870, which refers to the Carantanian Prince as the *dux*. The *Fredegarii Chronicum* mentions as the dux already the first prince of the ancestors of modern-day Slovenes known by name, Prince Valuk (Wallucus dux Vinedorum), who reigned the Slovene Caratanian Princedom (Marca Vinedorum) around 630 (11,18).

In his *History of the Lombards*, dating from the 8th century, the Lombard historian Paul the Deacon writes about the Bavarian king (duke) Tassilo, who, around 593, invaded the land of the Slovenes (in Sclaborum Provinciam), defeated them, and returned to his land with abundant plunder (17). In one of his other works, Paul the Deacon mentions that at approximately the

same time (around the year 595) the Bavarians attacked the Slovenes (Sclavos). With the help of the Avars, the Slovenes vanquished the Bavarians. Milko Kos (11) presumes that the name "Sclaborum Provincia" mentioned in Deacon's first report relates to Carantania (Fig. 1), which would in future sources be referred to as Sclavinia (Slovenia). Paul the Deacon also reports that the ancestors of modern-day Slovenes invaded (probably from Carniola) the Duchy of Friuli in the 7th century, and mentions their battle with the Lombards around the year 705.

In historical sources from the 6th, 7th, and 8th centuries, the ancestors of present-day Slovenes are most often called Sclavs, Sclabs, Sclauans, and Sclauons, and their land is referred to as Sclauinia/Sclavinia (11). The *Fredegarii Chronicum* refers to them as the Winedi (Vinedi). The biography of St. Columban, written around the year 618, employs the term "Venetii qui et Sclavi". The first ever historical mention of the name Sclaueni (Sclaveni) can be found in the manuscripts of Pseudo-Caesarius Nazianzenus dating from 525 (19). Procopius and Jordanes report that the Slavs (Venetharum Natio) were divided into several tribes, but they predominantly called themselves the Sclaueni (Sclaveni) and the Antes; they supposedly shared a common language (20,21,22). In his work from the middle of the 6th century, Jordanes, who was of Gothic descent, distinguishes among three different groups of the Slavs: the Venedi, the Sclavini, and the Antes (8). George Vernadsky (1887–1973), a historian of Russian descent and professor at Yale University, positioned the three major groups of the Slavs in the following regions: the Venedi/Venethi on the shores of the Baltic Sea and the region along the Vistula River, the Sclaveni between the Carpathian Mountains and the upper Dnieper River, and the Antes/Antae in the south, along the lower Dnieper and Don Rivers all the way to the Black Sea (23,24). It was proposed that the Sclaveni later migrated from the Dnieper River to the region around Lake Ilmen (23), which was also considered to be the territory of the north Slovenians/Slovenes in the 9th century, as presented in **Figure 4**.

Slovene ethnologist, anthropologist, and historian Niko Zupanič states that the Slavic people were referred to in written sources as the Venedi or the Venethi already in the first two centuries A.D., when C. Plinius Secundus placed them in the proximity of the Vistula River, the western border of European Sarmatia, while Cornelius Tacitus wrote about them as the eastern neighbours of the Germanic Svebs (21). In the manuscripts of the Alexandrine geographer Claudius Ptolemy (89–167), the Venedi are mentioned as one of the largest peoples in European Sarmatia (see also (21,25)), an expert in German linguistics, explains that the name Venedi (Venethi) meant "family" or "friends" to the Germanic tribes. The name has been preserved until the present day in the form of *Wenden* and *Windische*. Similarly, Slovenes are still known as Vends (Vendek) also in Hungary, although some Slovenes, such as for example those in Hungary, always referred to themselves as *Slovenes* (26). As written by F. Dvornik, the Slovenes are one of the rare nations who preserved their ancient name *Slovenes* (8), even though through history a large part of them adopted other territorial or state names, as for example the Carantanians (Carantani) (see **Figures 1** and **6**) or Carniolans.

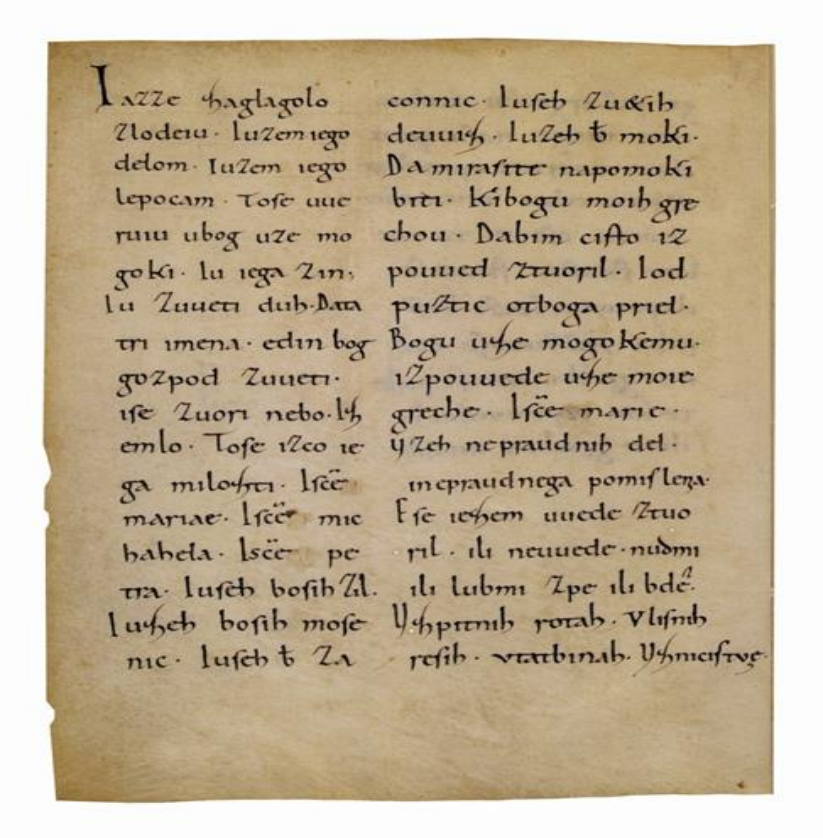

Figure 3. The Freising Manuscripts from the second half of the 10th century are the oldest known preserved manuscripts in the Slovene language and the oldest written record in the Latin alphabet in any Slavic language (12). The figure shows the first page of the 3rd Freising Manuscript, which is a penitential form that starts with the words "I renounce the devil ..." (27). The original is kept at the Bayerische Staatsbibliothek in Munich.

The names Winedi, Winidi, and Venedi/Venethi were used not only for the ancestors of present-day Slovenes but also for the ancestors of present-day Czechs, the Polabian Slavs, Slavs from Thuringia, and others (11,26); this notion is further reinforced by the Finnish name for Russia *Venäjä* (derived from *Venadä*), which was according to Zupanič acquired by the Finnish from the Germanic peoples (21). In relation to the Venedi being located on the shores of the Baltic Sea (23) and the region along the Vistula River (C. Plinius Secundus), it should be mentioned that even in the 16th century a map drawn by O. Magnus called the Gulf of Finland Sinus Venedicus, i.e. the Venedic Gulf (see also (22)). It is also interesting to note that between the 14th century and the year 1972, the Danish king held the title of "King of the Vends", which suggests that the Vends (Wends) settled in a broad area along the Baltic Sea.

The Slovene historian Janko Pleterski (28) gives credence to the hypothesis posed by J. Okulicz that the Veneti were originally an indigenous people in the region along the Baltic Sea (**Figure 4**), and that due to the pressure of the Germanic wave of colonisation they partially migrated to

the east, all the way to the Dnieper, where their culture and language fused with the native Baltic and Slavic population. The eastward migration of a group of Veneti (Venedi) is supposed to explain the previously mentioned writings by authors from the antiquity (Pliny, Tacitus, Ptolemy), who distinguished two groups of the Veneti, with the first located somewhere in the area between the Vistula and the Dnieper, and the second located along the coast of the Baltic Sea. This duality is also thought to be supported by the *Tabula Peutingeriana*, which mentions the Veneti and the Veneti-Sarmati (8), and by the hypothesis of the Slovene linguist France Bezlaj that the proto-Slavic language is actually a Baltic language infused with Venetian features (see also (28)). It cannot be disputed that the proto-Slavic language was closely related to the proto-Baltic language (there are hundreds of similar words between the two), the language of origin of today's Lithuanian language and the proto-Prussian language (which went extinct in the 18th century). Bezlaj's hypothesis on the proto-Slavic language as a Balto-Slavic language interspersed with Venetian features could explain why the Germanic peoples used a Venetian name for their eastern Slavic neighbours. If the first Baltic Veneti and the Veneti inhabiting the region along the Vistula River spoke closely-related languages, it is to be expected that the Germanic peoples (and the Finnic tribes) referred to both by the same name. What both of these groups – the Baltic Veneti and the Veneti along the Vistula River – called themselves remains unknown since there are no written records on the topic. Furthermore, unanswered questions remain as to the input of the Baltic Veneti in the formation of the ancient Slavs. With regard to Bezlaj's hypothesis on the proto-Slavic language as a Baltic language with Venetian features, it is quite possible that their impact was considerable. Over a longer period of time, it is common for a smaller group to adopt the language of the majority. This is a common occurrence regardless of social status, as was the case with the Normans in France, who adopted the French language and later on, after several hundreds of years, i.e. after settling down in England, the English Language, as well as with the Bulgarians. However, the opposite is also possible, i.e. that a larger group adopted the language of the minority, as we shall see in our later discussion.

Figure 4. The area populated by Slovenes in the 9th century A.D. in the regions of today's Slovenia and Austria, and in the northern part of today's Russia in the vicinity of Novgorod and lake Ilmen. Adapted from (23).

With regard to the Russians, it is worth mentioning the so-called Normanist Theory, formed by German historians in the 18th century (29), since similarly unfounded theories about Slovene history constructed by German historians from the 18th and 19th centuries are being adopted by many historians, including those from Western Europe, to this day. On the basis of a Russian Primary Chronicle from the beginning of the 12th century titled *Povest' vremennyh let*, the Normanist Theory of the above mentioned German historians states that the first rulers

reigning in what was to become present-day Russian territory belonged to the Scandinavian tribe Rus, who arrived in the area on the invitation of contending Slavic tribes (**Figure 4**), which most probably led to the domination of the Slavic tribes by the Scandinavian tribe. When 19th century archaeologists started to discover the advanced and highly developed civilisation of the Slavic tribes in southern Russia (23,24), it became clear that their civilisation extended all the way to the ancient Scyths, Goths, Romans, and Greeks (19,23,29), and that it was organised as a federation already prior to Scandinavian conquest (30).

According to Zupanič (21) the name Vends is furthermore contained in the name of the Ostrogoth king Vinitharius (the conqueror of the Vends, the Wendenkämpfer), who, as mentioned by Jordanes in the 4th century, defeated the western Antes and crucified their king Boz along with his sons and seventy nobles (23). Although Zupanič, who also signed his name Županič, attempts to show in the article *Boz rex Antorum* (21) that the name Boz is not of Slavic origin, Ammianus Marcellinus, a Roman officer of Greek descent from the 4th century, who wrote in Latin, refers to Vinitharius also by a different name, i.e. Vithimir, which does indeed sound Slavic. It is quite possible that Vinitharius actually had two names, a Gothic and a Slavic one (23).

With the names Venedi, Venethi, Venetii, Winedi, and Vinedi, neither Niko Zupanič nor Milko Kos refer to the antique tribe of the Veneti from the present-day Italian region of Veneto, who lived along the river Po, but rather to the Slavic tribe of the Venedi. It cannot be completely ruled out that prior to Slovene migrations in the 6th century, some Slavic Venedi already colonised today's Slovene territory (**Figure 4**), which was linked to the Baltic settlement area of the Venedi by the ancient Amber Road, already mentioned by Herodotus and Tacitus (22). Similarly, we cannot exclude the possibility that the antique tribe of the Veneti from the Po river basin had a connection to the indigenous Veneti from the region along the Baltic Sea – they might have been in contact via the ancient Amber Road even prior to the Baltic Veneti mixing with the neighbouring Slavic tribes.

The *Alpine* Slovenes (**Figure 4**) were not a uniform tribe that migrated as one to the regions of the central eastern and eastern Alps, the upper Sava River, and the Soča River; rather, they migrated in several waves (26,31). Grafenauer believes that the first settlement wave took place in the 6th century (32), proceeding first from the north (the western Slavic language group) and later on from the east; a testament to this fact is supposedly the current Slovene language, which has strong western Slavic roots (26). It should be noted that the preserved Slovene Carinthian dialects display many more typical western Slavic features than the Carniolan dialects, which represent the basis for the modern Slovene language (26).

In their new homeland, as recent genetic studies clearly show, the Alpine Slovenes at least partially mixed with the indigenous people (33,34). According to linguist Luka Repanšek, without the above mentioned intermixing of peoples, the Celtic heritage in the toponymy of

the southeastern Alpine region could not have been preserved (35). An analysis of pre-Slavic remnants in the names appearing in present-day Slovene territory has shown convincingly that the old claims according to which the Alpine Slovenes (Fig. 4) migrated to a virtually unpopulated area were baseless. In some mountainous regions, their harmonious coexistence with the indigenous population lasted even half a millennium. In some settlements, archaeologists recently found traces of uninterrupted continuity between antique and Slovene colonisation, which explains the fast cultural development of Slovenes after they settled down in their new homeland. The Slovene language preserved even some Romance and pre-Romance basic features, which cannot be found in any of the neighbouring Romance languages (26). Otto Kronsteiner, an Austrian Slavicist, therefore believes that the Slovenes, and partially their present-day language, are a mixture of Slavic, Celtic, and Romance (vulgar Latin) elements. As an interesting fact, it should also be noted that it is only with the Slovenes, the Irish, and the Scots (9) that cases of a particular metabolic disease were reported, while recent DNA analyses have shown a distinct percentage of Celtic genetic heritage in Slovenes (bearing in mind that the Celts settled in present-day Slovene territory around 300 B.C.). Furthermore, Bezlaj believed that the modern Slovene language includes many words of Illyrian descent, while the Polish scientist Lech Leciejewicz (36) indicated the presence of western Slavic groups also in northern Russia (**Figure 4**).

Most recent genetic studies (33,34,37,38) also confirm that the present-day Slovenes are to a considerable extent the descendents of indigenous peoples who lived in present-day Slovene territory and in the former Slovene territories in present-day Austria, Hungary and Croatia (**Figure 1**). This implies, similar to the situation in many other Slavic regions in Europe (39), the cultural and linguistic assimilation of indigenous populations by the arriving Slovene people as an important mechanism of the spread of the Slovene (Slavic) language after the decline and later fall of the western part of the Roman Empire. It therefore seems that Slovene and also Slavic expansions in general were to a large extent linguistic (40). The strong assimilation of indigenous peoples by the arriving smaller number of incoming Slovene people might have been possible because of the highly egalitarian culture of the arriving Slovene people, who among other things had no obligations to pay taxes (41), which may have been very attractive to indigenous populations (called Vlachs by the Slovenes).

It should also be stressed that the percentage of genetic in the modern Slovene population which has been inherited from indigenous peoples may vary considerably vary between the paternal and maternal lineages (33,34,37,38,42). The genetic continuity of several maternal lineages in central Europe from the times of the Bronze and Iron Ages does not seem to be connected to extensive demographic changes, regardless of the visible changes in the material culture of central Europe at the end of the Western Roman Empire (38). On the other hand, the genetic homogeneity of the paternal lineages of Poles, Slovaks, Czechs, Lusatians, Slovenes, western Croats, Belarusians, Russians and Ukrainians, extending from the Alps to the upper Volga (42), indicates that the Slavic expansion of predominantly male populations probably

started somewhere within the regions of the present-day states of the above mentioned Slavic nations. For example, in the middle Dnieper basin, as suggested by Rebała et al. (42). Based on the above cited studies, one can conclude that understanding the ethnogenesis of Slovenes requires a synthesis of autochthonous, allochthonous and cultural assimilation models (41).

French historian Francis Conte (43) suggested that the Gothic invasion of the region inhabited by the ancestors of present-day Slovenes (Sloveni, Slovenci) – namely, the region of Sclaveni between the Carpathian Mountains and the upper Dnieper River – divided the indigenous population into two groups. One group supposedly moved southward and settled down in the territory of today's Slovenia and Austria, while the other group supposedly moved northeast to the region of Lake Illmen, where they supposedly established the town of Novgorod (43), which corresponds to the similar view held also by Vernadsky (**Figure 4**). In ancient times, Lake Illmen was known as the Slovene Sea, and the town of Novgorod as Slovensk. There was also a district in Novgorod called the *Slovenski konec* (*Slovene District*) (22). The division of the Slovene tribe (or/and the Sclaveni) into two parts can also be supported by the finding of Bruno Volpi Lisjak (22), who discovered that the same name for the traditional monoxile boat (i.e. "čupa") was used only in the Slovene coastal villages between Trst/Trieste and the Timavo river (now in Italy) and in the Russian region near lake Ilmen close to Novgorod (Novgorod the Great). The Croats, who were the Slovenes' geographically closest Slavic nation as they populated the east Adriatic coast, already used a different name (i.e. "ladva") for the same type of monoxile boat (22). Also note that the craftsmanship of shipbuilding was highly developed at the east and north Adriatic Sea already before the ancient Romans occupied this region. Therefore, the appearance of the Slovene traditional monoxile boat "čupa" at the east Adriatic coast after the fall of the Western Roman Empire strongly supports the assumption of the arrival of a Slovene tribe into this region after the fall of the Roman Empire or maybe in small groups already in the final stage of the Roman Empire.

The case of the place name Vipolže and similar words found in Slovenia and northern Russia must also be mentioned, since there are 113 places in Russia, mostly in northern Russia around the town of Novgorod, called Vypolzovo (22,26). In old Russian, the phrase *vypolzovskije žiteli* denotes free people. In this case, possibly, parallels could be drawn with the so-called *kosezi* and Slovene place names such as Koseze. The Germans called the *kosezi,* a social class of the noble people (German: Edlinger), a special Slovene social class which formed a military retinue to the prince of the Slovene principality Carantania (18). Hence, we can guess that *kosez* is an old Slavic dialectal legal term that was brought to present-day Slovene territory by the same colonisation wave that formed the core of Novgorod Russia (26).

The previously mentioned Slovene linguist France Bezlaj composed a list of a wide array of Slovene words and toponymic roots typical of northern Russia (26). In line with the reasoning outlined above, there is more than enough evidence that what forms the linguistic basis of the Slovene language is in fact a northern Slavic language, which, however, developed under

constant southern Slavic influence ever since its beginnings, i.e. since the settlement of Carantania (26). Separate migrations resulted in the Slovene language having also strong western Slavic elements (26,39); this claim is supported by new archaeological findings on motorway construction sites in the vicinity of Maribor and in Prekmurje (9), as well as the most recent genetic analyses, which point to strong maternal and paternal genetic links between Slovenes and the western Slavic population, i.e. between the Slovenian, Slovakian and Czech populations (33,34,37), indicating, among others things, a common population of ancestors and/or a significant transfer of genes between the ancestors of Slovenes and Slovaks (37). The most recent genetic studies therefore confirm Bezlaj's hypotheses about the origin of the Slovene language and the Slovene nation (44). The links would probably be even stronger if the genetic studies included autochthonous Slovenes living in the territory of present-day Austrian Carinthia. Genetic studies also indicate strong links between Slovenes and Hungarians (33,37,44), which is to be expected if one takes into account that the Hungarians assimilated the Slovene (Carantanian) and the Moravian population (45) in the region of former Lower Pannonia under the rule of the Princes Pribina and Kocel (Fig. 1). It should also be noted that the most recent archaeological findings do not confirm the direct domination of the Avars over the ancestors of present-day Slovenes (9).

One could compose an expansive dictionary of Slovene words with suitable parallels found in northern Slavic languages but not in Balkan Slavic languages. This shows how strong the link between the Slovene language and the northern Slavic languages truly is, probably as a result of old Slavic migrations (26). All these observations in the Slovene language support the scientific views on different migration waves involved in the Slovene settlement in the Alps and their surroundings (**Figure 1**), which brought about the formation of the Slovene language as a Slavic-Baltic mix interspersed with features of indigenous languages. This does not exclude the possibility that, during their migration, a part of the ancestors of modern-day Slovenes intermixed, for example with what was left of the eastern Germanic tribes (26). Since there is a lack of written records, no other method besides genetic studies used in the research focusing on the old historic migrations of the ancestors of present-day Slovenes is more telling than the parallels that can be drawn between Baltic and northern Slavic words on the one hand and Slovene words on the other. One needs to bear in mind that the first smaller settlement waves of the Slavic people proceeding towards the region later known as Carantania happened earlier than in the 6th century (26). The Slavic people started to colonise the territory of today's Czech Republic already in the 4th century.

A wide array of Baltic-Slovene linguistic parallels and other archaisms speaks in favour of the assumption that the beginnings of Slovene linguistic individuality date back to at least the 4th century A.D. It is impossible to determine whether these beginnings go back to the old Slavic homeland (**Figure 4**) or if the first migration wave on Slovene (Carantanian) soil happened already a couple of centuries prior to 6th century migrations (26). Judging by linguistic studies,

the claims about a single migration of Slovenes to Carantania and its neighbouring regions (Fig. 6) are merely romantic, mythological stories (26).

On the basis of Bulgarian/Macedonian-Slovene linguistic parallels, Bezlaj presupposes that a part of the northern Slavic tribes migrated across the slopes of the Alps and the Carpathians and broke through all the way to the south and the Aegean Sea. In the old times, the name *Sloveni* was used only for the Slavic peoples in the vicinity of Thessaloniki, for the inhabitants of Novgorod, Russia, and for the Alpine Slovenes, where it also appears in place names. From the names of towns such as Slovenj Gradec, it can be inferred that the name *Sloveni* was used in the first centuries after the settlement of the ancestors of present-day Slovenes in a very general context (31). Slovenes from the regions of Prekmurje and Veneto were probably always called *Slovenje* (Slovenes) (26,46).

This could mean that the ancestors of present-day Slovenes, as members of the tribe Sloveni (in the region of *Sclaveni*), migrated in three main directions (23,43): to the north to the vicinity of Novgorod, to the south to the regions of Carantania, Posavska krajina and Lower Pannonia (Fig. 4), and then the latter group continued even further south in the third direction, to the vicinity of Thessaloniki (see also (47)).

Since the ancestors of present-day Slovenes were among all the Slavic nations the first to convert to Christianity, the Slovene Slavicist Franc Miklošič believed the language of Slovene Carantanians to have been the language of origin of so-called Old Church Slavic, i.e. the "language of worship" of all the Slavic people, who subsequently changed and transformed the language according to their needs. Later, it became clear that the basis of Old Church Slavic was a dialect of the Slavic people in the vicinity of Thessaloniki, who were probably also known as the Slovenes (26).

Since, according to F. Miklošič (German: von Miklosich), the ancestors of present-day Slovenes were the first among the Slavs to come in contact with the civilised world of the time in southern and western Europe, their (only marginally changed) name was used to designate all the Slavic peoples (see (48)). Note that the name Slovenes (Slovenians) at first referred to only one of the many Slavic tribes (**Figure 4**) (23).

The modern Slovene collective term "Slovani" (corresponding to English "Slavs") is an artificial neologism that was coined on the territories of today's Slovak Republic and Poland. In Slovenia, it was first used by Janez Bleiweis in his journal *Novice* just before the March Revolution in 1848. The name for the Slovene nation *Slovenci* (Sloveni, Slovenje) is an archaism, one of the many, which the Slovene language preserved the most out of all the Slavic languages. We can agree with the hypothesis of the Slovene art historian Janez Höfler that Trubar's 1555 manuscript "Lubi Slovenci" (English "Dear Slovenes") did not emerge out of nothing; it is rather a reflection of the old awareness of Slovene national identity (without political awareness),

which extends all the way back to the time of the Slovene principality Carantania (54), and even further back to a time before the ancestors of present-day Slovenes settled in the central and eastern Alps and Lower Pannonia (23,26). In accordance with Vernadsky, Bezlaj, Höfler, and also Miklošič, the historian Igor Grdina (49) believes that the Latin terms "Sclavinia" and "Sclavorum gentem" in the *Conversio Bagoariorum et Carantanorum* from the 9th century relate to Slovenia and Slovenes in Carantania and Lower Pannonia (**Figure 1**), as already discussed above. In addition, Höfler (50)(Höfler, 2009) lists further sources in which the name Sclavinia is discussed in relation to the regions of Carantania and Lower Pannonia (51,52) as the southern and southeastern neighbours of Bavaria. In one of the documents from the Court of Louis I, written in the period between 822 and 827, the list of lands, besides France, Burgundy, Provence, and Italy, also includes Baioaria (Bavaria) and Sclavinia (Slovenia) (53).

To the above mentioned Slovenes in Carantania and Lower Pannonia (**Figure 1**), we can also add the Slovenes in Sava March (**Figure 6**), the core of the later Duchy of Carniola. According to Grdina, the Sclavs should not be translated as "Slavic people" in today's sense of the word, but rather as Slovenes, since the Latin manuscript *Conversio Bagoariorum et Carantanorum* also mentions Moravians, who are designated by a Moravian name not hyponimic to the "sclavonic" name (49). The region of Slovenia (Latin: Sclavinia) is also mentioned in other medieval sources, including *Nestor's Chronicles*, which mentions the region on the same level as Croatian and Serbian regions (49). Therefore, the language of inauguration of Slovene Carinthian dukes after the 10th century cannot be defined as a Slavic language but rather as Slovene language (see also **Figure 2**) since the Slovene language at the time already developed some basic distinctive features in relation to other neighbouring and more distant Slavic languages (49).

In the 10th century, during the reign of Emperor Otto III, the only major Slavic people of the time included in the Holy Roman Empire were the Slovenes, who had their seat in Carinthia and its adjacent connected counties, such as the Carniolan March; this was roughly the region of the Carantanian Kingdom of Arnulf of Carantania from the 9th century (**Figure 6**), which recognised the privileged social group of the *kosezi*, a particularity known only to Slovenes, as discussed. Thus, in the famous illustration of the tribute of provinces Sclavinia, Germania, Galia, and Roma to Emperor Otto III (**Figure 5**, left panel), one can justifiably equate *Sclavinia* with *Slovenia* as the region of Slovenes within the Carantanian sphere of influence in the Holy Roman Empire in the 10th century (49,50), which comprised the regions of the future duchies of Carinthia, Carniola, Styria, the princely County of Gorica (Gorizia), and parts of northern Istria. Note that the origin of the Italian name Gorizia is the original Slovene name of the city Gorica, which means a small mountain.

In the same manner, Slovenia may be equated with Sclavinia also in a 14th century fresco found in St. Peter the Younger Church in Strasbourg (**Figure 5**, right panel), which depicts the tribute of European nations the Holy Cross. In it, Sclavinia represents the Slovene nation, alongside the nations of Germania, Italia, Gallia, Anglia, Scothia, Arragonia, Hungaria, Polonia, and others

(50,54). On the basis of everything mentioned above, it can be reiterated that Trubar's address "Lubi Slovenci" (English "Dear Slovenes") from 1555 was no novelty but rather a reflection of the ancient tradition dating back to the Carantanian period (54) and even further, (see also Fig. 4) back to the period before the ancestors of present-day Slovenes settled in the region of the eastern Alps and Lake Balaton (23,43,47).

Figure 5. In the famous illustration of the tribute of Sclavinia, Germania, Galia, and Roma to Emperor Otto III from the 10th century (left), Sclavinia can be equated with Slovenia, i.e. the region of the Slovenes of the time (Fig. 1) within the Holy Roman Empire (49,50), a region much bigger than present-day Slovenia. The original is kept at the Bayerische Staatsbibliothek in Munich. The figure on the right shows an illustration of Sclavonia, i.e. the Slovene nation, in the fresco of the Strasbourg Cathedral, which depicts a tribute of the European nations to the Holy Cross (50).

In the area of Novgorod (**Figure 4**), the name Sloveni (Slovenci) (English: Slovenes or Slovenians) disappeared completely after the 15th century; by contrast, the aforementioned name was still used by people in southern Istria in the 1960s (26). Bezlaj therefore suggests that if we decide to speak about the loss of Slovene ethnic territory in the north, we should also consider it in the south and southwest. In the 17th and partially also the 18th century, the Kaykavian and Chakavian Croats were still called Slovenci or Slovinci (26). Thus, even in the middle of the 19th century, Austrian ethnographers called them Sloveno-Croats. Kopitar regarded Kaykavian Croatian only as a dialect on the margin of the Slovene language area (12,55). Therefore, the historian Stane Granda believes that the naming of the great peasant revolt in 1473 as the Slovene and Croat revolt was politically motivated rather than substantive as all the participants were part of the same nation (9). The Kaykavians from Zagora were treated separately from other Slovenes mainly due to the border, not due to their language (9). It must

be added that the medieval region of Slavonia from the time when Croatia extended to the Sava River in the north (see also **Figure 1**) was ethnically different region than present-day Slavonia (a part of the Republic of Croatia), as it has undergone significant ethnic changes since the late Middle Ages due to various reasons, including the Turkish invasion. The name of present-day Slavonia originates from *Sclavonia*, therefore, prince Louis of Lower Pannonia (Liudewitus, dux Pannoniae inferioris) cannot be considered a Croatian prince and Lower Pannonia cannot be considered Pannonian Croatia (**Figure 1**). It was also logical that prince Louis of Lower Pannonia started his uprising/war against the Franks (bellum Liudewiticum) in the first half of the 9th century together with the Slovene Carantanians and Carniolans because they were all Slovenes (Latin: Sclaveni) from Sclavinia (**Figure 1**). Accordingly, even modern genetic studies indicate a closer genetic kinship between Slovenes and western Croats (42) than between other Croats and Slovenes.

With the Hungarian invasion and the foundation of the medieval Hungarian state, the Slovenes also lost the territorial link with their kinsmen in the present-day Slovak Republic (Slovak: Slovenská republika) as well as, at least partially, with present-day Croatian Slavonia (9) (**Figure 1**). Slovaks and Slovenes probably participated in the same tribal union established under the leadership of king Samo in the period from 623 to 658 A.D., see also (18).

The similarity between hand-made ceramic items found at some archaeological sites in the regions of old Slavic settlements from the 6th century in the territories of present-day Slovenia and the Slovak Republic and the ceramic items found in distant Ukraine (56) corresponds with the previously mentioned hypothesis that the ancestors of present-day Slovenes also included the Sclaveni (Slovenes) who migrated to the Alps (**Figure 4**) from the territory around lake Ilmen, arriving there from the region along the upper Dnieper River (23), i.e. from present-day Ukraine. High quality ceramic items from the Late Iron Age and from the Roman Empire period found at Slovene archaeological sites in present-day Slovenia speak in favour of the Slovene colonisation of the Alpine region. However, concerning Bezlaj's finding that the Slovene language contains words which appear across the entire Slavic world, some of them only in particular areas, it can be assumed that the ancestors of present-day Slovenes migrated to the settlement area of the Alpine Slovenes (Sloveni) in several waves and from different regions.

As already mentioned, the possibility of Slavic migration flows prior to the 6th century should not be completely ruled out (8,28). According to reports written by the Byzantine diplomat Priscus (23), some of the inhabitants of Pannonia (**Figure 1**) in the 5th century ate millet and drank beverages made out of "medos" (honey) and "kamos" (barley), they had "strava" (a funeral ceremony), and spoke a language that was neither Gothic nor Hun nor Latin, which suggests that they might have been Slavs (8,57). Jordanes (8) also reports the use of the word "strava" in the region along the Tisa (Tisza) River in the middle of the 5th century. Of great interest is also the Gothic origin of words such as *sword*, *helmet*, *regiment*, *prince* ("*meč*", "*šlem*", "*polk*", and "*knez*"), and others (23). The results of archaeological research in Moravia

and the Slovak Republic have shown that the Slavs were to a lesser extent present in those regions already in the 4th and 5th centuries A.D., which is in line with the reports of Procopius (*De Bello Gothico*), which state that between 508 and 514 A.D., the Herules, after their defeat in Langobardia (Lombardy), decided to return home, to the region of present-day Denmark, for which they received safe passage through the Slavic region (8).

The intermixing of various peoples in the formation of present-day modern European nations is not typical only of central European Slovenes but also of many other nations. In the Albanian language, for example, approximately 25 percent of the words are of Romance, 20 percent of Turkish, 15 percent of New-Greek, and 10 percent of Slavic origin, which means that the original vocabulary of said language does not contain more than 30 percent of words of lesser-known origin (10). The assumption about the Illyrian origin of the Albanian people can therefore be regarded only as a myth (58,10), established by some modern era historians.

It has been shown that a special type of hereditary dynastic succession within one family (11) was in effect in the land of the Alpine Slovenes (i.e. the Slovene Carantanians), which is probably the oldest known true Slavic state formation (**Figure 1**) (11,59) and existed in its initial form for about 200 years (from the first half of the 7th century until the third decade of the 9th century), in its last period under Frankish rule. The succession did not pass only from father to son, such as in the example of prince Borut (740–749), who was succeeded by his son prince Gorazd (749–752), but also to secondary family branches. Gorazd, for example, was succeeded by his cousin prince Hotimir (752–769) (59). Hotimir's successors, i.e. domestic Carantanian princes, were Valtunk, Pribislav, Semika, Stojmir, and Etgar, who was prince of Carantania until 828.

History also knows other Slovene princes, dukes or counts of Alpine Slovenes, for example prince/duke Domician, who lived in the 2nd half of the 8th century and the beginning of the 9th century (60,61) and is considered as the founder of the Millstatt Abbey church, located in present-day Austria. In 1992, archaeologists discovered in Millstatt Abbey the remnants of an original panel from the beginning of the 9th century placed over the grave of Domician, with the partially preserved inscription (QVIES)CIT DOMICIA(NVS) . . . (K)AROLI IMPERATORIS . . . (PA)GANITA(TEM), which confirmed that Domician lived in the time of Charlemagne (60)(Glaser, 1993), in agreement with the description of his life from the beginning of the 14th century, which is based on the previous texts from the 12th and 13th centuries (60,61,62). A Slovene nobleman, the blessed Domician is worshiped as a saint of the Catholic Church. Domician of Carantania (Carinthia).

The title *dux* puts Domician in the first line of the Carantanian upper class. However, his name is not found among those Carantanian princes who are mentioned in *Conversio Bagoariorum et Carantanorum* between the years 772 and 828. One could therefore assume that either Domician wasn't quite at the top of the country's nobility, or that there are other reasons for it.

Perhaps the name Domician is also the Christian baptismal name of a Slovene prince, who is listed in the *Conversio* under his Slovene name (63). It has also been suggested that Domician was a prince (duke, count) of Liburnia (64)(see also **Figure 6**). Both, Liburnia and Carantania Minor (the maternal principality of Carantania) can be considered as parts of the larger territory of Sclavinia, inhabited by Alpine Slovenes, in accordance with the spirit of *Conversio Bagoariorum et Carantanorum*, where Carantania and Lower Pannonia north of the Drava River (see also Fig. 1) are designated as Sclavinia, i.e. Slovenia.

After 828, Carantania (**Figure 6**) as a tribal princedom of Carantanian Slovenes within the Holy Roman Empire, existed until the 11th century. Members of the Carolingian dynasty Karlman (830–880) and his son Arnulf of Carantania (850–899), who was Carantanian on his mother's side, were also Carantanian rulers and signed documents with the title *Rex Carantanorum*, i.e. King of Carantania (65).

It has been proved that the Carantanian Prince had an accompanying landed nobility already in the middle of the 8th century, i.e. prior to the establishment of the Frankish social system (after 828). For the Carantanian society of the time, sources also mention other princeps (*principes*) and counts (*comites*). A document dating from 830 notes that the nobleman Baaz, who was a Carantanian Slovene, gave his heritage, including his unfree people and all of his properties, to the Diocese of Freising.

The first proprietary church of the Carantanian princes was the Church of St. Mary at Zollfeld (Slovene: Gosposvetsko polje), which was built by prince Hotimir (752–769) and consecrated by the provincial bishop Modestus.

After the removal of local Carantanian princes, the ownership of this ancient church passed to the crown and, through a deed of donation, to the Salzburg diocese. In a similar manner, approximately ten fortified manors/castles (*curtis*), which received their names already in the Frankish period, among them Krn Castle (German: Karnburg), Možberk Castle (German: Moosburg), Althofen, Ribnica, Breže (German: Friesach) and others. The construction of these original castles differed significantly from early feudal Frankish towers, representing the seats of old nobility from the reign of Slavic princes in Carantania. The name Carantania itself does not stem from a tribal name, as is the case with the majority of other Slavic nations; instead, it is associated with the seat of the Carantanian Prince at Krn Castle (curtis Carantana, civitas Carantana) (**Figure 6**).

Figure 6. The Carantanian Kingdom of Arnulf of Carantania (850–899) with its seat in Carantania Minor (dark green) included also the adjacent county (march) along the Sava River (Sava March, later Duchy of Carniola), Upper Pannonia and northern part of Lower Pannonia (see also (66)). Arnulf's Carantanian Kingdom in the 9th century, which almost entirely covered the settlement area of Slovenes/Carantanians (denoted by different shades of green), represented the basis for the subsequent formation of the duchies of Carinthia, Carniola (67), and Styria. Carantanian Kingdom can be considered as a Slovene, i.e. non-Germanic, tribal duchy within the Holy Roman Empire (68,69). Adaptad from (70).

In the scope of its extended Frankish territorial unit (**Figure 6**), Carantania, retaining the name of a considerably smaller territory, now called Carantania Minor (**Figure1**), united the greater part of the ancestors of present-day Slovenes up until its disintegration in the 11th century. The name

Carantanorum region (the county/principality of Carantanians), dating from 819 and designating the colonisation unit of Carantanians in Lower Pannonia north of the Drava River (**Figures 1 and 6**), shows that the Carantanians already shared some form of common awareness since it does not relate only to the population of Carantania Minor. In his 871 work, a priest from Salzburg who fled from Lower Pannonia (Fig. 6) treats the history of Lower Pannonia only as part of the history of Carantanians, and another source mentions the banishment of Archbishop St. Methodius from Lower Pannonia as banishment from the Carantanian region. Furthermore, Kocel (833–876), Count and Prince of Lower Pannonia, who succeeded his father Pribina, is in some documents referred to as *quidam Carantanus*, while in 880 Carantania also included Blatenski kostel (German: Moosburg, Latin: Urbs Paludarum, present-day *Zalavár*), the capital of Pribina and Kocel.

Between 869 and 874, Prince Kocel ruled as a completely independent sovereign. At Kocel's Court, they introduced services of worship in the local Slovene language and the use of the Glagolitic alphabet. Thus, Slovene linguist Jernej Kopitar (1780–1844) believed (as did Miklošič later on) that the so-called "korotanščina" (a Pannonian proto-Slovene language) was the basis of the Church Slavonic language of St. Methodius, i.e. Old Church Slavonic (48), which later proved to be false.

Kocel's Court (Fig. 1) was probably also where the first Slavic code of laws, "Zakon sudni ljudem", was written; it is supposed that the author of this work was Methodius. Per Kocel's wish, the Pope appointed Methodius the Archbishop of the reconstituted Pannonian Archdiocese (1,4). Following Kocel's fall, the Great Carantania also comprised the county (march) along the Sava River (Carniola) and both Upper and Lower Pannonia under the rule of Arnulf of Carantania **(Figure 6)**.

From the 9th century onwards, the name Carantanians was frequently used for all Alpine and Pannonian ancestors of present-day Slovenes, as well as for their land (68), although, as previously mentioned, Carantania was regarded as part of Sclavinia, i.e. Slovenia (**Figure 5**) (23,49,50). The manuscript on the conversion of Carantanians and Bavarians to Christianity *Conversio Bagoariorum et Carantanorum* refers to Carantanians as a "Slavic tribe" that lived in Carantania Minor and Lower Pannonia. In different records of the deeds of donation, both the phrase "regions of Bavaria" and the phrase "regions of Carantania" are used (65). From the 9th to the 13th century, European authors – from Russian to English and even Arabic authors – frequently used the word *Carantanians* to denote all Slovenes as a whole. The *Russian Chronicles* (*Letopisi*) called the 12th century Slovenes "Horutani" (i.e. Carantanians), while an Arabic geographer from Sicily described Carantania as a region comprising the entirety of eastern Alps and Pannonia. Furthermore, in 1168, a chronicler from Saxony included Carantanians in the group of *western Slavic nations*, besides the Poles, Czechs, Moravians, Prussians, and Polabian Serbs.

Figure 7. Slovene philosopher, mathematician, astronomer, astrologer, translator, and author Herman of Carinthia (Hermanus de Carinthia), born around 1100 in the Duchy of Carinthia (part of former Carantania) in the vicinity of St. Peter im Holz (Slovene: Sveti Peter v Lesu) in present-day Austrian Carinthia (71,72,73,74,75). Herman of Carinthia studied in Chartres and Paris, France, where he also died in 1160. Among other topics, Herman of Carinthia wrote an original treatise on roots in mathematics (76,77).

Around 880, the English King Alfred wrote that the Danube represented the northern border of Carantania with Moravia Magna, while in the east Carantania bordered on the Bulgarians, from which the Carantanians were separated only by the wilderness between the rivers Danube and Tisa/Tisza (32) (see also **Figure 1**). It is clear that present-day Austrian and Slovene Carinthia encompassed only the central part of the broader Carantanian territory (**Figure 6**); thus, in the geographical sense, they are two of the many successors of Carantania.

Austrian Slavicist O. Kronsteiner therefore states that the "existence of the thousand-year German Austria is a preconception and a lie." Austria has always been a multi-cultural land, and the predominant population in its central area were at first Slovenes. It was only much later that German gained ground and became the dominant language (78), especially in the northern Alpine regions, sparsely populated by the ancestors of present-day Slovenes, which later on resulted in the domination of Bavarian colonisers (79). Just the opposite occurred in the German colonisation areas south of the Drava River, which were almost completely taken by Slovenes. Only one large settlement area, that of the Kočevarji (see Figs. 36 and 40), who spoke a dialectal mixture of Thuringian and Frankish (79), was preserved; as can be deduced from the surnames on the commemorative plaque honouring the victims of WWI in the village of Stara Cerkev pri Kočevju, the Kočevarji (Gottscheers) mixed with Slovenes and were also partially germanised Slovenes.

All the regional names up to the boundary between Linz and East Tyrol that contain Slavic elements – which is a lot of them – are of Carantanian in origin, i.e. Slovene (78). In the early Middle Ages, the language of the largest part of present-day Austria was Slovene; Slovene was the main language not only in Carinthia but also in Styria, i.e. in the regions of former Great Carantania (**Figure 6**). Therefore, Kronsteiner believes that Austrian cultural history should be written anew. Carantanians may be regarded not only as the ancestors of present-day Slovenes but also, to some extent, as the ancestors of present-day Austrians (80).

So, between the 9th and 13th centuries, the name Carantanians in most cases referred to the ancestors of present-day Slovenes, even though Carantania Minor did not include some other (border) Slavene duchies (counties), such as Carniola along the upper Sava River and Liburnia west of the original Carantania (**Figure 6**). As previously mentioned, Carniola became a part of Great Carantania only later, in the second half of the 9th century, as a march (borderland) along the Sava River (Fig. 6). If the Bavarians, the Thuringii, the Saxons, and others are among the ancestors of present-day Germans, then the Carantanians are most definitely the ancestors of present-day Slovenes. The same could also be said of Carantanians with regard to present-day Austrians, since Slovenes, who were later on germanised, were up to the 19th century the predominant population in the former central Carantanian region. Between the 9th and the 13th century, the name Carantanians was a generally accepted name for all the ancestors of present-day Slovenes. During the renaissance of the Slovene people, the name "Carantanian" regained its old Latin meaning. Proof of this is, for example, the inscription on the tombstone of linguist Jernej Kopitar, which says "Bartholomeus Kopitar *Carantanus*", even though Kopitar was born in Carniola near Ljubljana. Moreover, in his poems about the Austrian revolution, Karl Marx referred to the Slovenes as Carantanians as late as in 1848, i.e. after Kopitar's death (see (81)).

Examples of the highest nobility having Carantanian (Slovene) names date back to the 7th century. The Princes Pribina and Kocel (**Figure 1**) were in their capital *Blatenski kostel* predominantly surrounded by Carantanian, i.e. Slovene nobility. The Carantanian Duke Arnulf of Carantania (850–899), a member of the Carolingian dynasty and great-grandson of Charles I, who signed with the title *Rex Carantanorum*, i.e. King of Carantanians, the same as his father, was the illegitimate son of a Carantanian woman and the Bavarian King Karlman I (65). His army consisted of Bavarians and Carantanians. With the help of said army, Arnulf of Carantania became King of East Francia and was the last Carolingian to be crowned Emperor of the Holy Roman Empire in 896. The Slovene Carantanian nobility largely fused with the Bavarian nobility (11) and gradually acquired Germanic names and surnames – a process that continued up to the 19th century; however, the Slovene nobility preserved its knowledge of the Slovene language.

Some Slovenes have the incomprehensible habit of forgetting or renouncing parts of their nation's history when it comes to those periods in which rulers were partially or completely of non-Slovene descent, even though the Carantanian Prince was granted a special right to use his

native Slavic language also while attending the Imperial Court (49). Because of the impact of German Slavophobic historiography, the Carantanian Kingdom of Arnulf of Carantania (Fig. 6), who was of Carantanian descent on his mother's side, was not regarded as Slovene tribal duchy. In some books, in fact, Arnulf is even referred to as Arnulf of Carinthia (instead of Carantania), despite the fact that the Duchy of Carinthia did not even exist at the time. If the English looked at their own history in the same way as some Slovenes, they should have renounced their history from William the Conqueror until the start of the Renaissance, and they would have had many more reasons to do so than Slovenes (see also (82)) since, as opposed to the Slovene lands, the high nobility of Norman descent in England used a foreign language, i.e. French, also as their colloquial language from the time of William the Conqueror until the Renaissance (i.e. three and a half centuries). And yet, the present-day English do not seem to be bothered by that. As an interesting aside, let us also note that, much later, even Empress Maria Theresa in her letters to her adult children did not use German (she only spoke the Viennese dialect) but French, the same as the majority of the Russian high nobility (29).

To conclude, in recent times, even foreign historians have appealed to the Slovene public not to forfeit their own history unnecessarily. The Austrian historian Kronsteiner states (78): "The Freising Manuscripts are of Slovene origin. The Slovene language was spoken in the region of present-day Austria. All regional names between Linz and East Tyrol which contain Slavic elements – and there are many – are not Slavic but Slovene in origin. And finally, Slovene was the provincial language in Carinthia, Styria, and Carniola, i.e. in the region of the former Kingdom of Great Carantania" (**Figure 6**).

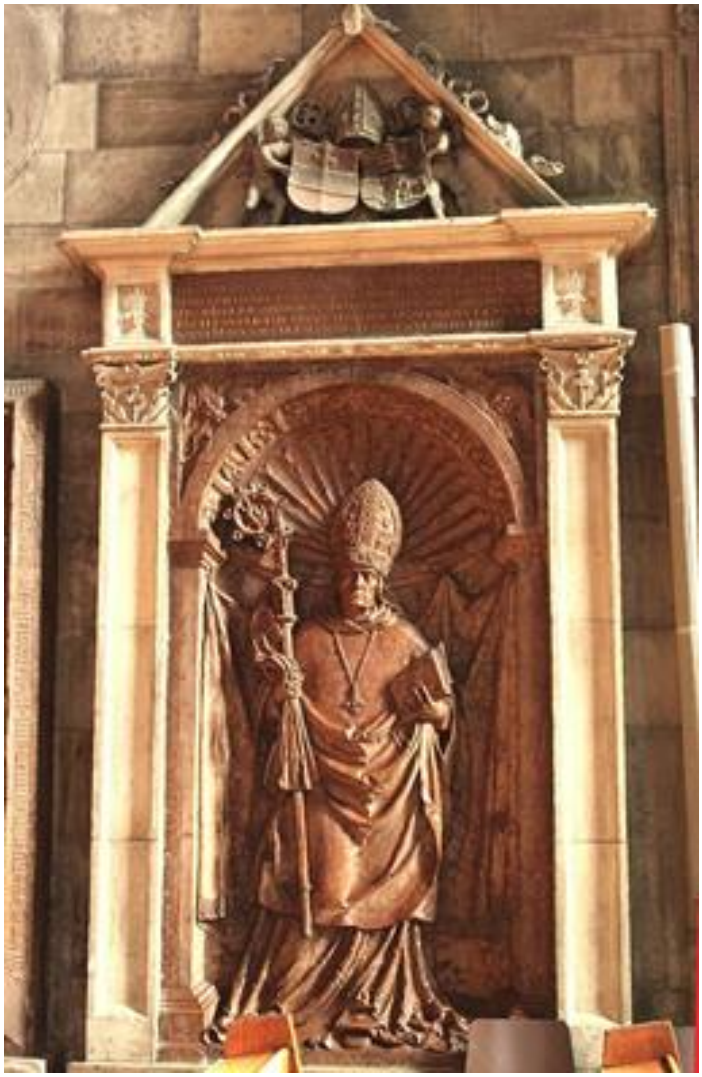

Figure 8. The tombstone of the Slovene conductor, composer, and Bishop of Vienna Jurij de Slatkonja (German: Georg von Slatkonia, Greek: Chrysippus) in St. Stephen's Cathedral in Vienna. The Latin epitaph starts with: Georgius Slatkonia, of Carniolan nationality, from Ljubljana, bishop with the Church of this city, etc. (83). Bishop Jurij (Georg) Slatkonja (1456–1522), a native of Ljubljana (84,85,86), was also a good mathematician and an amateur astronomer (83,87). He first attended school in Ljubljana, and then in Ingolstadt and Vienna, where he earned the title *baccalaureus*. In his coat of arms he had a golden horse (see this figure), derived from a most likely incorrect Slovene etymological analysis of his surname: slat (zlat) = gold and konj (Greek Chrysippus) = horse; although, due to the loose manner of writing down surnames in the 15th century, one can never really be sure how a surname was pronounced and what was its origin. In 1498, Slatkonja formed a boys' choir in Vienna, which went on to become the renowned Vienna Boys' Choir. Prior to becoming the Bishop of Vienna in 1513, Slatkonja was among other things a cantor at the Court of Vienna. The Jurij Slatkonja Music Conservatory in Novo mesto in Slovenia is named after him.

References

1. Iglič A.: Od nadškofije v Spodnji Panoniji do Ilirske metropolije. Oznanjenje : zbornik za krščanstvo, kulturo in umetnost, 37: 31–41, 2007.

2. Gruden J, Zgodovina slovenskega naroda – 1. del, Mohorjeva družba, Celje, 1992.

3. Cahill T, How the Irish Saved Civilization, Anchor Books, New York, 1995.

4. Grivec F, Slovanska apostola sv. Ciril in Metod, Apostolstvo sv. Cirila in Metoda, Ljubljana, 1927.

5. Bratož R, Vpliv oglejske cerkve na vzhodnoalpski in predalpski prostor od 4. do 8. stoletja, Zbirka Zgodovinskega časopisa – 8, Zveza zgodovinskih društev Slovenije, Znanstveni inštitut Filozofske Fakultete ter Inštitut za zgodovino Cerkve pri Teološki fakulteti, Ljubljana, 1990.

6. Iglič A, O Balkanu in koreninah sodobne evropske civilizacije, Dotiki zgodovine, Ampak: mesečnik za kulturo, politiko in gospodarstvo, 8: 32–34, 2007.

7. Nicol D.M, The Byzantine lady: ten portraits 1250–1500, Cambridge University Press, Cambridge, 1994.

8. Dvornik F, The Slavs and Their Early History and Civilization, Amer. Acad. of Arts and Science, Boston, 1956.

9. Granda S, Mala zgodovina Slovenije, Celjska Mohorjeva družba, Celje, 2008.

10. Grafenauer B, Kontinuiteta in vprašanje slovenskega srečanja s staroselci (Vlahi), Spremna besedila v Zgodovina Langobardov Pavla Diakona (Paul the Deacon: History of Lombards), Založba Obzorja MB, pp. 342–375, 1988.

11. Kos M, Srednjeveška zgodovina Slovencev, Slovenska matica, Ljubljana, 1985.

12. Grdina I, Od Brižinskih spomenikov do razsvetljenstva, Založba Obzorja, Maribor, 1999.

13. Leisering W, Historischer Weltatlas, Cornelsen Verlag, Berlin, 1999.

14. Iglič A, O uporabi slovenščine med plemstvom in meščanstvom v srednjem in novem veku (1.del), Dotiki zgodovine, Ampak : mesečnik za kulturo, politiko in gospodarstvo, 8: 32–34, 2007.

15. Iglič A, O uporabi slovenščine med plemstvom in meščanstvom v srednjem in novem veku (2.del), Dotiki zgodovine, Ampak : mesečnik za kulturo, politiko in gospodarstvo, 8: 34–36, 2007.

16. Žvanut M, Slovenski jezik: identiteta in simbol (Slovene language: identity and symbol), Narodni muzej Slovenije, Ljubljana, 2007.

17. Diakon P, Zgodovina Langobardov, ponatis in prevod: Založba Obzorja MB, 1988.

18. Luthar O, Grdina I, Šašel Kos M, Svoljšak P, Kos P, et al., The Lan Between – A History of Slovenia, Peter Lang Edition, Frankfurt am Main, 2013.

19. Županič N, Der Anten Ursprung und Name,.Extrait des Actes du III^{e} Congrès International des Etudes Byzantines, pp. 33l—339, Athènes, 1932. (published partially in French): Izvor in ime Antov, Etnolog VII, pp. 88–89, 1934. Printed again in: Županič N.: Izbrana dela iz historične etnologije in antropologije, AE Gradiva, vol. 1 (Editors: A. Iglič in V. Kralj–Iglič), pp. 59–72, Filozofska fakulteta, Ljubljana, 2006.

20. Županič N, Les premiìrs porteurs des noms Serbe, Croate, Tchéque et Ante, predavanje na III. kongresu Institut International d'Anthropologie v Amsterdamu, 20–29th September 1927, objavljeno v zborniku konference, pp. 238–243, Paris, 1928. Translated: Prvi nosilci etničnih imen Srb, Hrvat, Čeh in Ant, Etnolog II, pp.74–79, 1928. Printed again in: Županič N.: Izbrana dela iz historične etnologije in antropologije, AE Gradiva, vol. 1 (Editors: A. Iglič in V. Kralj-Iglič), pp. 73–79, Filozofska fakulteta, Ljubljana, 2006.

21. Županič N, Boz rex Antorum, Situla – Glasnik Narodnega muzeja v Ljubljani, 4, pp. 91–122, 1961. Printed again in: Županič N.: Izbrana dela iz historične etnologije in antropologije, AE Gradiva, vol. 1 (Editors: A. Iglič in V. Kralj-Iglič), pp. 15–59, Filozofska fakulteta, Ljubljana, 2006.

22. Volpi Lisjak B, Čupa, prvo slovensko plovilo in drevaki, Mladika, Trst, 2004.

23. Vernadsky G, Ancient Russia, Yale University Press, New Haven, 1969.

24. Vernadsky G, A History of Russia, Yale University Press, New Haven, 1986.

25. Much R, Die Germania des Tacitus, Heidelberg, pp. 414–415, 1937.

26. Bezlaj F, Eseji o slovenskem jeziku, Mladinska knjiga, Ljubljana, 1967.

27. Žniderščič M, Brižinski spomeniki, 2. izdaja, Slovenska knjiga, Ljubljana, 2004.

28. Pleterski A, Model etnogeneze Slovanov na osnovi nekaterih novejših raziskav, Zgodovinski časopis, 49: 537–556, 1995.

29. Figes O, Natašin ples : kulturna zgodovina Rusije, Modrijan, Studia humanitatis, Ljubljana, 2008.

30. Martin J, Medivial Russia 980–1584, Cambridge University Press, Cambridge, 2007.

31. Bezlaj F, Zbrani jezikoslovni spisi I–II, Furlan M. (Editor), Zbirka Linguistica et philologica, Založba ZRC, Ljubljana, 2003.

32. Grafenauer B, Karantanija – izbrane razprave in članki, Slovenska matica, Ljubljana, 2000.

33. Zupan A, Hauptman N, Glavač D, The maternal perspective for five Slovenian regions : the importance of regional sampling. Ann. Hum. Biol., 43: 57-66, 2016.

34. Delser PM Ravnik-Glavač M, Gasparini P, Glavač D, Mezzavilla M, Genetic landscape of Slovenians : past admixture and natural selection pattern. Frontiers in Genetics, 9: 1-8, 2018.

35. Repanšek L, Keltska dediščina v toponimiji jugovzhodnega alpskega prostora, Zbirka Linguistica et philologica (ured. A. Legan Ravnikar), vol. 33, Založba ZRC, ZRC SAZU, 331 pages, Ljubljana, 2016.

36. Leciejewicz L, Einige bemerkungen über die Kontakte der Ostsee-Slawen mit nördlicher Rus im Frühmittelalter, Folia Praehistorica Posnaniensia, III: 157–164, 1988.

37. Zupan A, Vrabec K, Glavač D, The paternal perspective of the Slovenian population and its relationship with other populations. Ann. Hum. Biol., 40: 515–526, 2013.

38. Mielnik-Sikorska M, Daca P, Malyarchuk B, Derenko M, Skonieczna K, et al., The history of Slavs inferred from complete mitochondrial genome sequences. PLoS ONE, 8: e54360, 2013.

39. Kushniarevich A, Utevska O, Chuhryaeva M, Agdzhoyan A, Dibirova K, Uktveryte I, et al., Genetic Heritage of the Balto-Slavic Speaking Populations: A Synthesis of Autosomal, Mitochondrial and Y-Chromosomal Data. PLoS ONE, 10: e0135820, 2015.

40. Riasanovsky NV, Russian Identities: A Historical Survey, Oxford University Press, Oxford, 2005.

41. Pleterski A, Etnogeneza Slavena - metode i proces (The ethnogenesis of the Slavs, the methods and the process), Starohrvatska prosvjeta. Ser. III, 40: 7-32, 2013.

42. Rebała K, Mikulich AI, Tsybovsky IS, Siváková D, Džupinková Z, et al., Y-STR variation among Slavs: evidence for the Slavic homeland in the middle Dnieper basin, J. Hum. Genet., 52: 406–414, 2007.

43. Conte F, The Slavs, East European Monographs, distributed by Columbia University Press, New York, 1995.

44. Zupan A, Genetska struktura Slovencev, kot jo razkrivajo polimorfizmi kromosoma Y in mitohondrijske DNA, doktorska disertacija, Biotehniška fakulteta, Univerza v Ljubljani, Ljubljana, 2014.

45. Kiss L, O medsebojnem jezikovnem vplivanju Slovanov in Madžarov. Jezik in slovstvo, 12: 167–171, 1967.

46. Fujs M, Oblikovanje narodne identitete pri prekmurskih in porabskih Slovencih, str. 204–207, v: Avstrija, Jugoslavija, Slovenija, Slovenska narodna identiteta skozi čas (ured. Nećak D.), Oddelek za zgodovino Filozofske fakultete, Ljubljana, 1997.

47. Iglič A, O severnoslovanskih koreninah slovenščine. Dotiki zgodovine, Ampak : mesečnik za kulturo, politiko in gospodarstvo, 9: 39–41, 2008.

48. Marn J, Kopitarjeva spomenica, Matica slovenska, Tiskarna J. Blaznik in nasledniki, Ljubljana, 1880.

49. Grdina I, Skupnost Slovencev in njih učena kultura v zgodnejših razdobjih srednjega veka, Zgodovinski časopis, 43: 183–192, 1989.

50. Höfler J, Trubarjevi »Lubi Slovenci« ali Slovenija pred 650 leti v Strasbourgu : o pojmu Slovenije v srednjem veku, samozaložba, Ljubljana, 2009.

51. Wojtecki D, Slavica beim Annalisten von Quedlimburg, v: Zeitschrift für Ostforschung: Länder und Völker im östlichen Mitteleuropa, XXX: 161–194, 1981.

52. Bertels K, Carantania: Beobachtungen zur politisch-geographischen Terminologie und zur Geschichte des Landes und seiner Bevölkerung im frühen Mittelalter, In: Carinthia I CLXXVII, pp. 87–196, 1987.

53. Zeumer K, Formulae Merowingici et Karolini Aevi, Monumenta Germaniae Historica: Legum sectio V. Formulae; published by Karl Zeumer, Hannover, 1886.

54. Höfler J, Trubarjevi »Lubi Slovenci« ali Slovenija pred 650 leti v Strasbourgu. Ampak : mesečnik za kulturo, politiko in gospodarstvo, 6: 46–49, 2005.

55. Kopitar J, Čop M., Izbrano delo, Kos J. ured, Zbirka Naša beseda, Mladinska knjiga, Ljubljana, 1973.

56. Guštin M, Tisti z vzhoda, Annales, Koper, 2005.

57. Barford PM, The Early Slavs: Culture and Society in Early Medieval Eastern Europe, Cornell University Press, 2001.

58. Altimari F, et al.: Albanci, Cankarjeva založba, Ljubljana, 1984.

59. Grafenauer B, Zgodovina slovenskega naroda, DZS, Ljubljana, 1978.

60. Glaser F, Eine Marmorinschrift aus der Zeit Karls des Großen in Millstatt. Carinthia I, 183: 303–318, 1993.

61. Pleterski A, Ecclesia demonibus addicta - Povedka o poganskem svetišču v Millstattu, Zgodovinski časopis, 48: 297-306, 1994.

62. Nikolasch F, Die Entwicklung der Legende des Domitian von Millstatt. In: Symposium zur Geschichte von Millstatt und Kärnten (Ed. Nikolasch F.), Millstatt 29-58, 1993.

63. Eichert S, Karantanische Slawen – slawische Karantanen. Überlegungen zu ethnischen und sozialen Strukturen im Ostalpenraum des frühen Mittelalters, In: Der Wandel um 1000. Beiträge der Sektion zur slawischen Frühgeschichte der 18. Jahrestagung des Mittel- und Ostdeutschen Verbandes für Altertumsforschung in Greifswald, Beiträge zur Ur- und Frühgeschichte Mitteleuropas 60 (Eds. F. Biermann, T. Kersting, A. Klammt), pp. 433-440, Beier and Beran, Langeweißbach (Germany), 2011.

64. Pleterski A, Lepi, grdi, zli. O metodah, Liburniji, Karantaniji, vojvodskem stolu, Konverziji in Brižinskih spomenikih, Zgodovinski časopis, 52: 215-277, 1998.

65. Zgodovina Slovencev (History of Slovenes), Cankarjeva založba, Ljubljana, 1979.

66. Pleterski A, Etnogeneza Slavena - metode i proces (The ethnogenesis of the Slavs, the methods and the process), Starohrvatska prosvjeta. Ser. III, 40: 7-32, 2013.

67. Komac A, Od mejne grofije do dežele, Ulrik III. Spanheim in Kranjska v 13. Stoletju, Zgodovinski Inštitut Milka Kosa ZRC SAZU, Ljubljana, 2006.

68. Korošec P, Alpski Slovani (Die Alpenslawen), Znanstveni inštitut Filozofske fakultete, Ljubljana, 1990.

69. Vilfan S, Pravna zgodovina Slovencev. Od naselitve do zloma stare Jugoslavije, Slovenska matica, Ljubljana, 1996.

70. Ilustrirana zgodovina Slovencev (Illustrated History of Slovenes), Mladinska knjiga, Ljubljana, 2001.

71. Nartnik V, Še nekaj o Hermanu s Koroškega. Slava : debatni list, 1: 110–114, 1987.

72. Nartnik V, Herman s Koroškega – Slovenec!. Koledar Mohorjeve družbe v Celovcu, pp. 77–82, 1990.

73. Šumrada J, Quelques recherches recentes en Slovenie sur Hermann de Carinthie. In: Le temps de Fulbert : enseigner le moyen age á partir d'un monument la cathédrale de Chartres, actes de l'Université d'été du 8 au 10 juillet 1996. Chartres: Societe Arheologique d'Eure-et-Loir: Association des Amis du Centre Médiéval Européen: Université d'été. 1996, pp. 115–119, 1996.

74. Caiazzo I, Hermann de Carinthie, XII S., pp. 671–672, In: Dictionnaire du Moyen Âge (Editors: Gauvard C., de Libera A. in Zink M.), PUF, Pariz, 2002.

75. Thomas D, Mallett A, Hermann of Carinthia, pp. 497–507, In: Christian-Muslim Relations. A Bibliographical History. Vol. 3 (1050–1200), Editors : D. Thomas, A. Mallett, Brill, Leiden, Boston, 2011.

76. Gantar K, Herman de Carinthia, Jezik in slovstvo, 10: 225–232, 1965.

77. Zečić D, Škamperle I, Vpliv Hermana iz Koroške na poznavanje srednjeveške arabske znanosti na Zahodu, Arhivi, 28: 7–13, 2005.

78. Kronsteiner O.: Avstrijci so že dolgo Slovenci, In: Pogovor z O. Kronsteinerjem, avstrijskim slavistom (wrote: J. Šutej Adamič), Delo, 11 October, p. 9, 2004.

79. Županič N, Slovenci, njih zgodovina in časnikarstvo, pp. 257–281, In: Županič N.: Slovensko časnikarstvo, separatni odtis (v slovenščini), iz: Zbornika "Jugoslovenska štampa", Beligrad, Državna tiskarna kraljestva Srbije, 1911.

80. Pleterski A, Knežji kamen s Sveškega polja v sklopu stare vere o tročanu, Delo, 16th December, p. 5, 2005.

81. Debeljak T, Slovenski narod in simboli njegove državnosti, Zbornik svobodne Slovenije, Buones Aires, 1971–1972.

82. Churchill WS, A History of the English-Speaking Peoples, vol. I–IV, Cassell, London, 2002.

83. Mantuani J, Jurij pl. Slatkonja, 20: 301–309, Dom in svet, 1907.

84. Höfler J, O nekaterih slovenskih skladateljih 16. stoletja. Kronika : časopis za slovensko krajevno zgodovino, 23: 87–94, 1975.

85. Höfler J, Ljubljana – Lubiana – Laibach um 1500: zur kultur-, kunst- und musikgeschichtlichen Situation in der Geburtsstadt Georg von Slatkonias und seiner Heimat im ausgehenden Mittelalter, pp. 37–48, In: Die Wiener Hofmusikkapelle I, Georg von Slatkonia und die Wiener Hofmusikkapelle (edit. Antonicek T., Hilscher E.T., Krones H.), Böhlau Verlag, Wien; Köln, Weimar, 1999.

86. Maček J, Prebivalstvo mesta Ljubljane v srednjem veku po svojih imenih in priimkih, Kronika slov. mest, pp. 160–163 and pp. 218–223, 1936.

87. Škulj E, Jurij Slatkonja na štirih upodobitvah, Kronika : časopis za slovensko krajevno zgodovino, 45, pp. 45–39, 1997.