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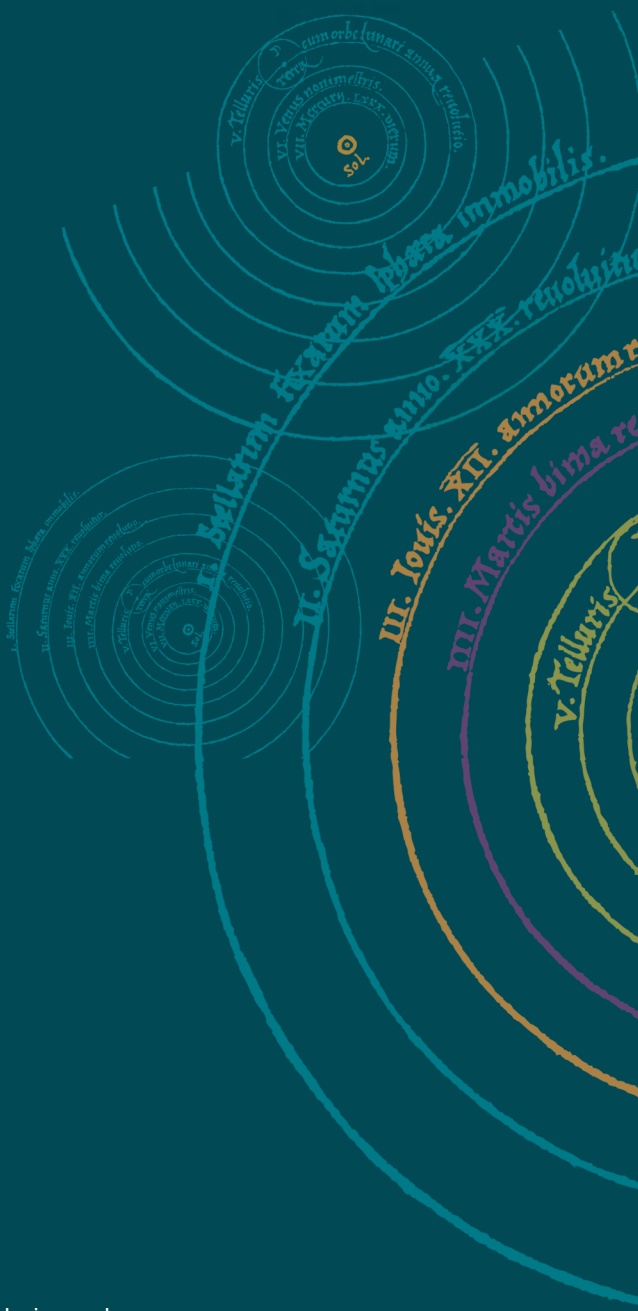

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EDITORIAL

Jacek Kubica

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Heart failure treatment according to the 2021 European Society of Cardiology Guidelines — experiences with SGLT2 inhibitors have changed the treatment strategy

Previously the treatment with β-blockers, angiotensin-converting enzyme inhibitors or angiotensin receptor blockers and mineralocorticoid receptor antagonists, was shown to provide benefits in terms of mortality and morbidity in heart failure (HF) patients with reduced ejection fraction (HFrEF) [1]. Later substantial improvement in outcomes with the angiotensin receptor-neprilysin inhibitor (sacubitril-valsartan) above the benefits provided by the angiotensin-converting enzyme inhibitor enalapril was shown [2]. Recently several clinical trials unexpectedly showed favorable impact on cardiovascular outcomes of some sodium-glucose co-transporter 2 inhibitors (SGLT2-I), namely: canagliflozin, dapagliflozin, and empagliflozin [3–8]. The positive cardiovascular effects of SGLT2 inhibitors became apparent within months from the beginning of treatment, suggesting that the mechanisms beyond improved glucose control and reduced atherosclerosis are involved in cardiovascular risk reduction [3 8].

In the Study to Evaluate the Effect of Dapagliflozin on the Incidence of Worsening Heart Failure or Cardiovascular Death in Patients with Chronic Heart Failure (DAPA-HF) 4744 patients with heart failure (NYHA II-IV) with reduced ejection fraction (< 40%) with diabetes (45%) or without (55%) were randomized to receive dapagliflozin 10 mg/day or placebo, on top of optimal standard therapy for heart failure. A significant reduction in the primary outcomes, defined as a composite of worsening heart failure or cardiovascular death, were achieved in patients receiving dapagliflozin (HR 0.74; 95% CI, 0.65 to 0.85; $p < 0.001$) [7].

In the Empagliflozin Outcome Trial in Patients with Chronic Heart Failure and a Reduced Ejection Fraction (EMPEROR-Reduced) 3730 patients with heart failure (NYHA II-IV) with reduced ejection fraction (< 40%) with diabetes (50%) or without (50%) were randomized to receive empagliflozin 10 mg/day or placebo, on top of optimal standard therapy for heart failure. The primary outcomes, defined as a composite of cardiovascular death or hospitalization for worsening heart failure, were significantly reduced in the empagliflozin arm (HR 0.75; 95% CI, 0.65 to 0.86; $p < 0.001$) [8].

The meta-analysis of these two large-scale trials including 8474 patients demonstrated a 13% reduction in all-cause death (HR 0.87, 95% CI 0.77–0.98; $p = 0.018$) and a 14% reduction in cardiovascular death (HR 0.86, 95% CI 0.76–0.98; $p = 0.027$) [9]. Moreover, a 26% reduction in the combined risk of cardiovascular death or first hospitalization for heart failure (HR 0.74, 95% CI 0.68–0.82; $p < 0.0001$) was observed in the patients treated with dapagliflozin or empagliflozin versus placebo [9].

After the publication of the DAPA-HF and the EMPEROR-Reduced trial results both showing the exceptional clinical benefits of dapagliflozin and empagliflozin respectively, applied on top of guidelines-recommended therapy for the treatment of patients with chronic HFrEF, regardless of coexistence of diabetes mellitus, some changes in ESC guidelines were expected [7, 8].

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Indeed, as expected, the recent ESC guidelines for the diagnosis and treatment of acute and chronic heart failure contain several changes that may be considered revolutionary [10].

To achieve three major goals of treatment defined as reduction in mortality, prevention of recurrent hospitalizations due to worsening HF, and improvement in clinical status, functional capacity, and quality of life, a new simplified treatment algorithm for HFrEF, according to phenotypes, has been introduced [10].

The cornerstone therapy with angiotensin-converting enzyme inhibitors (ACE-I) or an angiotensin receptor-neprilysin inhibitor (ARNI), beta-blockers (BB), and mineralocorticoid receptor antagonists (MRA) is recommended for patients with HFrEF unless the drugs are contraindicated or not tolerated. ACE-I should be replaced with ARNI in patients who remain symptomatic on ACE-I, beta-blocker, and MRA; however, ARNI may be also applied instead of an ACE-I as first-line therapy. Angiotensin-receptor blockers (ARBs) should be used in patients who are intolerant to ACE-I or ARNI. SGLT2-I - dapagliflozin or empagliflozin should be used on top of this cornerstone therapy in all patients with HFrEF unless contraindicated or not tolerated (class I of recommendations) [10].

The recommended four-component (ACE-I/ /ARNI + BB + MRA + SGLT2-I) first-line therapy has been proved to reduce the risk of HF hospitalization and death [7–9].

Additionally, according to the results of the Vericiguat Global Study in Subjects with Heart Failure with Reduced Ejection Fraction (VICTORIA) the oral soluble guanylate cyclase receptor stimulator, vericiguat, may be considered in patients in NYHA class II-IV who have had worsening HF despite treatment with three-component cornerstone therapy (without SGLT2-I) to reduce the risk of CV mortality or HF hospitalization (class IIb of recommendations) [10, 11].

The great emphasis in these guidelines was placed on prevention, including programs to improve outcomes, influenza and pneumococcal vaccinations, cardiac rehabilitation programs, and home telemonitoring in patients with heart failure and preserved ejection fraction [10].

Recommendations for the management of patients after hospitalization for HF highlights the need for adequate preparation for discharge from the hospital and cooperation with HF patients after discharge to improve adherence to treatment [10]. These guidelines create space for the use of already known and used tools and methods, including scales and self-reported questionnaires [11–21].

Summing up, the 2021 European Society of Cardiology Guidelines present a completely new strategy for the treatment of HF patients that offers a possibility to improve clinical outcomes and achieve long-term clinical improvement much faster than previously possible. The SGLT2-Is — dapagliflozin and empagliflozin play a key role in the new strategy.

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INVITED EDITORIAL

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De-escalation of antiplatelet therapy after acute coronary syndrome — a way to improve medication adherence?

According to European Society of Cardiology (ESC) guidelines [1–3], dual antiplatelet therapy (DAPT) with a P2Y12 inhibitor and aspirin is recommended for 12 months after acute coronary syndrome (ACS) to prevent adverse thrombotic events. Earlier DAPT termination is justified only in high bleeding risk patients [1–3]. Recently, Kubica et al. [4] proposed a DAPT de-escalation strategy based on the pathophysiological premises providing a rationale for a randomized clinical trial. They designed the Evaluation of safety and efficacy of two ticagrelor-based de-escalation antiplatelet strategies in acute coronary syndrome — a randomized clinical trial (ELECTRA-SIRIO 2), to assess the influence of ticagrelor dose reduction with or without continuation of aspirin versus DAPT with standard-dose ticagrelor in reducing clinically relevant bleeding and maintaining anti-ischaemic efficacy in ACS patients [4]. The authors stressed that an increased ischaemic risk occurs in the early period after ACS, with elevated rates of clinical events clustering during the first month, while the bleeding risk is related to the duration and dose of the antiplatelet treatment and the majority of bleeding events occur after 30 days post-ACS [5]. Therefore, in the earliest phase after ACS potent antiplatelet treatment is justified, whereas after the clinical stabilization occurs, de-escalation of the antiplatelet therapy may be a better option. Previously published studies showed that reduction of ticagrelor bioavailability significantly decreases the antiplatelet effect of ticagrelor in patients with acute myocardial infarction (MI), but not in the stable setting [6–8].

Moreover, a pharmacodynamic randomized study provided evidence that reduced ticagrelor maintenance dose of 60 mg b.i.d. provides comparable antiplatelet effect to the standard 90 mg b.i.d. dose in stable patients one month after MI [9, 10]. This observation was in line with results of the PEGASUS-TIMI 54 sub-study showing similar platelet inhibition with reduced (60 mg b.i.d) and standard (90 mg b.i.d) maintenance doses in stable patients more than 1 year after MI [11]. It should be underlined that in the PEGASUS-TIMI 54 study both ticagrelor doses showed comparable clinical efficacy, however, better tolerability of treatment with the lower dose of ticagrelor resulting in better adherence to medication was observed [12, 13].

According to the results of the TWILIGHT study, replacement of standard DAPT (ticagrelor plus aspirin) with ticagrelor alone resulted in a substantially lower bleeding rate than in the DAPT arm, without an increase of ischaemic events over a 1 year of follow-up [14, 15]. Moreover, adherence to ticagrelor treatment one year after randomization was slightly better in the ticagrelor-plus placebo arm than in the ticagrelor-plus-aspirin arm (87.1% and 85.9%, respectively) [14].

The ELECTRA-SIRIO 2 trial has been designed taking into account all these premises [4]. Patients with ACS will be randomised in a 1:1:1 ratio into one of three arms: standard-dose ticagrelor (90 mg b.i.d) with aspirin (100 mg q.d.) for 12 months; low-dose ticagrelor (dose reduction to 60 mg b.i.d. after one month) with aspirin group, low-dose ticagrelor (dose reduction to 60 mg b.i.d. after one month) with the placebo group (aspirin cessation after three months). The primary safety composite endpoint of this trial is the first occurrence of type 2, 3 or 5 bleeding according to the BARC criteria within 12 months after ACS. The primary efficacy endpoint is the composite of death from any

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cause, first nonfatal MI, or first nonfatal stroke [4]. To date, the de-escalation of antiplatelet therapy in ACS patients based on lowering the dose of ticagrelor with or without discontinuation of aspirin has never been tested in a large randomised clinical trial. It should be highlighted, that this groundbreaking trial has been made possible thanks to the support of financial support from the Medical Research Agency.

The primary hypothesis of the ELECTRA-SIRIO 2 trial is that monotherapy with low-dose ticagrelor will lead to improved safety (reduction of clinically relevant bleeding) with the same efficacy (no increase of adverse ischaemic events) in comparison to standard-dose ticagrelor with aspirin in ACS patients [4].

Both strategies applied in the trial — ticagrelor dose decrease and aspirin cessation are expected to improve adherence to treatment [16–33]. This effect is expected to be enhanced by the Multilevel Educational and Motivational Intervention in Patients After Myocardial Infarction (MEDMOTION) project, including assessment with the Readiness for Hospital Discharge after Myocardial Infarction Scale (RHD-MIS) at the end of hospitalization, and with the Functioning in Chronic Illness Scale (FCIS) during follow-ups [34–41]

In summary, the tested antiplatelet strategy, which is expected to be safer in comparison to standard treatment may also be more effective in the prevention of ischaemic events due to better adherence to study medication.

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VIA MEDICA

ORIGINAL ARTICLE

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Study on the utility and efficacy of clinical and instrumental tests in the follow-up of COVID-19 patients

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ABSTRACT

Introduction: The disease caused by the SARS-CoV-2 virus (COVID-19) frequently leads to serious complications and prolonged hospitalizations requiring effective care after discharge.

Aim of the study: Aim of this study was to identify feasible and cost-effective predictors of outcome among clinical characteristics, functional status, laboratory, echocardiographic and lung ultrasound data of COVID-19 patients.

Material and methods: Patients affected by COVID-19 who experienced a prolonged hospitalization due to a severe form of the disease and that have been discharged from the COVID-19 rehabilitation unit (RU) were prospectively enrolled between April 6th and May 22nd, 2020. All the patients underwent a 6-minute walk test (6MWT) at the 30-day follow-up. Baseline characteristics, laboratory, functional exercise tests, echocardiographic and lung ultrasound (LUS) data collected between hospitalization, admission to RU, discharge from RU and follow-up were compared. Correlations with the predicted distance covered at the 6MWT (6MWD) were made.

Results: 40 patients met inclusion criteria and presented to follow-up (13 women [32.5%] and 27 men [67.5%]; mean age 66 ± 10 years). Among all variables analysed, only functional tests at discharge showed a remarkable correlation with the 6MWD. Significant improvement in lung ultrasound score (LUSS) was also observed however without correlation with 6MWD.

Conclusions: functional tests at discharge from RU identified patients with different 30-day outcomes that could deserve a stricter long-term follow-up. This may help in planning a personalized follow-up. The costs and effort were minimal. The severity of the acute phase did not significantly influence functional recovery. LUS was useful to identify subclinical lung damage and its evolution over time, however without clear functional correlation.

Key words: COVID-19, lung ultrasound, rehabilitation, SARS-CoV-2

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How these fit in:

- Little is known about the long-term functional status of discharged COVID-19 patients that experienced a severe disease needing rehabilitation.
- Given the variability of clinical pictures led by COVID-19 and the continuous increase of patients' discharge, cost-free and easy to use, efficient tools for planning the follow-up and stratify prognosis are needed.
- In a selected population of COVID-19 patients with loss of functional autonomy that required a rehabilitation period, commonest functional tests showed to correlate well with 6MWT performance at 30 days from discharge.
- Lung ultrasound sonography is a safe, feasible and cost-effective method to monitor lung damage over time.

Introduction

The coronavirus disease of 2019 (COVID-19) is a viral illness caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with large variability of clinical pictures [1]. Considering the high potential severity burden of long-term complications [2] and the need for prolonged hospitalizations [3], the establishment of rehabilitative departments dedicated to most compromised patients was deemed necessary [4–6]. In this context, an amount of literature discussing patient in-hospital management was published [7]. Nevertheless, remain a lack of evidence about adequate follow-up planning [8] because insight about the long-term clinical outcome and functional status is still missing [9, 10]. Considering the enormous number of outpatients to manage and the limited resources, the research should be oriented towards the identification of harmless, feasible and cost-effective tools that easily provide information on individual risk and help to select patients who need closer follow-up. Other than physiological characteristics, many laboratory and instrumental tests are used as risk predictors of mortality during hospitalization and at discharge [11], however without clear evidence of long-term benefit. Chest computed tomography (CT) is a critical tool for the diagnosis and inpatient management, with higher sensitivity than a swab sample [12] capable to identify lung alterations even at 60 days of follow-up [13] in asymptomatic individuals [14]. Despite this, it may not be a feasible method for the routine follow-up of discharged COVID-19 patients because of radiation exposure, costs, availability, and logistic issues. On the other hand, lung ultrasound sonography (LUS) appears to be a useful, rapid, harmless and low-cost alternative to CT, with similar sensitivity in COVID-19 [15, 16] and capable to identify subclinical residual lung damage in patients with severe COVID-19 that met discharge criteria [17]. Exercise tests and functional scales are quantitative validated tools to assess and train the functional reserve in several rehabilitation units, being useful to stratify patients risk according to their fitness. Among them, the 6 Minute Walk Test (6MWT) is a submaximal exercise test used to assess aerobic capacity and endurance, providing a measure of functional status and outcome in patients affected by different lung and cardiac diseases [18–20]. So far, the only test that found application in the context of the COVID-19 pandemic was the 6MWT, which demonstrated effectiveness in assessing oxygenation reserve in non-hypoxic patients at rest and help in looking for discharge preparedness [27, 28]. Moreover, in the largest cohort study with the longest follow-up available today, 6MWT at 6 months was decreased proportionally to the severity of illness [29]. This study aimed to identify feasible and cost-effective predictors of outcome among clinical characteristics, laboratory and functional tests, echocardiographic and LUS data in a selected high-risk cohort of COVID-19 patients who needed a rehabilitative recovery. This might help to identify frailer patients deserving a stricter follow-up strategy and to avoid the routine use, even during the acute phase of the disease, of useless laboratory and diagnostic tests with a huge waste of resources. The authors used the 6MWT as a functional endpoint, being this an effective, and easy to perform predictor of prognosis, largely validated in several lung and cardiac diseases [18, 19].

Material and methods

Study population

We prospectively enrolled consecutive COVID-19 patients who were hospitalized during the acute phase of the disease in the Emergency Room (ER), Intensive Care Units (ICU), Respiratory High Dependency Care Units (RHDCU) or Infectious Diseases units of the San Raffaele Hospital, that were subsequently admitted to a dedicated Rehabilitation Unit (RU) and underwent a 30-day post-discharge follow-up between April 6 and July 2, 2020. Criteria to admit COVID-19 patients in the RU were: positive swab for SARS-CoV-2, stable $SatO_2$ and respiratory rate, no need for respiratory assistance or no more than 2 l/min O_2, absence of fever, presence of areas of dependence at the FIM evaluation (FIM score < 100) [4]. For the present study, exclusion criteria were the presence of acute cardio-pulmonary or inflammatory conditions, not COVID-19 related (*i.e.,* acute heart failure, COPD exacerbation, pulmonary embolism), concurrent condition influencing functional tests (i.e., traumatic injury), patient refusal or loss at the 30-day follow-up and death. All patients gave their informed consent, and the study was approved by the local Ethics Committee.

Laboratory tests

Based on the reported haematological findings of COVID-19 patients, the following inflammatory indexes were considered: white blood cells (WBCs) and subtypes counts, C-reactive protein (CRP), serum ferritin, and D-dimer [30]. The N-terminal pro-brain natriuretic peptide (NT-proBNP) level was also assessed, which is an independent risk factor for in-hospital death in patients with severe COVID-19 [31]. Laboratory data were assessed during the hospitalization (acute phase of the disease) before admission to the RU and were part of the COVID-BioB Study [32].

Transthoracic echocardiography

Wide spectrum cardio-pulmonary involvement is common in COVID-19 [33]. Transthoracic echocardiography at discharge from RU and follow-up was performed by a trained cardiologist blinded to the patient's clinical characteristics, aimed at identifying signs of right ventricle (RV) dysfunction and/or pressure overload. The authors evaluated RV longitudinal systolic function by tricuspid annular plane systolic excursion (TAPSE) and lateral tricuspid annular tissue Doppler imaging (S' TDI). Pulmonary artery systolic pressure (PASP) was estimated by a sum of tricuspid regurgitation jet gradient and estimated right atrial pressure derived from analysis of the inferior vena cava (IVC) dimensions and response to inspiration. Images were obtained with GE VividS60 (GE-Healthcare, Chicago, Illinois) equipped with a 3Sc-RS sector transducer probe.

Lung US (LUS)

Lung US at admission and discharge from the RU and at 30-day follow-up was performed with the patient in a sitting position by a single trained operator blinded to the patient's clinical characteristics. Findings were classified according to a validated quantitative LUS Score (LUSS) [34]. Three areas per hemithorax were identified (anterior, lateral, and posterior) by using the anterior and posterior axillary lines as anatomical landmarks. Each area was then divided into two, superior and inferior [35]. Therefore, a total of 12 thoracic areas was considered. Each area was given a score from 0 to 3 according to the following criteria: 0, normal aeration; 1, more than 2 B-lines occupying 50% of the pleura or less; 2, more than 2 B-lines occupying greater than 50% of the pleura; and 3, tissue-like pattern. Therefore, the total LUSS ranged from 0 to 36. Lung US images were obtained with a Prosound α6 system (Hitachi Aloka Medical Systems, Tokyo, Japan) equipped with a UST-9123 convex transducer.

Functional evaluation

Functional status and independence in daily living activities were assessed at the admission of the RU, discharge from RU and 30-day follow-up with tests used in Rehabilitation Units and selected for this kind of patient [6]: 6MWT [36], TUG [21] and 30CST [23]. Timed up and go test (TUG) [21] is a measure of functional mobility validated in patients affected by chronic obstructive pulmonary disease and pulmonary arterial hypertension [22]. It evaluates the time a patient takes to rise from a chair, walk three meters, turn around, walk back to the chair and sit down. The Thirty-second chair-stand test (30CST) evaluates leg strength and endurance in older adults assessing the number of stands that a person can complete in 30 seconds [23, 24]. Activities of daily living (ADL) is commonly assessed using the Functional Independence Measure (FIM) scale, an 18-item measurement tool that explores the level of a patient's disability and indicates how much assistance is required for the individual to perform daily living activities [25]. By adding the points for each item (1 = total assist and 7 = complete independence), the level of independence ranges from 18 (lowest) to 126 (highest) [26]. The 6MWT can be expressed as absolute distance and percentage of the predicted 6-minutes walking distance (6MWD). A validated reference equation was used for the prediction of the total distance walked during six minutes for healthy adults [38]. The distance covered over a time of 6 minutes is used to compare changes in performance capacity.

Statistical analysis

For each continuous variable, normal distribution by the Shapiro-Wilk test was verified. Normally distributed variables were described as mean ± standard deviation while non-normally distributed ones were described as medians (interquartile range). The comparisons between groups were performed using t-tests or Wilcoxon sum-rank tests, as appropriate.

The categorical variables were described as frequencies (percentages) and compared by Chi-squared tests. The relationship between variables was exhibited by using the Spearman Rank Correlation coefficient. A two-sided P value < 0.05 was required for statistical significance. Data were analysed with R software version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Forty patients (13 women [32.5%] and 27 men [67.5%]; mean age 66 ± 10 years) met inclusion criteria and presented to the follow-up (Fig. 1). Baseline demographic and clinical characteristics of the population are reported in Table 1. Eighteen patients (45%) required ventilatory support during the acute disease phase. Comorbidities were present in 57.5% of patients, with hypertension being the most prevalent one (50%). 36 patients (90%) received at least 1 antimicrobial or immunosuppressant COVID-19 treatment, with hydroxychloroquine being the most frequent one (77.5%). Patients baseline characteristics, treatment during the acute phase (hospitalization), laboratory findings, functional tests, echocardiographic data and LUSS at discharge from RU were correlated with the distance covered at the 6MWT at the 30-day follow-up.

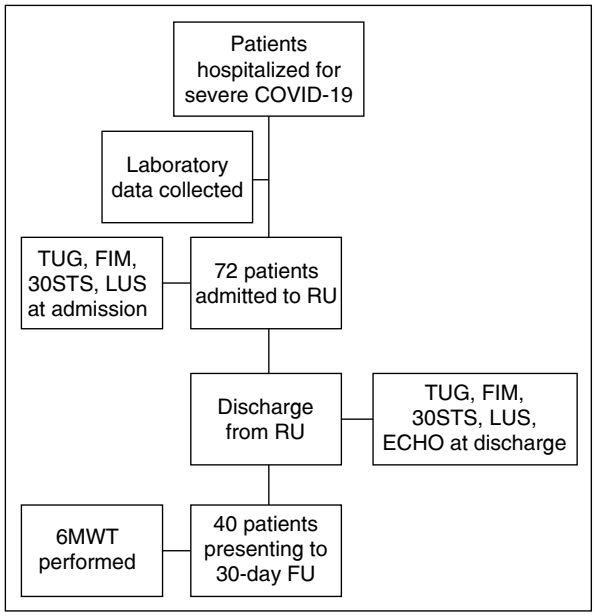

Figure 1. Flow diagram of study structure.
30 STS — 30 seconds sit-to-stand; TUG — time up-and-go; FIM — functional independence measure; LUS — lung ultrasound; ECHO — transthoracic echocardiography; RU — rehabilitation unit; FU — follow-up

(Tab. 3). Considering comorbidities, diabetes was associated with the worst performance at 6MWT (p = 0.02). Patients treated with lopinavir-ritonavir combination or IL-6 inhibitors performed significantly better at the 6MWT at follow-up. No difference was observed between patients treated with other drugs. Eighteen patients (45%) scored a 6MWT lower than the predicted for age, weight and height. Interestingly, a patient needing ventilatory support during the acute phase did not perform worse at the 30-day 6MWT (p = 0.399). Regarding functional evaluation, this analysis showed that all TUG, FIM and 30CTS at discharge from RU significantly correlated to the 6MWD at follow-up (Tab. 3, Fig. 2) and markedly improved throughout the observation period from discharge from RU to the 30-day follow-up (Tab. 2). No correlation was found between laboratory tests, echocardiographic parameters at discharge from RU and follow-up and the distance covered at the 6MWT.

Similarly, no correlation was observed between LUSS at discharge from RU, LUSS at follow-up and 6MWT. About Lung US findings, median LUSS at follow-up was significantly lower when compared with LUSS at discharge from RU (8.0 *vs.* 3.0, p < 0.001; Tab. 2). The same trend was observed both in patients that needed ventilatory support during the acute phase of the disease (8.0 *vs.* 4.0 p < 0.001) and those who did not (2.5 *vs.* 0.0 p = 0.05) and it was significantly higher in the first group at discharge from RU and follow-up

Table 1. Baseline demographic and clinical characteristics of the population

Variable	
No. Patients, n	40
Age, mean ± DS	66 ± 10
Female, n (%)	13/40 (32.5%)
Body mass index, Kg/m2 ± DS	25.7 ± 4.9
Current smoking status, n (%)	3/40 (7.5%)
Need for endotracheal intubation (n%)	18/40 (45%)
6MWT lower than the predicted	18/40 (45%)
Comorbidities, n (%)	
Hypertension	20/40 (50%)
Diabetes	9/40 (22.5%)
Chronic obstructive lung disease	2/40 (5%)
Coronary artery disease	5/40 (12.5%)
Chronic kidney disease	5/40 (12.5%)
Chronic therapy with ACE-i or ARBs	16/40 (40%)
COVID-19 treatment, n (%)	
Hydroxychloroquine	31/40 (77.5%)
Antibiotics (Azithromycin)	20/40 (50.5%)
Antiviral drugs (Lopinavir — Ritonavir)	26/40 (65%)
IL-1 inhibitor (Anakinra)	4/40 (10%)
IL-6 inhibitors (Tocilizumab, Sarilumab)	7/70 (10%)
Laboratory findings at discharge, median (IQR)	
White blood cell count, ×109/L	5.7 (2.7)
Lymphocyte count, ×109/L	1.7 (1.0)
C-reactive protein, mg/L	5.95 (7.7)
Serum ferritin, μg/L	401.50 (650)
D-dimer, μg/L	0.42 (0.305)
NT-pro-BNP, pg/mL	167 (461)

ACE-I — Angiotensin-converting enzyme inhibitors; ARBs — Angiotensin II receptor blockers; COVID-19 — coronavirus disease 2019; IL — Interleuchine

(Fig. 3). A positive trend from admission to RU (median 8, IQR 9) to discharge from RU (median 8, IQR 8, p = 0.0413) was also found.

Discussion

Summary

Our study led to the following results: 1) Among a variety of clinical, laboratory and instrumental data in a selected population of Covid-19 patients with loss of

Table 2. LUSS, echocardiography and functional test findings of patients at discharge from COVID-19 Rehabilitation Unit and after 30 days

Variable	Discharge	Follow-up	P-value
Lung ultrasound, median (IQR)			
LUS score, points	8 (8)	3 (5.75)	< 0.001
Transthoracic echocardiography of the RV, mean ± DS			
Estimated PASP, mmHg	28.9 ± 4.8	27.2 ± 8.0	0.328
TAPSE, mm	23.3 ± 4.6	21.5 ± 4.6	0.092
Lateral tricuspid S' TDI, cm/s	17.5 ± 4.5	14.8 ± 4.4	0.008
Functional Tests, median (IQR)			
30 STS test, No. of repetitions	10.5 (5)	12 (6)	0.0036
TUG test, seconds	11 (5)	9 (5)	0.00038
FIM scale, points	110 (14.5)	125 (7.75)	< 0.00001

Bold for statistically significant values at $p < 0.05$. IQR — interquartile range; LUS — lung ultrasound; RV — right ventricle; PASP — pulmonary arterial systolic pressure; TAPSE — tricuspid annular plane systolic excursion; TDI — tissue doppler imaging 30 STS, 30 seconds sit-to-stand; TUG — time up-and-go; FIM — functional independence measure

functional autonomy that required a rehabilitation period only functional evaluation through TUG, FIM and 30CTS showed to correlate well with the prognosis evaluated by 6MWT at 30 days from discharge; 2) the severity of the acute phase of the disease did not influence functional recovery; 3) LUSS significantly improved from admission to RU to discharge form RU and 30-day follow-up, however without functional correlation at the 6MWT.

Follow-up and long-term outcome of severe COVID-19 patients

Nowadays there is a poorness of shared strategies for the follow-up of patients that suffered from severe COVID-19 requiring strict monitoring. There is also little knowledge about functional long-term outcomes, the presence of residual lung damage and its clinical correlation. In this pilot single-centre study, even limited by low sample size and a short-term follow-up, it was looked for a correlation between a variety of clinical and functional tests, evaluated before discharge from the rehabilitation unit of the hospital, and the functional outcome using the 6MWT, a simple, economic, reproducible and largely validated prognostic predictive test. In a population of patients that presented with clinical indications for functional rehabilitation (FIM < 100) shortly after the onset of the disease and immediately after the acute phase of the infection, this analysis showed that functional tests performed at discharge (TUG, 30-CTS, FIM) positively correlated with the distance covered at the 6MWT at the follow-up, helping to identify frailer patients needing a stricter and personalized follow-up strategy after discharge. On the other hand, no laboratory test before discharge (white blood cell and subtype counts, C-reactive protein, serum ferritin, D-dimer and N-terminal pro-brain natriuretic peptide) nor echocardiographic parameters (right ventricular function, PASP) correlates with 6MWD at the 30-day follow-up.

Ventilatory support and functional recovery

An interesting aspect that emerged from this analysis is that patients that needed ventilatory support during the acute phase of the disease, experiencing a more severe pulmonary involvement also confirmed by higher LUSS, performed worse at the 30-CTS test before discharge, but no difference emerged at the 30-day follow-up. Similarly, there was no difference between the two groups and the 6MWT performance at the 30-day follow-up. This result may signify that the severity of the acute phase, once discharge criteria are met, does not influence the functional long-term outcome. This data is in line with a recent study that highlighted the incongruity between the severity of respiratory disease and cognitive outcome [38]. The authors hope that this notable acknowledgement would be confirmed in larger population observational studies.

Lung ultrasound in the follow-up of discharged patients

Lung ultrasound is a central tool for the diagnosis and management of hospitalised COVID-19 patients, owning several advantages in terms of safety, costs, comfort and availability in respect to radiological instruments [6, 26, 27]. In the present study, the 30-day LUSS was significantly lower compared to LUSS at discharge. Thus, there was a clear reduction of parenchymal involvement within 30 days both in the group of patients that needed mechanical ventilation and those who did not. Furthermore, patients experiencing a more severe

Table 3. Correlations between variables in the study and the percentage of the predicted value of 6 minutes walking test

Qualitative Variables	P-value
Need for endotracheal intubation	0.399
Current smoking status	0.082
Chronic therapy with RAAS Inhibitors	0.98
Comorbidities	
Hypertension	0.37
Diabetes	0.02
Chronic obstructive lung disease	0.91
Coronary artery disease	0.09
COVID-19 treatment	
Hydroxychloroquine	0.36
Antibiotics (Azithromycin)	0.31
Antiviral drugs (Lopinavir–Ritonavir)	0.059
IL-1 inhibitor (Anakinra)	0.82
IL-6 inhibitors (Tocilizumab, Sarilumab)	0.027
Quantitative Variables	**r coefficient**
Body mass index	0.4
Laboratory findings at discharge	
White blood cell count	–0.2
Lymphocyte	–0.2
C-reactive protein	–0.14
D-dimer	–0.04
Serum ferritin	0.004
NT-pro-BNP	–0.21
Lung Ultrasound	
LUSS at discharge	–0.04
LUSS at follow-up	–0.14
Echocardiography of the RV at discharge	
Estimated PASP	0.52
TAPSE	0.24
Lateral tricuspid S' TDI	0.04
Echocardiography of the RV at Follow-up	
Estimated PASP	–0.16
TAPSE	0.22
Lateral tricuspid S' TDI	0.37
Functional Tests at discharge	
30 STS test	0.58 (95CI 0.25–0.79; p = 0.002)
TUG test	–0.66 (95CI 0.42–0.82; p < 0.001)
FIM scale	0.57 (95CI 0.30–0.75; p < 0.001)
Functional Tests at follow-up	
30 STS test	0.65
TUG test	–0.55
FIM scale	0.48

Discharge refers to discharge from the rehabilitation unit. Bold for statistically significant values at p < 0.05. RAAS — Renin Angiotensin Aldosterone System; COVID-19 — coronavirus disease 2019; IL — Interleuchine; LUS — lung ultrasound; RV — right ventricle; PASP — pulmonary arterial systolic pressure; TAPSE — tricuspid annular plane systolic excursion; TDI — tissue doppler imaging; 30 STS — 30 seconds sit-to-stand; TUG — time up-and-go; FIM — functional independence measure

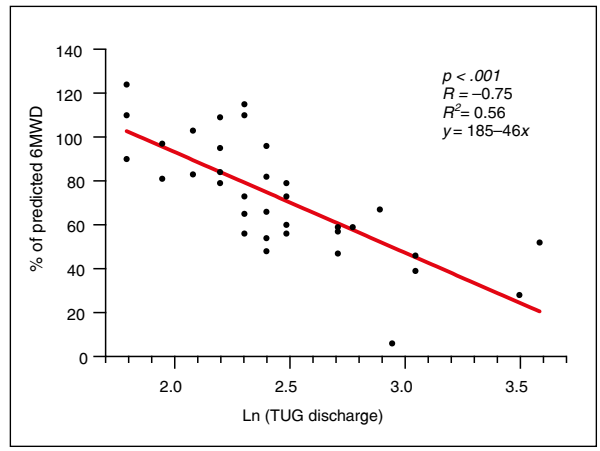

Figure 2. Correlation between TUG (values expressed as natural logarithm) and the distance covered at 6MWT

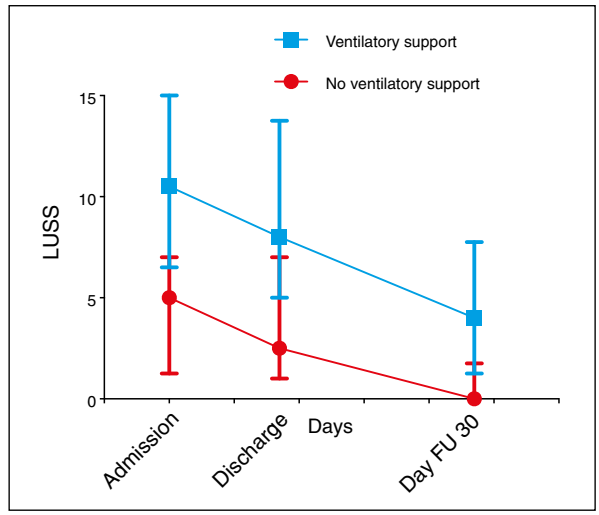

Figure 3. Median LUSS from admission to discharge and follow-up in patients needing and not needing ventilatory support during the acute phase of the disease. The median LUSS was higher in patients needing ventilatory support (p < 0.05)

disease (needing ventilatory support) had a higher LUSS at the admission of the RU, discharge from RU and 30-day follow-up. However, no correlation was found between LUSS at discharge, LUSS at follow-up and 6MWT. This interesting result may be explained by the high sensitivity of the method, revealing residual lung involvement even without clinical and/or functional correlation.

Implications for research and/or practice

Most of the tests commonly used to predict outcome in COVID-19 during the acute phase failed in predicting

the long-term outcome availed at the follow-up by the 6MWT, a well-validated predictor of mortality. Therefore, new risk predictors to consider for customized follow-up planning aimed at the optimization of available resources may be needed. Routine use of instrumental or expensive tests (i.e., pro-BNP, echocardiography, contrast-enhanced CT, LUS etc.) in the absence of specific condition (i.e., heart failure, signs of pulmonary hypertension, pulmonary embolism) should be avoided, as they failed to correlate with the clinical and functional outcome of the patient and, as in the case of echocardiogram, may raise the risk of infection spread among healthcare professionals. On the contrary, all the functional tests that were considered (TUG, 30CTS, FIM) seemed to be the most useful and strongest predictors of outcome at 30-day.

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VIA MEDICA

ORIGINAL ARTICLE

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Improvement of the quality of cardiopulmonary resuscitation performed with Real CPR Help® device among medical students and medical workers

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ABSTRACT

Introduction: This study aimed to compare the quality of CPR performed with real-time feedback device with CPR delivered without it by medical students and practising medical workers.

Material and methods: Studied group consisted of 96 participants. Real CPR Help® technology providing real-time feedback was used. The following parameters were measured: mean depth, mean frequency, adequate depth rate, adequate frequency rate, and general compressions quality. Participants performed one-minute cycles of CPR with and without the feedback.

Results: Mean compression depth lowered with the feedback (6.1 ± 1.3 cm *vs.* 5.3 ± 0.4 cm; $p < 0.001$) and the number of participants with adequate depth increased (25% *vs.* 78,1%; $p < 0.001$). Mean compression frequency lowered after the use of the device (119.8 ± 16.8 cpm *vs.* 111.9 ± 6.9 cpm; $p < 0.001$) and the number of participants performing CPR with recommended compression frequency increased (50% *vs.* 86.5%; $p < 0.001$). Overall quality increased significantly with the feedback (0.0; IQR: 0.0–13.7 *vs.* 55.1; IQR: 31.5–78.8; $p < 0.001$). Similar CPR quality was observed in the student group *vs.* medical workers without the feedback (0.81; IQR: 0.0–16.2 *vs.* 0.0; IQR: 0.0–12.7; $p = 0.27$) and after the device implementation (61.26; IQR: 38.16–80.0 *vs.* 49.54; IQR: 30.06–65.84; $p = 0.21$).

Conclusions: The use of the Real CPR Help® device in the simulated test improved the overall quality of CPR. There were no differences concerning CPR quality between students and medical workers after the device implementation.

Key words: cardiopulmonary resuscitation, CPR, chest compressions, feedback, training

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Introduction

Out-of-hospital cardiac arrest (OHCA) remains a relevant health problem in Poland with an incidence estimated as 0.57–1.7 per 1000 inhabitants per year and still unsatisfactory survival [1–4]. Various treatment options, e.g implementation of mild therapeutic hypothermia, have been investigated to improve the patients' survival and clinical outcome [5–7]. Nevertheless, the basic action to improve the survival of patients after OHCA is to introduce cardiopulmonary resuscitation (CPR) as soon as possible. Early bystander CPR forms the basis of the chain of survival along with early recognition and call for help, defibrillation (if needed), and the implementation of advanced resuscitation procedures [8].

Both the American Heart Association [9] and the European Resuscitation Council (ERC) [8] strongly stress the importance of good-quality chest compressions during CPR. The recommended frequency and depth of chest compressions is 100–120 per minute and 5–6 cm, respectively. The American and European guidelines also emphasize full chest relaxation

after each compression to achieve optimal perfusion pressure [8]. Complete chest relaxation helps create a pressure gradient that allows venous blood to return to the right atrium [10]. The importance of good quality CPR is incontestable since only chest compressions performed with adequate depth, frequency, and chest relaxation enhance patient survival [9, 11]. Previous studies revealed that too shallow chest compressions result in poor coronary perfusion and therefore reduce patient survival [11]. On the contrary, too deep compressions are associated with significantly higher rates of complications such as rib fractures [12].

Systematic training is required among the entire community to increase the quality of CPR. Particular emphasis should be put on the CPR training of medical school students, including future physicians, nurses, midwives, physiotherapists, etc. Previous studies showed that the quality of CPR performed with real-time feedback devices was better in comparison to standard resuscitation [13–18]. Furthermore, those devices proved to enhance the learning process of how to perform CPR properly [18–20]. Good quality compressions are essential also for practising medical workers (physicians, nurses etc.) since it is much more likely for them to perform CPR in comparison to the general population.

This study aimed to compare the quality of CPR performed with real-time feedback device with CPR delivered without it by medical students and practising medical workers.

Material and methods

The study group

The study group consisted of 63 students from Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun and 33 medical workers from Antoni Jurasz University Hospital No. 1 in Bydgoszcz who volunteered for a CPR training session. All participants provided informed consent to use obtained data in the presented study. Students represented various medical fields (medicine, nursing, emergency medical science, laboratory medicine) and different study years. The medical workers' group was heterogeneous and consisted of practising physicians, nurses, and an electroradiology technician.

Measurements

The training was carried out using a ZOLL® R Series® defibrillator with Real CPR Help® technology that provided real-time feedback, triggering a monitor alert whenever chest compression depth or frequency were beyond values recommended in the ERC guidelines [8].

All participants performed CPR twice on a training manikin. The first no-feedback round was followed by a second attempt with the implementation of real-time feedback on the depth and frequency of chest compressions. The effectiveness of the resuscitation, as assessed by adequate depth and frequency of compressions, was measured in one-minute periods for each person. The following parameters of chest compression quality were recorded: mean depth (cm), mean frequency [compressions per minute (cpm)], adequate-depth compression rate (%), adequate-frequency compression rate [%] and adequately-delivered compression rate (%) (only when both the depth and frequency were within the guideline-recommended ranges a compression was considered to be adequately delivered). The study was conducted by the Declaration of Helsinki.

Statistical analysis

Statistical analysis was performed using IMB SPSS Statistics version 23. Testing for normality of data distribution was performed with the Shapiro-Wilk test. Continuous variables were presented as means ± standard deviation (SD) or medians with interquartile range (IQR) and categorical variables as counts with percentages. Statistical analysis for continuous variables was performed using the t-Student or Mann-Whitney U test as appropriate according to data distribution. Differences between categorical variables were calculated with the chi-square test. Differences were considered significant at $p < 0.05$.

Results

A total of 96 participants were included in the study, with the majority of medical students 65.6% (n = 63). Median age of the study group was 24.0 (IQR: 23,0–37,0) with older participants among medical workers group (46.0; IQR: 36.5–54.0 *vs.* 23.0; IQR: 22.0–24.0; $p < 0.001$). Females predominated in the group (n = 72; 75.0%) with no differences between medical workers and medical students (69.8% and 84.8% respectively; $p < 0.139$). Both groups differed in regard to participants profession ($p < 0.001$) with the majority of medicine students in the students' group (n = 53; 84.1%) and nurses in the medical workers group (n = 21; 63.6%),

Mean depth was 6.1 ± 1.3 cm without the feedback and 5.3 ± 0.4 cm after implementation of the feedback device (Fig. 1). The number of participants who performed compressions with adequate depth was 3-fold higher in the feedback group (25%; n = 24 *vs.* 78.1%; n = 75; $p < 0.001$). Only half of the participants (n = 48) without the feedback performed CPR with the recom-

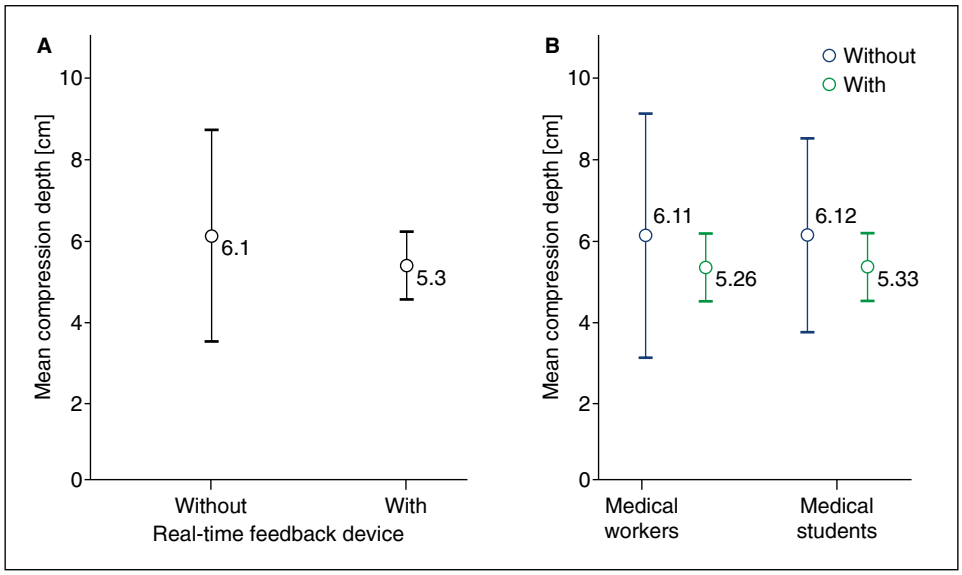

Figure 1. Mean compression depth with and without feedback for the entire study group (**A**) and separately for medical workers and medical students (**B**). Error bars show ± 2 standard deviations

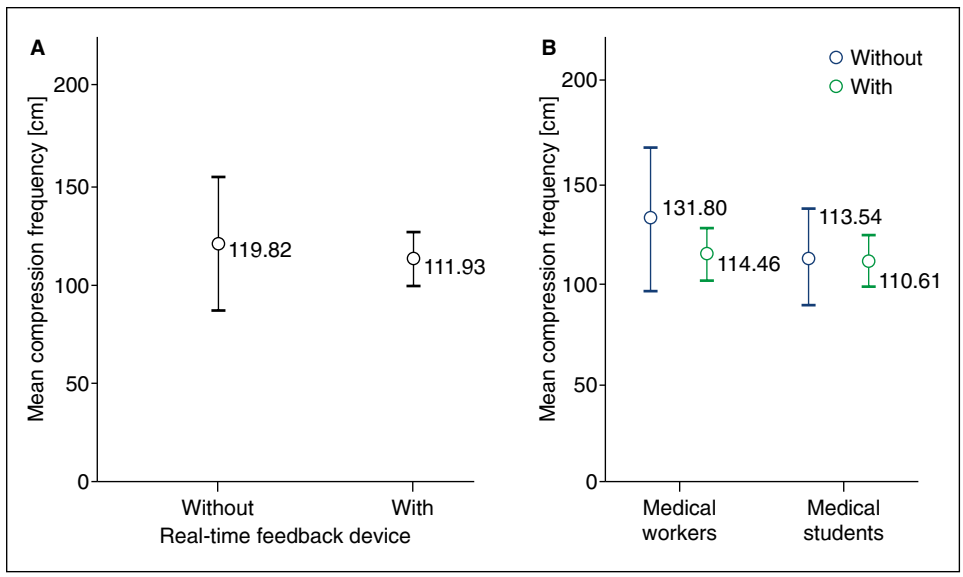

Figure 2. Mean compression frequency with and without feedback for the entire study group (**A**) and separately for medical workers and medical students (**B**). Error bars show ± 2 standard deviations

mended compression frequency in comparison to 86.5% (n = 83) with the feedback device ($p < 0.001$). The feedback group demonstrated a significantly lower average frequency of chest compressions (Fig. 2), as well as significantly higher rates of adequate compression depth (11.06; IQR: 0.0–38.91 *vs.* 70.81; IQR: 51.12–84.92; $p < 0.001$), adequate compression frequency (54.96; IQR: 6.35–84.33 *vs.* 83.32; IQR: 65.61–94.75; $p < 0.001$) and adequately delivered compressions (Fig. 3).

Students *vs.* medical workers

Implementation of the feedback device was associated with a decrease in the mean depth and frequency of compressions as well as with a significant improvement in CPR quality in both groups (Fig. 1–3). Improvement was observed in adequate compression depth rate (9.4; IQR: 0.0–32.4 *vs.* 76.3; IQR: 51.03–86.1; $p < 0.001$) in the student group with the feedback device. The difference in adequate compression fre-

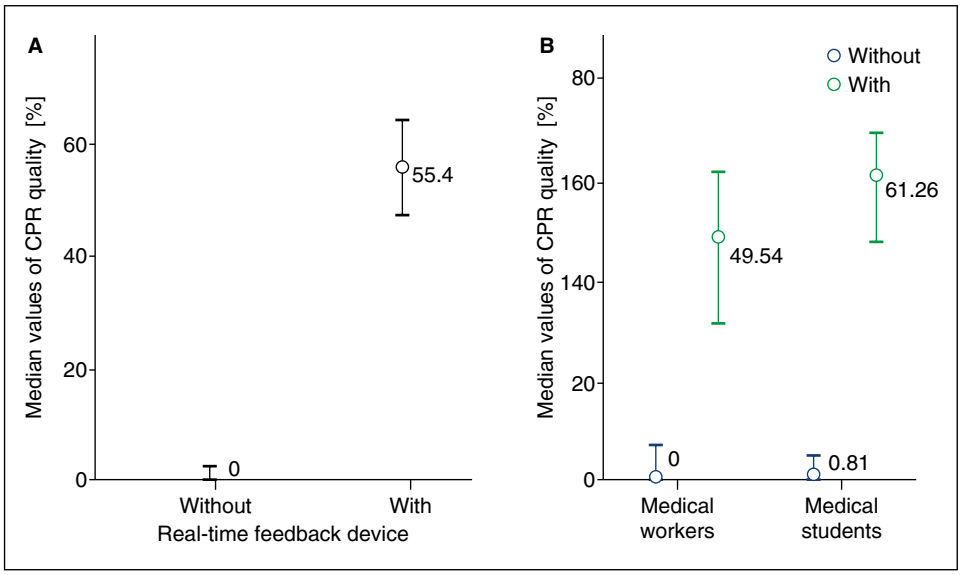

Figure 3. Median values of CPR quality with and without feedback for the entire study group (**A**) and separately for medical workers and medical students (**B**). Error bars show 95% Confidence Interval

Table 1. Comparison of CPR performance among students and medical workers with and without real-time feedback

	Students	**Medical workers**	
	Mean ± SD / Median (IQR)		
	Without real-time feedback		**P-value**
Mean depth [cm]	6.12 ± 1.20	6.11 ± 1.52	0.981
Mean frequency [cpm]	113.54 ± 12.06	131.80 ± 18.1	< 0.001
Quality [%]	0.81 (0.0–16.19)	0.0 (0.0–12.72)	0.265
Adequate depth rate [%]	9.4 (0.0–32.36)	12.71 (0.0–49.97)	0.660
Adequate frequency rate [%]	73.17 (18.68–88.29)	10.53 (0.0–76.77)	< 0.001
	With real-time feedback		
Mean depth	5.33 ± 0.41	5.26 ± 0.41	0.238
Frequency	110.61 ± 6.89	114.46 ± 6.38	0.009
Quality	61.26 (38.16–80.0)	49.54 (30.06–65.84)	0.210
Depth [%]	76.34 (51.02–86.09)	66.67 (49.77–79.07)	0.227
Frequency [%]	85.32 (67.37–96.26)	77.98 (57.89–92.55)	0.093

quency rate was also significant, but less marked in this group (73.2; IQR: 18.7–88.3 *vs.* 85.3; IQR: 67.4–96.3; p = 0.001). Similar results were found in the group of medical workers — increase in the rates of adequate compression depth (12.7; IQR: 0.0–50.0 *vs.* 66.7; IQR: 50.0–79.1; p < 0.001) and adequate compression frequency (10.5; IQR: 0.0–77.8 *vs.* 78.0; IQR: 57.9–92.6; p < 0.001).

The rate of adequate compression frequency (73.17; IQR: 18.68–88.29 *vs.* 10.53; IQR: 0.0–76.77; p < 0.001) without the feedback was higher in the student group in comparison with medical workers (Tab. 1). Furthermore, medical students performed CPR with a significantly lower mean compression frequency at baseline (Fig. 2.) After the implementation of the real-time feedback device medical workers still performed CPR with a significantly higher mean compression frequency, however, both results were following current guidelines (110.61 ± 6.89 *vs.* 114.46 ± 6.38; p = 0.009). There were no differences regarding other measured parameters between students and medical workers when the device was used (Tab. 1).

Discussion

In the presented study, implementation of the real-time feedback device significantly improved all measured parameters of CPR (mean compression depth, mean compression frequency and the rates of proper-quality CPR, adequate compression depth and adequate compression frequency) for the total study cohort and each of the investigated subgroups. Despite the improvement in the overall quality of CPR, the results remained unsatisfactory since the median rate of proper-quality CPR in terms of compression depth and frequency was merely 55.4%. When performing CPR without the feedback device, the medical students failed to achieve the recommended depth but successfully maintained the recommended frequency of chest compressions, while medical workers tended to overdo, both in terms of compression depth and frequency, with the mean frequency exceeding 130 compressions per minute. However, the overall quality of CPR at baseline was similar in both groups. The implementation of the feedback device resulted in the improvement of CPR quality in either of the groups.

Our results are consistent with some previously published studies which demonstrated improvement in CPR quality with the use of real-time feedback devices [13–18]. OHCA remains one of the most challenging medical conditions due to a very low survival rate. Previous reports showed that return of spontaneous circulation was achieved in 33% of cases, however, only 8% survived until hospital discharge [21]. Two main components of adequately performed CPR are compression depth and frequency. The adequate depth range recommended in the guidelines is 5-6 cm [8]. In a meta-analysis by Wallace et al. [22], it was demonstrated that deeper chest compressions improved the prognosis of patients after cardiac arrest. Similar results were reported in another meta-analysis by Talikowska et al. [23], who showed that deeper chest compressions were related both with a greater chance of return of spontaneous circulation and survival. Vadeboncoeur et al. [24] investigated 593 cases of OHCA and found incremental odds of survival with an increase in compression depth by each 5 mm (aOR: 1.29; 95% CI 1.00–1.65). Deeper chest compressions were also proven to improve haemodynamics (assessed with coronary perfusion pressure, carotid and renal blood flow) in the animal model [25]. However, in a study by Stiell et al. [26] an inverse association between the depth and compression rate was found. In the present study, the mean compression depth without the feedback was slightly higher than the range recommended in the guidelines, but it reached the appropriate range (5.3 ± 0.4 cm) after the implementation of the feedback device.

The importance of performing chest compressions with an adequate frequency was demonstrated in several previous studies. Current guidelines recommend maintaining the pace between 100 and 120 compressions per minute [8]. In the above-mentioned meta-analysis by Talikowska et al. [23] the frequency of 100–120 cpm was associated with survival till hospital discharge. Of note, even within this range, a higher frequency was observed in the non-survivor group [23]. The analysis of data collected from a monitor-defibrillator recording of 10 371 OHCA patients revealed the greatest survival odds for rates of 100–119 cpm in comparison with the other four analysed rate ranges (< 80, 80–99, 120–139, and ≥ 140) [27]. Furthermore, increasing compression frequency was found to be accompanied by a simultaneous significant decrease in compression depth. Similar results were presented by Monsieurs et al. [28], who investigated compression depth for 3 rate categories (< 80/min, 80–120/min, and > 120/min) in 133 patients undergoing CPR. Rates >120 cpm were associated with decreased compression depth, hoverer in this study the deepest compression was 4.5 cm at the frequency of 86/min, therefore was insufficient in terms of the current guidelines. On contrary, Lee et al. [29] reported a proportional relationship between chest compression depth and frequency, however, the frequency > 120 cpm was associated with the highest rate of incomplete chest relaxation. Both higher frequency and deeper compressions are related to a greater chance of injury including ribs and sternum fractures [11, 12]. In the present study, the mean compression frequency remained within the recommended range regardless of the feedback present or absent, however, it significantly decreased when the device was used (119.8 ± 16.8 *vs.* 111.9 ± 6.9; $p < 0.001$). Without the feedback, over half of all compressions were performed with the recommended frequency, but only in 11% of cases, the compression depth was adequate.

In this study, an increase in the quality of feedback-assisted CPR was present for both investigated groups analysed either together or separately. After the implementation of the feedback device, a significantly higher mean compression frequency was observed in the medical workers' group, however, both results were following current guidelines. Besides the above-mentioned, no differences were found between those groups. Without it, the major difference between students and medical workers concerned compression frequency, in favour of the student group. However, the overall quality of CPR was similar in both groups. During their courses, medical students more often can perform CPR training in a controlled setting, when they are supervised by the instructor. On the other hand, medical workers perform CPR in a real-life setting, which is a major source of stress and there is no supervision

available. Those conditions can potentially lead to higher compression frequency and therefore impaired quality of CPR. The introduction of post-cardiac arrest debriefing sessions has been proven to improve the CPR parameters (ventilation, frequency, and depth of chest compressions) and the chances to achieve ROSC [30]. Presented results also support the need for continuous CPR training for all social groups. Performing CPR with real-time feedback devices should also be considered a part of medical training to support the education process and improve the quality of CPR training [18–20].

Limitations

The study has several limitations to be mentioned. Firstly, it was conducted under simulated conditions. However, a similar study performed on real patients after OHCA might be related to unacceptable risk for their life and health. Furthermore, simulated conditions allowed proper standardization of the study. Secondly, no data on chest relaxation were provided, making the analysis of this CPR-vital parameter impossible. Thirdly, CPR duration was confined to two one-minute runs, thus eliminating the effect of exhaustion-related CPR quality deterioration seen with prolonged real-life CPR.

Conclusions

The use of the Real CPR Help® real-time feedback device in a simulated test improved CPR quality. Even with real-time feedback, the overall CPR quality remains unsatisfactory, mandating additional training for CPR skills improvement. There were no differences concerning CPR quality between students and medical workers after the implementation of the real-time feedback device.

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VIA MEDICA

ORIGINAL ARTICLE

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Rationale and design of a clinical trial to evaluate the safety and efficacy of gum Arabic in patients with nephrolithiasis and renal cyst simultaneously

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ABSTRACT

The elderly group is one of the most heterogeneous and vulnerable groups of the population in developed countries with a greater risk of suffering from imbalances, deficiencies and nutritional problems. Diet and nutritional status have a great influence particularly on the prevention or treatment of various diseases that affect these groups. Long-term accumulation of waste in the body and age-related changes in metabolism create many problems that shorten their life expectancy. Their diet and gastrointestinal function play a key role in their Urine composition. It seems that the gastrointestinal microbiome has a great influence on the metabolization and absorption of the ingredients of the diet. In this clinical trial, the authors concluded that oral administration of gum arabic dissolved in orange juice could conceivably wash out the renal stones and eliminate renal cysts which in the long-term did not raise any safety concerns. The oral administration of gum arabic reduces kidney failure and slows its progression, which might be ascribed to their antioxidant and free radical-scavenging properties. Gum arabic could be considered as an important natural medicinal compound, actually a fascinating one because of its high therapeutic capabilities. Therefore, a prospective observational study has been designed and aimed to assess the efficacy and safety of treatment with gum arabic in patients with nephrolithiasis and renal cysts.

Key words: gum arabic, prebiotic, orange juice, kidney cysts, renal lithiasis, chronic kidney disease

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Introduction

Kidney cysts and stones when occurring in the elderly are risk factors for developing chronic kidney disease (CKD), which is seen globally. CKD recorded a global prevalence of 9.1%, which is more prevalent in men, in elderly subjects, and subjects with CVD or cardiovascular risk factors. The prevalence of kidney stones and cysts among chronic kidney disease patients is 11.2% and 10.7% respectively.

Kidney cystic diseases

The kidney is one of the places in the body where cysts develop frequently [1]. In 1988, Gardner suggested that a tubular dilation four times the normal diameter (more than 200 μm) should be called a cyst. Cystic kidney disease is often discovered either during the workup for kidney failure through ultrasound, or incidentally during an imaging test or family investigation. The most common forms of kidney cysts are simple cysts and acquired cystic disease (usually associated with dialysis). In general, the association with symptoms has been considered a mere coincidence. They can be detected by ultrasound and radiological studies indicated for urological problems, arterial hypertension or haematuria. In 1930, Hepler proposed that its aetiology could be a tubular obstruction, which distends and become cystic due to the continuous flow of urine, however, this is not the only mechanism involved in its formation [2].

Some fundamental aspects for its development have been also mentioned: 1) abnormal proliferation and/or lack of differentiation of renal epithelial cells; 2) continuous flow of fluid (urine); 3) Abnormalities in the tubular basement membrane and/or extracellular matrix [3].

The other proposed mechanism is chronic hypokalaemia, which has been described as causing structural and functional abnormalities including increased renal cell growth [4], renal condensing capacity, interstitial fibrosis, chronic inflammation, and defects [5]. This type of cyst formation has been reported in primary hyperaldosteronism [6, 7], distal renal tubular acidosis (dRTA) [8, 9], Bartter's syndrome [10, 11] and in apparent mineralocorticoid excess syndrome [12]. Meanwhile, acquired renal cysts are observed in chronic kidney disease [13]. A renal cyst is a cavity filled with fluid, limited by a single layer of epithelial cells (may be cuboidal or flattened), the origin of which is a dilation of any part of the nephron or the collecting tubule. The presence of cysts in the kidneys is a common occurrence and its incidence increases with age. In the autopsy series, more than 50% of subjects 50 years of age present at least one microscopic renal cyst. The alteration of the structure and function of the primary cilia appears to be a common factor in the pathogenesis of renal cystic diseases.

Simple cyst

Simple, solitary, or multiple kidney cysts are usually harmless and detected incidentally. They are common cystic lesions in adulthood. They are located in the cortex, are usually unilateral and their highest incidence is observed after 40 years of age [14, 15]. They have a predilection for the male sex (M:F — 2:1), the left kidney and the upper renal pole [16]. However, they are rare in infants and children, where they usually appear as solitary lesions. Sonographically, they appear in the renal cortex, they are usually less than 1 cm in diameter thin-walled, predominantly unilocular and without echo-refringence in their interior [17, 18]. They are not hereditary, but little is known about the contribution of genetic and environmental factors to their formation. In the natural history of simple kidney cysts, they can be a few millimetres or several centimetres in diameter (their size ranges are between < 0.1 to > 10 cm), which sometimes increase in size over time. Microscopically, a single layer of cuboidal or flattened cells surrounded by dense fibrous tissue lines cyst. In computerized axial tomography, the characteristic of a simple cyst is clearly separated from the surrounding parenchyma, it has a thin and smooth wall, the cyst fluid is homogeneous, with a density similar to water, and there is no evidence of intensification of the cyst mass after contrast administration [19].

Renal lithiasis

Urate stones have particularly been shown to be associated with diabetes, cardiovascular disease, hypertension, and chronic kidney disease [20]. Some studies show that simple renal cysts are a tubular development anomaly rather than an acquired secondary lesion. They hypothesise that both entities, renal cysts, and genetic predisposition to kidney stones, are related. Since simple renal cysts are assumed to be acquired, a possible first causal mechanism would be mechanical, that is; the cyst would occur secondary to an intratubular obstruction, caused by crystalluria in the other word kidney stones could be a risk factor in the presence of simple renal cysts [21]. Previous studies show that gum arabic can reduce serum uric acid levels and reverse the process of kidney failure. However, the radical treatment of simple kidney cysts with gum arabic has not been investigated.

Role of diet in influencing the urine composition

Key urinary parameters that might be affected by the microbiome include oxalate and citrate. The intestinal microbiome contains numerous obligate and generalized oxalate degrading bacteria. Evidence advises that the faecal content of *Oxalobacter formigenes,* the best-studied oxalate degrader, vary and is linked to stone formation risk and urinary oxalate excretion. To date, all attempts to destroy oxalate by probiotics, including those containing *Oxalobacter*, *Lactobacillus*, and/or *Bifidobacterium spp*. have been disappointing. From this study, it was concluded that oral administration of gum arabic dissolved in orange juice could conceivably wash out the renal stones and eliminate renal cysts. The oral administration of gum arabic reduces kidney failure and slows its progression, which might be ascribed to their antioxidant and free radical-scavenging properties.

What is gum arabic?

Naturally, gum arabic (GA) is white-yellow to brownish-yellow colour crystal shape. To use in the food and pharmaceutical industries, after collecting the exudates, grinding and purification by dissolution in the water, pasteurization and ultrafiltration, and dried by spray drying and is packed as a form of pre-hydrated powder. The product obtained is non-toxic, easily soluble in water, colourless, does not have a strong taste and odour and, above all, does not distort the taste and odour of the food system. Gum arabic is a unique polysaccharide, which has excellent emulsifying properties and, despite its relatively high molecular weight (460,000), forms solutions of surprisingly low viscosity,

has a bland taste and is odourless best tolerated by the intestine and its metabolism by bacteria is gentle and progressive. Even at high doses, it does not produce bloating or fermentation. This fibre also has a regulatory action on the water content of the stool, favourably influencing the regularity of intestinal transit. Gum arabic is widely used in various fields of the food industry as an effective stabilizer of disperse systems, structure and texture control of foods, a film-forming agent, a material for microencapsulation etc. These functional properties exist thanks to the characteristics of gum arabic chemical structure. There is a great deal of experimental evidence that insufficient intake of dietary fibre in the diet by humans leads to a high risk of diabetes, obesity, hypertension, digestive tract, cardiovascular system, renal and others disease. Conversely, a large number of clinical data confirm that the consumption of dietary fibre protects against these diseases. In addition, according to strictly confirmed epidemiological data, clinical and research evidence on the physiology of dietary fibre intake improves gastrointestinal function, glucose homeostasis and serous exudate lipid content [22, 23]. It has antioxidant and anti-inflammatory properties used in the treatment of various kidney, cardiovascular and gastrointestinal diseases [24]. Used as an emulsifier and stabilizer in the food and pharmaceutical industries. As a "dietary fibre", gum arabic contains various carbohydrates such as l-arabinose, l-rhamnose, and d-glucuronic acid. The backbone structure of gum arabic is mainly composed of 1, 3-linked β-d-galactopyranosyl units which resist hydrolysis by human digestive enzymes, but can be fermented by the colonic microflora, cause a greater increase in bifidobacteria and lactobacilli and mostly excreted in the faeces. The GA solutions contained particularly high concentrations of Ca_2^+, Mg_2^+, and K^+. Because of enhanced uptake, treatment with GA significantly increased both the intestinal and renal excretion of Mg_2^+ and Ca (2^+). The latter was accompanied by decreased urinary excretion of inorganic phosphate and decreased plasma concentrations of 1.25 dihydroxy vitamin D (1.25 $(OH)_2$ D). Moreover, GA significantly increased faecal weight and Na^+ excretion [25].

Health benefits of gum arabic or acacia gum

While gum arabic has been extensively investigated for its properties as a hydrocolloid with various food applications, it has also been the subject of more recent research for its ability to improve human health. Because gum arabic can reach the large intestine and resist digestion in the small intestine, it can be classified as an indigestible carbohydrate or dietary fibre. Gum arabic can also be classified as a prebiotic. In the large intestine, gum arabic is fermented by bacteria that produce short-chain fatty acids (SCFA), particularly propionic acid, as by-products of fermentation that are associated with significant improvements to human health [26].

Bifidogenic

Fermentation of gum arabic has been shown to selectively increase the proportions of lactic acid-producing bacteria and bifidobacteria in study subjects. It also increases the water content of the stool and increases the production of stool.

Gum Arabic is known to feed several different strains of indigenous bifidobacteria, including B. longum [27], and was shown to increase *Bifidobacterium animalis* subsp. lactis BB-12® significantly better than inulin and glucose [28].

Prebiotic

Gum arabic can selectively increase the proportions of lactic acid bacteria and bifidobacteria in healthy subjects. It ferments slowly, with digestibility of around 95%. Gum arabic also increases stool production by increasing the water content of the stool. It is well tolerated in high daily doses and is consumed without any adverse intestinal events. Evidence shows that acacia gum acts as a prebiotic at a dose of 10 g/day [29].

Short-chain fatty acids (SCFAs)

Other research has shown that bacterial fermentation with gum arabic produced more SCFAs such as butyrate and propionate in vitro and in vivo than other well-known prebiotics, such as pectin, inulin, and alginate. This is unequivocal evidence that gum arabic is an indigestible prebiotic polysaccharide [30].

Therefore, the simple addition of gum arabic improved food metabolically for human use. Treatment with gum arabic increased urinary Ca_2^+ excretion and decreased plasma phosphate concentration and plasma urea concentration. The urinary flow rate, natriuresis, phosphaturia, glycosuria, proteinuria, and blood pressure (BP) in diabetic mice were also decreased. These results suggest that the effect of gum arabic on intestinal glucose transport may be useful in the prophylaxis and treatment of metabolic disorders such as obesity and diabetes [31].

Nephroprotective

Gum arabic increases creatinine clearance, increases renal excretion of antidiuretic hormones, decreases plasma phosphate concentration, increases renal secretion of antidiuretic hormone, and is used as a treatment for chronic kidney disease and in a terminal stage in Middle Eastern countries. Gum arabic may also serve as a treatment for kidney disease due to its ability to trap bile salts along with its relatively high effect on bu-

tyrate production, which has been shown to suppress TGF-beta1 cytokine production [32–34].

Material and methods

The objective of the proposed clinical trial is to evaluate the benefit/tolerance ratio and to determine the optimal dosage of gum arabic in patients with nephrolithiasis. The low efficacy of available treatments for kidney stones and persistent renal cysts is the main reason for the design and conduct of this clinical trial.

The study design

This clinical trial was conducted by the medical teams of researchers in Dubai Pharmacy College with the collaboration of Shiraz University of Medical Sciences, Iran. This clinical trial launched in 2016, and proceed in 5 years (Fig. 1).

— Primary efficacy endpoint: ultrasonographic assessment of kidney stones and cysts at one year after enrolment into the study;
— Primary safety endpoint: occurrence of any side effect of evaluated treatment at one year after enrolment into the study;
— Secondary efficacy endpoint: ultrasonographic assessment of kidney stones and cysts five years after enrolment into the study;
— Secondary safety endpoint: occurrence of any side effect of evaluated treatment five years after enrolment into the study;
— Study population: adult patients (age ≥ 18 years) with a history of nephrolithiasis and proteinuria meeting inclusion criteria.

Safety variables and analysis

Monitoring/evaluation in particular of adverse drug reactions/adverse events/toxic effects/periodical assessment on clinical laboratory parameters. Including microalbuminuria dipstick, BUN, creatinine, ALT, AST, uric acid, total bilirubin and alkaline phosphatase, by using standard forms, including symptoms, date and time of onset, first observation, diagnosis, end of episode and outcome, as recommended by the protocols

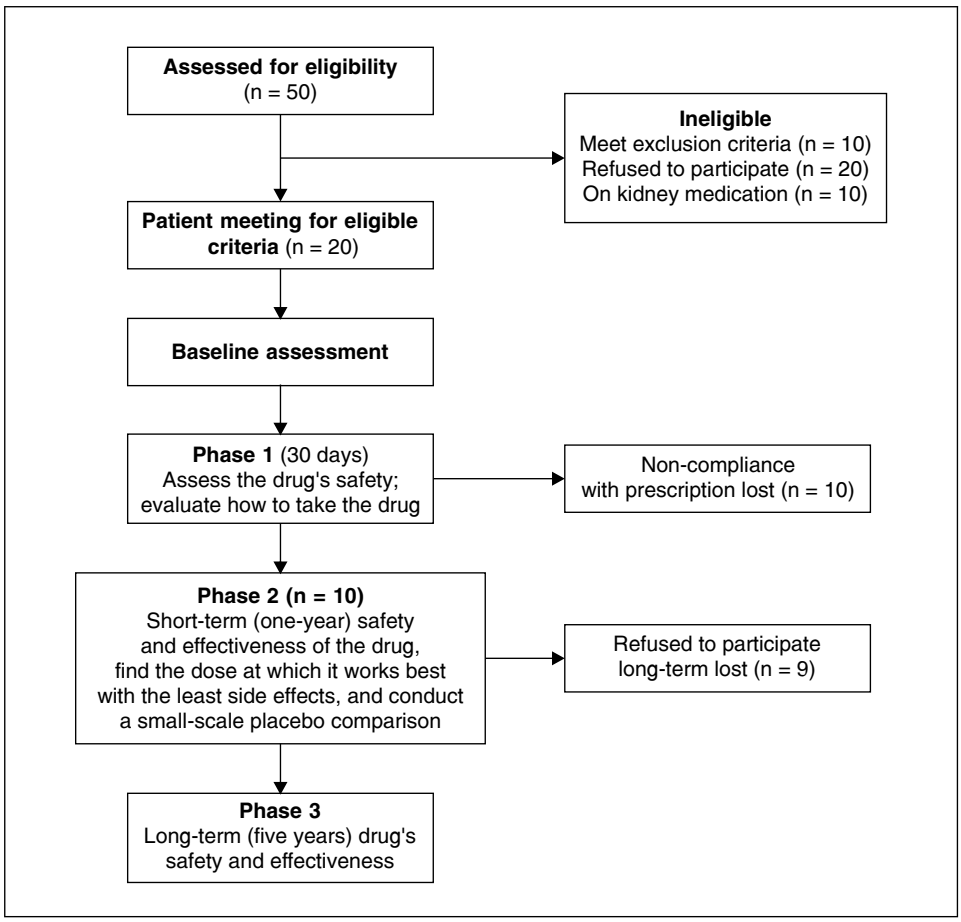

Figure 1. Clinical trial flowchart including the number of patients assessed so far

Table 1. Urine analysis and blood biochemical parameters of the patient before and after of the gum Arabic treatment

	Normal range 2016	Before treatment 2021	After treatment
Microalbuminuria dipstick	0–30 mg/dL	10–300 mg/dL	0
BUN	7–20 mg/dL	18.1	19
Creatinine	0.74–1.35 mg/dL	0.98	1 mg/dL
ALT	29–33 IU/L	18	20
AST	5–40 U/L	14	16
Uric acid	4.0–8.5 mg/dL	8.6	3.4
Bilirubin total	1.2 mg/dL	1	0.4
Alkaline phosphatase	44–147 IU/L	74	68

BUN — blood urea nitrogen; ALT — alanine aminotransferase; AST — aspartate aminotransferase

on safety, efficacy, standardization, and documentation of herbal medicine (IUPAC technical report).

Inclusion criteria for gum arabic hypothesis:

— Adult patients (age ≥ 18 years);
— History of nephrolithiasis and proteinuria;
— Willing to participate in the study and provide informed consent;
— Not enrolled in another trial;
— Estimated survival of at least 5 years.

Exclusion criteria for gum arabic hypothesis:

— Known major complicated disease;
— Haemodialysis patients or severe renal insufficiency defined as CrCl < 30 mL/min);
— Lack or withdrawal of informed consent;
— Not adhering to this study prescriptions;
— Any allergic reaction to gum arabic;
— Use of other medication for renal disease.

Pharmaceutical formulation

A total of 15 grams of acacia Senegal powder dissolved in 250 mL fresh orange juice had it with breakfast daily. For the first 3 months, it was used to add in milk then changed to fresh orange juice.

Preliminary results — case report

At the end of phase three, long-term (five years), in on an abdominal ultrasound examination, it was found that the patient's chronic cyst and kidney stone have disappeared. The 10mm kidney cyst in the renal cortex was discovered incidentally during a training check-up in 1990. It persisted until 2015 without changing in size. Since 1990, now and again he has suffered from kidney stones and proteinuria (+ to +++) frequently. An abdominal ultrasonography scan in December 2020 unexpectedly showed that there was no trace of kidney cyst or stone.

Our results showed that the renal cyst and stone were completely disappeared simultaneously. It was found to be a safe, effective, and economic method for the treatment of renal cysts and stones. The test results in Table 1 show that gum arabic has no side effects for long-term consumption.

Discussion

Chronic kidney disease (CKD) is a pathology of various origins affecting kidney function. One strategy to prevent the progression of CKD is to decrease the uremic retention molecules (URMs) using prebiotics. This study aimed to evaluate the administration of gum arabic dissolved in fresh orange juice for the elimination of kidney cysts and stones hence the potential of reducing the progression of CKD. Factors such as hypercalciuria (resorptive, renal leak, absorptive, and metabolic diseases), hyperuricosuria, Oxidative stress, excessive protein intake, hyper-oxaluria, hypo-citraturia, hypo-magnesuria, and hyper-cystinuria, contribute to kidney stone and cyst formation [35–37].

Changes in urinary pH and Proteinuria

The study reported that both entities, kidney cysts and genetic predisposition to kidney stones, are related [38–41]. Excessive protein intake increases the urinary excretion of uric acid, calcium, oxalate, and decreases urinary pH and citric excretion. Uric acid crystals form at acidic pH. Therefore, this type of crystals can form, even in the presence of normouricemia, in situations of persisting acidic urinary pH, especially in those patients with an excess intake of proteins of animal origin. When the urinary pH is persistently below 5.5, uric acid is in the undissociated and insoluble form, so it can crystallize as a pure form. A disorder, generally of familial origin, has been described, consisting of a decrease in ammonia synthesis by the renal proximal tubular cell and, consequently, decreased urinary ammonia. The deficit of this urinary buffer gives rise to an excess of free hydrogen ions in the urine that would reduce its pH [42]. Orange

juice contains a series of strong antioxidants including flavonoids (hesperetin and naringenin predominantly as glycosides), carotenoids (xanthophylls, cryptoxanthins, carotenes), and ascorbic acid and additionally other beneficial phytochemicals, like folate. All of these are believed to be significant contributors to the preventive effects of orange juice in the inflammatory process, which leads to chronic kidney disease. In addition, the combination of orange juice and gum arabic leads to the production of potassium and magnesium citrate. These compounds are useful for alkalizing urine in patients prone to develop urate and cystine stones.

Uric acid and NOD-LRR-and pyrin domain-containing protein 3)NLRP3 (inflammasome

The inflammatory response makes it possible to fight against the "non-self" such as infectious organisms or exogenous particles. The inflammasome is an intracytoplasmic multi-protein complex activated by cell stresses or infections and is responsible for the release of pro-inflammatory cytokines, including IL-1β. The NLRP3 inflammasome, the most studied of the inflammasomes, is involved in inflammatory pathologies such as Crohn's disease, rheumatoid arthritis or gouty arthritis. Uric acid is a damage-associated molecular pattern (DAMP), released from ischemic tissues and dying cells which, when crystallized, can activate the NLRP3 inflammasome, is associated with water ingress leading to cell swelling [43].

Histopathological study of atherosclerotic lesions reveals the presence of inflammatory cells (activated T lymphocytes and macrophages), as well as abundant pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNF-α, INF-γ, etc.), which modulate the local inflammatory response, altering plaque stability and favouring the development of acute cardiovascular events. However, the role of anti-inflammatory cytokines has not been as well studied.IL-10 is an anti-inflammatory cytokine capable of inhibiting the synthesis of pro-inflammatory cytokines by T lymphocytes and macrophages, as well as other inflammatory functions of these cells [44, 45]. IL-6 is a critical point in the inflammatory cytokine network. Under conditions of autoimmunity and chronic inflammation, elevated levels of IL-6 can affect the homeostasis of multiple physiological processes and contribute to chronic inflammation and disease progression [46–49]. IL-6 could be produced by renal resident cells, including podocytes, mesangial cells, endothelial cells, and TECs. Meantime, all these cells, as well as immune and inflammatory cells will actively respond to IL-6 via classic or/and trans-signalling pathways.

The role of albumin in the formation of kidney stones has been described, acting as a heterogeneous nucleant and favouring the crystallization of calcium oxalate and sodium urate [50–52]. A large number of patients with proteinuria showed multiple kidney cysts on abdominal ultrasonography. Bilateral kidney cysts were detected in 56% of patients with proteinuria, whereas no patient with negative proteinuria showed kidney cysts or other radiological abnormalities [53]. Proteinuria can cause kidney cysts by tubular obstructions, or kidney stones acting as a heterogeneous nucleant, vice versa.

Gum arabic simultaneously reduces proteinuria and serum uric acid hence motivates the elimination of renal cysts and kidney stones.

Oxidative stress

Reactive oxygen species (ROS) can damage lipids, nucleic acids, and proteins, thereby altering their functions. Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and the ability of a biological system to detoxify reagent intermediates or to repair the resulting damage. Oxidative stress has been linked to the pathogenesis of a variety of diseases, including kidney and cardiovascular disease, atherosclerosis, hypertension, cancer, diabetes, arthritis, neurodegenerative diseases (i.e., Alzheimer's and Parkinson's disease), and ageing. For its elimination, the body has an antioxidant defence system made up of enzymatic elements (superoxide dismutase, glutathione peroxidase, catalase) and non-enzymatic elements (glutathione, ascorbic acid, α-tocopherol). Oxidative stress, a situation in which there is an excess of these highly reactive molecules with oxidative capacity, has been related to important deleterious actions, such as lipid peroxidation, protein oxidation, nucleic acid damage, induction of transcription factors such as NF-κB, stimulation of cell hypertrophy and proliferation, or induction of apoptosis [54, 55]. Oxidative stress stimulates the sympathetic nervous system and increases glomerulosclerosis, renal fibrosis, and proteinuria. On the contrary, adiponectin protects the kidney by reducing podocyte dysfunction. Oxidative processes are increased in patients with renal failure and especially in patients with end-stage renal failure on dialysis. Gum arabic is said to have an antioxidant effect and this will decrease the harmful effect of free radicals in haemodialysis patients [56]. The kidney has a very important endothelial surface and recently much importance has been given to vascular damage that produces ischemia of kidney tissue and progression of chronic kidney disease. Therefore, the preservation of vascular integrity and the endothelial wall not only prevents the cardiovascular events associated with chronic kidney disease but is also a very important aspect in slowing the progression of kidney disease. The endothelial cells of the vascular tree respond to signals such as endocrine or paracrine hormones, cytokines and growth factors, exogenous and endogenous toxins, including traditional and non-traditional vascular risk factors. On the other hand, the endothe-

lium also responds to rheological and hemodynamic changes. However, of all of them, oxidative stress and inflammation are the most important elements that produce endothelial and vascular dysfunction in patients with chronic kidney disease. Following the above, the consumption of foods rich in antioxidants will prevent the manifestation of oxidative stress and, therefore, will provide us with a good quality of life [57–59].

Several studies carried out reveal that gum arabic had nephron-protective properties, among which the authors can mention nephrotoxicity induced by gentamicin (antibiotic) and cisplatin [60] or by cardiotoxicity induced by doxorubicin, which was carried out on rats [61]. Tromer and Neubert have shown that the treatment of GA with the addition of polysaccharides decreases lipid peroxidation [62]. It is believed that the antioxidant capacity of GA may be related to its ability to bind biomolecules such as lysine, tyrosine and histidine [63]. In other studies, using GA with tap water (15% w/v) have shown reduced plasma urea and creatinine concentrations linked to adenine-related chronic kidney disease (RCF) and a significant decrease in the negative effects induced by adenine [64]. The protective effects of GA on kidney tissue greatly reduced urea nitrogen and creatinine concentrations in patients with diabetic nephropathy [32].

Serum phosphorus

High serum phosphorus levels are strongly and independently associated with the rate of progression of renal function deterioration in patients with advanced CKD, as well as cardiovascular morbidity and mortality [65–67]. Hyperphosphatemia has been associated with increased blood pressure and hyperdynamic circulation [68]. Phosphorus overload has also been observed to damage the podocyte in experimental animals [69]. These two mechanisms could alternatively explain a causal relationship between phosphorus and the magnitude of proteinuria.

The magnitude of proteinuria and the degree of metabolic acidosis, factors that are associated with higher phosphorus concentrations, have also been implicated as determinants of the rate of progression of CKD [70, 71]. Gum arabic acts as a phosphate-binding agent is a safe treatment of hyperphosphatemia in the intestinal tract [23]. The theory of the lack of inhibitors establishes that the absence or deficiency of natural inhibitors of lithogenesis (magnesium, citrate, pyrophosphates, acid glycoproteins and some trace metals) would be responsible for the formation of kidney stones [72–76]. The magnitude of proteinuria is the main modifiable factor that decisively influences prognosis and clinical decision-making and is an independent factor for cardiovascular risk. It has a direct renal toxic effect, induces inflammation and tubule-interstitial fibrosis, and contributes to the loss of the nephron mass [77–83].

Salt & kidney stones

Calcium excretion is directly linked to sodium excretion, i.e., sodium intake. A high salt diet is a risk factor for kidney disease, also associated with kidney stones. It can increase the amount of calcium lost in the urine, which can cause kidney stones [84–88]. Approximately, a dietary increase of 100 mmol of sodium generates an increase in urinary calcium of one mmol. This physiologic feature does not make much difference between different races [89]. In postmenopausal women, the occurrence of kidney stones is associated with a history of hypertension and a low dietary intake of magnesium and calcium, as they increase urinary oxalate excretion [90]. Gum arabic not only increased faecal weight, compatible with the action of dietary fibres, but also it seems that it bounds free water, which results in a reduction of intestinal fluid absorption. Possibly, due to decreased intestinal water uptake, the plasma Na^+ concentration is increased during gum Arabic treatment. The increased extracellular Na^+ concentration may have accounted for the stimulation of ADH release, which is reflected by increased urinary ADH excretion. Consequently, the ADH stimulates renal water reabsorption, thus decreased urine volume. Gum arabic further enhances the intestinal elimination of Na^+, resulting in a major reduction of renal Na^+ excretion. The impaired intestinal Na^+ absorption may be thanks to the Na^+ binding ability of the gum. Magnesium forms complexes with oxalate, reducing the supersaturation of calcium oxalate. Furthermore, magnesium oxalate complexes reduce the intestinal absorption of oxalate. At physiological oxalate concentrations, magnesium reduces both stone nucleation and growth rates [91]. Citrate salts therapy is available in most prescriptions for kidney stones to raise urine pH to an optimal level and increase urinary citrate levels. Citrate supplementation like potassium and magnesium citrate is prescribed for the patients with recurrent calcium lithiasis, uric acid and hyperuricosuria, cysteine stones, renal tubular acidosis, chronic diarrheal syndrome, essential hypocitraturia or secondary to thiazides. Hypocitraturia and hypercalciuria are entities that have been treated with potassium citrate with satisfactory results. Citrate is known to be a potent inhibitor of kidney stone formation. In the urine, several substances facilitate the formation of soluble complexes with cations and are generically known as crystallization inhibitors. The decrease or absence of these inhibitors facilitates the formation of stones. Citrate retards the crystallization of calcium salts through two mechanisms: a) it complexes with calcium and reduces the concentration of ionic calcium in the urine; b) citrate directly inhibits the crystallization of calcium oxalate and calcium phosphate. Hypocitraturia is a common finding in patients with calcium oxalate renal lithiasis. Numerous articles show that the decrease in urinary citrate in pa-

tients with renal lithiasis varies between 19% and 63%. In the Dallas study, isolated hypocitraturia was found in 5% of patients, and associated with other abnormalities in 50% of patients with lithiasis [92–95].

Potassium citrate causes a decrease in urinary calcium and the saturation of calcium oxalate; in addition, it increases the inhibitory activity against calcium oxalate crystallization, while sodium citrate does not alter urinary calcium, nor does it have any inhibitory effect on calcium oxalate or calcium phosphate crystallization [96]. However, consumption of citrate salts has many side effects, such as gastrointestinal disturbances, black or tarry stools, convulsions, severe diarrhoea, nausea, or vomiting, severe stomach pain, difficulty breathing, hyperkalaemia which may result in muscle cramps or weakness, dizziness, confusion or restlessness, bradycardia, arrhythmias, tingling of extremities and cold skin. Citrate salts are contraindicated in pregnant or breastfeeding women, bleeding disorders, kidney impairment, uncontrolled diabetes, severe cardiac disease and citrate allergy. While these problems are not found in, the consumption of gum arabic mixed in fresh orange juice. Gum arabic is a more palatable and less expensive alternative for citrate supplementation. Gum arabic plays an important antioxidant and protective role to combat many diseases such as renal, cardiovascular, gastrointestinal and respiratory diseases, due to its remarkable effect on oxidative stress and DNA damage. Study shows that gum arabic has a powerful immunomodulatory effect. Patient safety is a discipline of health care that emerged with the evolution of the complexity of health care systems and the consequent increase in harm to patients in health centres. A cornerstone of the discipline is a continuous improvement based on avoiding medicine adverse events. Patients with chronic kidney disease (CRF) are at high risk for developing side effects and drug interactions. Inadequate drug prescribing in a patient with CRF may be toxic or ineffective. The elderly are particularly at risk. The chronic renal disease will alter glomerular blood flow, glomerular filtration rate, tubular secretion and reabsorption, as well as renal metabolism. This will cause changes in the absorption, bioavailability, protein binding, and volume of distribution and metabolism of the drugs. In the examination of empirical evidence of safety, from a toxicological point of view, research authorities have led them to conclude that there is no restriction on the use of gum arabic and did not raise any safety concerns [97]. The present clinical trial results are consistent with previous research.

Conclusions

In conclusion, today many scientific data are reinforcing the importance of diet for the establishment, composition, structure and functional activity of the human intestinal microbiota. Studies identifying microbiota-health associations in humans provide evidence pointing to the role of diet in the pathogenesis of certain diseases through its effects on gut microbial communities. The modulation of dysbiotic microbiota with diet or the use of probiotics and prebiotics will surely help us prevent diseases such as obesity, renal stone, metabolic syndrome or cancer and, in general, many inflammatory processes. Arabic gum could be considered an important natural medicinal compound, because of its high therapeutic capabilities. Available evidence strongly suggests that gum arabic is an alternative therapeutic approach for patients with renal simple cysts and stones. This supports the idea to conduct a prospective observational study aimed to assess the efficacy and safety of treatment with gum arabic in patients with nephrolithiasis and renal cysts.

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VIA MEDICA

ORIGINAL ARTICLE

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Influence of dexamethasone and doxorubicin on inhibition of hypoxia--induced metastatic potential in HepG2 cell line

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ABSTRACT

One of the commonly applied methods in the case of median to advance stages of liver cancer is the transarterial chemoembolization (TACE) procedure. It involves the administration of relatively high doses of cytostatics to the tumour-supplying artery followed by the embolization of the vessel. It limits the drug action almost only to the tumour mass. However, this also reduces the availability of oxygen, which stimulates cell migration. Therefore, the study aimed to assess how the introduction of an additional drug — dexamethasone and its combination with doxorubicin will impact the viability and migration of HepG2 cells under hypoxia-mimic conditions. To assess the basic response of the cells to the drugs and evaluate the interaction between them MTT assay and apoptosis assay were used. To analyse the migratory potential transwell migration assay was applied. Epithelial-mesenchymal transition (EMT) markers and apoptosis-related proteins were studied using Western blot assay. Hypoxia-mimic conditions were induced using pretreatment with cobalt chloride. The obtained results suggest that the developed doxorubicin: dexamethasone combination limits hypoxia-induced increase in the migratory potential of HCC cells, which is connected with the inhibition of the EMT process and directing cells to death on the cellular level.

Key words: hepatocellular carcinoma, doxorubicin, dexamethasone, metastasis, hypoxia

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Introduction

Hepatocellular carcinoma (HCC) is in fifth place among the most common cancers in the world. The most effective method of HCC treatment is radical surgery, however, it is rarely possible. Another therapeutic option is transarterial chemoembolization (TACE) [1]. The procedure consists of administering cytostatics directly to the tumour-supplying artery and embolization of the vessel. This limits blood flow to the tumour, thereby reducing the oxygen level inside the tumour mass. Moreover, the direct delivery of the drug results in an enhanced anti-tumour effect and simultaneously reduces adverse reactions [2]. The most widely used cytostatic for TACE in HCC is anthracycline doxorubicin (DOX) [3]. The use of DOX in TACE is generally accepted, although drug resistance is often seen in HCC [4]. A significant cause of chemoresistance is hypoxia. This is especially important considering the TACE procedure, which results in the induction of acute hypoxia [5]. Many reports indicate that the heterodimer of the hypoxia-inducible factor 1α (HIF-1α) signalling pathway induces epithelial-mesenchymal transition (EMT) in cancer cells [6]. During the EMT process, tumour cells lose epithelial cell traits and acquire mesenchymal characteristics, which favours growth, invasion, and metastasis of cancer cells [7].

Considering the huge impact of HIF-1α on drug resistance and cancer development, compounds able to affect the protein are searched. One of the chemical

compounds with such properties is dexamethasone (DEX). Many reports indicate that the level of HIF-1α was decreased after the treatment of DEX in colorectal and breast cancer cells [8].

Hence the study aimed to indicate the possibility of reducing the EMT process by using combination therapy composed of DOX and DEX in hypoxia-mimic conditions in the HepG2 cell line.

Materials and methods

Cell culture and treatment

HepG2 cell line was obtained from the Department of Histology and Embryology, Medical University in Wrocław, Poland. The cells were grown in EMEM (LONZA, Basel, Switzerland) with the addition of 10% (v/v) fetal bovine serum (FBS, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and 50 μg/mL gentamycin (Sigma-Aldrich) in standard culture conditions. For the cell viability analysis cells were treated with 3, 4, 5, 6, 7 μM DOX (Sigma-Aldrich); 3, 4, 5, 6, 7 μM DEX (Sigma-Aldrich) or the combination of DOX and DEX in the 1:1 ratio for 24h. Control cells were grown under identical conditions but in the absence of DOX and DEX. Based on the MTT assay, for the other experiments, 3, 6μM DOX; 3, 6 μM DEX and the combination 3:3 μM DOX/DEX and 6:6 μM DEX were used. The hypoxic microenvironment was mimicked using cobalt chloride ($CoCl_2$, Sigma-Aldrich). To assess whether $CoCl_2$ impacts cell viability and HIF-1 level, the cells were incubated with 60, 80, 100, 120, 140 μM concentrations of $CoCl_2$. Based on the obtained results 80 μM concentration was selected to induce hypoxia during further experiments.

MTT assay and interaction between the drugs

To determine the cytotoxic effect of DOX, DEX, and their combination the colourimetric MTT assay was used. After 24h, cells were washed with PBS and incubated with MTT working solution for 3 h in standard culture conditions. The formazan crystals were dissolved in 2 mL isopropanol (10 min, 37°C; Avantor, Gliwice, Poland). The absorbance of each dose was read using a spectrophotometer (Spectra Academy, K-MAC, Korea) at a wavelength of 570 nm. The dye absorbance of the control cell was assumed as 100% and constituted a reference point in assessing the viability of the cells from the studied group.

The data obtained from the MTT assay was used to evaluate the potential interaction between the drugs. For this purpose, the Chou-Talalay median effect principle and CompuSyn software were applied. The method is based on the determination of combination indexes (CI) relative to the level of cytotoxicity. The analysis classifies relationships into three groups – synergistic (CI < 1), additive (CI = 1) and antagonistic (CI > 1) [9]. The results were presented as the f_a–CI plot which was constructed by simulating CI values over a range of fraction affected (f_a) levels from 0.1 to 0.6.

Cell death analysis

To analyse cell death double staining with the Apoptosis Assay Kit containing Annexin V Alexa Fluor 488 and Propidium Iodide (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was performed according to instructions included by the manufacturer. The analysis was performed using a Guava 6HT-2L Cytometer (Merck KGaA) and FlowJo vX 10.3 software.

Western blot

The semi-quantitative protein levels of vimentin, β-catenin, N-cadherin, E-cadherin, HIF-1 (Thermo Fisher Scientific), Bax (Abcam, Cambridge, United Kingdom) and Bcl-2 (Abcam) were performed using the standard protocol described in a previous study [10].

Transwell migration assay

To evaluate the migration potential of HepG2 cells transwell inserts (Corning, New York, USA) were used. Transwells were placed in a 24-well plate, where each well (the lower chamber) contained a 700 μM EMEM medium with 15% FBS as a chemoattractant. After 24h incubation, cells from the upper surface of the membrane were removed. The migration cells which adhered to the lower surface of the membrane were fixed in 3.6% PFA, incubated with 100% methanol and stained using 0.4% crystal violet. The preparations were examined using the Eclipse E800 microscope (Nikon) equipped with DS–5Mc–U1 CCD camera (Nikon) and NIS–Elements image analysis system (version 3.30; Nikon).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The results were considered statistically significant when $p < 0.05$. For data obtained from MTT assay and Western blot analysis of HIF-1α, the Wilcoxon test was used where doses of DOX, DEX, and their combinations were compared to the hypothetical value for the control group absorbance considered as 100%. In apoptosis analysis, the non-parametric Kruskal-Wallis with Dunn's post hoc test was used. In the case of the transwell migration assay and Western blot assay, 2way ANOVA with Dunnett's or Sidak's multiple comparisons test was performed. All data are presented by

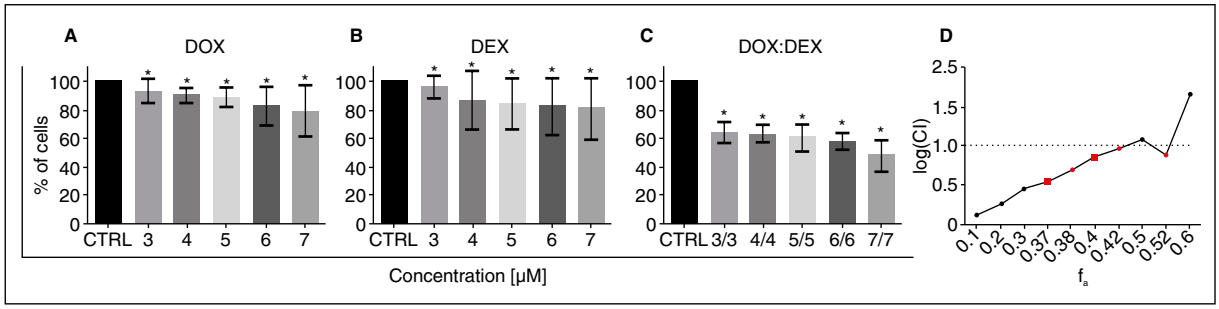

Figure 1. The cytotoxic effect of doxorubicin (DOX) and dexamethasone (DEX) individually and in combined treatment on cell viability of HepG2 cells. The cell viability analysis was based on the results obtained from the MTT assay. HepG2 cells were treated with DOX (**A**) and DEX (**B**) at concentrations from 3–7 μM for 24h and their combination in ratio 1:1 (**C**). The data represent mean values ± SD of 3 independent experiments (n = 3). Statistically significant differences were marked with '*' ($p < 0.05$; Wilcoxon test) (**D**). The combination index plot for DOX and DEX co-treatment in HepG2 cells in the range of f_a from 0.1 to 0.6. CI < 1– synergism, CI =1 — additive effect, CI > 1 — antagonism. For real measuring points, the values have been marked in red

means ± standard deviation (SD) of three independent experiments (n = 3).

Results

The cytotoxic effect of DOX and DEX individually and in combined treatment on cells viability

As shown in Figures 1A and 1B, the use of DOX and DEX at doses from 3 to 7 μM resulted in a dose-dependent decrease in cell viability. In the case of using DOX/DEX combination in the 1:1 ratio, it was also noted to decrease the population of live cells (Fig. 1C). All results obtained were statistically significant compared to the control (Fig.1A–C). The IC_{50} (half maximal inhibitory concentration; $f_a = 0.5$) value for 24h incubation was calculated in the CompuSyn program and reached 17.77 μM for DOX and 13.19 μM for DEX. Analysis of the type of interaction showed a CI value of < 1, which was characteristic of synergism (Fig. 1D).

Treatment of HepG2 cells with DOX, DEX and the combination of these drugs increased the population of apoptotic cells

The analysis of cell death after treatment of HepG2 cells for 24h with DOX, DEX, and their combination in a 1:1 ratio resulted in a decrease in the percentage of live cells (AV–/PI–) in comparison to untreated cells. In the case of 24h incubation with 6 μM DOX, 6 μM DEX and a combination of 6 μM DOX with 6 μM DEX the population of living cells also decreased. Except for the 3 μM DEX, all results obtained were statistically significant (Fig. 2A). Figure 2A presented an increase in the percentage of apoptotic cells (AV+/PI– and AV+/PI+). All obtained results were statistically significant (Fig. 2A). The analysis also showed the necrotic population (AV–/PI+) (Fig. 2A). The representative plots were presented in Figure 2B.

The MTT analysis was also used to assess the effect of various doses of $CoCl_2$ on the cell viability of the HepG2 line. The compound induces hypoxia *in vitro* by stabilizing HIF-1α. After 24h incubation with $CoCl_2$, the cell viability increased slightly but was statistically significant (Fig. 3A). Due to the HIF-1α being the major regulator of response to hypoxia, the level of this protein was assessed using the Western blot method (Fig. 3B). The densitometric analysis showed that the highest level of HIF-1α protein was recorded after using 80 μM $CoCl_2$ (Fig. 3C). Considering the obtained results during further experiments 80 μM $CoCl_2$ concentration was used to induce the hypoxia-mimic conditions.

The effect of DOX and DEX individually and in combined treatment on migration potential of HepG2 cells

The presented study mainly aimed to investigate the effect of DOX, DEX, and the combination of drugs in ratio 1:1 on the migration potential of HepG2 cells in both, normoxic and hypoxic conditions. For this purpose, recommended methods such as transwell migration assay and measurement of EMT markers with Western blot assay were used.

Figure 4A presents representative images of HepG2 cells with high migratory potential. For statistical analysis cells in normoxic and hypoxic conditions were counted on the underside of the transwell inserts. In the case of CTRL in normoxia, data was estimated as 100% and the results obtained for the drugs are presented

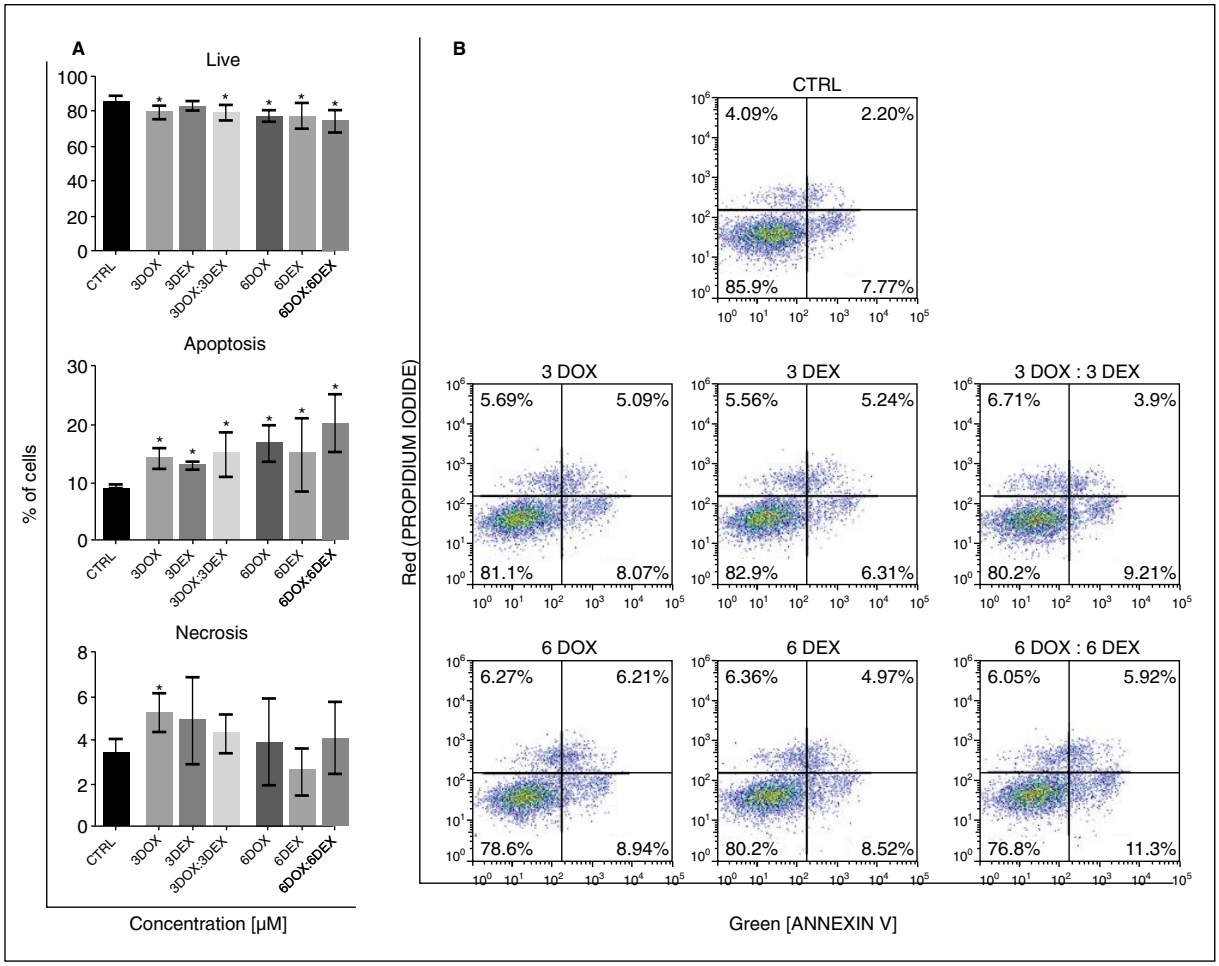

Figure 2. The effect of doxorubicin (DOX) and dexamethasone (DEX) individually and in combined treatment on cell death of HepG2 cells. The cytometric analysis of cell death using Annexin V Alexa Fluor 488 and PI double staining assay (**A**). The percentage of live, apoptotic, and necrotic cells. The HepG2 cells were treated with different concentrations of DOX and DEX (3 μM or 6 μM) for 24h, and the combination of both drugs in ratio 1:1. '*' indicate statistically significant differences in comparison to control cells ($P < 0.05$; the non-parametric Kruskal-Wallis with Dunn's post hoc test) (**B**). The representative plots

as an average percentage in comparison to untreated cells (Fig. 4B). The obtained results suggest that the combination of 6 μM DOX and DEX was more effective in reducing cell migration than 3 μM DOX: 3 μM DEX cotreatment in hypoxic conditions.

Data obtained in the Transwell migration assay was supported by Western blot analysis of selected EMT markers (Fig. 5). All results obtained in the analysis were evaluated in comparison to the HepG2 control cells without the $CoCl_2$ pretreatment (estimated as 1). Assessment of vimentin level in HepG2 cells grown in normal conditions resulted in a decrease compared to CTRL. After treatment with DOX and DEX in ratio 1:1 increase was observed. Following incubation with 6 μM DOX, a statistically significant increase in vimentin was noticed. In hypoxic condition, 3 μM DOX, 3 μM DEX and the combination of drugs significantly decreased protein level in comparison to untreated HepG2 cells (Fig. 5A). Under both conditions, 6 μM DEX alone and combined with 6 μM DOX resulted in a significant decrease in the protein level in HepG2 (Fig. 5A).

Western blot evaluation of β-catenin showed that 3 μM DOX and 6 μM DEX promotes an increase in protein expression in comparison to untreated cells, respectively (Fig. 5B). The reductions in protein levels were observed for both combinations of drugs. All of the results were statistically significant. Moreover, a significant increase in β-catenin level for HepG2 with addition $CoCl_2$ for CTRL, combined of 3 μM DOX and DEX and 6 μM DEX Fig. 5B) was noted.

Figure 5C presents the changes in the N-cadherin level in normoxic and hypoxic conditions. Following treatment with 3 μM DOX: 3μM DEX and 6 μM DOX increase in N-cadherin level was noticed. The untreated

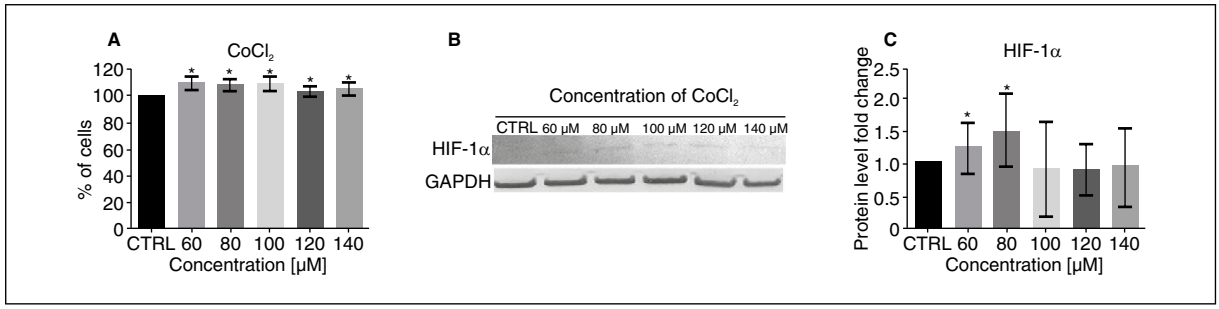

Figure 3. The effect of $CoCl_2$ on the viability of HepG2 cells and the level of HIF-1α. (A) The cell viability analysis was based on the results obtained from the MTT assay. The HepG2 cells were treated with different concentrations of $CoCl_2$ (60–140 μM) for 24h. The data represent mean values ± SD of 3 independent experiments (n = 3). Statistically significant differences are marked with '*' ($p < 0.05$; Wilcoxon test); (**B**) Western blot analysis results of HIF-1α and GAPDH in HepG2 cells following $CoCl_2$ treatment for 24h; (**C**) Densitometric analysis of HIF-1α. Protein levels were normalized to untreated cells and the results were expressed as fold change vs. control (estimated as 1). Statistically significant differences are marked with '*' ($p < 0.05$; Wilcoxon test). Data presented as mean ± SD

HepG2 cells in hypoxic condition were characterized by an increase in protein expression in comparison to CTRL. Under both conditions, a statistically significant decrease in protein expression was observed for 3 μM DOX, 3 μM DEX 6 μM DEX and 6 μM combination of drugs (Fig. 5C).

The last of the analysed protein was E-cadherin. A statistically significant decrease was observed in the level of E-cadherin following treatment with 3 μM DOX and 3 μM DOX: 3 μM DEX in comparison to untreated HepG2 cells in normoxia. In the same conditions, after 24h incubation with 3 μM DEX and 6 μM DEX E-cadherin levels increased (Fig. 5D). The addition of $CoCl_2$ and 3 μM DOX, 3 μM DEX, 6 μM DEX and combination of cytostatics at concentrations 3 μM and 6 μM resulted in a statistically significant increase in E-cadherin level in comparison to control in standard conditions. The significant reductions in protein level in untreated HepG2 cells in hypoxic condition was noticed (Fig. 5D).

In the next step, Western blot analysis was applied to examine the expression of Bax and Bcl-2, which are involved in apoptosis (Fig. 6A, B). As previously, evaluated HepG2 cells were treated with DOX, DEX, and a combination of drugs in ratio 1:1 in both, normoxia and hypoxia. Figure 6D presented that DOX at concentration 3 μM and 6 μM induced a statistically significant decrease in Bax/Bcl-2 ratio in comparison to CTRL in normal conditions Data obtained for HepG2 cells cultured with the addition of $CoCl_2$ presented an increase in the Bax/Bcl-2 ratio compared to untreated cells in normoxia. Moreover, a statistically significant increase in Bax/Bcl-2 ratio for 24h incubation with 6 μM DOX, 6 μM DEX and a combination of cytostatics were also noticed (Fig. 6D). These results may explain the inhibition of HepG2 migration potential after treatment in hypoxic conditions.

Discussion

Although many studies have been conducted on how to improve the TACE treatment strategy, the results are still not fully satisfying [11]. In the present study, it is suggested that the combination of a cytostatic commonly used during TACE — DOX with corticosteroid drug — DEX has the potential to improve TACE procedure outcome.

One of the problems arising during the TACE procedure is hypoxia. The cells under hypoxic conditions produce hypoxia-inducible angiogenic factors, e.g., VEGF, which stimulates the surrounding endothelial cells to form new blood vessels. Moreover, the lack of oxygen is one of the triggers for EMT [12]. This, in turn, is the basis for metastasis formation. As was shown by Li et al., the TACE procedure followed by a liver transplant results in a higher incidence of pulmonary metastasis in comparison to patients with liver transplants alone [13]. This implicates that poor response to the procedure may be connected with metastasis occurrence.

As indicated by Buschauer et al., HepG2 cells after DOX treatment undergo selection rather than death [14]. Moreover, the population of cells, which survived was characterized by an increased migratory potential and a decreased susceptibility to drug retreatment. In the case of this study, the authors applied 3 μM and 6 μM doses of DOX. Another drug applied in the study was DEX, which is a commonly used corticosteroid drug. However, some literature reports indicate its inhibiting effect on the migratory potential of cancer cells and blocking the hypoxia-induced EMT process [15]. Furthermore, the combination of DEX and DOX along with other cytostatics has been extensively tested for many types of cancer [16, 17]. As was indicated by Ogasawara et al., administration of DEX before TACE limits

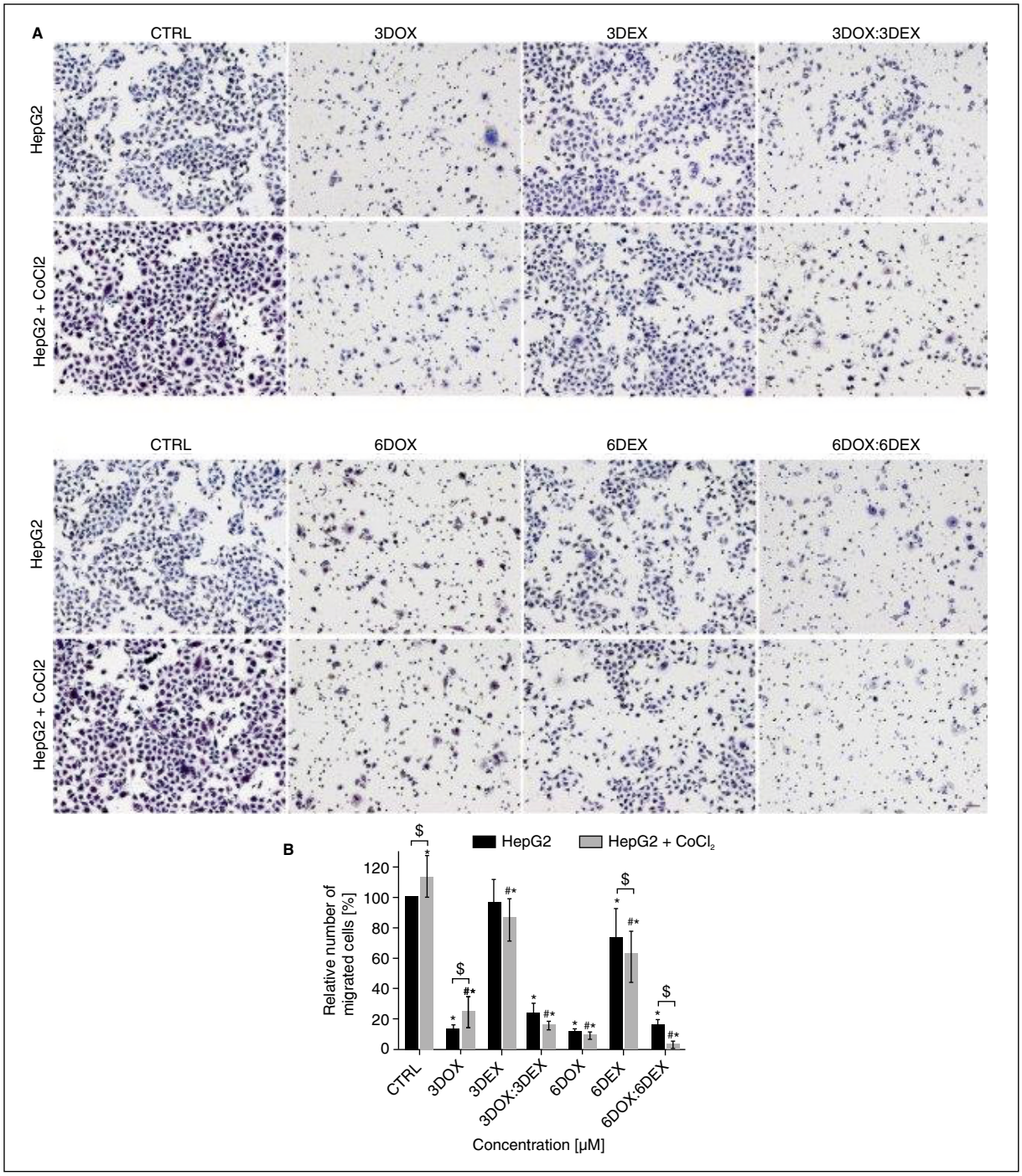

Figure 4. The effect of doxorubicin (DOX) and dexamethasone (DEX) individually and in combined treatment on migration potential of HepG2 cells — Transwell migration assay. HepG2 cells were treated with DOX, DEX, and the combination of drugs in ratio 1:1 in normoxia (HepG2) and hypoxia (HepG2 + $CoCl_2$) for 24h; (**A**) The representative images of the results obtained in Transwell migration assay. Magnification 10x, Bar = 50μm; (**B**) The average percentage of cells with high migratory potential relative to control cells in normoxia (estimated as 100%). '*' indicate statistically significant differences in comparison to control cells in normoxia, '#' to hypoxia, and '$' between appropriate doses in normoxia and hypoxia ($P < 0.05$; 2way ANOVA with Dunnett's or Sidak's multiple comparisons test). Data presented as mean ± SD

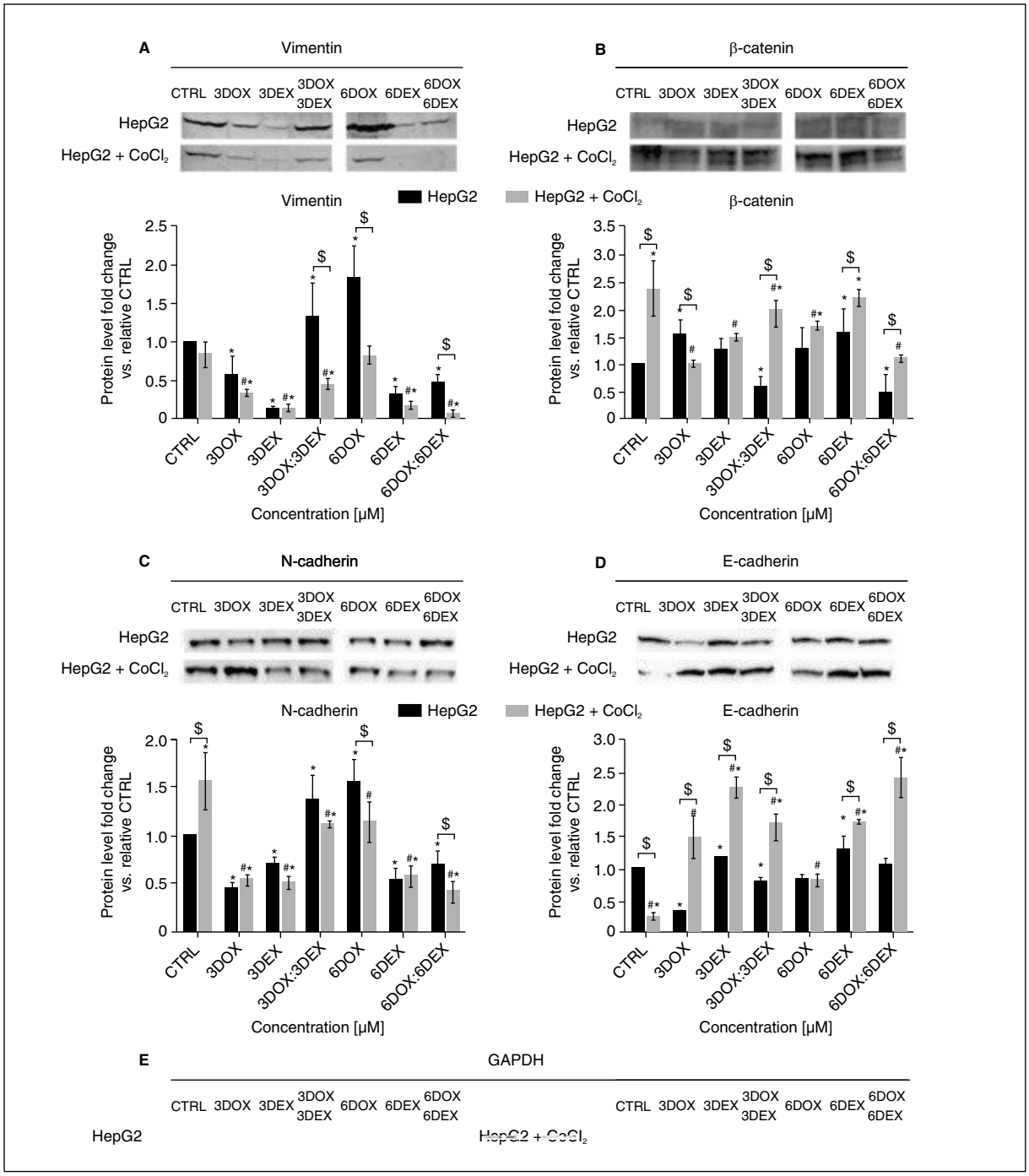

Figure 5. The effect of doxorubicin (DOX) and dexamethasone (DEX) individually and in combined treatment on migration potential of HepG2 cells — Western blot assay. HepG2 cells were treated with DOX, DEX, and the combination of drugs in ratio 1:1 in normoxia (HepG2) and hypoxia (HepG2 + $CoCl_2$) for 24h. Representative pictures of membranes and graphs of (**A**) vimentin, (**B**) β-catenin, (**C**) N-cadherin, (**D**) E-cadherin, and (**E**) GAPDH are presented. Levels of the proteins were normalized to untreated cells and results were expressed as fold change vs. control in normoxia (estimated as 1). On the graphs '*' indicate statistically significant differences for control cells in normoxia, '#' in hypoxia, and '$' between normoxic and hypoxic conditions ($P < 0.05$; 2way ANOVA with Dunnett's or Sidak's multiple comparisons test). Data are presented as mean ± SD

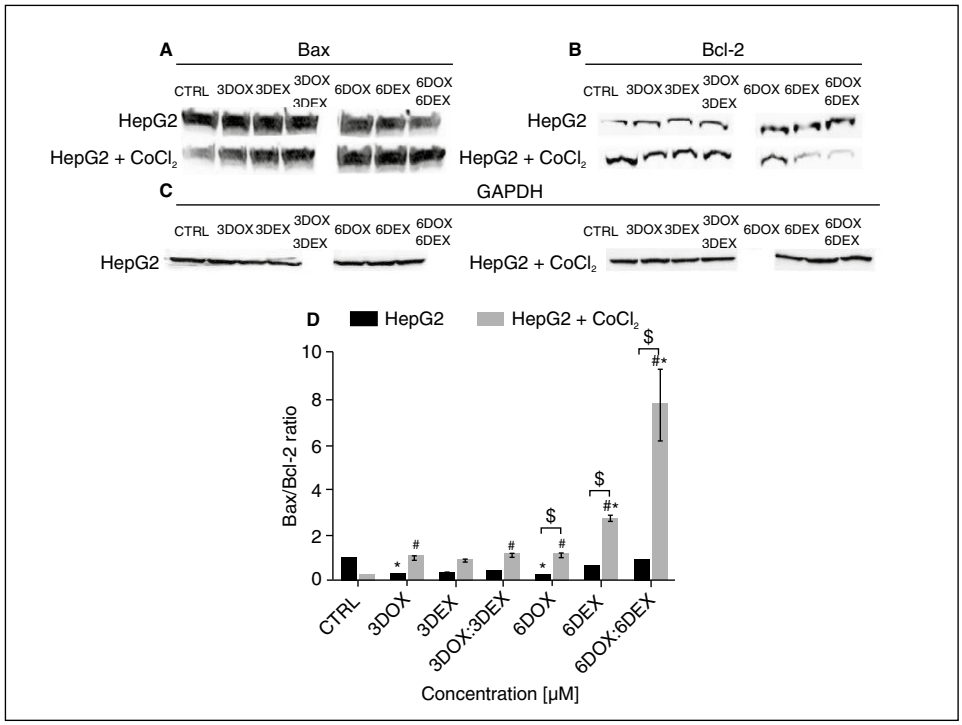

Figure 6. The effect of doxorubicin (DOX) and dexamethasone (DEX) individually and in combined treatment on the expression level of Bax and Bcl-2 in HepG2 cells — Western blot assay. HepG2 cells were treated with DOX, DEX, and the combination of drugs in ratio 1:1 in normoxia (HepG2) and hypoxia (HepG2 + CoCl2) for 24h. Representative membranes of (**A**) Bax, (**B**) Bcl-2, (**C**) GAPDH and a graph of Bax/Bcl-2 ratio in HepG2 (**D**) are presented. Levels of the proteins were normalized to untreated cells and results were expressed as fold change vs. control in normoxia (estimated as 1). '*' indicate statistically significant differences for control cells in normoxia, '#' in hypoxia, and '$' between appropriate doses in normoxic and hypoxic conditions ($P < 0.05$; 2way ANOVA with Dunnett's or Sidak's multiple comparisons test). Data are presented as mean ± SD

the incidences of fever, anorexia, and nausea/vomiting and also reduces recovery time [18].

In the case of this study, both DOX and DEX caused a dose-dependent decrease in the viability of HepG2 cells after 24h treatment. However, the greatest reduction was visible in the case of the 1:1 combination of the drugs. The obtained value of CI < 1 indicated synergistic interaction. Subsequent analyses confirmed a statistically significant increase in the percentage of apoptotic cells with a particular indication of a combination of cytostatics at a dose of 6 μM. This is consistent with the studies carried out by Dubbelboer et al. who showed that doxorubicin treatment results in a dose-dependent increase in the percentage of apoptotic cells [19]. The dose-effect disproportion between the studies may be connected with the phenomenon known as confluence dependent resistance, which involves a differentiated cell response to a drug depending on the confluence at the time of treatment. This is especially problematic considering the morphology of HepG2 cells, which tend to grow in clusters, making it difficult to assess the real confluence. To the authors' knowledge currently, there are no studies on the effect of DEX on liver cancer cells. However, apoptosis induction by the drug was noted in the case of colon cancer (LoVo) [20]. In turn, it did not show any significant effect on cell apoptosis in the case of non-small cell lung cancer (A549) cells, while for human ovarian cancer cells (HO-8910 and SKOV3) DEX treatment caused drug resistance to further chemotherapy [21, 22]. The diverse response of different cancer cell types to DEX treatment results in its frequent use in combination therapy rather than a single therapeutic agent. A combination with DOX has also been used for MCF-7 breast cancer cells [17]. However, the DOX: DEX combination in the context of liver cancer is innovative.

Numerous studies show that hypoxia promotes the migration and invasion of cancer cells [23]. The hypoxic conditions lead to the stabilization of HIF-1, which in turn causes an increase in the VEGF level [24]. VEGF promotes angiogenesis and EMT — both processes strictly connected to metastasis. Therefore, the main goal of the project was to assess the impact of the developed combination of drugs in the context of this

process. To induce hypoxic condition, $CoCl_2$ is often applied [25]. It mimics hypoxic conditions through the stabilization of HIF-1 protein. The present study also observed enhanced cell migration caused by hypoxic conditions. Transwell migration assay showed that pretreatment with $CoCl_2$ resulted in intensified cell passage through the inserts pores. Moreover, in the case of DEX used in mono treatment enhanced cell migration was observed in comparison to the appropriate control, while for the combination of the drugs this effect was not visible. This is consistent with the observations of Guan et al. (2018) who noted increased migration of C6 glioma cells in response to DEX despite a reduction in cell proliferation [26]. In turn, Wu et al. (2019) indicate inhibition of HepG2 migration in response to DEX, however, both dose and incubation time were significantly greater [27]. The goal of the present research was to obtain the most efficient dose-effect ratio for the combination of the drugs. Selected doses of DOX and DEX, and in particular their combinations, led to a decrease in the number of cells with high migratory potential compared to the corresponding samples in normoxia.

The induction of hypoxia and the high migratory potential of cancer cells is closely related to EMT [28]. The main markers of this process are N- and E-cadherin, vimentin, and β-catenin. For the cells pretreated with $CoCl_2$, the level of E-cadherin is lower than for cells cultured in normoxic conditions, while the level of N-cadherin is exactly the opposite. This suggests that also in the case of HepG2 cells, hypoxia-mimic conditions induced by $CoCl_2$ result in an enhanced EMT process. This effect is limited by the developed combination of drugs, especially in the case of the 6 DOX: 6 DEX combination, which is manifested by the N/E-cadherin switch. Similarly, lower levels of vimentin expression after incubation with a combination of compounds were noted. It is commonly known that high levels of vimentin correlate with high cell mobility [29].

Hypoxia-induced migration may follow various pathways in cancer cells. Some studies point to the role of the Wnt/β-catenin pathway. Moreover, this pathway is abnormally activated in the case of HCC. For this reason, it was also decided to assess the level of β-catenin. The authors suggest that elevated β-catenin levels in hypoxia may correlate with the activation of this pathway, which has also been shown in the study by Zhang et al. (2013) [30]. Moreover, the authors indicate the connection between the Wnt/β-catenin and hypoxia-induced EMT, which is also visible in the present study. In turn, the developed combination inhibited this effect, which also translates into a decrease in Bcl-2 (antiapoptotic) and an increase in BAX (proapoptotic). Overexpression of Bcl-2 favours the EMT process through inhibition of E-cadherin. The obtained results show that the combination of DOX and DEX used in hypoxic conditions may inhibit migration due to the promotion of apoptosis.

Conclusions

In summary, this study demonstrated that the developed innovative combination of DOX and DEX in the 1:1 ratio may limit hypoxia-induced enhancement in the migratory potential of HCC cells. The observed effect is connected with the inhibition of the EMT process and directing cells to death on the cellular level.

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VIA MEDICA

ORIGINAL ARTICLE

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Comorbidities of patients with chronic obstructive pulmonary disease (COPD): thyroid abnormalities in stable COPD

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ABSTRACT

Objective: The aim of the study is to evaluate the prevalence of thyroid abnormalities in patients with stable chronic obstructive pulmonary disease (COPD) and the relationship between thyroid disorder and ventilatory function tests and arterial blood gas analyses.

Material and methods: This cross-sectional study was conducted with 60 patients with stable COPD without diagnosed thyroid disorder before the study (37 males and 23 females aged 40–75) as the Investigated Group (IG) and 30 subjects from the general population without COPD as the Control Group (CG). They were matched by age, gender and body mass index with the IG. All patients underwent laboratory tests, thyroid hormones –free thyroxin (fT4), thyroid-stimulating hormone (TSH), and free triiodothyronine (fT3), pulmonary function tests (FEV_1, FVC%, FEV_1/FVC, FEF25–75%), and ABG parameters (PH, $PaCO_2$, PaO_2, HCO_3, O_2 saturation). The severity level in patients with COPD was determined according to GOLD (Global Initiative for Chronic Obstructive Lung Disease) criteria and classified into four stages GOLD I, II, III, and IV.

Results: Our results presented a statistically significant difference between prevalence of thyroid hormones abnormalities in stable COPD compared to controls 18 (30.0%) vs 3 (10.0%), $p = 0.0355$; $p < 0.05$). Thyroid dysfunction among COPD patients was more common in females than males. Serum level of TSH was lower than the normal range in 18 patients (30.0%) from the IG and in 3 (10.0%) from the CG, with a statistically significant difference, $p = 0.0355$; $p < 0.05$. Thyrotoxicosis with low serum TSH and a higher serum level of fT3, according to the referent range, was present in 8 patients (13.3%), and in no patients from the CG 0 (0.0%), $p = 0.0375$; $p < 0.05$. The prevalence of subclinical hyperthyroidism with low serum TSH and normal serum level of fT3 was higher in the IG –10 patients (16.7%) compared with 3 (10.0%) of the CG, but the difference was not statistically significant $p = 0.3970$. Acute exacerbation frequency of IG was significantly higher than in the CG (1.6 ± 0.42 and 0.82 ± 0.79 respectively; $p < 0.0001$). A positive significant relationship between acute exacerbation frequency and TSH values was found ($p < 0.0001$; $r = 0.82$). The mean values of fT3 in the IG were significantly increasing with the increased severity of COPD. The degree of airflow limitation in COPD (FEV_1 as a percentage of the predicted value, FEV_1%pred) was significantly negatively correlated to fT3, Pearson correlation, ($R = -0.525$; $p = 0.000$; $p < 0.01$). FEV_1%pred was positively correlated with TSH ($R = 0.358$; $p = 0.005$; $p < 0.01$). Significant negative correlations were present between fT3 levels and both PaO_2 and SO_2 in the IG, and elevation of fT3 was associated with higher PaCO2.

Conclusion: The present study confirms that both clinical and subclinical hyperthyroidism was higher in patients with COPD compared to the non-COPD group. TSH and fT3 are related to lung function. A better understanding of the correlation between thyroid gland disorders and COPD may contribute to better care of patients.

Key words: COPD, comorbidity, thyroid gland, thyroid hormone, hyperthyroidism

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Introduction

Chronic obstructive pulmonary disease (COPD) is a common, preventable, and treatable chronic airway inflammatory disease. COPD has been the third leading cause of death in the world since 2020. COPD is a respiratory disease with systemic complications, which is characterized by chronic airflow limitation due to the destruction of lung parenchyma and airways. It is a multicomponent disease with extrapulmonary effects, such as cardiovascular disease, anemia, polycythemia, malnutrition, muscle disorder, osteoporosis, metabolic syndrome, diabetes, gastroesophageal reflux, anxiety, depression, hormonal imbalance, infections, lung cancer, and thrombosis. Many systems including the endocrine system are affected by COPD. Comorbidities not only affect symptom burden, health status, and quality of life, but they also carry the risk of hospitalization and mortality in patients with COPD [1_5]. Some comorbidities, such as coronary artery disease or metabolic syndrome are well-recognized in COPD patients. But thyroid diseases, which are more common in COPD patients compared to patients who do not have COPD, are underestimated despite important clinical consequences [6]. Abnormalities in thyroid hormone regulation are encountered frequently in nonthyroidal diseases; these include normal or decreased total and free thyroxine (TT4 and FT4, respectively), decreased total (TT3) and free (FT3) triiodothyronine, along with usually normal thyroid-stimulating hormone (TSH) levels [7]. Neurohormones, blood gas abnormalities, glucocorticoid administration also disturb the hormonal balance [8]. Hypoxia and hypercapnia cause destruction in sella turcica and pituitary gland dysfunction. During the course of COPD with hypoxia, peripheral metabolism of thyroid function changes, and thyroid hormone levels decrease in patients with very severe COPD [9]. Frequently drugs used by COPD patients to treat comorbidities, such as amiodarone, may be a cause of thyroid disorder [10]. Systemic inflammation may express the link between COPD and thyroid diseases. Supporting this, Karadag et al. [11] found a positive correlation between interleukin 6, which is a systemic inflammation marker, and total triiodotironine (TT3) and TT3/TT4 (total tyhroxine) in patients with stable COPD [11]. Smoking increases systemic inflammation independently from associating COPD and may affect thyroid functions [12]. Higher levels of serum total triiodothyronine (TT3) were found in young healthy smokers compared to non-smoking control subjects, which may suggest that smoking acts independently of coexistent diagnosis of COPD [13]. On the other hand, hormones may affect regulation of breathing. Some hormones act on the level of the central nervous system, some have an impact on peripheral chemoreceptors, others may contribute to this process by influencing the metabolism rate, and others exert their effect directly on receptors in the respiratory tract. Hypothyroidism may also cause alveolar hypoventilation, decreased lung volumes, upper airway obstruction, depression in respiratory stimulus and respiratory failure [14]. A cross-sectional study from Spain showed that patients with COPD had a higher prevalence of thyroid disease (14.2%) than the expected standardized prevalence of chronic diseases (11.06%), and the prevalence of thyroid disease was higher in female than male patients (24.6% vs 10.9%) [15]. Gumus et al. [16] found a decrease in triiodothyronine (T_3; 18.2%) and thyroid-stimulating hormone (TSH; 30.3%) in patients with exacerbation of COPD. The exacerbation frequency was higher in patients with COPD with hypothyroidism than in those without hypothyroidism. The frequency of COPD exacerbation was positively correlated with TSH levels [16].

The objective of the study is to evaluate the prevalence of thyroid abnormalities in patients with stable COPD and the relationship between thyroid disorder and ventilatory function tests and arterial blood gas analyses.

Material and methods

Study design and setting

A cross-sectional study aimed to evaluate the prevalence of thyroid abnormalities in patients with stable COPD and the relationship between thyroid disorder and ventilatory function tests and arterial blood gas analyses was performed at the City General Hospital "8mi Septemvri", Skopje.

Study subjects

The study population included 60 patients with stable COPD (37 males, 23 females), diagnosed according to the actual Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria and 30 subjects without COPD matched to the COPD group by sex, age, body mass index (BMI), and smoking status. All enrolled subjects gave their written informed consent before entering the study.

Inclusion criteria for Investigated Group (IG)

Both sexes, aged 40–75 years, with diagnosed COPD according to the actual GOLD criteria, had a history of current or former smoking (equal or more than 10 pack-years) and were clinically stable condition at least 6 weeks prior to involvement.

Exclusion criteria for Investigated Group (IG)

The exclusion criteria were age less than 40 years and more than 75 years, other chronic respiratory diseases (asthma, bronchiectasis, active tuberculosis, sarcoidosis, lung carcinoma, pulmonary fibrosis, sleep apnea syndrome), BMI > 35kg/m^2, electrolyte imbalance, hepatic, renal failure, anemia. We excluded patients with immunosuppressive therapy, patients during acute exacerbation of COPD, and those who received systemic corticosteroids, medications containing iodine, amiadorone and/or contrast material within the prior two months, those who could not perform the pulmonary function tests, those with thyroid surgery, other endocrine diseases (including diabetes mellitus), neuromuscular and cardiovascular diseases, symptoms of any infections or using anti-inflammatory medications. Patients who did not agree to participate were excluded from the study.

Inclusion criteria for the Control Group (CG)

We included patients aged from 40 to 75 years, with a smoking history ≥ 10 pack-years, current or former smokers, patients with normal spirometry, in stable clinical condition, without significant difference in sex, age, BMI, and patients who signed consent for participation.

Methods

All patients underwent laboratory testing and pulmonary function tests. The severity level in patients with COPD was determined according to GOLD criteria.

The BMI as a measure of body fat, based on height and weight that applies to the adult population, was determined in all study subjects by computed calculation using a BMI calculator [17].

Classification of smoking status was done as per the World Health Organization (WHO) recommendations by the Brinkman Index, as a clinical quantification of cigarette smoking is used to measure a person's exposure to tobacco, and it is calculated as number of pack-years = (number of cigarettes smoked per day / 20) × number of years smoked [17].

Pulmonary evaluation

The pulmonary evaluation included: dyspnea severity assessment, baseline, and post-bronchodilator spirometry, arterial gas analysis, and chest X-ray (to exclude respiratory disease other than COPD).

The baseline spirometry, including measures of forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), FEV_1/FVC, and maximal expiratory flow at 75%, 50%, 25%, and 25–75% of FVC (MEF_{75}, MEF_{50}, MEF_{25}, and $MEF_{25–75}$, respectively), was performed in all subjects using electronic spirometer Spirobank G USB Spirometer (Medical International Research, Roma, Italy) – the best result from three measurements the values of FEV_1 were recorded. The difference between two spirometry values of FEV1 was less than 5%. The results of spirometry were expressed as percentages of the predicted values, according to the actual recommendations of the European Respiratory Society and American Thoracic Society [18]. Bronchodilator test was performed by spirometric measurements before and 20 minutes after administration of 400 mg salbutamol by metered-dose inhaler through a spacer. The post-bronchodilator value of the FEV_1/FVC ratio was less than 0.70 and indicated persistent airflow limitation [18]. According to the actual GOLD recommendations, COPD was considered by finding of a post-bronchodilator FEV_1/FVC ratio less than 0.70 in symptomatic subjects (dyspnea, chronic cough, and/or sputum production) with a history of exposure to risk factors for the disease (noxious particles and gases). In addition, according to the FEV_1 value, airflow limitation in the subjects with COPD, i.e. severity of the disease, was classified as mild (FEV_1 value higher than 80% of the predicted value), moderate (FEV_1 value higher than 50% but lower than 80% of the predicted value), severe (FEV_1 value higher than 30% but lower than 50% of the predicted value), and very severe (FEV_1 value lower than 30% of the predicted value [1]). Gas analysis was performed with SIEMENS RAPIDPOINT 405 System (Siemens Healthineers, Australia).

Laboratory evaluation

Analysis of venous blood was taken in the morning at 8 a.m (following an overnight fast) and biochemistry, sedimentation rate, C-reactive protein, thyroid hormones were analyzed. Thyroid-stimulating hormone (TSH) (normal range: 0.35–4.0 mIU/L), free triiodothyronine (fT3) (normal range: 2.3–6.7 pmol/L), and free tetraiodothyronine (fT4) (normal range: 10.2–24.4 pmol/L) were measured using electrochemiluminescence immunoassay (E170, Mannheim, Germany).

Statistical analysis

Statistical analysis was done using the SPSS Statistics 20 software package (SPSS. Inc., Chicago, IL, USA). The results of the tests were usually expressed with numerical values, so the comparison between them was performed using a correlation with the Pearson Correlation test. To test hypotheses involving multiple samples, a standard Student t-test for two or more sam-

ples was used. The Mann-Whitney U-test was used to test two independent samples. In the case of more than two samples, a Kruskal-Wallis H test of K-independent samples was used, which is a one-way analysis of the variants of independent samples (one-way ANOVA on ranks). The level of statistical significance was set at p value less than 0.05.

RESULTS

Demographic data and pulmonary function tests of the study subjects are presented in Table 1. The two groups were similar regarding the gender and age distribution of the included subjects, as well as their smoking status, mean BMI, and height.

Diagnosis of an exacerbation relies on the clinical presentation of the patient complaining of worsening symptoms (dyspnea, cough, or sputum production) and leading to an increase in the use of maintenance medications and/or supplementation with additional medications that is beyond normal day-to-day variations [1]. This data was collected based on the medical history of each patient. Age, BMI, and height were not different between study groups.

The mean values of spirometry parameters (FVC, FEV_1, and FEV_1/FVC ratio) were significantly lower in COPD patients than in non-COPD controls ($p < 0.0001$).

Acute exacerbation frequency of IG was significantly higher than CG (1.6 ± 0.42 and 0.82 ± 0.79 respectively; $p < 0.0001$).

According to the severity of airflow limitation, i.e. to the post-bronchodilator value of FEV_1, COPD patients were categorized into four stages: mild (GOLD I), moderate (GOLD II), severe (GOLD III), and very severe COPD (GOLD IV) (Tab. 2).

Laboratory findings of thyroid function tests are presented in Table 3. The number of patients with abnormal thyroid hormone status was significantly higher in stable COPD than control group 18 (30%) vs 3 (10.0%); $p = 0.0355$; $p < 0.05$. Thyrotoxicosis with low serum TSH and higher serum level of fT3, according to the referent range, was present in 8 patients (13.3%) and in none patient in the CG 0 (0.0%); $p = 0.0375$; $p < 0.05$. The prevalence of subclinical hyperthyroidism with low serum TSH and normal serum level of fT3 was higher in the IG – 10 patients (16.7%) compared with 3 (10.0%) in the CG, but the difference was not statistically significant $p = 0.3970$.

The effect of thyroid function on COPD exacerbation frequency was examined. Only TSH was found to be significantly associated with acute exacerbation frequency. A positive significant relationship between acute exacerbation frequency and TSH values was found ($p < 0.0001$; $r = 0.82$) (Fig. 1). The mean values of fT3 in the IG were significantly increasing with the increased severity of COPD. The degree of airflow limitation in COPD (FEV_1 as a percentage of the predicted

Table 1. Demographic data and pulmonary function test

Characteristic	COPD patients (n = 60)	Non-COPD subjects (n = 30)	P-value
Gender			
Males	37 (61.7%)	21 (70.0%)	p = 0.4406
Females	23 (38.3%)	9 (30.0%)	
Mean age (years)			
Male	64.9 ± 6.2	65.8 ± 4.6	p = 0.4837
Female	65.9 ± 5.4	64.7 ± 5.2	p = 0.3172
Smoking status			
Active smokers	35 (58.3%)	18 (60%)	p = 0.8779
Former smokers	25 (41.7%)	12 (40%)	p = 0.8779
Pack-year smoked	66.1 ± 25.8	67.4 ± 25.5	p = 0.8216
Mean BMI value (kg/m^2)	26.8 ± 3.5	27.1 ± 3.2	p = 0.6944
Height (cm)	164.6 ± 6.3	166.4 ± 6.9	p = 0.2191
Mean baseline values of spirometry parameters	75.8 ± 10.3	113.2 ± 15.1	p < 0.0001
FVC (% pred)	47.2 ± 16.1	91.2 ± 13.9	
FEV_1 ((% pred)	0.6 ± 0.05	0.9 ± 0.05	
FEV_1/FVC ratio			
Acute exacerbation freq uency (/years)	1.6 ± 0.42	0.82 ± 0.79	p < 0.0001

BMI — body mass index; COPD — chronic obstructive pulmonary disease; FEV_1 — forced expiratory volume in one second; FVC — forced vital capacity; %pred — percentage of the predicted value

value, FEV_1%pred) was significantly negatively correlated to fT3, Pearson correlation, (R = –0.525; p = 0.000; p < 0.01). FEV_1%pred was positively correlated with TSH (R = 0.358; p = 0.005; p < 0.01).

Results of gas analyses in COPD and non-COPD subjects are presented in Table 4. Significant negative correlations were present between fT3 levels and both PaO_2 and O_2 saturation in the IG and elevation of fT3 was associated with higher PaCO2.

Discussion

COPD is a respiratory disease that is prevalent worldwide and has chronic airway inflammation as its major characteristic. In addition to airway inflammation, patients with COPD have inflammation in other body systems. It is not only a pulmonary disease but a systemic disease with numerous extrapulmonary manifestations. It is associated with many comorbidities that further increase hospital costs and reduce lifespan. The GOLD guidelines state that COPD is often complicated by many diseases, but thyroid dysfunction may be an underestimated complication of COPD due to insufficient clinical data [1, 19]. In our study male patients dominated, which is probably due to the fact that men are main cigarette consumers, and smoking is the main risk factor. In the study 61.7% of participants were men, and 37.3% women, out of 60 COPD patients, current smokers constituted 58.3% and former smokers 41.7%. The mean age of patients with COPD was for men 64.9 ± 6.2 and for women 65.9 ± 5.4. According to the GOLD criteria, they were predominantly in GOLD stage II and III on the basis of spirometry (FEV_1%pred 30–80%). Our results presented a statistically significant difference between prevalence of thyroid hormones abnormalities in stable COPD compared to controls, 18 patients (30.0%) vs 3 (10.0%); p = 0.0355; p < 0.05). Previous studies showed that hypothyroidism was more common than hyperthyroidism in COPD patients, and its frequency showed positive correlation with the stage of COPD [20, 21].

Table 2. Distribution of the COPD patients by degree of airflow limitation

COPD severity	COPD patients (n = 60)
GOLD 1 - mild ($FEV_1 \geq 80\%$ pred)	4 (6.7%)
GOLD 2 – moderate (FEV_1 = 50% – 79% pred)	22 (36.7%)
GOLD 3 – severe (FEV_1 = 30% – 49% pred)	21 (35.0%)
GOLD 4 – very severe (FEV_1 < 30% pred)	13 (21.7%)

COPD — chronic obstructive pulmonary disease; GOLD — Global Initiative for Chronic Obstructive Lung Disease; FEV_1 — forced expiratory volume in one second; %pred — percentage of the predicted value

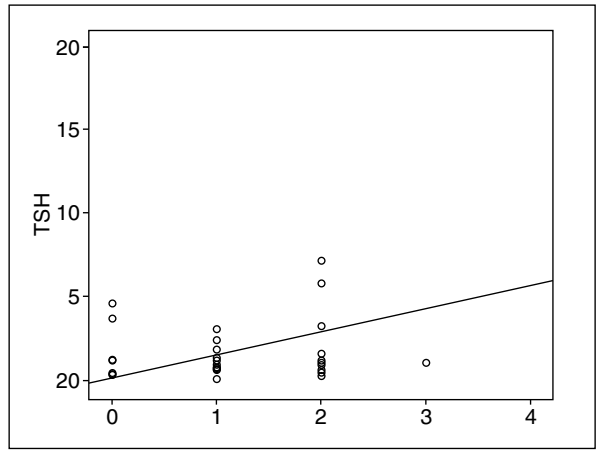

Figure 1. Relationship between thyroid TSH level and frequency of acute COPD exacerbation
COPD — chronic obstructive pulmonary disease; TSH — thyroid stimulating hormone

Table 3. Presentation of thyroid hormone status in COPD and non-COPD subjects

Thyroid hormones	COPD patients (n = 60)	Non-COPD subjects (n = 30)	P-value
Thyroid hormone status			
TSH (mIU/mL)	4.7 (0.3–18.89)	0.7 (0.4–4.5)	p < 0.0001
fT4 (pmol/L)	2.35 ± 3.42	2.47 ± 3.32	p = 0.8745
fT3 (pmol/L)	3.37 ± 1.21	2.33 ± 0.44	p < 0.0001
Normal thyroid status	42 (70.0%)	27 (90.0%)	p = 0.0355
Hyperthyroidism	8 (13.3%)	3 (10.0 %)	p = 0.6539
Subclinical hyperthyroidism	10 (16.7%)	3 (10.0%)	p = 0.3970

COPD — chronic obstructive pulmonary disease; fT4 — free tetraiodothyronine; fT3 — free triiodothyronine; TSH — thyroid stimulating hormone

Table 4. Presentation of gas analyses in COPD and non-COPD subjects

Gas analyses	COPD patients (n = 60)	Non-COPD subjects (n = 30)	P-value
PaO_2 (mmHg)	48.41 ± 9.9	64.4 ± 6.4	$p < 0.0001$
$PaCO_2$ (mmHg)	41.1 ± 10.8	31.4 ± 7.6	$p < 0.0001$
O_2 saturtion (%)	78.3 ± 14.9	88.1 ± 4.2	$p = 0.0007$
pH	7.39 ± 0.2	7.45 ± 0.4	$p = 0.3440$
HCO_3 (mmol/L)	27.2 ± 5.9	22.6 ± 2.1	$p = 0.0001$

COPD — chronic obstructive pulmonary disease; HCO_3: bicarbonate; O_2 — oxygen; $PaCO_2$ — partial pressure of carbon dioxide; PaO_2 — partial pressure of oxygen; pH: potential of hydrogen

In our study hyperthyroidism was more frequent, serum level of TSH was lower than the normal range in 18 patients (30.0%) of the IG and in 3 (10.0%) in the CG, with a statistically significant difference, $p = 0.0355$; $p < 0.05$. Thyrotoxicosis with low serum TSH and higher serum level of fT3, according to the referent range, was present in 8 patients (13.3%) and in none patient in the CG 0 (0.0%); $p = 0.0375$; $p < 0.05$. A cross-sectional study from Spain showed that patients with COPD had a higher prevalence of thyroid disease (14.2%) than the expected standardized prevalence of chronic diseases (11.06%), and the prevalence of thyroid disease was higher in female than male patients (24.6% vs 10.9%) [22]. The increase in TT3/TT4 and fT3 in COPD patients was previously reported in the study of Ulasli et al. [23]. El-Yazed et al. [24] demonstrated that the increase in fT3 showed a negative correlation with PaO_2 and a positive correlation with $PaCO_2$. The elevation of fT3 was associated with higher COPD severity. Bacakoglu et al. [25] demonstrated that low fT3 and fT4 levels increase the rates of invasive mechanical ventilation and mortality in patients with respiratory failure. Exacerbations of COPD are important events in the course of the disease, as exacerbations negatively affect the quality of life, accelerate the decline of pulmonary function and are associated with higher socioeconomic costs and mortality. Development of strategies to prevent exacerbations is an important goal in COPD management [1]. In our study, acute exacerbation frequency of IG was significantly higher than CG (1.6 ± 0.42 and 0.82 ± 0.79 respectively; $p < 0.0001$). A positive significant relationship between acute exacerbation frequency and TSH values was found ($p < 0.0001$; $R = 0.82$). Both cigarette smoke and chronic inflammatory and COPD can impair the thyroid gland and lead to abnormal thyroid hormone production. Karadag et al. [11] demonstrated that the prevalence of non-thyroidal illness syndrome (abnormal thyroid hormone levels not due to thyroid disease) was 14–20% in patients with stable COPD and 70% in patients with acute exacerbation COPD.

Conclusions

This trial shows that thyroid abnormalities are not uncommon in COPD patients. The present study confirms that both clinical and subclinical hyperthyroidism was higher in patients with COPD, compared to the non-COPD group. A positive significant relationship between acute exacerbation frequency and TSH values was found. TSH and fT3 are related to lung function. A better understanding of the correlation between thyroid gland disorders and COPD may contribute to better care of patients.

The findings of the present study are subject to some limitations. First, only one center is included, and the relatively small size of the study subjects could have certain implications on the evaluated data and their interpretation. Second, the unequal distribution of COPD patients by degree of airflow limitation could have a certain influence on the data obtained and their interpretation.

Conflict of interest: *None.*

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VIA MEDICA

ORIGINAL ARTICLE

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The Oncotype DX recurrence score impact on the management of ER-positive, HER2-negative, node-negative breast cancer

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ABSTRACT

Introduction: Oncotype DX recurrence score is used to categorize estrogen-receptor-positive, human epidermal growth factor receptor-2 negative, lymph-node negative early breast cancer in high- or low-recurrence risk groups. It has a guiding significance for whether post-operative chemotherapy or only hormonal manipulation is selected as an adjuvant treatment.

Aims: Assess the impact of the Oncotype DX recurrence score on adjuvant chemotherapy-related management decision-making in the cases of ER+ve, HER2–ve, LN–ve early breast cancer in our local unit.

Material and methods: A cohort of 76 patients with early breast cancer were included, two had bilateral disease. All were operated for estrogen-receptor-positive, human epidermal growth factor receptor-2 negative, lymph-node negative early breast cancer. Tumor grade, Ki67 proliferative index, PREDICT and Oncotype DX recurrence score results were obtained in addition to the offered treatment information for each case.

Results: After the primary tumor surgery and an Oncotype DX recurrence score assessment, 18 patients (24%) were eligible for adjuvant chemotherapy; out of them, 10 patients (56%) had a chemotherapy absolute survival benefit of > 15% at 10 years, where 5 patients (30%) had a chemotherapy relative survival benefit of ~ 6.5%, 3 patients (17%) had a chemotherapy relative survival benefit of ~1.6%. In this cohort, 10 patients (13%) had a low Oncotype DX recurrence score; however, they received adjuvant chemotherapy based on other clinico-biological parameters. The other 48 patients (63%) with a low recurrence risk were spared potential adverse events related to the systemic therapy. Based on the menopausal status, every patient had received suitable hormonal manipulation therapy. The data also revealed the absence of a relationship between the Ki67 proliferative index and the Oncotype DX recurrence score ($p = 0.06$); moreover, the size of the tumor did not correlate with the Oncotype DX recurrence score ($p = 0.5$).

Conclusion: The Oncotype DX recurrence score provides a credible prediction of distant disease recurrence risk in early breast cancer; however, it does not correlate with other prognostic markers, such as the Ki67 proliferative index as well as the tumor size. In this cohort, the use of the Oncotype DX recurrence score led to a 24% rate of treatment recommendations in the direction of adjuvant chemotherapy in addition to anti-hormonal therapy for estrogen-receptor-positive, human epidermal growth factor receptor-2 negative, lymph-node negative early breast cancer.

Key words: breast cancer, Oncotype DX, estrogen receptor, Ki67, HER2, chemotherapy

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Introduction

Worldwide, breast cancer is regarded as the most frequent malignancy; however, it occupies the fifth place on the list of cancer-related mortality after lung, colon, liver, and stomach cancers. According to the 2017 UK data, in both genders, it is reported to be the most diagnosed cancer with over 55,000 new cases diagnosed yearly. It accounts for 15% of all newly diagnosed cancers and also is considered to be the most

common female cancer. Up to 80% of these cases are categorized as early breast cancer [1, 2].

The standard treatment of estrogen-receptor-positive (ER+ve), human epidermal growth factor receptor-2 negative (HER2–ve), lymph-node negative (LN–ve) early breast cancer is hormonal manipulation therapy administered in addition to chemotherapy and radiotherapy as appropriate. Adjuvant-chemotherapy treatment decision in breast cancer may be affected by different biological and molecular parameters, such as tumor size, nodal status, histological grade, oestrogen hormone status, the HER2 receptor expression or the Ki67 proliferative index (Ki67-PI) (Fig. 1). The systemic treatment recommendation is also supported by online tools, such as PREDICT and NPI (Nottingham Prognostic Index) – these tools utilize demographic patient characteristics as well as tumor's biological markers [3]. Few molecular technologies have been developed to aid in decision-making when dealing with moderately aggressive tumors, and the cancer-biology characteristics do not provide enough justification to proceed with adjuvant chemotherapy. Such technologies include the Oncotypoe DX recurrence score (RS) and Mammaprint70 [4]. The RS is an RT-PCR (real-time reverse transcriptase-polymerase chain reaction) based multigene assay, involving 21 genes, performed on fixed paraffin-embedded tissues of the excised tumors. Its result is used for the prediction of adjuvant chemotherapy benefits and disease prognosis.

Material and methods

The patients that were enrolled in this study, according to the inclusion criteria for had (1) operable estrogen-receptor positive, human epidermal growth factor receptor-2 negative and lymph-node negative (including micro-metastatic disease) operable early breast cancer, (2) an intermediate risk of distant recurrence using a validated tool such as PREDICT or the NPI (Nottingham Prognostic Index). A cohort of 76 patients with operable early breast cancer was included; two of them had bilateral disease, and the RS was examined for 78 tumors. The patients' age ranged from 37 to 74 years, with a median age of 55 (Fig. 2). All the patients underwent tumor surgical excision with breast conservation surgery or mastectomy in addition to axillary node staging via sentinel lymph node biopsy or axillary node sampling. Tumor size, grade, Ki67-PI, PREDICT, and Oncotype DX recurrence score results, and the information related to the offered treatment was obtained and analyzed. The value of 20% was used as a cut-off point for the Ki67-PI level, a low Ki67-PI is < 20%, whereas it is high if the level is ≥ 20%.

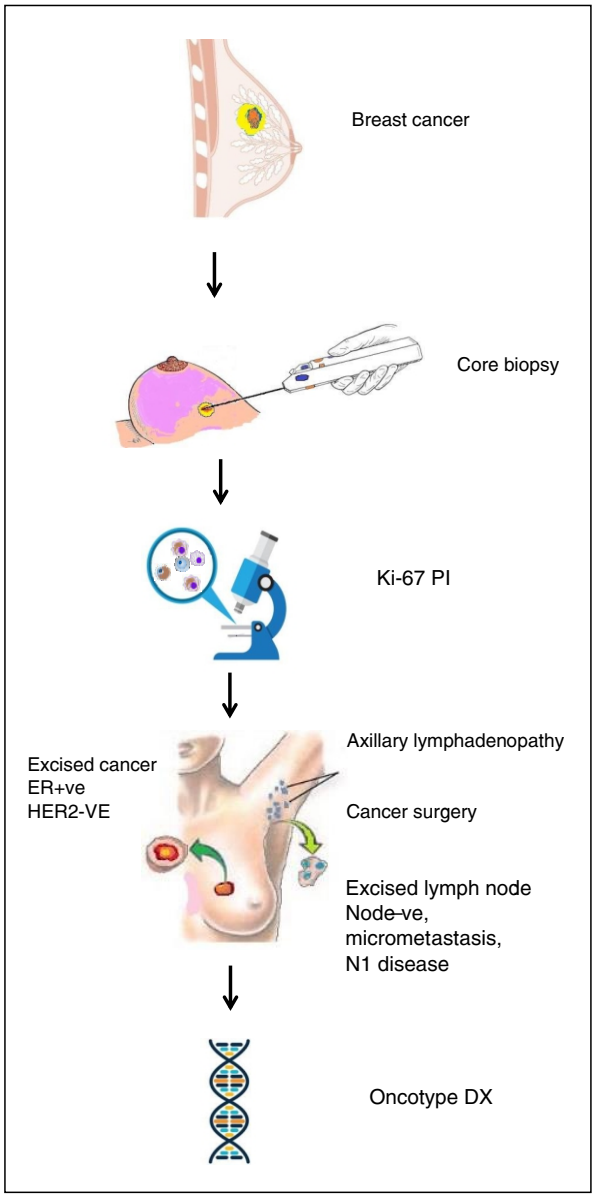

Figure 1. Oncotype DX and Ki67-PI in breast cancer diagnosis workup (the illustration was created by Dr. Abdalla Saad Abdalla Al-Zawi)

Results

The Ki67-PI analysis categorized most of the cohort within the low Ki67-PI group < 20% (68%), where only 25 tumors showed high Ki67-PI expression ≥ 20% (32%). There were only 17 (22%) tumors with a high Ki67-PI and a low Oncotype DX recurrence score; on the contrary, 8 patients had a low Ki67-PI with a high RS (Tab. 1). Our data revealed the absence of a relationship between the Ki67-PI and the RS ($p = 0.06$). Looking at the tumor size, T3 tumors (>50mm) were detected in 5 lesions (6%); 37 of them had a low RS. However, 53 lesions (68%)

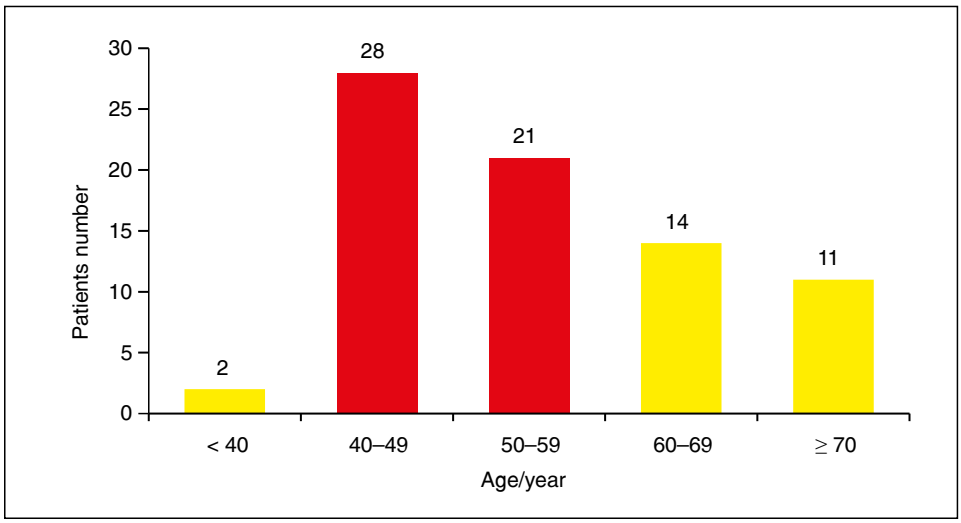

Figure 2. Age distribution of 76 patients treated for early breast cancer

Table 1. The Oncotype DX recurrence score and the Ki67 proliferative index in 78 excised tumors

Age group ≤ 50 years			
Oncotype DX recurrence score	**Chemotherapy (CTH) benefit**	**Low Ki67-PI group***	**High Ki67-PI group****
0–15	No CTH benefit	12	6
16–20	~1.6% CTH benefit	2	4
21–25	~6.5% CTH benefit	4	2
26–100	15% absolute CTH benefit	1	3
Age group > 50 years			
0–25	No CTH benefit	24	13
26–100	15% absolute benefit	2	5

*Low Ki67-PI < 20%; **High Ki67-PI ≥ 20%

were T2 tumors (20–50 mm). Out of these, 37 (70%) had a low RS, and only 8 (15%) showed a high RS (p = 0.5). This means that tumor size is not associated with an Oncotype DX recurrence score. Regarding tumor grade, G2 lesions were seen in 59 tumors (76%), only 5 (6%) were associated with RS > 20. Sixteen lesions (21%) were G3 tumors, only 3 (4%) had a high RS (p = 0.46).

By using the PREDICT online tool, two (3%) patients in the age group of ≤ 50 years had a high PREDICT and RS; one patient aged ≤ 50 had a low PREDICT score and a high RS. In the age group > 50 years, 4 patients (5%) had a high PREIDCT with a low RS, 4 patients (5%) a low PREDICT and RS; only only 2 had high both the PREDICT and the RS. After primary tumor surgery and an RS assessment, in total, 28 patients (36%) were eligible for adjuvant chemotherapy (Tab. 2). Adjuvant chemotherapy was determined by RS in 18 patients (24%). Out of them, ten patients (56%) had a chemotherapy absolute survival benefit of > 15% at 10 years, where 5 (30%) patients had a chemotherapy relative survival benefit of ~6.5%, 3 patients (17 %) patients had a chemotherapy relative survival benefit of ~1.6%. Ten patients (13%) had a low RS; however, they had chemotherapy based on other clinico-pathological parameters. This means 48 patients (63%) with a low recurrence risk had been spared potential adverse events related to the systemic therapy.

Discussion

The Oncotype DX recurrence score is a 21-multigene real-time reverse transcription polymerase chain reaction assay (Fig. 3). It is performed on the excised tumor cells, and the outcomes are presented as numerical results (0–100%) (Tab. 3). It is recommended to be used as a systemic recurrence predictive tool for guiding adjuvant-chemotherapy decisions in

Table 2. The Oncotype DX recurrence score and chemotherapy indication for the cohort

Age group ≤ 50 years			
Oncotype DX recurrence score	**Chemotherapy (CTH) benefit**	**Total patients number (%)**	**Patients for CTH(%)**
0–15	No CTH benefit	17 (22)	4 (5)
16–20	~1.6% CTH benefit	6 (8)	3 (4)
21–25	~6.5% CTH benefit	7 (9)	5 (7)
26–100	15% absolute CTH benefit	3 (4)	3 (4)
Age group > 50 years			
0–25	No CTH benefit	36 (47)	6 (8)
26-100	15% absolute CTH benefit	7(9)	7(9)

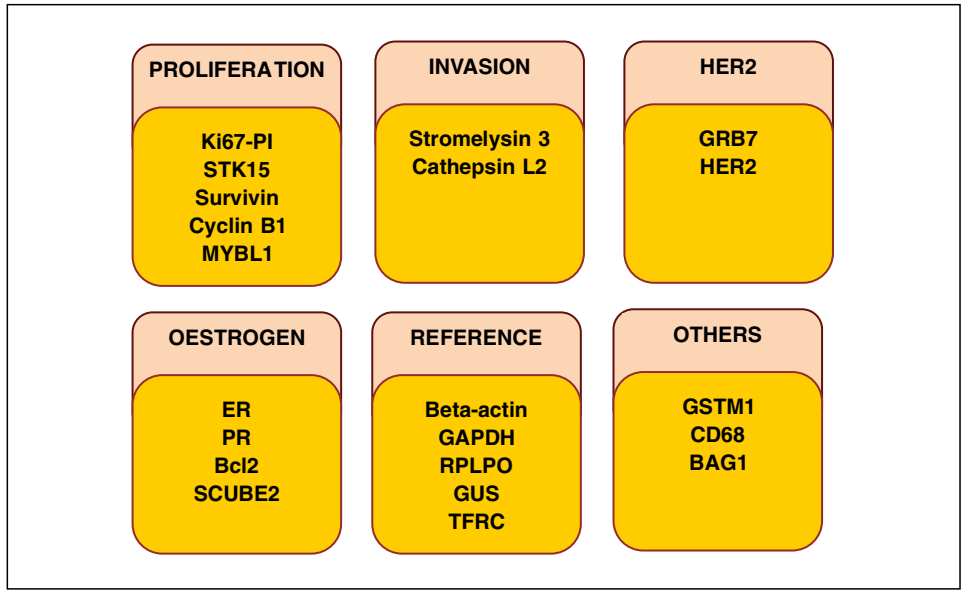

Figure 3. The 21 genes tested by RT-PCR (real-time reverse transcriptase-polymerase chain reaction) to generate the Oncotype DX recurrence score

moderately aggressive ER+ve, HER2–ve, LN–ve early breast cancers [5]. This gene panel is formed of five reference genes and sixteen cancer-related genes, the latter include those associated with tumor invasion, cell proliferation, and hormone receptors expression. The test result will have a recurrence score between 0 and 100. This score will give an idea about the likelihood of disease recurrence within 10 years after diagnosis [6], and it estimates the chemotherapeutic benefit in ER+ve, HER2–ve, LN–ve early breast cancer [7]. After the introduction of the Oncotype DX recurrence score used to measure the systemic recurrence score risk on the basis of tumor genomic signature, the adjuvant-chemotherapy indications in ER+ve, HER2–ve, LN–ve breast cancer disease have been changed notably. The genomic assay results (the Oncotype DX recurrence score) are used as a decision-making tool and to aid in determining whether the patient will be only treated with adjuvant hormonal therapy (a low RS) or, additionally, with adjuvant chemotherapy (a high RS), so that overtreatment can be avoided in the earlier group. The Oncotype DX recurrence score also has a place in male breast cancer management [8] because male breast cancer, compared with female breast cancer, is usually diagnosed at a later stage, has a larger tumor size, more lymph node-positive disease, higher rates of estrogen-receptor positivity and lower rates of HER2 expression.

The Oncotype DX recurrence score results interpretation is categorized according to age (Tab. 3), for patients > 50 years of age, it is classified into two groups:

(I) **Oncotype DX recurrence score of 0–25**: There is a low risk of recurrence and benefits of adjuvant chemotherapy are not likely to outweigh the risks of side effects.

Table 3. Interpretation of Oncotype DX recurrence score and the chemotherapy survival benefit at 10 years

Age group ≤ 50 years	
Oncotype DX recurrence score	**Chemotherapy (CTH) benefit**
0–15	No CTH benefit
16–20	~1.6% CTH benefit
21–25	~6.5% CTH benefit
26–100	15% absolute CTH benefit
Age group > 50 years	
0–25	No CTH benefit
26–100	15% absolute CTH benefit

(II) **Oncotype DX recurrence score of 26–100**: Breast cancer is associated with a high risk of recurrence, so adjuvant chemotherapy benefits are likely to be greater than the risks of side effects.

For patients aged 50 years and younger, the Oncotype DX recurrence score results interpretation is categorized into four groups (Tab. 3):

(I) **Oncotype DX recurrence score of 0–15**: The cancer is associated with a low recurrence risk, and chemotherapy benefits are not likely to outweigh the risks of chemotherapy adverse effects.

(II) **Oncotype DX recurrence score of 16–20**: Breast cancer possesses a low to medium recurrence risk, and chemotherapy benefits are not likely to outweigh the risks of chemotherapy adverse effects.

(III) **Oncotype DX recurrence score of 21–25**: Breast cancer has a medium recurrence risk, and chemotherapy benefits are likely to be greater than the risks of side effects.

(IV) **Oncotype DX recurrence score of 26–100:** Breast cancer is associated with a high recurrence risk, and chemotherapy benefits are likely to be greater than the risks of adverse effects [9].

A number of studies have, indeed, reported different rates of treatment recommendations supporting adjuvant chemotherapy in ER+ve, HER2–ve, and LN–ve early breast cancer. In TAILORx (Trial Assigning Individualized Options for Treatment) phase III clinical trial, it was reported that adjuvant chemotherapy can be omitted for ER+ve, HER2–ve, and LN–ve breast cancer patients aged ≤ 50 years with the Oncotype DX recurrence score ≤ 15, in addition to patients aged > 50 years and RS ≤ 25 [9]. In 2019, Dieci et al. [5] published the ROXANE Italian prospective study, which evaluated the impact of the Oncotype DX recurrence score on adjuvant treatment decision for patients with early breast cancer; 251 patients were included in the study. The authors found that 15% of the patients had a post-RS test recommendation to chemotherapy and hormonal manipulation. Overall, the change in management recommendation from pre-RS testing to post-RS testing occurred in 30% of patients, most frequently from hormonal treatment and chemotherapy to hormonal therapy alone [5]. In other reports, the rates ranged from 13 to 28%, in our cohort the rate was 24%, which is within the range of the published data [10–13]. The Southwest Oncology Group in phase III of SWOG-8814 trial investigated whether the RS was prognostic in breast-cancer patients treated with tamoxifen alone and whether the RS identified those who might not benefit from anthracycline-based chemotherapy despite higher recurrence risks. The full title of this trial is "Tamoxifen with or without Combination Chemotherapy in Postmenopausal Women who have Undergone Surgery for Breast Cancer". The study showed that adjuvant chemotherapy with cyclophosphamide, doxorubicin, and fluorouracil (CAF) prior to hormonal manipulation of tamoxifen (CAF-T) added survival benefit to the treatment option with tamoxifen alone [14]. This trial was followed by a retrospective study done by Albain et al. [15] in 2010, which assessed the impact of the Oncotype DX recurrence score on disease-free survival by treatment option group (tamoxifen only vs CAF-T). It was found that the Oncotype DX recurrence score is prognostic for node-positive disease treated with tamoxifen alone as there was no benefit of CAF in the cases with a low recurrence score. The high Oncotype DX recurrence score predicts the significant benefit of CAF in breast cancer, associated with an improvement in disease-free survival for patients belonging to this group. A low Oncotype DX recurrence score identifies patients who might not benefit from anthracycline-based chemotherapy, despite the presence of nodal disease [15].

In 2010, Dowsett et al. [16] found in a cohort of 1231 patients that the Oncotype DX recurrence score assessment is an independent predictor of distant disease recurrence in node-negative and node-positive, hormone-receptor-positive breast cancer patients treated with aromatase inhibitors (AI), adding value to estimates with standard clinico-pathologic indicators. Generally speaking, compared with clinical and other biomarkers, the Oncotype DX recurrence score is a more reliable indicator in predicting disease recurrence as well as distant metastasis risk in early ER+ve, HER2–ve, and LN–ve early breast cancer patients. In the patient group associated with a high-risk RS, the additional chemotherapy benefit when combined with hormonal therapy is higher compared to hormonal manipulation alone, where the benefit is minimal in the low-risk group.

Conclusion

The Oncotype DX recurrence score use is an effective move toward precision medicine, it allows picking up the group of patients with unfavorable tumor biology to receive the systemic treatment, where a significant number of patients will be spared unnecessary overtreatment. In this cohort, the use of RS testing led to a 24% rate of recommendations to administer adjuvant chemotherapy for ER+ve, HER2–ve and LN–ve early breast cancer.

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VIA MEDICA

ORIGINAL ARTICLE

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Observational, retrospective study evaluating the temporal variability of out-of-hospital cardiac arrests (OHCA) in the district of Bydgoszcz in a 24-month period

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ABSTRACT

Introduction: The incidence of out-of-hospital cardiac arrests (OHCA) varies periodically. The aim of our study was to assess the temporal variability of OHCA occurrence in adult population of Bydgoszcz district.

Material and methods: A retrospective analysis of 782 cases of OHCA, which occurred between January 1st, 2018, and December 31st, 2019, was performed. The temporal variability of OHCA occurrence was assessed during the day (within twenty-four 1-hour periods and four 6-hour time intervals), weeks, months, and seasons of the year.

Results: The incidence of OHCA in the analyzed population was 84 per 100,000 inhabitants/year. The highest incidence of OHCA was observed between 08:00 and 08:59 and between 15:00 and 15:59. The lowest number of OHCA occurred at night (n = 84; 10.7%; $p < 0.001$). During the week, the lowest number of OHCA was noted on Saturday (12.4%) and the highest on Monday (16.5%), with no significant differences between days. The highest incidence of OHCA was observed in winter and the lowest in summer [225 (28.8%) *vs.* 171 (21.9%), $p = 0.006$]. December was the month with the highest number of OHCA cases, and July the lowest.

Conclusions: The present analysis confirms that the occurrence of OHCA demonstrates circadian, monthly, and seasonal rhythm. The highest incidence of OHCA was in the morning and afternoon, and in winter, especially in December. The lowest occurrence of OHCA was at night and in the summer, particularly in July. There was a weekly pattern with the highest occurrence of OHCA on Mondays; however' no significant differences between weekdays were achieved.

Key words: acute coronary syndrome, chronobiology, circadian rhythm, out-of-hospital cardiac arrest, resuscitation

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Introduction

Out-of-hospital cardiac arrest (OHCA) remains a significant issue worldwide. It is defined as the loss of functional cardiac mechanical activity in association with an absence of systemic circulation, occurring outside of hospital [1]. OHCA is the leading cause of death in developed countries [1, 2]. The average occurrence of OHCA in adults worldwide is 95.9/100,000 per year, while European incidence varies according to sources from 16 to 119/100,000 per year [2]. In Poland, the estimated total number of OHCA is 15200 per year [3]. The predominant cause of OHCA is coronary artery disease [1]. According to the analysis by Berdowski at al., the global average incidence of OHCA of presumed cardiac cause was 55 adult per 100,000 person-years [4]. Large

differences in reported rates may result from the use of different OHCA definitions, investigated populations, differences in Emergency Medical Services (EMS) in various regions, as well as research methodology. Regardless of the reported number, the survival rate after OHCA is frighteningly low with the average at 7% in the adult population [4].

Circadian rhythms — well established in humans — affect not only hormone, cytokine, and fibrinogen changes, but also influence endothelial function, vascular tone, and blood pressure [5]. That, in turn, translates into occurrence of acute coronary syndromes throughout the day [6]. Furthermore, it was demonstrated that the incidence of OHCA of cardiac etiology shows not only daily, but also weekly, monthly, and seasonal variation [7]. OHCA due to its unexpected nature and life-threatening features remains at the center of interest of researchers and healthcare professionals. A better understanding this complex phenomenon will enable more effective assistance to our patients and improve long-term outcomes. Hence, the need for further research in this area.

The aim of our study was to assess the temporal variability of OHCA occurrence in the adult population of Bydgoszcz district.

Material and methods

A retrospective analysis of dispatched cards from EMS in Bydgoszcz district between January 1st, 2018, and December 31st, 2019, was performed. Dispatch cards were compatible with the Utstein template. Bydgoszcz district includes the city of Bydgoszcz and surrounding towns and occupies 1395 km^2 [8]. During the study period, it was inhibited by approximately 470000 citizens [8, 9]. The district includes 176 km^2 of urban areas inhibited by 75.5% of the population and 1219 km^2 of suburban areas.

An OHCA was defined as a sudden and unexpected event leading to a cardiac arrest. The mechanism of cardiac arrest was determined based on the first recorded heart rhythm: shockable (ventricular fibrillation [VF] or ventricular tachycardia [VT]) or non-shockable (asystole or pulseless electrical activity [PEA]). Patients with OHCA due to trauma, younger than 18 years of age and with late signs of death (i.e., rigor mortis, decomposition) were excluded from the analysis.

To maintain comparability with other studies a similar pattern of division was used to analyze the OHCA occurrence. Circadian rhythm was investigated within twenty-four 1-hour periods and four 6-hour time intervals: night (0:00– 5:59), morning (6:00–11:59), afternoon (12:00–17:59), and evening (18:00–23:59). The weekly variation was analyzed for days of the week and monthly variation was analyzed for individual months. The seasons were defined as: spring (March, April, May), summer (June, July, August), autumn (September, October, November), and winter (December, January, February).

The present study was approved by the Ethics Committee of the Nicolaus Copernicus University in Torun, Collegium Medicum in Bydgoszcz and was conducted in accordance with Declaration of Helsinki and Good Clinical Practice guidelines.

Statistical analysis

IBM SPSS Statistic software version 27 was used to perform the statistical analysis. The data distribution was checked using the Shapiro-Wilk test. Categorical variables were presented as counts and percentages. Continuous variables were reported as means with standard deviation (SD) or medians with interquartile range (IQR). The distribution of OHCA cases was generally non-normal and the differences between medians were small even when statistically significant. Therefore, it was decided to present the data as percentages to better visualize observed differences. The differences between variables were tested using the Mann-Whitney test or the Kruskal-Wallis test, as appropriate. Two-sided p-value < 0.05 was applied for statistical significance.

Results

General characteristic

There were 782 cases of OHCA included in the present analysis. The incidence of OHCA in the present population was 84 per 100,000 inhabitants per year. Among the enrolled patients, the majority were men (63.6%, $p < 0.001$). The mean age of patients was 69.2 ± 14.2 years. Non-shockable rhythm as the first recorded rhythm was present in 79% cases. The majority of OHCA events occurred in the urban area (84.5%, $p < 0.001$). Baseline characteristics of the study population is presented in Table 1. The higher incidence of OHCA was observed in the population > 60 years of age ($p < 0.001$) (Fig.1).

Circadian variation of OHCA

The highest incidence of OHCA was observed between 08:00–08:59 and then between 15:00–15:59 (Fig. 2). The fewest events of OHCA were observed during the night between 01:00–04:59 (Fig. 2). When analyzing 6-hours intervals (Fig. 3), the lowest number of OHCA occurred at night (n = 84; 10.7%; $p < 0.001$). Numerically, the highest incidence of OHCA was registered in the afternoon (n = 254; 32.5%). However, there were no significant differences between the number of OHCA in the morning (n = 239; 30.6%), afternoon (n = 254; 32.5%), and evening intervals (n = 205, 26.2%) (Fig. 3).

Table 1. Baseline characteristics of the study population

Variable	Study population
Number of cases	782
Mean age [years]	69.2 ± 14.8
Female	36.4%
Initial rhythm	
VF/VT	21%
Asystole/PEA	79%
Location	
Urban	84.5%
Suburban	15.5%

Weekly variation of OHCA

The lowest number of OHCA was noted on Saturday (n = 97, 12.4%) and the highest on Monday (n = 129, 16.5%) (Fig. 4). However, no significant differences between particular days regarding the number of OHCA events were observed.

Monthly and seasonal variation of OHCA

Figure 5 demonstrates monthly distribution of the number of OHCA. A gradual decrease in the number of OHCA was observed from May to July, with the fewest events in the latter (July: n = 50), while the highest num-

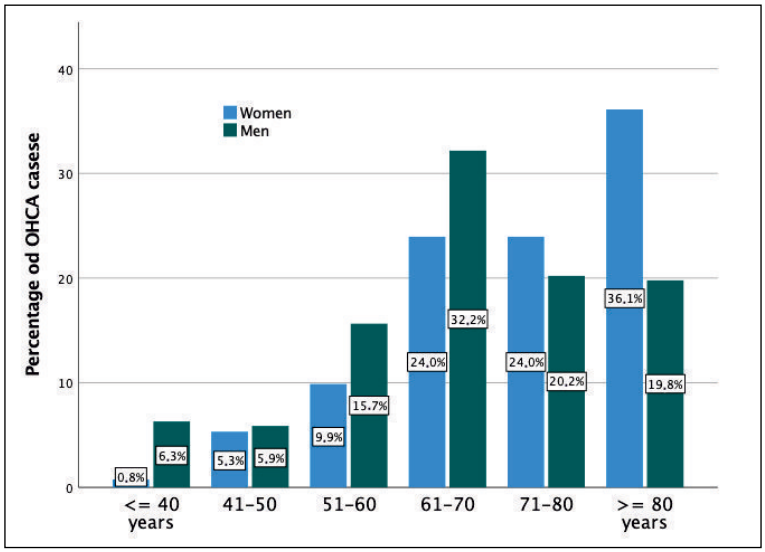

Figure 1. Distribution of out-of-hospital cardiac arrest (OHCA) in different age groups depending on gender

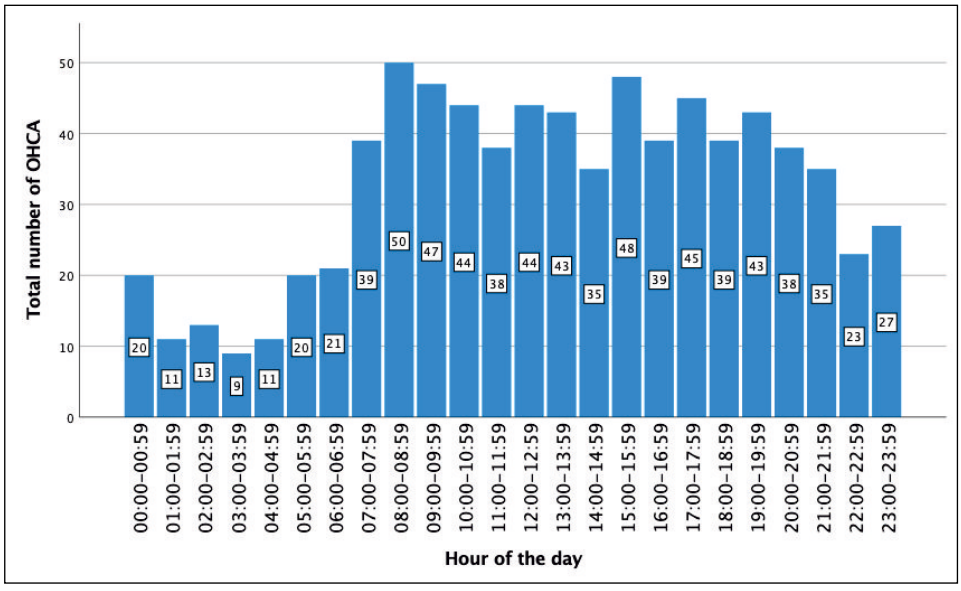

Figure 2. Circadian distribution of out-of-hospital cardiac arrest (OHCA) occurrence divided into 1-hour periods

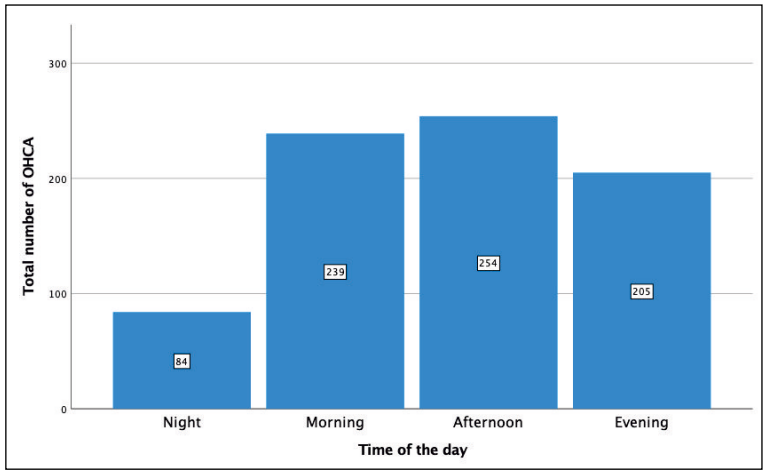

Figure 3. Number of out-of-hospital cardiac arrest (OHCA) in 6-hour intervals. Night *vs.* Morning: $p < 0.001$; Night *vs.* Afternoon: $p < 0.001$; Night *vs.* Evening: $p < 0.001$; Morning *vs.* Afternoon: $p = 0.242$; Evening *vs.* Afternoon: $p = 0.038$; Evening *vs.* Morning: $p = 0.365$

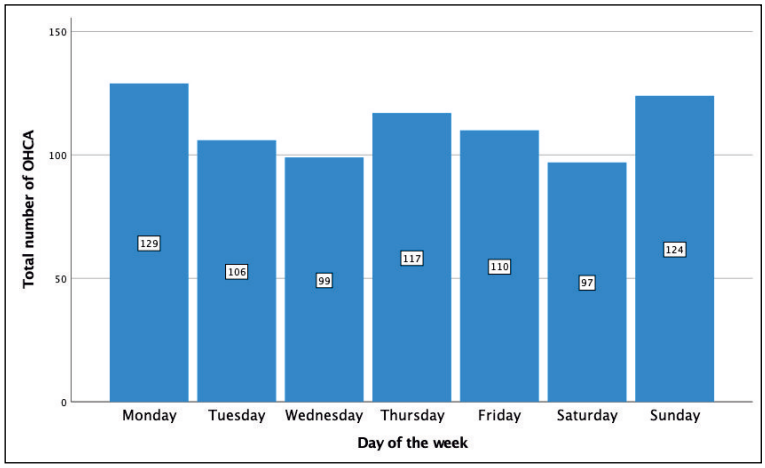

Figure 4. Distribution of out-of-hospital cardiac arrest (OHCA) events in subsequent days of week ($p = 0.095$)

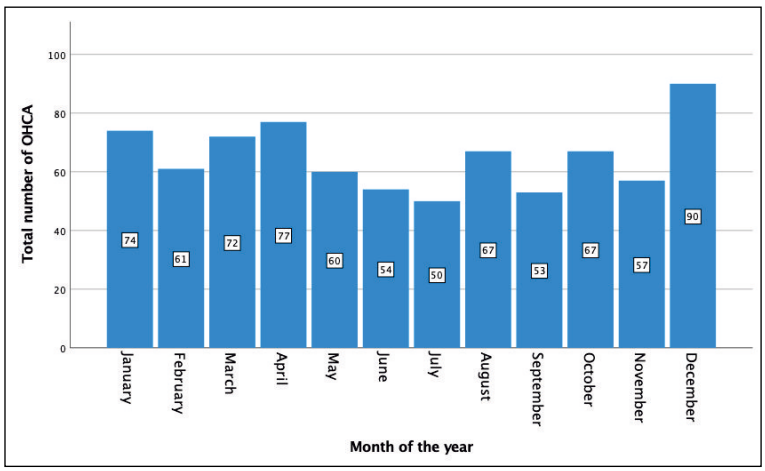

Figure 5. Distribution of out-of-hospital cardiac arrest (OHCA) events in subsequent months of the year ($p = 0.52$)

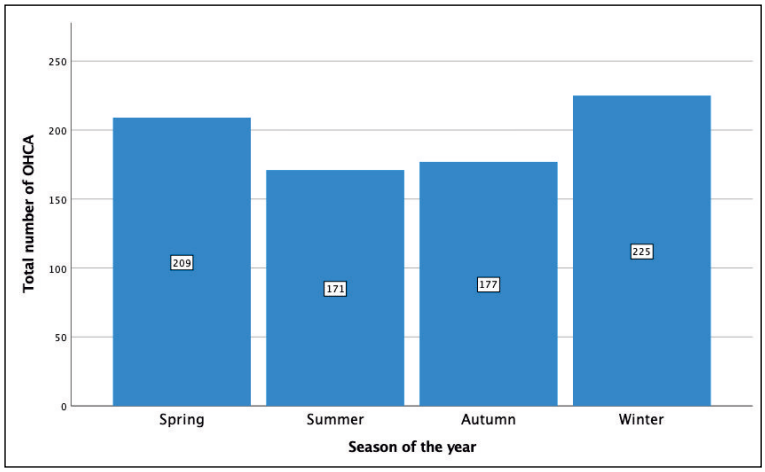

Figure 6. Seasonal distribution of out-of-hospital cardiac arrest (OHCA) events

ber of OHCA cases was registered in December [July *vs.* December: 50 (6.4%) *vs.* 90 (11.5%), p = 0.002]. The variability in subsequent months, according to number of OHCA, was statistically insignificant (p = 0.52).

Considering the seasons of the year, we observed different results. There were significant seasonal differences between number of OHCA in the spring vs. summer [209 (26.7%) *vs.* 171 (21.9%), p = 0.021), autumn *vs.* winter [177 (22.6%) *vs.* 225 (28.8%), p = 0.047], and summer *vs.* winter [171 (21.9%) *vs.* 225 (28.8%), p = 0.006) (Fig. 6). No significant difference in the number of OHCA events was observed between spring and winter [209 (26.7%) *vs.* 225 (28.8%), p = 0.645].

Discussion

Our study evaluating temporal variability of OHCA confirmed circadian rhythm of OHCA incidence, as well as monthly and seasonal patterns. To the best of our knowledge, it is the first study investigating temporal variation of OHCA conducted in the Bydgoszcz district.

The incidence of OHCA in our study was 84/100,000 inhabitants per year, which corresponds to the European indicators within the range 16–119/100,000 inhabitants [2]. The rate in our study is lower than demonstrated by Szczerbinski 156/100,000 [10, 11] and Gach 170/100,000 [3]. However, the population analyzed by Gach [3] was older, and it has been demonstrated that the number of OHCA increases with age.

The circadian rhythm of OHCA was associated with the lowest number of events at night. Our results are consistent with other studies in this regard [3, 7, 10, 12–14]. The highest incidence of OHCA in our study was noted in the morning (08:00–08:59) and in the afternoon (15:00–15:59). Similar results were demonstrated in numerous studies [15–20], where two-peak occurrence curve was shown. However, the afternoon peak in the mentioned studies was later (16:00–20:00) in comparison to our result [15–20]. Other reports presented three peaks of OHCA occurrence: morning (08:00–10:59), afternoon (14:00–15:59), and evening peak (18:00–21:59) [10, 21]. Morning increase in the incidence of OHCA is associated with an increased incidence of acute coronary syndromes during this time. These, in turn, are the most common causes of cardiac arrest and occur as a result of diurnal changes in blood pressure, vascular tone, heart rate, endothelial function, platelet aggregability, and catecholamines [5, 10, 14, 22–24]. The authors agree that the early morning peak of OHCA may be related to the fact that these arrests occurred without witnesses and patients were found by family members in the morning or after returning home from work in the afternoon [10, 14]. Although, in our study the analysis of witnessed and non-witnessed OHCA was not performed.

When analyzing individual days of the week, the highest number of OHCA was recorded on Monday, and the lowest on Saturday. Although, the weekly variability did not reach statistical significance in our study. A similar distribution with the Monday peak in OHCA incidence was demonstrated by Herlitz et al. [7] in the Swedish population, Naknishi et al. [15] among the Japanese, and Ong et al. [16] in the population of Singapore. Contrary to our analysis, Brooks et al. [17] found the highest incidence of OHCA on Saturdays, while Allegra et al. [25] found both Monday and Saturday with the highest occurrence of OHCAs. Another analysis concerning the Polish subpopulation from the Opole district showed an increased frequency of OHCA on Saturdays, Sundays, and Mondays [10]. However, in a long-term follow-up of 13 years, Szczerbinski et al. [12] demonstrated the highest frequency of sudden cardiac arrest on Mondays. A possible explanation for the Monday peak in the OHCA occurrence is related to human behavior, work patterns, and the stress level

[16]. A similar pattern has previously been shown for the incidence of myocardial infarctions [26, 27].

The incidence of OHCA also shows seasonal variability, with a significantly greater number of events in winter and the lowest in summer. According to monthly variability, the highest occurrence of OHCA was noted in December, and the lowest in July. Our observations are in line with the results of other researchers' [3, 7, 12, 15, 17, 25]. Meanwhile, observations from Singapore [16] did not confirm the seasonal variability in the incidence of OHCA. However, the equatorial climate of Singapore has a constant temperature and weather throughout the year [16]. Meanwhile, studies conducted in a moderate climate, where we observe seasonal changes in temperature, air pressure, and precipitation, clearly indicate the influence of atmospheric conditions on the occurrence of OHCA. A possible explanation is the effect of low temperature on arterial blood pressure [16, 28], arterial spasm, platelet and red blood cells count, blood viscosity [16, 29], and serum cholesterol levels [16, 30]. Low temperature affects hemodynamic processes, including an increase in systemic vascular resistance and oxygen consumption by myocardium [16, 31].

The last two years have unexpectedly brought another threat – related to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic. As reported by the latest registers from around the world, the incidence of OHCA increased significantly during the pandemic [32, 34–39]. Moreover, performed meta-analyzes demonstrated that not only the incidence but also mortality following OHCA were higher during the COVID-19 pandemic [32, 33, 40]. Borkowska et al. [40] explained that reduced survival rate after OHCA in suspected or diagnosed COVID-19 patients was probably due to the lower rate of shockable rhythms in COVID-19 patients [32, 40]. Further studies on the impact of COVID-19 on the OHCA occurrence, taking into account previously demonstrated risk factors and treatments, are necessary [41–45].

Cardiac arrest is a complex process that requires a prompt and professional action to restore a haemodynamically efficient heart rhythm, followed by continued treatment for the causes of the condition [46, 47]. Hence, further intensive research on this phenomenon is necessary to improve outcomes after OHCA. The COVID-19 pandemic has revealed how much remains to be done.

Conclusions

The present analysis confirms that the occurrence of OHCA demonstrates circadian, monthly, and seasonal rhythm. The highest incidence of OHCA was in the early morning and afternoon and in winter, especially in December. The lowest occurrence of OHCA was at night and in summer, particularly in July. There was a weekly pattern with the highest occurrence of OHCA on Mondays; however, no significant differences between days of the week were achieved.

Limitations: *The present study has several limitations. Firstly, our study is a retrospective analysis which makes it difficult or even sometimes impossible to recover some data. Secondly, the analyzed period covered only two years and a longer follow-up would certainly be more reliable. Third, the collected data concerned only the district of Bydgoszcz and larger scale studies are necessary.*

Conflict of interest: *None.*

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VIA MEDICA

INVITED EDITORIAL

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Functioning of patients with post-COVID syndrome — preliminary data

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ABSTRACT

Introduction: Post-COVID syndrome is a common finding during the first year after SARS-CoV-2 infection affecting the daily living of many patients.

The aim of this study was to assess the functioning of patients with post-COVID syndrome.

Material and methods: A self-reported questionnaire — the Functioning in Chronic Illness Scale (FCIS) — was applied in 79 (30 women, 49 men) patients (mean age of 62.7 ± 13.6 years), suffering from post-COVID syndrome 5.8 ± 2.3 months after discharge from hospital.

Results: The mean FCIS score was 86.2 ± 12.8 points, corresponding to medium functioning level. The mean score in the first, second and third subscale was 27.0 ± 6.4; 27.5 ± 3.7; and 31.7 ± 4.3 points respectively. Better functioning was observed in men vs women: the FCIS score 88.59 ± 10.95 vs 82.20 ± 14.71; $p = 0.02$ and in the youngest patients: first (< 59 years) vs second (59–67 years) vs third tercile (> 67 years): FCIS score 92.76 ± 14.84 vs 83.15 ± 11.64 vs 83.07 ± 9.68; $p = 0.01$). The amount of time from COVID-19-related hospitalisation did not affect the FCIS score.

Conclusion: Symptoms of post-COVID syndrome influencing patients' functioning persist within the first year regardless of the time elapsing from the disease. Men and younger patients demonstrate better functioning abilities.

Key words: functioning of patients, FCIS, post-COVID syndrome

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Introduction

A substantial proportion of COVID-19 survivors persistently complain of symptoms or development of new symptoms related to SARS-CoV-2 infection [1, 2]. Most of the patients experience at least one symptom during their convalescence. The clinical presentation of post-COVID syndrome is heterogeneous [3, 4]. The most common symptoms are fatigue, dyspnoea, chest pain, joint pain, palpitations, anosmia and dysgeusia, hair loss, cognitive symptoms (memory and attention deficits) and psychosocial distress (loneliness, anxiety, depression and sleep disorders) [4]. Moreover, the symptoms may persist, fluctuate, or appear and be replaced by other symptoms, strongly influencing functioning of patients and requiring dedicated rehabilitation [5]. Post-COVID syndrome meets criteria for a chronic disease according to the World Health Organization (WHO) and U.S. National Center for Health Statistics (USNCHS) [6, 7]. WHO states that chronic diseases are of long duration, generally slow progression and show no person-to-person transmission [6]. According to the USNCHS definition, chronic diseases last at least 3 months, cannot generally be prevented by vaccines or cured by medication, nor do they just disappear [7]. Therefore, for the assessment of the functioning of patients with post-COVID syndrome we applied the Functioning in Chronic Illness Scale (FCIS).

Material and methods

The self-reported FCIS questionnaire was applied in 79 patients suffering from post-COVID syndrome. Patient characteristics are presented in Table 1. The FCIS has been designed to evaluate the impact of

the disease on the patient, the patient's impact on the disease and the impact of the disease on patient attitudes [8, 9]. This tool allows comprehensive assessment of physical and mental functioning in chronic diseases. This self-reported questionnaire consists of 24 questions divided into 3 subscales. The value of the a-Cronbach coefficient for the entire questionnaire is 0.855, indicating its reliability and homogeneity. The value of the determinant of the correlation matrix was 0.001, K-M-O parameter was 0.843 and the Bartlett's test of sphericity was statistically significant [10–13]. Answers for each questionnaire question are graded 1 to 5 points. The maximal score is 120 points. For each section of the questionnaire, the maximal score is 40 points. The score of less than 79 points for the entire questionnaire indicates low functioning, 79–93 points — medium functioning and > 93 points — high functioning [12]. In the first subscale evaluating the impact of the disease on the patient, scores < 23 points indicate low level, 24–33 — medium level and > 34 points — high level of functioning. The respective scoring categories for the second subscale assessing the patient's impact on the disease, are: < 24 points, 25–29 points and > 30 points. The impact of the disease on patient attitudes is evaluated in the third subscale, where the score intervals for low, medium and high functioning are: < 27 points, 28–33 points, and > 33 points [12].

Results of the FCIS were analysed according to patients' gender, age and time from hospitalisation due to COVID-19.

Statistics

Statistical analysis was carried out using the Statistica 13.0 package (TIBCO Software Inc., Palo Alto, California, USA). Continuous variables were presented as means with standard deviations, medians with interquartile range, minimum and maximum value. The Shapiro–Wilk test demonstrated non-normal distribution of the investigated continuous variables. Therefore, non-parametric tests were used. Comparisons between 2 groups were performed with the Mann–Whitney unpaired rank sum test. For comparisons between 3 or more groups, the Kruskal–Wallis one-way analysis of variance and multiple comparison test were used. Results were considered significant at p-value < 0.05.

Results

The mean FCIS score obtained in 79 patients was 86.2 ± 12.8 points, corresponding to medium functioning level (Tab. 1). The mean scores obtained in the first (impact of the disease on the patient), second (patient impact on the disease) and third (impact of the disease on patient attitudes) subscales were 27.0 ± 6.4; 27.5 ± 3.7, and 31.7 ± 4.3 points respectively. Of note, as many as 50 (63.3%) patients, referring to the first statement of the first part of the FCIS (My physical capacity is similar as prior to the illness), answered "definetely NOT" (n = 19) or "rather not" (n = 31). Referring to the

Table 1. Patients' characteristics and the FCIS results

Parameter		All patients	
		N-value	% / SD
Gender	Female	30	38%
	Male	49	62%
Age	Years	62.7	13.6
Time from COVID-19	Months	5.8	2.3
FCIS total score	Low level	18	22.8%
	Medium level	37	46.8%
	High level	24	30.4%
FCIS 1st subscale score	Low level	21	26.6%
	Medium level	42	53.2%
	High level	16	20.3%
FCIS 2nd subscale score	Low level	16	20.3%
	Medium level	40	50.6%
	High level	23	29.1%
FCIS 3rd subscale score	Low level	13	16.5%
	Medium level	40	50.6%
	High level	26	32.9%

Table 2. The FCIS results according to gender

FCIS	Gender	FCIS score								P-value
		N	Mean	SD	ME	Q1	Q3	Min	Max	
Total score	Male	49	88.59	10.95	86.00	81.00	99.00	65.00	109.0	0.0204
	Female	30	82.20	14.71	79.50	72.00	95.00	57.00	111.0	
1st subscale	Male	49	27.90	5.99	28.00	25.00	32.00	14.00	38.00	0.0854
	Female	30	25.50	6.94	24.50	21.00	31.00	11.00	38.00	
2nd subscale	Male	49	27.90	3.38	28.00	25.00	30.00	21.00	35.00	0.2414
	Female	30	26.93	4.25	26.50	24.00	30.00	18.00	36.00	
3rd subscale	Male	49	32.80	3.59	33.00	30.00	36.00	27.00	40.00	0.0042
	Female	30	29.77	4.76	29.00	27.00	33.00	23.00	37.00	

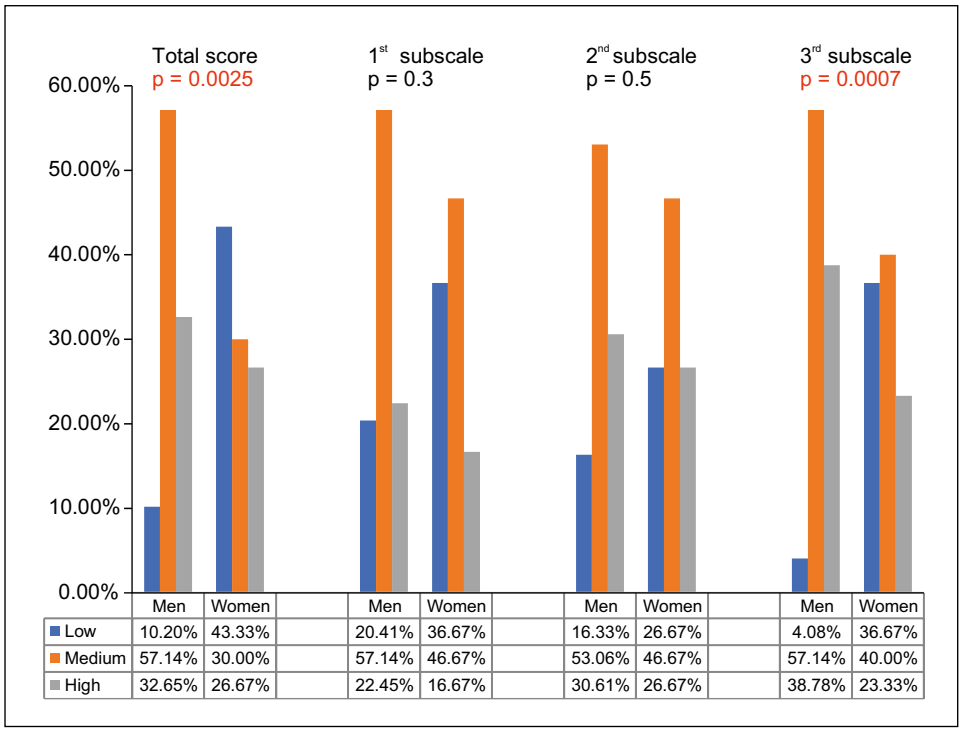


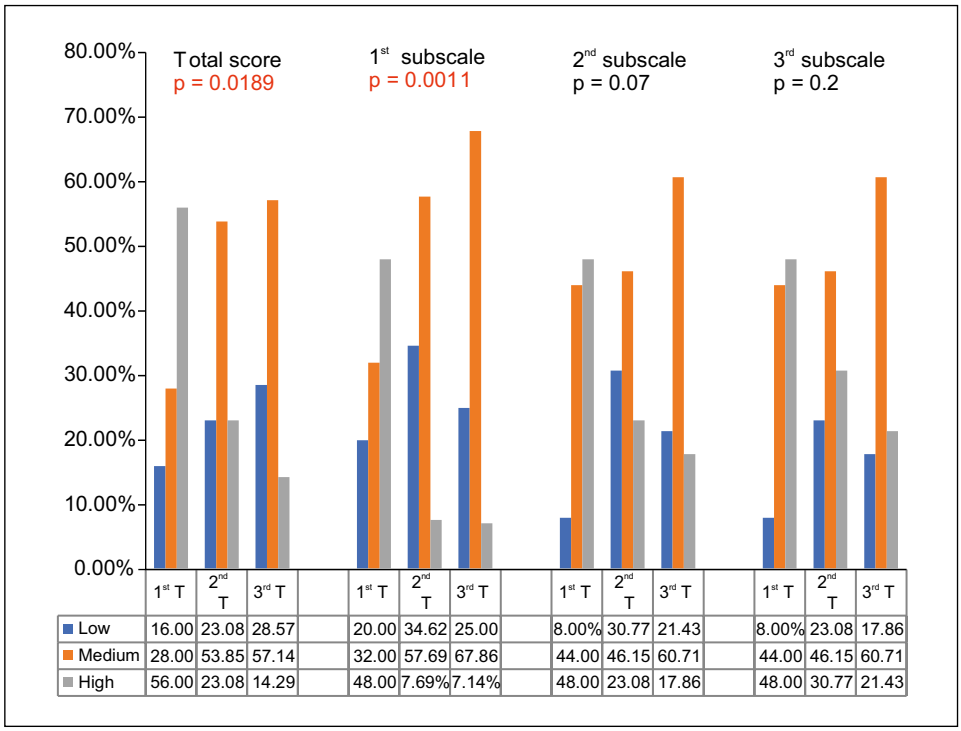
Figure 1. The prevalence of low, medium and high levels of FCIS score according to gender

other statement of the second part of the questionnaire (I am primarily responsible for my future well-/ill-being) a vast majority of patients (n = 59; 74.7%) answered "rather yes" (n = 38); "definitely YES" (n = 21). It also needs to be highlighted that referring to the first statement of the last part of the questionnaire (My illness made me actively take care of my health) 67 (84.8%) patients rendered a positive answer, choosing the "rather yes" (n = 38) or "definitely YES" (n = 29) option.

Men demonstrated higher levels of functioning than women, with significant differences in the total FCIS score and in the third subscale (Tab. 2). Consequently, the prevalence of low levels of FCIS score was significantly higher in women, while medium and high level scores were more common in men (p = 0.0007). This was mainly driven by the differences observed in the third subscale (Fig. 1). A substantial impact of age on the functioning level of patients with post-COVID syndrome was also noted. Higher FCIS scores were acquired in the youngest subset of patients (first tercile: < 59 years), compared with the second (59–67 years) and third tercile (> 67 years) (Tab. 3), with a significant difference between the first and the second tercile (p = 0.0372) and between the first and the third tercile (p = 0.0323). This was reflected by adequate prevalence of low,

Table 3. The FCIS results according to age

FCIS	Age	FCIS score								P-value
		N	Mean	SD	ME	Q1	Q3	Min	Max	
Total score	1st tertiary	25	92.76	14.84	99.00	80.00	105.00	59.00	111.00	0.0149
	2nd tertiary	26	83.15	11.64	82.00	78.00	92.00	60.00	103.00	
	3rd tertiary	28	83.07	9.68	83.50	77.00	89.00	57,00	100.00	
1st subscale	1st tertiary	25	29.84	7.85	32.00	25.00	36.00	11.00	38.00	0.0167
	2nd tertiary	26	25.69	6.03	26.00	22.00	30.00	14.00	35.00	
	3rd tertiary	28	25.64	4.47	25.00	23.50	28.00	16.00	36.00	
2nd subscale	1st tertiary	25	29.68	3.79	29.00	27.00	33.00	23.00	36.00	0.0071
	2nd tertiary	26	26.54	3.22	27.00	24.00	29.00	20.00	32.00	
	3rd tertiary	28	26.54	3.43	26.50	25.00	29.00	18.00	35.00	
3rd subscale	1st tertiary	25	33.24	4.74	33.00	30.00	37.00	23.00	40.00	0.0581
	2nd tertiary	26	30.92	4.30	30.50	28.00	36.00	23.00	38.00	
	3rd tertiary	28	30.89	3.61	30.50	28.00	33.00	23.00	38.00	



	1st T	2nd T	3rd T		1st T	2nd T	3rd T		1st T	2nd T	3rd T		1st T	2nd T	3rd T
Low	16.00	23.08	28.57		20.00	34.62	25.00		8.00%	30.77	21.43		8.00%	23.08	17.86
Medium	28.00	53.85	57.14		32.00	57.69	67.86		44.00	46.15	60.71		44.00	46.15	60.71
High	56.00	23.08	14.29		48.00	7.69%	7.14%		48.00	23.08	17.86		48.00	30.77	21.43

Figure 2. The prevalence of low, medium and high levels of FCIS score according to age

medium and high levels of FCIS score in subsets of patients divided according to age (Fig. 2). The difference in the total FCIS score (p = 0.0189) was mainly influenced by the result of the first subscale (p = 0.0011). Contrary to gender and age, the time from COVID-19-related hospitalisation did not affect the FCIS score (Tab. 4).

Discussion

According to our best knowledge, this is the first report using the FCIS questionnaire in a post-COVID-19 population. While a significant impact of gender and age on the functioning of patients with post-COVID syndrome could be observed, no such

Table 4. The FCIS results according to the time from hospitalization due to COVID-19

FCIS	Age	FCIS score								P-value
		N	Mean	SD	ME	Q1	Q3	Min	Max	
Total score	1st tertiary	15	84.73	11.40	81.00	76.00	93.00	70.00	111.00	0.7316
	2nd tertiary	29	85.76	13.06	83.00	80.00	96.00	59.00	107.00	
	3rd tertiary	35	87.11	13.43	85.00	78.00	100.0	57.00	109.00	
1st subscale	1st tertiary	15	26.60	6.60	27.00	24.00	31.00	14.00	38.00	0.9916
	2nd tertiary	29	26.93	6.50	28.00	23.00	32.00	11.00	36.00	
	3rd tertiary	35	27.20	6.48	26.00	23.00	32.00	15.00	38.00	
2nd subscale	1st tertiary	15	27.13	3.78	27.00	25.00	29.00	21.00	36.00	0.8665
	2nd tertiary	29	27.55	4.05	28.00	25.00	30.00	20.00	35.00	
	3rd tertiary	35	27.69	3.55	27.00	26.00	30.00	18.00	36.00	
3rd subscale	1st tertiary	15	31.00	3.76	31.00	28.00	33.00	23.00	37.00	0.6048
	2nd tertiary	29	31.28	4.22	31.00	28.00	36.00	23.00	37.00	
	3rd tertiary	35	32.23	4.62	32.00	29.00	36.00	23.00	40.00	

relation was demonstrated for the time elapsing from COVID-19-related hospitalisation. We have demonstrated strong influence of post-COVID syndrome on the daily living of a majority of patients, as after 6 months since acute SARS-CoV-2 infection, the FCIS score was high only in 30% of patients. Almost 80% of survey participants obtained low or medium score in the first subscale. This result suggests a significant impact of the disease on physical and mental functioning. According to the result of the second subscale, 29% of patients believe that they have a significant impact on the course of illness, while 20% negate this possibility. Furthermore, according to the result of the third subscale, 33% of responders hold a very optimistic view for the future, while 16% remain pessimistic. Our functional assessment suggests that a vast majority of patients suffering from post-COVID syndrome need urgent and dedicated rehabilitation providing both a physical and mental coverage. Our results are in line with some previous studies showing severe impairments in physical functioning and during activities of daily living in post-COVID-19 patients [14–16]. In a large study assessing 1733 post-COVID-19 patients 6 months after discharge from hospital, more then 60% of survivors reported fatigue or muscle weakness, sleep difficulties, and anxiety or depression [16]. Patients with a more severe in-hospital course of COVID-19 had more severe impairment of pulmonary diffusion capacities and abnormal chest imaging manifestations. These patients are indicated in literature as the main target population for intervention [16, 17]. As shown in this preliminary report, due to the variety of symptoms of post-COVID syndrome, a personalised approach is indispensable. An experienced physician should assess the patient, and after thorough assessment of the patient's clinical condition, a dedicated intervention should be set up in collaboration with the rehabilitation team [17]. We have demonstrated a stronger impact of post-COVID syndrome on patient functional status in females than in males. The difference was mainly driven by the evaluation of the impact of the disease on patient attitudes (the third FCIS subscale). Men were significantly more optimistic regarding their future than women. We have also demonstrated better functional status in the youngest patients (< 59 years). We found a significant difference in the first and second FCIS subscales reflecting patient's believes respectively regarding the impact of post-COVID syndrome on their lives, and the possibility of influencing the course of the disease. Long-term persistence of symptoms was confirmed by similar FCIS results irrespective of the time from hospitalisation due to COVID-19.

An integrated, comprehensive rehabilitation programme is recommended for post-COVID patients, involving a multidisciplinary and multi-professional team providing neuromuscular, cardiac, respiratory, and swallowing interventions, and psychological support, in order to improve patients' quality of life [14, 17]. Similarly to other chronic diseases, rehabilitation should be complemented by patient education and strengthening of patient motivation [11, 18–22]. We are convinced that, despite the logistic difficulties related to the epidemiological situation, education of patients should be initiated before discharge from hospital [23, 24]. A personalised approach to rehabilitation often requires pharmacological support, mainly due to frequent comorbidities. Therefore, monitoring of adherence to medication is also an important issue in this specific subset of patients [25–33]. The results of this preliminary

report were used to plan a personalised rehabilitation programme for patients with post-COVID syndrome. We are going to assess the effectiveness of our rehabilitation programme with comprehensive assessment of patient functional status using the FCIS, both before and after the rehabilitation.

The main limitations of this study are the small number of assessed patients and the limited number of factors that could influence the FCIS score.

Conclusion

Symptoms of post-COVID syndrome influencing patient functioning persist within the first year regardless of the time elapsing from the disease. Better functioning was observed in men and younger patients.

Conflict of interest: *None.*

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VIA MEDICA

ORIGINAL ARTICLE

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Implementation of therapeutic recommendations in high cardiovascular-risk patients. The Polish population of EUROASPIRE V survey

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ABSTRACT:

Introduction: Poor medication adherence is associated with unsatisfactory health outcomes, elevated mortality, and high costs of medical care. This study aimed to assess the implementation of therapeutic recommendations in high cardiovascular-risk patients based on self-report questionnaires.

Material and methods: The study included 194 patients from the Cardiology Outpatient Clinic. Two self-reported questionnaires were used to assess medication adherence: the Medication Adherence Questionnaire (MAQ) and the Adherence in Chronic Diseases Scale (ACDS).

Results: Antihypertensive drugs were prescribed to 65.46% of the patients. According to the MAQ, 54.33% of them reported high adherence, 21.26% medium adherence, and 24.41% low adherence to the treatment. Lipid-lowering drugs were prescribed to 46.39% of the patients, all of whom were treated with statins. Among this group, 34.44% reported high adherence, 27.78% medium adherence, and 37.78% low adherence to pharmacotherapy. According to the ACDS, the majority of patients (45.55%) received a score indicating medium adherence (21–26 points), 39.27% high adherence (> 26 points), and 16.75% low adherence to treatment (< 21 points). A high level of adherence was declared by 61.54% of the patients that reached the therapeutic goal of lipid-lowering therapy, defined as LDL-C of < 2.6 mmol/L (< 100 mg/dL). On the other hand, among the patients whose LDL-C remained elevated, 23.44% declared high adherence to treatment. There were no significant differences in achieving the intended therapeutic goal of blood pressure (BP ≤ 140/90 mmHg) in the groups with high, medium and low adherence (26.53% *vs.* 23.47% *vs.* 50.00%; p = 0.1880).

Conclusion: Despite higher adherence to treatment in the patients with hypertension compared to patients with hyperlipidemia, the latter more often achieved the therapeutic goal. Declarations regarding high adherence to medication in the MAQ and in the ACDS are consistent in patients with hyperlipidemia and hypertension.

Key words: adherence, self-report questionnaires, hyperlipidemia, hypertension

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Introduction

Poor medication adherence is associated with unsatisfactory health outcomes, elevated mortality, and high costs of medical care [1, 2]. According to various studies, 30% to 50% of patients do not adhere to medical recommendations [3–8]. In secondary prevention of myocardial infarction, the adherence to pharmacotherapy decreases over time, for nearly all classes of drugs [3, 13]. The pharmacotherapy of hypertension, dyslipidemia, and diabetes reduces the risk of cardiovascular events [9]; yet, adherence to preventive pharmacotherapy is even lower compared to secondary prevention [9–12]. In a meta-analysis comparing adherence to pharmacotherapy of coronary heart disease in primary *vs.* secondary prevention, Naderi et al. [12] reported an adherence rate of 50% (CI, 45–56) and 66% (CI, 56–75), respectively (p = 0.012). Overall, the adherence in the study was 57% during a median treatment of 24 months [12].

A number of validated medication-adherence scales have been described in the literature [8, 13–15]. These scales, based on self-reported questionnaires, are low-cost and easy to use in routine clinical practice [8].

This study aimed to assess the implementation of therapeutic recommendations in a group of patients treated for hypertension, hyperlipidemia, and/or diabetes with the use of two self-report questionnaires.

Material and methods

The data were collected as a part of the EUROASPIRE V survey, a prospective observational study conducted between 2016 and 2017 in European countries. The study included 200 patients (66.50% women and 33.50% men), with a mean age of 51.49 years, from the Cardiology Outpatient Clinic of the Department of Cardiology and Internal Medicine of the University Hospital No. 1 in Bydgoszcz, Poland. The following inclusion criteria were applied: adult (18–80 years old), diagnosed with hypertension, hyperlipidemia or diabetes, without documented cardiovascular diseases. All the patients provided written informed consent. Baseline characteristics of the patient population are presented in Tables 1 and 2.

The final analysis comprised 194 patients treated with one or more medications for a period of 6–24 months (lipid-lowering drugs, antihypertensive drugs, oral antidiabetic medicines, and/or insulin). Six of the patients were treated with a diet for diabetes, and therefore were excluded from the analysis.

Questionnaires

Two self-reported questionnaires were used to assess medication adherence: the Medication Adherence Questionnaire (MAQ) and the Adherence in Chronic Diseases Scale (ACDS) [13–15].

In the MAQ, the patients were asked to answer the question "How often do you take your medications as

Table 1. Characteristics of the study population

Parametr	Mean	SD	Median	Q1	Q3	Min	Max
Age [years]	51.49	13.63	52.00	43.00	60.00	20.00	81.00
SBP [mmHg]	127.23	14.47	125.00	118.00	135.00	97.00	183.00
DBP [mmHg]	76.52	9.32	77.50	70.00	82.00	54.00	100.00
Waist circumference [cm]	88.01	12.20	87.00	80.00	95.50	54.00	126.00
Height [cm]	169.47	9.62	169.50	162.00	176.00	147.00	200.00
Weight [kg]	75.77	14.79	74.00	64.75	86.00	44.00	118.00
BMI [kg/m2]	26.35	4.06	26.00	23.90	28.73	17.10	42.20
T-CH [mg/dL]	217.42	41.78	214.85	190.84	241.69	105.60	344.50
LDL-CH [mg/dL]	129.09	38.13	127.10	103.80	154.55	42.20	253.00
TG [mg/dL]	121.04	71.32	106.80	79.96	137.50	40.20	630.90
Glucose [mg/dL]	100.60	19.74	97.60	90.75	106.35	52.20	207.70
ACDS — score	24.35	3.67	25.00	22.00	28.00	12.00	28.00

ACDS — Adherence in Chronic Diseases Scale; BMI — body mass index; DBP — diastolic blood pressure; LDL-CH — low-density lipoprotein cholesterol; SBP — systolic blood pressure; SD — standard deviation; T-CH — total cholesterol; TG — triglyceride

Table 2. Qualitative variables in the study population

Parameter	Count	Percent
Gender (female/male)	133/67	66.5/33.5
Hypertension	127	63.5
Hyperlipidemia	90	45
Diabetes	41	20.5
Smoking	76	38
Any pharmacotherapy	194	97
Antihypertensive drugs	127	63.5
Lipid-lowering drugs	90	45
Hypoglycemic drugs	34	17

prescribed by the doctor. Based on their response, they were then classified into one of three groups depending on the level of adherence: high (taking medication 100% of the time as prescribed), medium (90% of the time), low (75% of the time or less).

Another tool used to evaluate adherence to pharmacotherapy was the ACDS. This scale consists of 7 questions relating to behaviors influencing adherence, either directly (questions 1–5) or indirectly (questions 6 and 7). The maximum score in the ACDS is 32 points. A score above 26 points indicates high adherence, while a score under 21 points indicates low adherence; the remaining score range of 21–26 points is classified as medium adherence [13–15].

Statistics

The statistical analysis was carried out using the Statistica 13.0 package (TIBCO SoftwareInc, California, USA). Continuous variables were presented as means with standard deviations, medians with interquartile range, minimum and maximum value. The Shapiro-Wilk test demonstrated the non-normal distribution of the investigated continuous variables.

Therefore, non-parametric tests were used for statistical analysis. For comparisons of ACDS scores and MAQ levels, the Kruskal-Wallis one-way analysis of variance and a multiple comparison test was used. Categorical variables were expressed as counts and percentages. Categorical variables were compared using the $\chi 2$ test. Results were considered significant at $p < 0.05$.

Results

Among 200 patients included in the study, there were 127 cases of hypertension, 90 of dyslipidemia, and 41 of diabetes. The clinical and anthropometric data of the study population are shown in Tables 1 and 2.

Table 3. Distribution of adherence levels according to the MAQ in the study population

MAQ levels	Count	Percent
MAQ hypertension (n = 127)		
Low level	31	24.41
Medium level	27	21.26
High level	69	54.33
MAQ hyperlipidemia (n = 90)		
Low level	34	37.78
Medium level	25	27.78
High level	31	34.44
MAQ diabetes mellitus (n = 34)		
Low level	18	52.94
Medium level	3	8.82
High level	13	38.24

Antihypertensive drugs were prescribed to 65.46% of patients. The most common medications were angiotensin-converting enzyme inhibitors (ACEI; 45.50%), followed by beta-adrenolytics (20%), calcium channel blockers (7%), angiotensin receptor blockers (ARBs; 7%), and diuretics (4.5%). According to the MAQ, 54.33% of the patients on antihypertensive drugs reported high adherence, 21.26% medium adherence, and 24.41% low adherence to the treatment. Lipid-lowering drugs were prescribed to 46.39% of the patients, all of whom were treated with statins. Among this group, 34.44% reported high adherence, 27.78% medium adherence, and 37.78 % low adherence to pharmacotherapy. Antihyperglycemic drugs were prescribed to 17.53% of the patients. Based on the MAQ, 38.24% of them reported high adherence, 8.82% medium adherence, and 52.94% low adherence. Due to the low number of patients on glucose-lowering drugs, the results were not analyzed statistically. The distribution of adherence levels according to the MAQ is presented in Table 3.

According to the ACDS, the majority of patients (45.55%) received a score indicating medium adherence, 39.27% high adherence, and 16.75% low adherence to the treatment (Tab. 4).

Among the hypertensive patients who achieved blood pressure of ≤ 140/90 mmHg, only half declared high adherence to treatment while in the patients with elevated blood pressure (> 140/90 mmHg) as many as 68.97% declared taking medication 100% of the time as prescribed. The high level of adherence was declared by 61.54% of the patients who reached the

therapeutic goal of lipid-lowering therapy defined as LDL-C of < 2.6 mmol/L (< 100 mg/dL). On the other hand, among the patients whose LDL-C remained elevated, 23.44% declared high adherence to treatment. In patients with diabetes, who achieved fasting blood glucose concentration of < 100 mg/dL, only 8.33% declared high adherence to medication while 83.33% declared low adherence. Due to the low number of patients on glucose-lowering drugs, the results were not analyzed statistically. The distribution of adherence levels according to the MAQ in relation to the treatment goal achievement is presented in Table 5.

In pharmacologically treated patients with hypertension and hyperlipidemia, as well as in the entire study population, the results of the MAQ were consistent with those of the ACDS (Tab. 6). The patients who declared high adherence in the MAQ obtained the highest mean ACDS scores, while the patients with low adherence, according to the MAQ, were characterized by the lowest mean ACDS scores.

Discussion

Poor adherence to recommended medication is a well-documented problem in the pharmacological treatment of chronic conditions, such as coronary artery disease, diabetes, hyperlipidemia, hypertension [2–8, 15, 16]. It is hard to define medication adherence with a clear quantitative threshold below which a patient is classified as effectively non-adherent [17]. In the literature, the level of actual adherence ≥ 80% is typically considered relevant for the effectiveness of long-term pharmacotherapy [3, 17–19]. In this study, the patients with indications for chronic pharmacotherapy due to hypertension, diabetes, and/or hyperlipidemia, were classified as having low, medium, or high declared adherence. It is extremely important to differentiate between the actual adherence and the declared adherence, as the latter is usually higher than the former [16, 34–37]. Insufficient or low adherence was defined as taking drugs 75% of the time or less according to the MAQ or as a score of < 21 points in the ACDS [13–15]. In our study, 24.41% of the hypertensive patients and 37.78% of the patients with hyperlipidemia declared a low level of adherence in self-reported MAQ. Among the patients that were treated for hypertension and achieved the intended therapeutic goal of blood pressure ≤ 140/90 mmHg, only 50% declared high adherence in the MAQ. A slightly higher percentage was observed in the patients with hyperlipidemia; in this group, 61.54% of patients who reached the LDL-C of < 2.6 mmol/L (< 100 mg/dL) declared high adherence to the lipid-lowering treatment. The patients with high adherence reached their therapeutic goals significantly more often than those who were considered non-adherent to statin therapy (p = 0.0005).

Adherence to medications is a long process consisting of initiation, implementation, and persistence. The problem of non-initiation is estimated at 4-5% in clinical trials [19, 20]. In routine clinical practice, over 20% of patients with hypertension, as well as those with dyslipidemia, never start their treatment [20–23]. However,

Table 4. Distribution of adherence levels according to the ACDS in the study population

ACDS	N = 194	Percent
Low (score < 21)	32	16.50
Medium (score 21–26)	87	44.85
High (score > 26)	75	38.65

Table 5. Distribution of adherence levels according to the MAQ in relation to treatment goal achievement in the study population

	MAQ			P-value
	Low	Medium	High	
Hypertension (n = 127)				
BP > 140/90 mmHg	17.24%	13.79%	68.97%	0.1880
BP ≤ 140/90 mmHg	26.53%	23.47%	50%	
Hyperlipidemia (n = 90)				
LDL > 2.6 mmol/L (> 100 mg/dL)	48.44%	28.13%	23.44%	0.0005
LDL < 2.6 mmol/L (< 100 mg/dL)	11.54%	26.92%	61.54%	
Diabetes (n = 34)				
Glucose > 100 mg/dL	36.36%	9.09%	54.55%	na*
Glucose < 100 mg/dL	83.33%	8.33%	8.33%	

*Due to the small number of patients on glucose-lowering drugs, the results were not analyzed statistically

Table 6. Comparison of adherence levels with the score in the ACDS

	ACDS			P-value
	N	Mean	SD	
Hypertension (n = 127)				
MAQ Low level	31	21.29	3.95	< 0.0001
MAQ Medium level	27	23.93	3.01	
MAQ High level	69	26.01	2.34	
Hyperlipidemia (n = 90)				
MAQ Low level	34	22.74	4.63	0.0024
MAQ Medium level	25	24.76	3.05	
MAQ High level	31	26.19	2.50	
Diabetes (n = 34)				
MAQ Low level	18	24.80	2.93	na*
MAQ Medium level	3	20.00	1.73	
MAQ High level	13	26.38	3.55	
Overall (n = 191)				
MAQ Low level	64	22.25	4.09	< 0.0001
MAQ Medium level	47	24.62	3.01	
MAQ High level	80	25.97	2.61	

*Due to the small number of patients on glucose-lowering drugs, the results were not analyzed statistically

it is the discontinuation of treatment that seems to be a greater issue [13, 22–26].

In many European countries, lipid control remains poor and most patients with dyslipidemia do not achieve the treatment goals recommended by the ESC guidelines [27]. This could be attributed to a high percentage of discontinuation of lipid-lowering treatment. Among patients with coronary heart disease in the EUROASPIRE IV survey, 90.4% were on statins, and only 19.3% achieved values of LDL-C < 1.8 mmol/L; statin was discontinued in 11.6% of cases [28]. Statins were also the most common medication class to be discontinued one year after myocardial infarction [24]. Only 50% of patients after myocardial infarction continued the therapy with statins in one-year follow-up [27–29].

Adherence to pharmacotherapy of hypertension is necessary to achieve optimal levels of blood pressure control. Discontinuation of antihypertensive therapy is a common problem as more than 50% of patients stop their treatment after one year [16, 20]. In addition, the rate of omitted doses is reported to be 10% per day [20]. A much higher adherence rate was observed in clinical trials with frequent clinical visits and where pill count was used to assess adherence [19]. In our study, there were no significant differences in achieving the therapeutic goal of blood pressure (BP ≤ 140/90 mmHg) in the groups with high (100%), medium (90%), and low adherence (≤ 75%) (26.53% *vs.* 23.47% *vs.* 50.00%; p = 0.1880). Apart from low adherence, failure to achieve therapeutic goals might be related to inadequate therapy, as well as suboptimal dosage of prescribed medications.

As there are multiple reasons for non-adherence, efficient and simple tools are needed to determine patients' adherence in clinical practice [16, 26, 30–33]. Questionnaires are subjective methods and generally tend to overestimate true adherence when compared with more objective measures [34–37, 40]. The relatively low specificity and sensitivity may occur due to incorrect data reporting by patients. However, the simplicity, practicality, low cost, and real-time feedback have caused a widespread use of self-report questionnaires in clinical practice [16, 38, 40, 41]. Yet, adherence determined by questionnaires correlates with objective adherence measures and clinical outcomes, such as lipid levels, blood pressure, and blood glucose control [17]. Moreover, questionnaires can be useful tools to complement more objective methods of measurement as they may identify patients' concerns and reasons for non-adherence. Declarations regarding high and medium adherence in the MAQ and the ACDS were consistent; the patients with hyperlipidemia and hypertension with high adherence in the MAQ received an average of > 26 points in the ACDS. The higher the adherence level declared in the MAQ, the higher was the score in the ACDS. The ACDS is designed to reflect various aspects of patient adherence [14, 15, 34–36].

This information can help implement an appropriate therapeutic plan, including additional personalized education and motivation methods [26, 39, 40].

Limitations of the study

The presented study had some limitations. The patients included in the study were not representative of all high cardiovascular-risk patients. Due to the small number of diabetic patients on glucose-lowering drugs, the results were not analyzed statistically. For evaluating medication adherence, only subjective methods in the form of self-report questionnaires were used. Patients' self-reported drug intake is often overestimated [34, 35]. Since there is no single method of choice in evaluating adherence, selecting at least two different methods (objective and subjective) can yield more reliable results. Finally, other factors that could have affected the therapeutic goals were not analyzed. In some cases, insufficient control of risk factors might have resulted from suboptimal doses of prescribed drugs.

Conclusion

Despite higher adherence to treatment in the patients with arterial hypertension compared to the patients with hyperlipidemia, the latter more often achieved the therapeutic goal. Declarations regarding high adherence to medication in the MAQ and in the ACDS are consistent in patients with hyperlipidemia and hypertension.

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VIA MEDICA

REVIEW ARTICLE

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Does SARS-CoV-2 infect cardiomyocytes directly? Yes, it does

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ABSTRACT

Introduction: COVID-19 (Coronavirus disease 2019) appeared in Wuhan, China, at the ending of 2019. The SARS-CoV-2 virus which causes the illness has spread all over the world and caused a pandemic. The first target of the virus is the respiratory tract; however, the COVID-19 may present different types of course. It is known that the SARS-CoV-2 affects multiple organs, including the heart. Cardiac manifestations of COVID-19 include myocarditis, myocardial infarction, heart failure, acute coronary syndrome, arrhythmia. The authors know about the patients who had only cardiovascular complications due to the COVID-19. Several mechanisms of heart injury are considered and so is the direct infection.

Aim of the study: The present review aimed to find out if the SARS-CoV-2 may infect the heart directly and in which mechanism. The review is an information collection considering the SARS-CoV-2 impact on the heart.

Material and methods: The authors have made research using the PubMed search engine to find studies and case reports considering the cardiovascular implications of COVID-19. The signs and symptoms in patients with cardiac implications were studied. The authors have also checked if studies explaining does the SARS-CoV-2 affects the heart directly were conducted.

Results: SARS-CoV-2 brings several cardiovascular signs such as changes in imaging tests and elevation of several laboratory markers. The changes may suggest myocarditis or mimic cardiac infarction. The SARS-CoV-2 may affect cardiomyocytes indirectly by causing hypoxia and cytokine storm. As the heart tissue presents a high level of ACE2 which is the target of the virus, the SARS-CoV may infect cardiomyocytes directly. The hypothesis was confirmed in endomyocardial biopsies, autopsy, and in vitro studies.

Conclusions: The SARS-CoV-2 impacts several organs. The heart may be injured indirectly (hypoxia and cytokine storm) and directly (ACE2 present in the heart), which gives consequences in a clinical course. The direct injury was confirmed in a variety of ways.

Key words: SARS-CoV-2, COVID-19, cardiomyocyte

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Introduction

The history of coronavirus disease 2019 (COVID-19) has begun in Wuhan in China at the ending of 2019 where inhabitant doctors encountered pneumonia of unknown aetiology. On 8 January 2020, the first information that the novel coronavirus could be responsible for this unknown pneumonia appeared [1]. Two days later, the genome sequence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was obtained [2]. The World Health Organization declared a pandemic on 11 March 2020 [3]. Since the beginning of the pandemic, SARS-CoV-2 has spread all over the world. To date, there have been 160,040,871 confirmed cases of COVID-19, including 3,4494,758 deaths, reported to WHO [4]. The COVID-19 has varied clinical courses which can be divided into asymptomatic infection, mild upper respiratory tract illness, moderate upper respiratory tract illness, severe upper respiratory tract illness, and even death. The severity of clinical manifestation is associated with risk factors, such as gender, age whether the presence of comorbidities like diabetes, cardiovascular disease, and immunological disorders [5]. The main sign due to SARS-CoV-2 infection concerns the

respiratory tract, however, cardiovascular involvement has been noticed in several studies. Cardiac manifestations of COVID-19 include myocarditis, myocardial infarction, heart failure, acute coronary syndrome, arrhythmia. The previous coronavirus epidemic has also shown cardiovascular complications as hypotension, arrhythmia, and sudden cardiac death. Influenza virus affects the heart similarly to SARS-CoV-2 as well [6]. Furthermore, COVID-19 may cause renal dysfunction, gastrointestinal complications, liver dysfunction, neurological and haematological disorders, and vascular abnormalities. Extrapulmonary symptoms have been reported in previous coronavirus epidemics, which have been associated with increased mortality [7]. In this review, the authors describe cardiovascular involvements of SARS-CoV-2 infection, their possible pathogenesis, and signs and try to prove that SARS-CoV-2 can affect cardiomyocytes directly.

Materials and methods

A literature search was conducted using PubMed to identify relevant studies. Keywords used during searching are in Table 1. The date of publication was between 01.01.2020 and 19.05.2021, the texts written in English. The accepted studies included original research, review, and letters.

The primary inclusion criteria were autopsy, biopsy, and in vitro studies regarding the presence of viruses in heart tissue. Review articles were excluded. Review articles were used to write the introduction and propose mechanisms of myocardial damage during COVID-19. If the abstract did not contain any information about research virus genome into cardiomyocytes the articles were excluded as well.

To write the introduction the PubMed database was searched using “SARS-CoV-2” or “COVID-19”, and “Cardiomyocytes” keywords. Subsequently, the most interesting articles in the authors’ opinion were chosen.

Discussion

Viral infections are known to affect cardiac functions. The heart is the second target of SARS-CoV-2 after the lungs [8]. Nevertheless, the impact of Sars-CoV-2 has not been widely described yet.

During the Sars-CoV-2 pandemic, differential diagnosis of cardiovascular diseases (CVD) should include COVID-19 even in patients without typical symptoms of COVID-19 such as fever or cough [9]. Clinicians have known several signs of cardiac injury caused by SARS-CoV-2.

In several cases the electrocardiography test showed ST-segment elevation in lateral (I, aVL) leads [9–12], ST-segment depression in aVR [9, 10], T-wave inversion [10], low QRS voltage [9, 10, 12] and sinus tachycardia [8, 10]. The important fact is that these patients did not have a history of heart diseases [8, 10, 12]. Patients with CVD presented similar findings in ECG [9, 11] Moreover, new arrhythmias appeared in the COVID-positive patients [13, 14]. SARS-CoV-2 is proven to lead to arrhythmias significantly more frequently than other coronaviruses [15]. Diffuse elevation of ST-segment may appear in patients with myocarditis [16] which is a possible cause of cardiac injury connected with COVID-19 [17]. However, ST-segment elevations should be interpreted carefully because they may mimic a STEMI [11]. Cardiac arrhythmias such as AV block, VF, and VT are risk factors of death in patients with myocarditis [8].

One of the most valuable tests in describing COVID-19 impact on the heart is echocardiography. Features include severely depressed left ventricular ejection volume (LVEF) — about 30–40% [8–10, 12], hypokinesis of the left ventricle, heart apex [9–11] and right ventricle [9], enlarged left ventricle [8, 9], pulmonary hypertension [8], increased wall thickness [9, 10, 18] and pericardial effusion [10, 18]. The findings were not present in previous echocardiograms [11] and disappeared after recovery [9, 10, 12]

Table 1. Keywords used during the searching

Virus	COVID-19	Heart	Intervention
• SARS-CoV-2 • 2019-nCoV • Severe acute respiratory syndrome Coronavirus 2 • SARS Coronavirus 2 • SARS virus • Coronavirus	• COVID-19 • SARS-CoV-2 Infection • 2019-nCoV Infection • 2019-nCoV Disease • Pneumonia	• Heart • Cardiomyocyte • Myocarditis • Endocarditis • Pericarditis • Heart failure • Cardiac failure • Cardiogenic shock • Myocardial Ischemia • Myocardial infarction • ACE-2 • RAAS	• Endomyocardial biopsy • Cardiosphere • Heart slice • Engineered heart tissues • Autopsy • Echocardiography • Histopathology

Table 2. Information about original studies

Authors	Type of study	Methods used in studies
L. Bailey et al. [27]	Original research article	*In vitro*
D. Bojkova et al. [28]	Original research article	*In vitro*
A. Sharma et al. [29]	Original research article	*In vitro*
P. Wenzel et al. [30]	Research letter	Endomyocardial biopsy
F. Escher et al. [31]	Original research article	Endomyocardial biopsy
G. Tavazzi et al. [32]	Case report	Endomyocardial biopsy
Z. Varga et al [33]	Research letter	Autopsy
D. Lindner et al. [34]	Original research article	Autopsy
M. Pesaresi et al. [35]	Original research article	Autopsy
B. T. Bradley et al. [36]	Original research article	Autopsy
G. Pietro Bulfamante et al. [37]	Original research article	Autopsy

Table 3. TnT level impacts several laboratory parameters [13] which is shown in the table below

	Elevated TnT levels	Normal TnT levels
HDL	Did not differ	
LDL	Did not differ	
Triglyceride	higher	Lower
CRP	Significantly higher	Lower
Procalcitonin	Significantly higher	Lower
globulin	Significantly higher	Lower
CK-MB test	Significantly higher	Lower
Myoglobin	Significantly higher	Lower
NT-proBNP	higher	Lower
Lactic acid	higher	Lower
Respiratory dysfunction	More severe	Mild
Creatinine	higher	Lower
AST	higher	Lower
ALT	Did not differ	

ALT — alanine aminotransferase; AST — aspartate aminotransferase; CK-MB test — creatine kinase–myocardial band test; CRP — C-reactive protein; HDL — high-density lipoprotein; LDL — low-density lipoprotein; NT-proBNP — N-terminal pro-brain natriuretic peptide; TnT — troponin T

Magnetic resonance imaging (MRI) may be used to confirm myocarditis in patients with COVID-19 [11, 18]. Lake Louise Criteria include injury of myocardium on T1-weighted image with late gadolinium enhancement and myocardial oedema on T2-mapping sequences [17]. It may present biventricular myocardial oedema, biventricular hypokinesis, increased thickness of the wall, and decreased LVEF [10, 18]. MRI is an alternative test to myocardial biopsy [12].

Laboratory findings are important factors of heart injury. High-sensitivity troponin T (TnT) was often increased in patients with COVID-19 [10–13]. Myocardial injury, which is associated with a severe course of COVID-19, may lead to cardiac dysfunction and arrhythmias. TnT level above the 99th percentile confirms the diagnosis [19]. In the case series study conducted by T. Guo et al. [13], the myocardial injury appeared in 27.8% of patients with COVID-19. The scientists confirmed the relationship between TnT level in patients with myocardial injury and mortality. Out of patients without underlying cardiovascular diseases (CVD) died 7.62% with normal TnT levels and 37.5% with elevated TnTs. The highest mortality was in patients with elevated TnTs and underlying CVD (69.44%). In comparison to patients without TnT elevation, increased TnT level was associated with more frequent complications. The authors indicated possible

risk factors of TnT elevation — i.e. older age, men sex, comorbidities such as hypertension, cardiomyopathy, coronary disease, chronic obstructive pulmonary disease, chronic kidney disease [13].

Another predictive factor is the N-terminal pro-brain natriuretic peptide (NT-proBNP) [13] which may be used as a myocardial injury marker [8]. Elevated levels of NT-proBNP were observed in several patients with COVID-19-induced myocarditis [8, 10, 11, 13].

Speed of TnT and NT-proBNP concentration changes is also significant. Dynamic elevation appeared in patients who ultimately died while gradual changes were evident in those who survived. [13]

D-dimer may be elevated in patients with COVID-19 and be associated with multiorgan dysfunction [12]. A Higher D-dimer level is associated with a fatal course of COVID-19 and is taken as a death risk factor [20]. Scientists hypothesize that elevated D-dimer may be a result of microvascular thrombosis connected with inflammation [12]. Elevation of D-dimer was seen in several patients with cardiovascular complications of COVID-19 [9, 11–14]. A possible mechanism of heart injury connected with thrombosis is acute coronary syndrome [18].

Interleukin-6 is an inflammatory cytokine that marks cytokine storm [12, 18], as previously mentioned. Interleukin-6 levels were elevated in several cases [8, 9, 11, 12, 18] as well as C-reactive protein and ferritin which also appear in cytokine-induced cardiac injury [12, 18]. Moreover, the elevation of Interleukin-6 and ferritin levels were connected with a higher risk of death [20].

SARS-CoV-2, as well as SARS-CoV, infects cells thanks to the ability to bind to angiotensin-converting enzyme 2 (ACE2) [21]. SARS-CoV-2 similarly to other coronaviruses has 4 structural proteins (spike-S, envelope-E, membrane-M, nucleocapsid-N). Protein S acts the main role in infecting cells. When coronaviruses enter the target cell the protein S is cleaved at S1 and S2 sites. The fusion of viral and cellular membrane is possible thanks to subunit S2, which binds with ACE2 and it causes virus endocytosis. Protein S is divided by a cellular protease such as Transmembrane protease serine 2 (TMPRSS2), Cathepsin B (CTSB), Cathepsin L (CTSL), but only TMPRSS2 activity is necessary for viral spread and pathogenicity whereas cathepsin B/L activity is dispensable [22, 23]. ACE2 is present in every part of the human body. The heart is one of many organs with a high level of ACE2 expression. SARS-Cov-2 has a much bigger affinity to ACE2 than SARS. In one autopsy study, scientists discovered copies of the SARS-CoV-2 from 16 of 22 patients who died due to COVID-19. In the second study, a high viral load was detected among 39 people who died due to COVID-19. On the other hand, 38% of these patients had not SARS-CoV-2 in the heart. Such myocardial injury, reflected by an increased level of troponin is a risk factor for in-hospital mortality. The concentration of troponin correlates with mortality. Also, patients with an elevated level of troponin demonstrate a higher concentration of inflammatory markers. Myocarditis is a severe complication of COVID-19. Unlike SARS, SARS-CoV-2 creates a lymphocyte inflammatory response. Several cases of myocarditis imitate a heart attack. Except for direct impact on myocardiocytes, SARS-CoV-2 may affect endothelial cells [24]. SARS-CoV-2 attachment to ACE2 may lead to a change of ACE2 signalling pathway causing lung and heart injury [25]. During SARS-CoV-2 infection, the serum level of inflammatory mediators increases, such as C-reactive protein (CRP), ferritin, IL-6, IL-2, TNF, IL-10, lactic dehydrogenase (LDH). All of them may cause cytokine storms. The highest concentration of these markers occurs in severe upper respiratory tract illness and is related to immune response and inflammatory reactions which are dynamic [5].

Immune reactions can be divided into 3 phases: early infection, a pulmonary phase, and a severe hyper inflammation phase. Most patients do not develop a severe hyper inflammation phase.

Unfortunately, several per cent of sick people get into the most severe phase. During this phase, the human immune response develops cytokine storm which may injure organs different than lungs [26]. The respiratory tract is the main beneficiary of SARS-CoV-2 infections. Infection and immune response are responsible for pulmonary tissue injury, endothelial permeability, vasodilatation, and leukocyte recruitment which lead to lung damage and hypoxemia. Ten per cent of patients with lung damage and hypoxemia develop acute respiratory distress syndrome (ARDS) [6]. According to what is written several mechanisms responsible for the cardiac injury can be identified.

1. Indirect myocardial injury:
   a. Severe hypoxia caused by lung damage and acute respiratory distress syndrome — hypoxia is responsible for cardiac injury;
   b. Cytokine storm caused by the huge immune response — immune cells and immune proteins damage cardiomyocytes.
2. Direct myocardial injury caused by direct cardiomyocytes infection by SARS-CoV-2 — the heart has a high level of ACE2 expression making the heart a target for the virus.

Results

A. L. Bailey et al. [27] tested samples derived from 4 patients suffering from COVID-19 and myocarditis. These have been obtained thanks to autopsy and

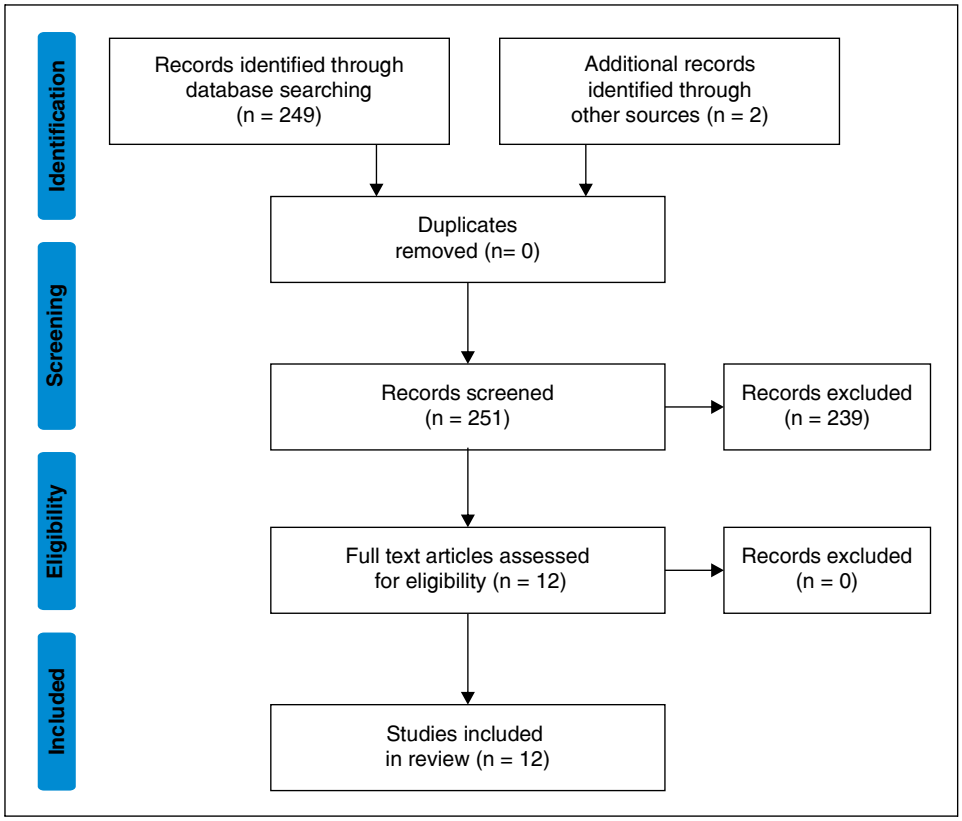

Figure 1. Flow diagram

endomyocardial biopsy. Coronary angiography was normal, with no signs of luminal stenosis or thrombosis what was confirmed in the autopsy. SARS-CoV-2 infection was detected by polymerase chain reaction (PCR) from nasopharyngeal samples. In postmortem microscopic examination of the left ventricle images of necrosis, degenerative vacuolization of cardiomyocyte cytoplasm, and mononuclear cell infiltration were obtained. Negative controls were autopsied heart samples from patients with metastases and neurodegenerative diseases. The researchers detected SARS-CoV-2 spike and nucleocapsid RNA in the samples of myocardium in every patient with COVID-19-related myocarditis. Viral transcripts were mainly detected within cells that were morphologically compatible with cardiomyocytes. These transcripts were also detected in lung airway epithelial cells, rarely in myocardial pericytes and adipocytes. The transcripts were located in cytoplasmic and perinuclear locations. The scientists checked the susceptibility of different myocardial cells to SARS-CoV-2 and if the virus can develop in myocardium bred combinations of fibroblasts, macrophages, and human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) with the virus. Only combinations with hiPSC-CMs cardiomyocytes showed the production of viruses and viral RNA. The researchers proved also that numerous genes in different cells were regulated upon SARS-CoV-2 infection. What is interesting, the cells which did not show viral replication also had changed gene expression. It may mean that the SARS-CoV-2 can change gene expression without direct viral infection. Overall, genes responsible for immune cell activation were upregulated and genes related to metabolism, mitochondrial function, muscle contraction showed downregulations. The scientists used engineered heart tissue (EHT) in their research. They wanted to check if EHTs mimics aspects of COVID-19 myocarditis. The examinations showed that SARS-CoV-2 infected EHTs showed a reduced speed of contraction and relaxation. This may be caused by left ventricular dysfunction reported during COVID-19-related myocarditis. Many mechanisms may be responsible for the decline in cardiac contractility, including cell death, sarcomere structure, immune responses, metabolism dysfunction. The researchers confirmed ACE2 mRNA expression in cardiac tissue.

D. Bojkova et al. [28] also cultured cells with SARS-CoV-2. The results show that the virus can undergo a complete replication cycle in human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs). Moreover, the conducted laboratory tests showed the activation of various signalling pathways, including interferon. 3D tissue demonstrated signs of tissue injury. The virus was found in cardiomyocytes using electron microscopy. The researchers performed an endomyo-

cardial biopsy in one patient with COVID-19 myocarditis symptoms and detected the virus by electron microscopy as well. The scientists also confirmed ACE2 mRNA expression in heart tissue. What is interesting, the mRNA expression was not present in undifferentiated iPS cells and ACE2 expression in cardiomyocytes was lower and localized more to perinuclear and cytoplasmic regions as against other TMPRSS2-positive cells.

Conclusions which are provided by research by A. Sharma et al. [29] are that SARS-CoV-2 may invade hiPSC-CMs *in vitro.* The virus enters the cells via ACE2. Cytopathic effect and replication of the virus trigger apoptosis of cardiomyocytes. hiPSCs were extracted from peripheral blood mononuclear cells and differentiated into cardiomyocytes. The differentiation was confirmed by levels of cardiac troponin T and αactinin. The hiPSC-CMs were exposed to SARS-CoV-2 infection for 72h. The results in infected cells were compared to the mock cells. Infected cells were positive for viral spike protein — it means that SARS-CoV-2 is can cardiomyocytes directly. The cytopathic effect occurred after 72h and was verified by cleaved caspase-3, which is an apoptosis marker, and by the double-stranded DNA (dsDNA). The infected cells were positive for both which suggests virus-induced apoptosis. After 72h of infection, the hiPSC-CMs discontinued beating, possibly because of cell-cell gap junctions disruption. What is interesting, previous treatment of infected cells with an ACE-2 antibody reduced the level of cleaved caspase-3 and inhibited apoptosis. However, the virus was still able to infect the cells — it may suggest variant ways of internalization.

P. Wenzel et al. [30] took an endomyocardial biopsy from two patients with COVID-19-related myocarditis symptoms and who were admitted to the hospital. Both patients had signs of COVID-19 up to 4 weeks before the biopsy. SARS-CoV-2, influenza A and B, metapneumovirus, parainfluenza virus, respiratory syncytial virus were excluded using laboratory tests on the day of admission. Pathomorphological tests revealed myocardial inflammation with the presence of lymphocytes (mainly cytotoxic T cells) and macrophages without cardiomyocyte necrosis. Furthermore, the real-time polymerase chain reaction (RT-PCR) assay confirmed the presence of the SARS-CoV-2 genome in biopsy samples. Lower or negative viral load for CMV, EBV, adenovirus, parvovirus B19, HHV6, and Coxsackie virus were also detected. During hospitalization, IgG antibodies for SARS-CoV-2 were detected.

F. Escher et al. [31] obtained endomyocardial biopsies of 104 patients. Every patient had angiography to exclude coronary artery disease. Most of the patients were men (76%), mean age was 57.9 ± 16,4, the diagnosis: active myocarditis 13.4%, inflammatory cardiomyopathy 32.6%, borderline myocarditis 2.9%, dilated cardiomyopathy 41.3%, amyloidosis 9.6%. SARS-CoV-2 genome was detected in the myocardial biopsy in 5 of 104 patients (RT-PCR). Parvovirus B19 genome was detected in 70 of 104 patients, HHV6 genome in 8 patients, EBV in 4 patients, and Coxsackie virus in 1 patient. In SARS-CoV-2 positive samples, only parvovirus B19 was detectable. Histological images of SARS-CoV-2-positive samples showed: 1 active myocarditis (according to the Dallas criteria), 1 borderline myocarditis (according to the Dallas criteria), and 3 inflammatory cardiomyopathies. An inflammatory infiltration consisting of macrophages, T lymphocytes, and memory T cells (CD45R0) was found in 4 of 5 SARS-CoV-2 positive samples. All of these 5 patients had elevated numbers of cell adhesion molecules (CD54/ICAM-1).

The case report performed by G. Tavazzi et al. [32] has proved myocardial localization of the SARS-CoV-2. Scientists have described a case of a 69-year-old patient who suffered from COVID-19. The clinical manifestation suggested severe acute myocarditis. An endomyocardial biopsy was performed. The biopsy demonstrated CD68-positive macrophages with damaged membrane, particles of the SARS-CoV-2 virus in interstitial cells which lost cytoplasmatic membrane integrity; and low-grade myocardial inflammation. Viral particles were not observed in endothelia and myocytes, however, cardiomyocytes showed focal lysis of myofibrils and lipid droplets. The presence of macrophages may suggest the migration of infected macrophages from the respiratory tract to extra-pulmonary tissues. The case report may be taken as evidence of SARS-CoV-2-connected direct heart damage and also damage associated with the infected macrophages migration in patients with COVID-19 presenting clinical manifestation of acute myocarditis.

Z. Varga et al. [33] proved that SARS-CoV-2 can infect endothelial cells and cause endothelial inflammation as well. The findings revealed the presence of SARS-CoV-2 elements within endothelial cells, inflammatory infiltration, apoptosis, and pyroptosis. COVID-19 endotheliitis may be responsible for the systemic impaired microcirculatory function.

D. Lindner et al. [34] have conducted an autopsy study that included patients fatal because of COVID-19 (confirmed *post mortem* by RT-PCR). 39 patients in a median age of 85 were included in the study. Almost 90% (35 of 39) of them died because of pneumonia. The cause of death of the rest of the patients was unknown. Myocarditis wasn't documented among the group (according to the Dallas criteria). Some of the patients suffered from comorbidities, including coronary artery disease, hypertension, and diabetes. 2–4 days after death, samples of cardiac tissue were collected. Scientists performed quantitative

RT-PCR for SARS-CoV-2. For paraffin sections *in situ* hybridization was conducted. SARS-CoV-2 was found in 61.5% (24 of 39) patients: in the 8 cases virus load was below 1000 copies per microgram RNA and in the 16 cases virus load was more than 1000 copies. In 5 patients who had the highest virus load, replication of SARS-CoV-2 was detected. In situ hybridization showed the localization of SARS-CoV-2 — interstitial cells and macrophages invading the myocardium.

M. Pesaresi et al. [35] have used Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy to identify the SARS-CoV-2 particles in samples from 3 organs: heart, lung, and kidney. The samples were collected from 2 dead patients with COVID-19 (confirmed *post mortem* by RT-PCR). In one of the cases, viral particles were present in the cardiomyocytes adjoining myofibrils. The finding was observed in TEM and then confirmed by SEM analysis. TEM showed dissimilar morphology of the viral particles, which may suggest a different state of viral replication or tissue damage. Moreover, the SARS-COV-2 virus was also identified in the heart using RT-PCR.

B.T. Bradley et al. [36] have conducted a post-mortem examination on 14 patients who died with COVID-19. All patients suffered from comorbidities and were in the median age of 73.5 years. Half of the patients were examined by *in-situ* dissection and the rest of them by standard autopsy procedure. The scientists used light and electron microscopy, immunohistochemistry, and quantitative RT-PCR. SARS-CoV-2 RNA was detected in cardiac tissue in 2 patients. In one of them, lymphocytic myocarditis was detected. The sample of myocardial tissue taken from the patient was positive for SARS-CoV-2 RNA by PCR, however, immunohistochemistry and electron microscopy did not confirm the finding. The scientists made a hypothesis that the presence of viral RNA in several extra-pulmonary tissues may be a result of contamination by the virus which is circulating.

G. Pietro Bulfamante et al. [37] conducted 6 autopsies — 5 males and 1 female with COVID-19. The mean age was 59.5. Every patient died of respiratory failure due to COVID-19 and did not show clinical signs of left ventricle damage. PCR assay of heart tissue samples confirmed the presence of the SARS-CoV-2 genome. The viral genome was found in lung extracts as well. The tests were negative in healthy control. In the light microscopy, researchers confirmed the presence of SARS-CoV-2 nucleoprotein and spike protein. The viral proteins were discovered within cardiomyocytes and in cytosolic areas of lipofuscin. Thanks to the RNAScope assay, scientists were able to determine, if the SARS-CoV-2 is transcriptionally active in cardiomyocytes. The test proved that the virus can replicate in cardiomyocytes. In this study, in contrast to the study by Z. Varga et al., it was not shown endothelial cytopathic effects nor endotheliitis. Electron microscopy corroborated evidence on the presence of SARS-CoV-2 in cardiomyocytes.

Conclusion

The study limitation is that one cannot exclude an important role for hypoxemia, thrombosis, and inflammation. The research material was not big, these studies were performed on a small number of samples. Moreover, the COVID-19 pandemic does not last long. The longer it lasts, the bigger number of research will be conducted. Also, long-term effects are probable to be seen further in the future.

Fortunately, the studies were of high quality, which gives greater confidence that the pieces of evidence are authentic. Cited examples prove that SARS-CoV-2 may affect cardiomyocytes directly.

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VIA MEDICA

REVIEW ARTICLE

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Liquid biopsy in targeting gene polymorphism related to the response within immunocheckpoint inhibitors therapeutic regimen

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ABSTRACT

Immunotherapy belongs to the group of targeted therapies; it is based on natural immune mechanisms which axis can be promoted or blocked at appropriate points. Breast cancer is the world's most common cancer among women and in March 2019 the FDA approved the first immunopharmaceutical Atezolizumab, for the treatment of breast cancer. So far, the only registered marker for classification for checkpoint inhibitor therapy has been the presence of PD-L1 receptor expression in tumour cells.

A comprehensive search of the literature to elucidate the correlation between PD-1/PD-L1 single nucleotide polymorphism (SNP) and cancer, especially breast cancer or other diseases susceptibility and PD-1/PD-L1 expression.

Seven susceptibility loci was considered: rs41386349, rs7421861, rs36084323, rs11568821, rs2227981, rs10204525, rs2227982. Three of them may be taken into account as potentially helpful in breast cancer patient treatment tailoring: rs36084323, rs2227981, rs2227982.

Key words: PD-1, PD-L1, immunocheckpoint inhibition, immunogenetics, single nucleotide polymorphism, breast cancer, liquid biopsy

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Introduction

Liquid biopsy (LB) is the most superior diagnostic method for the determination of the tumour signature in blood [1].

Breast cancer is the most common female cancer worldwide. Only in 2018, it reached the top rate among 25 cancers for 40 countries in Europe (523,000 cases, within 3.91 million new cases of cancer per year in both genders) [2]. In simplified words, the prognosis depends on the tumour type (lumina, basal-like, triple-negative) and its stage (histological grade, axillary lymph node involvement and distant metastasis) at the time of diagnosis. Despite low immunogenicity in breast cancer [3], there is a rising interest in anti-programmed dead receptor 1 and programmed dead ligand 1 (anti-PD1/anti-PD-L1) treatment in a special subset of triple-negative breast cancer (TNBC) and human epidermal growth factor receptor 2 positive (HER-2 positive) [4–6]. In March 2019, FDA approved Atezolimumab (Tecentriq) for adult patients with unresectable, locally advanced or metastatic triple-negative breast cancer (TNBC) the tumours of which express PD-L1 as determined by an FDA-approved test [7].

PD-1 firstly identified by Ishida in 1992 [8] is a transmembrane glycoprotein located in tumour cells, cytotoxic T-lymphocytes, natural killer (NK), B-lymphocytes, monocytes and other tissues infiltrating lymphocytes (TILs) [9]. PD-1 receptors linked with their ligands PD-L1 identified in 1999 [10] on an antigen presenting cell (APC) inhibit antitumour activity. PD-1/ PD-L1 axis block activation of cytotoxic T-cell. This blockade plays a critical role in tumour resistance mechanisms. The suspension of PD-1/PD-L1 axis can restore T-cell and promote immunity against tumour. Antitumour effect exerted via immunocheckpoint inhibitors (Atezolizumab,

Avelumab, Nivolumab, Pembrolizumab) is based on the promotion of a cell-mediated immune response. To date, for the purpose of pretreatment qualification, FDA-approved only the PD-L1 expression level, as determined by an IHC based on the VENTANA assai [7] for a primary or metastatic tissue source. It is still unclear if PD-1(PDCD1)/PD-L1(Pdcd1l2) gene single nucleotide polymorphism (SNP) could be a predictor for immunocheckpoint inhibition treatment, especially in the context of breast cancer risk and prognosis [11, 12]. The frequencies of rs36084323 GG genotype and rs2227981 CT genotype can affect the susceptibility of breast cancer [12]. The human gene encoding PD-1 is located on 2q37.3 [12].

Programmed death-1 (costimulatory molecule, PD-1, CD279) gene polymorphism/ *PDCD1* (coding for programmed cell death-1)

Loci of *PD-1* gene might be a potential biomarker for predicting susceptibility to therapeutic markers for cancer treatment. However, the identification of biomarkers able to predict a clinical benefit of PD-1/PD-L1 inhibitors seems to be a challenge. To apply liquid biopsy into minimal invasive stratification, single nucleotide polymorphism (SNP) should be designated. Seven susceptibility loci for immunotherapy effect in breast cancer patients can be considered: rs41386349, *PDCD1* (rs7421861), PD-1.1 (rs36084323), PDCD-1.3 (rs11568821), PDCD-1.5 (rs2227981), PDCD-1.6(rs10204525), PDCD-1.9 (rs2227982). Previous studies have discussed methods and combined biomarkers from different points of view: tumour mutation and neoantigens burden as well as some oncogene mutations like EGFR, ALK, KRAS and STK11 [13].

However, PD-L1 expression measured using the IHC is the only FDA-approved test. Lower PD-1 expression has been found to be associated with G of rs10204525 [14].

Certain single nucleotide polymorphisms (SNPs) in genes such as PDCD1 (coding for programmed cell death)

Rs41386349, this SNP was previously considered as a hot point for Grave's disease and Addison's disease [15], rheumatoid arthritis (RA) [16], Kawasaki disease [17], multiple myeloma [18] and variant A for rs41386349 affects susceptibility of chronic HBV [19]. Whereas, only PD-L1 rs1411262C/T gene polymorphism in the case of Grave's disease and Addison's disease [15], increased rheumatoid factor seropositivity [16] and a higher probability of Kawasaki disease [17] was discovered. The multiple myeloma occurs more often with a PDCD1 GCC/GCC haplotype (rs36084323/rs41386349/rs2227982) variant [18].

PDCD1 (rs7421861) has been studied in case of esophageal cancer [20] and chronic HBV infection, its oncogenicity [19] as well as RA [16]. *PDCD1* gene polymorphisms influence the severity of a disease and are associated with distant metastasis, higher TNM stage, higher PD-1 gene and plasma levels in esophageal cancer patients and risk of esophageal cancer in general [20]. This gene variability may play a role in hepatocarcinogenesis caused by chronic HBV infection [19]. Tseng CC et al. have found higher expression of PDCD1 in RA compared to controls. However, PDCD1 gene polymorphisms were similar in this study [16].

PD-1.1 (rs36084323) has been considered in a case of breast cancer [21], epithelial ovarian cancer [22] esophageal cancer [20], NSCLC [23], overall cancer risk [24], RA [16] pregnancy losses [25] and aplastic anemia [26]. Therefore, significantly lower frequencies of PD-1.1 GG have been presented in women with breast cancer [21]. Rs36084323 polymorphism predicts epithelial ovarian cancer development [22]. No association with esophageal cancer metastasis [20], no differences in the distribution of the PD-1.1 alleles in NSCLC [23], no association between overall cancer risk have been obtained [24]. For autoimmune diseases, such as RA, rs36084323 decreased an inadequate response to conventional synthetic disease-modifying antirheumatic drugs [16]. Hayashi Y. et al. have evaluated the association of genetic variants of PD1 with recurrent pregnancy loss with significantly higher frequencies of rs36084323 in women with two or more pregnancy losses [25]. Polymorphism of PD-1.1 has been rare in the case of patients with aplastic anaemia [26].

PDCD-1.3 (rs11568821) gene polymorphism has been considered to be related to an overall cancer risk [24, 27] and SLE [28]. Researchers have found a decreased overall cancer risk in case of PDCD-1.3, variant TC [24, 27]. PD1.3GG genotype and G allele have been significantly more frequent in SLE patients [28]. Prokunina et al. have found that this genetic variant would affect PD-1 mRNA level by changing the binding affinity of RUNX (a transcriptional factor of PD-1) [29].

PDCD-1.5 (rs2227981) variant TT has been associated with a decreased risk of cancer [24, 27]. CT genotype has been significantly lower in breast cancer women [21], whereas an increase in the risk of cervical [30], T-allele of rs2227981 gene polymorphism reduced risk of epithelial ovarian cancer [22], lung [31], gastric [32], colon [33], thyroid cancers [34] and CC variant was significantly more frequent in patients suffering from SLE [28].

PDCD-1.6 (rs10204525) has been explored with respect to the associations between *PD-1.6* gene polymorphisms and esophageal cancer [20, 35], overall cancer risk [24], aplastic anemia [26], juvenile idiopathic arthritis [36] and HBV infection [37]. GG genotype of rs10204525 polymorphism has increased the risk of esophageal cancer in contrast to the more common AA genotype which was associated with distant metastasis and higher PD-1 gene and plasma levels [20, 35]. Even in some other work, there is no relation between this SNP and overall cancer risk [24]. Furthermore, some variants of rs10204525 have been linked to aplastic anaemia [26], while the CT variant was associated with juvenile idiopathic arthritis and linked to Anti-CCP antibodies, RF, and the CHAQ score [36]. Next, subjects carrying minor allele G had a significantly decreased risk of getting infected with HBV and were associated with lower PD-1 expression [14], variant AA of PD-1 rs10204525 was prone to higher PD-1 expressions in tumour tissues, peri-tumour tissues and cirrhotic tissues [37]. GG genotype variant altered tumour necrosis factor-α (TNF-α) to increase levels in HBV patients [38].

PDCD-1.9 (rs2227982) has been evaluated in terms of breast cancer [39], ovarian cancer [40], ankylosing spondylitis [41], multiple myeloma [18] and chronic HBV infection and its oncogenity[19]. This polymorphism increases the probability and severity of the disease in case of ankylosing spondylitis and breast cancer [39, 41]. Simultaneously, the same reserchers have found that C > T variant reduces the risk of breast cancer [39] and raises the risk of ovarian cancer [40]. CC genotype variant was significantly correlated with a higher frequency of osteolysis [18]. This gene polymorphism may have a significant influence on hepatocarcinogenesis [19]. However, CC of rs2227982 variant had a shielding role in HBV infection [14].

Conclusions

In summary, there are no reports on PD-1 gene polymorphism and its association with immunotherapy favourable outcome susceptibility. Seven susceptibility loci was considered: rs41386349, rs7421861, rs36084323, rs11568821, rs2227981, rs10204525, rs2227982. The important question for developing next-generation anti-PD-1/PD-L1 antibodies is whether the therapeutic effect can be predicted with SNP analysis using liquid biopsy. Nevertheless, three SNP may be taken into account in the breast cancer patient: rs36084323, rs2227981, rs2227982.

Conflict of interest: *The authors have no conflicts of interest to declare regarding this study*.

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VIA MEDICA

REVIEW ARTICLE

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Health in the context of psychological flexibility and acceptance and commitment therapy

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ABSTRACT

Losing health is associated with an imbalance in one or more areas that are important to an individual. The spectacular achievements of modern medicine have made people believe that the only source of health is the treatment and reduction of symptoms. The ability to function in conditions of unavoidable discomfort can bring back an individual's loss of balance. The article aims to present the model of Psychological Flexibility and its relationship with selected health aspects of an individual. According to the statement of the founders of Acceptance and Commitment Therapy (ACT), suffering in various forms is an integral part of human life. In situations of discomfort that cannot be avoided, and with which an individual must learn to function on a daily basis over a longer period of time, ACT proposes the development of Psychological Flexibility, which strengthens the psyche and body's immune resistance. The Covid-19 pandemic, which has been going on for a year, has disrupted the macro balance. At the micro-scale, this balance can be disturbed by chronic disease states such as pain, anxiety and depression. The above conditions reduce the quality of life and health. Psychological Flexibility shapes a set of skills that improve the quality of life and also affect balance despite perceived discomfort. Psychological Flexibility is a base that has a wide application and significance for the quality of life and health. Its higher resources help an individual live a rich, worthwhile life despite suffering from pain, anxiety or pandemic stress.

Key words: psychological flexibility, pain, anxiety, depression, pandemic, Covid-19

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Introduction

Health is one of the most important values for individuals and the whole society [1]. There are over a hundred definitions of health [2]. They are often based on various, separately treated planes: biological, psychological, or social. Health can be presented as the absence of disease and suffering; as biopsychosocial well-being; as a state of the balance; as a function; as a social role; as the ability to adapt; or as a value norm or asset [3]. These planes are linked by the holistic, still valid, definition of the World Health Organization (WHO), according to which health is perceived as "a state of total physical, mental and social well-being" [4].

Regardless of the adopted definition, loss of health is associated with an imbalance (distraction of well-being) in one or more areas that are important for an individual. This, in turn, makes it difficult, or impossible, to function, and deteriorates the quality of life [5]. Western civilization glorifies the absence of physical and mental discomfort. The spectacular achievements of modern medicine have made people believe that the source of health is treatment and the reduction of symptoms [6]. In the field of health psychology, prophylaxis and health promotion, effective methods are being sought, which are based on scientific evidence, and which improve the health level of individuals and the entire society [7].

Scientific research shows that Acceptance and Commitment Therapy (ACT) is effective in promoting healthy behavior, while at the same time improving the quality of life and mental well-being of cardiological, cancer, and irritable bowel syndrome patients who suffer from anxiety and affective disorders [8–13].

ACT belongs to the "third wave" of the Cognitive Behavioral Therapy (CBT) trend. Both CBT and ACT base their effectiveness on scientific evidence [14]. According

to the statement of the founders of Acceptance and Commitment Therapy, suffering, disease, pain, loss, grief, fear and disappointment are inseparable elements of human life [15]. However, they do not have to interfere with an individual's quality of life if they learn to respond to them with awareness. ACT teaches a person to lead an attentive and conscious life and changes the relationships of an individual with undesirable symptoms in such a way that their occurrence does not interfere with leading a life that is consistent with values. The "side effect of therapy" is often the reduction of symptoms. In turn, the main goal of ACT is to increase PF — a construct that is responsible for the ability of an individual to act in line with values, regardless of the experienced physical or mental discomfort (e.g. pain, anxiety) [15, 16]. PF negatively correlates with such personality trait as neuroticism, whereas it positively correlates with an openness to experience and self-control. PF influences a person's health globally and helps to smoothly maintain a balance between important areas in their life, which appear to be the specific mental resilience of an individual [17]. Therefore, PF seems to be important in the context of an individual's resource in situations of discomfort that cannot be avoided, and also in which one has to learn to function on a daily basis over a longer period of time.

This article presents the model of Psychological Flexibility (PF) and its relationship and protective effect on selected health aspects of an individual. The theoretical assumptions of ACT and the PF model are presented on the basis of leading references in this field [15, 16]. Additionally, the relationship between PF and chronic pain, anxiety and depression, as well as stress caused by the Covid-19 pandemic, was analyzed. Scientific databases were reviewed using the following keywords: psychological flexibility, pain, anxiety, depression, stress, pandemic, Covid-19.

Psychological flexibility and acceptance and commitment therapy

PF is a construct that is responsible for the ability of an individual to freely choose an action, the direction of which is compatible with the goals and values of the individual, regardless of the experienced difficult thoughts, emotions and sensations. PF has developed in the area of Contextual Behavioral Science (CBS), the source of which has its origin in the classical behavioral analysis [18]. The main goal of CBS is the scientific prediction of behavior in a specific context and its modification [19]. Through the prism of PF, thoughts, emotions and experiences do not have a good or bad label, with their evaluation taking place in a specific context for a given situation. The lower the PF, the more the person, under the influence of an experienced discomfort (e.g. anxiety or pain) reduces (slows down) their actions (while losing the sense of their meaning) [15]. The higher the level of PF, the more an individual can consciously choose a course of action that is consistent with their goals and life values, regardless of their emerging thoughts, emotions or impressions. Moreover, they can persevere in this action [15].

Participation in Acceptance and Commitment Therapy increases and strengthens PF. Through its motivational nature, ACT helps to make an individual aware of which values mean a lot to them and also provides direction to their life [16]. It teaches careful observation and acceptance of one's emotions, thoughts and feelings, which in turn improves the quality of life and functioning of an individual with psychiatric, somatic or social problems [20–22]. Meta-analyzes have shown that ACT has a greater potential to induce psychological benefits and effectiveness in the long term perspective when compared to classical CBT methods [23, 24].

The PF model consists of six processes, such as acceptance, cognitive defusion, flexible focus on the present moment, self-as-context, values, and engaged action. They are responsible for shaping PF and taking action in accordance with the values adopted in life. In turn, their opposition, i.e., avoidance of experience, cognitive fusion, lack of flexible attention, the conceptualized self, the lack of awareness of values, passivity, impulsiveness, and persistent avoidance are responsible for adaptive abilities [15]. PF shapes psychological skills or a set of skills that have a wide application and go beyond a single state of mental or physical health [22]. For example, it appears as a mechanism that explains the influence of personality on the well-being of an individual [25]. The correlation of PF with personality traits, which influence the quality of life, shows the possibility of developing adaptive resources along with the development of PF, regardless of the basic structure of personality [26]. A higher PF is associated with a better mental well-being of obese people, with a higher quality of life in people with type I diabetes, and it also positively correlates with a higher mental resistance of people after trauma [27–29].

Psychological flexibility versus pain

The Central Statistical Office (CSO) in Poland [30] lists among the six most common chronic diseases/health diseases: chronic back, neck and joint pain. Chronic pain can be caused by damage to the nervous system, undetectable pathology, or psychogenic pain. This type of pain proves the changes that have taken place in the body and becomes a disease that is often very difficult to treat [31]. Chronic pain affects all areas of an individual's life. It may contribute to the development of depression [32]. If it is not possible to eliminate it,

an important element of functioning will be adapting to bothersome symptoms. Patients report a slowdown in life as a consequence of chronic pain. Pain accompanied with anxiety, as a variable closely related to fear and avoidance, has a significantly detrimental effect on the quality of life of patients [33]. Some data show that it is not the level of pain, but the attitude towards it, that affects the functioning of a person [34].

The chronic low back pain (CLBP) model assumes that fear of pain is related to avoiding painful movements. It was originally developed to explain the transformation of acute back pain into chronic pain [35]. The CLBP model is currently used for research that links the subject of anxiety and pain with the development of disabilities of a broader scope than just chronic back pain [36].

A study on a sample of 252 people with chronic pain showed that PF is an important mediating factor in the relationship between symptoms (pain and anxiety intensity) and the functioning of the respondents. People with lower PF showed sickness absenteeism much more often than people with higher PF [37]. Similar results were obtained in other studies concerning the relationship between PF and the adaptive functioning of people with chronic pain. Higher PF, reflected in acceptance, mindfulness, cognitive defusion, and acting on values, was responsible for more frequent use of health care and more frequent undertaking of jobs [38]. The studies of Rhodes [39] found that chronic pain patients with a higher PF used fewer opioids. Various PF measures have proved to be significant mediators of therapeutic treatment in patients with chronic pain [40]. The participation of patients with chronic pain in ACT increases their PF and acceptance of pain, improves their daily functioning, and reduces their level of depression [41].

Psychological flexibility versus anxiety and depression

In Poland, in 2018, neurotic disorders (approx. 30%) and affective disorders (approx. 20%) were ranked in the first two places among people suffering from mental and drug-free behavior disorders [42]. Coexisting anxiety and depression are responsible for the mental condition of patients - exacerbating the chronicity and severity of any psychiatric and somatic diagnosis, reducing the quality of life, hindering professional development, and increasing the risk of suicide [43–45]. In a longitudinal study, anxiety predicted subsequent depression, which was measured after 12–14 years [45].

There is evidence that higher PF and adaptive emotional schemas show a negative correlation with anxiety [46]. A study on a group of HIV-infected people indicates that higher PF is responsible for lower mental health rates (lower levels of anxiety and depression), as well as a higher quality of life [47]. Higher PF scores were reliable predictors of mental health (lower anxiety and depression) in homosexual men who underwent screening for anal cancer risk. It should be added, however, that the relationship between PF and mental health in this study was mediated by Difficulty Identifying and Describing Feelings (DIDF). Low levels of PF may increase DIDF, and this, in turn, leads to higher levels of anxiety and depression [48].

A study by Masuda and Tully [49] indicated that lower PF in the American student population was associated with higher levels of depression and anxiety. Psychological Inflexibility (PI) in Turkish students partially mediates in the relationship between anxiety against negative evaluation and psychological susceptibility associated with a wide range of mental disorders (with the dominant style of avoidance reactions) [50]. The anxiety of public speaking reported by the student correlated with lower PF in the domains of openness to experience and higher cognitive fusion [51].

Psychological flexibility versus functioning during the Covid-19 pandemic

In Poland, more than two and a half million infections and over 60,000 deaths have been recorded since the beginning of the Covid-19 pandemic (from March 4, 2020, to this day) [52]. Undoubtedly, the Covid-19 pandemic has had a negative impact on the mental health of citizens, which is measured by the level of anxiety and depression [53, 54]. Research indicates PF as an important factor in mental resilience to stress caused by the current Covid-19 pandemic. PF negatively correlates with anxiety, depression, insomnia and suffering from the pandemic, and positively correlates with well-being and coping with avoidance [55, 56]. A lack of PF increases the risk of suicide in the pandemic era [57], whereas a greater openness to experience, and behavioral awareness (as measures of PF) were associated with lower general and peri-traumatic distress in the context of the pandemic [58].

A study conducted during the lockdown in Italy on a group of 1,035 adults showed that global PF and its four subtypes (self-as-context, defusion, values, and engaged action) reduced the destructive impact of Covid-19 risk factors (such as duration of isolation, increase in domestic violence and unhealthy behavior, and infection of relatives with Covid-19) on mental health [59]. A longitudinal study based on 3 measurements and conducted during the lockdown in Spain showed that the lack of PF in the first measurement indirectly predicted symptoms of mental health in the last measurement through autoregressive parallel paths and directly in the same measurement [60]. A study of patients with chronic pain during the Covid-19 pandemic

showed that PF processes such as pain acceptance, self-as-context, and engaged action can play a protective role in demonstrating anxiety and avoidance [61].

An American study of family functioning during pandemic stress found that a lack of parental PF was associated with depression, Covid-19 stress, discord between family members, toxic parenting, and greater suffering for both parents and their children. Similarly, higher PF measures were associated with greater family cohesion and the use of constructive parenting strategies such as inductive, democratic behavior and positive and supportive parenting practices [62].

Conclusions

PF is a factor of mental resilience that has wide applications and is relevant to the quality of life and health. Its higher resources help an individual live a rich, worthwhile life despite suffering from pain, anxiety or pandemic stress. The strength of this construct is that it can be developed and strengthened within ACT, which in turn may affect all areas of mental, physical and social health, including self-care and raising children [21, 41, 63–65]. The advantage of ACT is its empirical, scientific verifiability. ACT can be used by psychologists, psychotherapists, doctors, and other people involved in improving the health of individuals [22]. Scientific and diagnostic work may be facilitated by the availability of free, reliable research tools that are validated on the Polish population [66, 67]. Therefore, it is worth considering the study of PF on the Polish population and the use of ACT intervention techniques that can improve the health condition of Polish society in times of a pandemic.

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REVIEW ARTICLE

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Novel vitamin D_3-hydroxyderivatives as candidates for the therapy against skin-aging and photo-aging: bioinformatical analysis

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ABSTRACT

Vitamin D3 acts through its most active form, calcitriol, 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3] as agonist of one of the receptors involved in this ligand action, vitamin D receptor (VDR), which is also a transcription factor. Numerous modifications of calcitriol at its side-chain, C-ring, A-ring, triene system, alone or in combination, as well as nonsteroidal mimics provided new VDR agonists and some antagonists with biological activity and possible therapeutical potential. Some of the D3 metabolites, including 20,23(OH)2D3 and 20(OH)D3 are able to inhibit RORE-mediated transactivation, as well as the interaction between the RORα/γ ligand-binding domain (LBD) with an LXXLL coactivator peptide. Our analysis of recently reported microarray data on vitamin D3 (D3) induced changes in cultured human keratinocytes indicated that D3 hydroxyderivatives stimulate the expression of genes involved in anti-aging activities. Furthermore, we noted upregulation of the kallikrein gene family by 1,25(OH)2D3 after 24-hour treatment, including stimulation of KLK6, KLK13, KLK3, KLK9, KLK5, KLK7, and KLK10. Also, after 6-hour incubation with 1,25(OH)2D3, the upregulation of KLK6, KLK13, and KLK3 was seen. Interestingly, ACEIs administered to hypertensive rats doubled the lifespan of these animals. In humans, ACEIs prevent hallmarks of aging, such as organ fibrosis and cardiac hypertrophy. We noted also that vitamin D3-hydroxyderivatives act against oxidative stress through upregulation of thioredoxin reductase (TXNRD1) and heme reductase-1 (HMOX-1) gene expression in keratinocytes treated for 24h. Another mechanism of anti-aging properties of inverse agonist RORa/γ is the resolution of inflammation caused by T helper (Th17) lymphocytes and switching the immune response into T regulatory (Treg) lymphocytes activation, with silencing of the inflammation state and reducing the inflammation process. The gene connected with inflammatory response, AKR1C3 (which encodes prostaglandin F synthase) is also strongly downregulated by 20,23(OH)2D3 in keratinocytes after incubation for 24 h. We suggest that vitamin D3 analogs, such as 20,23(OH)2D3, 1,25(OH)2D3, and 20(OH)D3 may have anti-aging properties through action on different pathways connected with DNA repair.

Key words: skin aging, photo-aging, ceramide, melanosis, atopic dermatitis, vitamin D_3-hydroxyderivatives

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Introduction

An immune profile during aging was established to uderstnad better which compounds may be beneficial for promoting healthy aging. T cells from the older group of patients (60-year-old people) produced more cytokines associated with Th17 in in-vitro studies, a group of T lymphocytes, which promotes inflammation occurring in many diseases, including cancer cardiac disease, and neurodegeneration [1, 2].

Some of the vitamin D_3 analogs with anti-inflammatory properties that can modulate the immune response, including $20,23(OH)_2D_3$ and $20(OH)D_3$, are able to inhibit the interaction between an LBD of rexinoid orphan receptor a/γ (RORa/γ) with an LXXLL coactivator peptide and can act as inverse agonists of RORα/γ [3–14].

In the current paper, we describe the impact of vitamin D_3-hydroxyderivatives on the inhibition of inflammaging and a decrease of gene expression associated with the aging process.

Structure and chemical properties of vitamin D analogs

Vitamin D_3 acts through its active form, calcitriol, 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] as an agonist of one of the receptors involved in this ligand action, vitamin D receptor (VDR), which is also a transcription factor [15]. Numerous modifications of calcitriol at its side-chain, C-ring, A-ring, triene system, alone or in combination, as well as nonsteroidal mimics, provided new VDR agonists and some antagonists with biological activity and a possible therapeutical potential [16]. About 150 crystal structures of VDR's ligand-binding domain were discovered with various vitamin D derivatives, which allows molecular studies of the dependences between the structure of the particular compound and their mechanism of action (Fig. 1) [15]. The VDR is a nuclear receptor and an endocrine receptor. The mechanisms of its action are comparable to those connected with receptors for estrogen and glucocorticoids. VDR's ligand-binding domain (LBD) is considered as structurally conserved and comprises 11–15 α-helices, varies solved crystal complexes. The LBD structure depends on the folding of an intrinsically disordered region included between α-helices H1 and H3 and a presence of the helix HX between α-helices H11 and H1228 [17, 18]. The lower part of the LBD contains a ligand-binding pocket (LBP), which has a structure of a cavity of ~700 Å^3 volume formed by some of the 40 mostly nonpolar amino acids [19–21]. Within the LBP, the three pairs of polar amino acids fix by hydrogen bonds each one of the three particulars OH groups (at C-1α, C-25, C-3β position) of $1,25(OH)_2D_3$. The group of 1α-OH interacts with helix H5 (S278), as well as helix H1 (Y143), and the 3β-OH group links helix H3 (S237) and helix H5 (R274). The 25-OH group interacts with H305 (the loop located between helices H7 and H6) and H397 (in helix H11) [22, 23].

The ligands of VDR have induced a conformational shift to the LBD, which allows the replacement of co-repressor molecules by coactivator proteins. This interaction is responsible for the effect connected with ligand binding that induces a different protein-protein interaction profile of the VDR receptor [24]. This finding is also connected with changes in the gene expression profile in many biological processes including aging and is connected also with the strength of biomedical activity. The agonists of VDR cause an efficient co-repressors dissociation from the LBD and are responsible for specific binding of the mediator complex and coactivators. Additionally, coactivators attract the chromatin-modifying enzymes, which erase write or read post-translational modifications of histones, such as with methyl and acetyl groups, changing histone proteins of nucleosomes within the genomic VDR binding sites [24].

Interaction of vitamin D3--hydroxyderivatives with rexinoid orphan receptor a/γ (RORa/γ)

Isoforms of RORs possess different biological functions. Human RORγ has two isoforms (RORγ1 and RORγ2), and RORα-4 isoforms (α1–4) [25, 26]. The described isoforms differ only in their N-terminus. The molecules have different patterns of expression, therefore regulating different genes, as well as biological processes. RORγ1 isoform is expressed in liver, adipose, kidney, muscle tissue, where the receptor regulates glucose and lipid metabolism and circadian rhythm [27–29]. However, RORγ2 is selectively expressed in immune cells, such as T helper Th17 cells that promote inflammation, CD4+CD8+ (DP) thymocytes, type 3 innate lymphoid (ILC3) cells, the proinflammatory immune cells, and lymphoid tissue inducer cells (LTi) [30,31]. RORγ2's physiological function is the regulation of the DP thymocytes' survival and apoptosis. The molecule is also crucial for the development of both ILC3 and Th17 cells and the production of the proinflammatory cytokine, IL-17 [32, 33].

Vitamin D_3 metabolites, such as $1,20(OH)_2D_3$, $20(OH)D_3$, and $20,23(OH)_2D_3$ possess the ability to inhibit the interaction between a RORα/γ LBD with an LXXLL coactivator peptide and RORE-mediated transactivation [34, 35]. Molecular modeling with the established crystal structures of LBDs of RORγ and RORα showed that the inverse agonists possess high docking scores revealing the interaction of the vitamin D_3- hydroxyderivatives with a ligand-binding pocket of RORα/γ [29, 34, 36, 37]. Therefore, these produced endogenously noncalcemic vitamin D_3 metabolites can act as RORα/γ inverse agonists and further modulate RORs functions and activity. We expect a decrease of the inflammaging process through the reduction of inflammation mediated by Th17 lymphocytes. This action is due to inverse the agonism of the vitamin D_3 analogs in relation to RORα/γ.

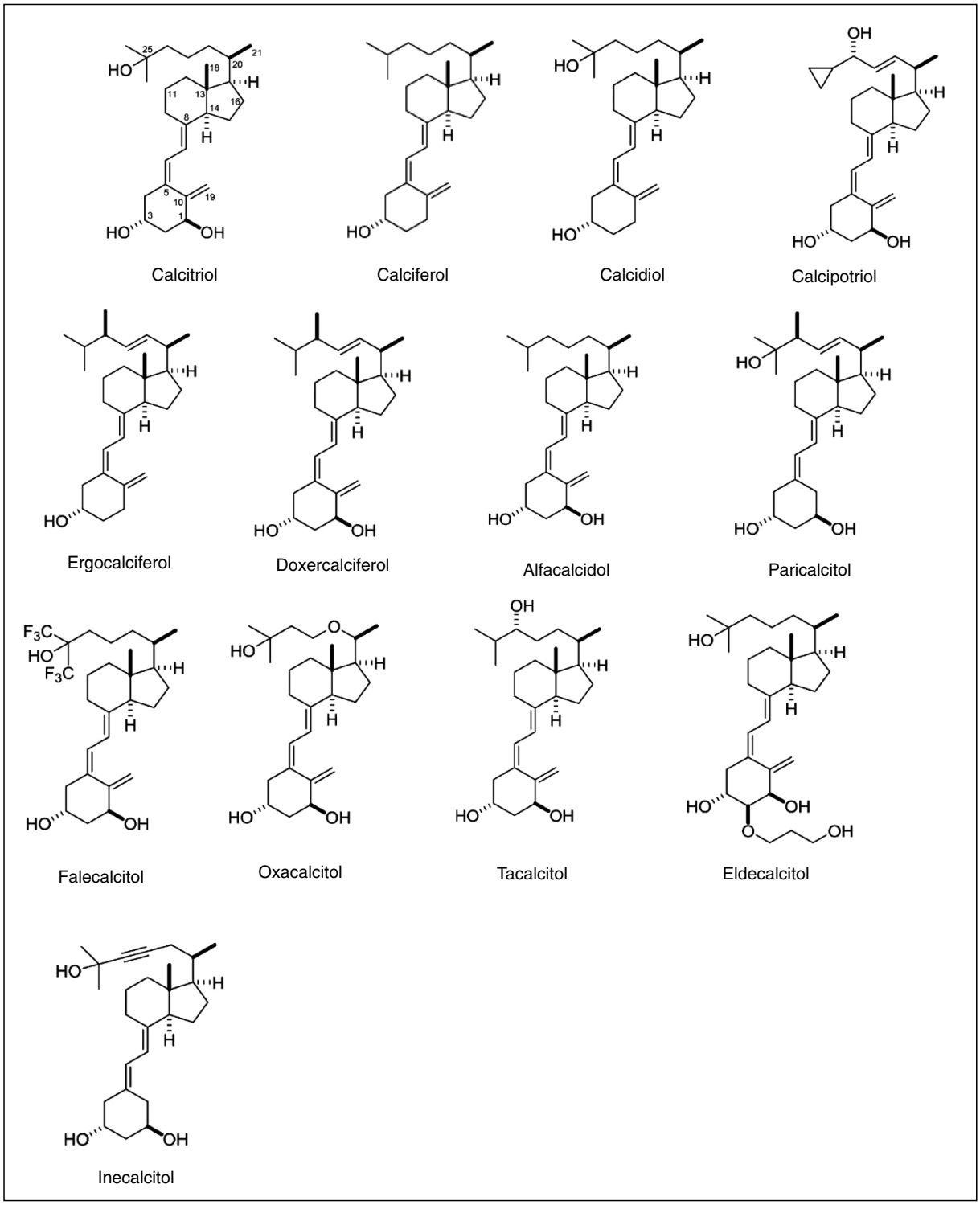

Figure 1. Examples of vitamin D compounds. Vitamin D_2 (ergocalciferol) used in the prevention of vitamin D deficiency and associated diseases, like as rickets [15]; Vitamin D_3 (calciferol) is used worldwide in the prevention of vitamin D deficiency and associated diseases, such like also rickets; Calcidiol (25(OH)D3) is used in the treatment of chronic hypocalcemia, renal osteodystrophy, rickets; Calcitriol [1,25(OH)2D3] is prescribed for renal osteodystrophy and psoriasis; Calcipotriol [22-ene-26,27-dehydro-1,25(OH)2D3] is used for psoriasis; Doxercalciferol [1α(OH)D2] prescribed for secondary hyperparathyroidism; Alfacalcidol (1α(OH)D3) is used for renal osteodystrophy, secondary hyperparathyroidism, osteoporosis as well as rickets; Tacalcitol (1α,24(OH)2D3), prescribed for psoriasis; Paricalcitol [19-nor-1,25(OH)2D2], used for secondary hyperparathyroidism; Oxacalcitriol (22-oxa-1,25(OH)2D3), used for secondary hyperparathyroidism treatment and psoriasis in Japan; Falecalcitriol [1,25(OH)2-26,27-F6-D3], prescribed for secondary hyperparathyroidism in Japan; Eldecalcitol [2α-(3-hydroxypropoxy)-1,25(OH)2D3] [15]

Physiology of vitamin D_3-hydroxyderivatives

The immediate precursor of cholesterol, 7-Dehydrocholestrol (7DHC), absorbs ultraviolet B radiation (UVB) producing pre-vitamin D_3 that isomerizes to vitamin D_3 (D_3) [56, 83–85]. Within the canonical pathway of calcitriol biosynthesis, enzymes 25-hydroxylase (CYP27A1) and 1α-hydroxylase (CYP27B1) activate D_3 for producing $1,25(OH)_2D_3$. $25(OH)D_3$, as well as $1,25(OH)_2D_3$, are degraded by sequential oxidation reactions of the side-chain catalyzed by CYP24A1 to produce calcitroic acid [56, 86, 87]. D_3 is also activated by CYP11A1 in a novel noncanonical pathway [56, 87, 88], with initial hydroxylation at C20 with the generation of $20(OH)D_3$ [56, 89, 90] or C22 [56, 91] and further subsequent reactions of hydroxylation at the positions of C20, C22, C23 and in some compounds at C17 [92]. The generated products of these reactions are selectively hydroxylated by different kinds of cytochromes: CYP24A1, CYP24A1, CYP27A1, CYP2R1, and/or CYP3A4, with additional hydroxylation at the position of C1α for all de novo synthesized compounds, with the exemptions of those which have a hydroxyl group at C17 [93–98]. Many of the described intermediates can be synthesized in the placenta, skin cells, as well as adrenal glands, and some are present in the pig adrenal gland and human serum and/or epidermis [99–102]. Native vitamin D_2 also undergoes reaction of hydroxylation through CYP11A1 with the generation of products, such as $20(OH)D_2$, $17,20,24(OH)_3D_2$, $17,20(OH)_2D_2$ [103, 104], leading to hydroxylation of $20(OH)D_2$ by CYP27B1 to $1,20(OH)_2D_2$ compound [105]. The described vitamin D_2 metabolites are also produced by pig adrenal glands, as well as in human skin cells and the placenta [106].

7-dehydrocholesterol (7-DHC), as well as lumisterol (L_3), also became substrates for CYP11A1 [56]. Finally, in the skin, the 5,7-dienal compounds with a shortened or full-length side-chain undergo a UVB-induced photoisomerization conversion to give the corresponding L_3, D_3 or T_3 products [56].

More of the UVB photons can be absorbed by the stratospheric ozone layer with an increase in the solar zenith angle [107]. While the zenith angle of the sun is so oblique that very few UVB photons penetrate to the surface of the earth, this phenomenon causes little, if any, vitamin D3 cutaneous production. This is a cause of little production (if any) of vitamin D3 in the skin during the winter at latitudes above or below 35°N and 35°S [108]. A season of the year, time of day, altitude, and latitude all markedly affect the vitamin D3 cutaneous production [109]. Cutaneous levels of 7-dehydrocholesterol decrease with the age. This markedly reduces the capacity of the aging skin to produce vitamin D_3 [110]. On the other hand, despite the up-to-fourfold reduction in the production of vitamin D_3 in the 70-year-olds compared to the 20-year-olds, the skin has such a high capacity to produce vitamin D_3 that elders with exposure to sunlight can produce an adequate amount of vitamin D to meet their vitamin-D needs [109, 111–114].

The concentration of 10^{-7} M for *in vitro* treatment of keratinocytes is an amount that refers to *in vivo* concentration of vitamin D_3 analogs, such as $20,23(OH)_2D_3$ and $1,25(OH)_2D_3$ in the physiological state [38]. Native vitamin D_3 (cholecalciferol) is widely used both as a nutritional supplement and a drug for the treatment of vitamin-D deficiency and its complications like rickets, osteoporosis, or decreased immunological resistance.

However, in the pathological conditions, the development of some types of cancer, like hepatocellular carcinoma (HCC) is associated with vitamin-D deficiency, and vitamin-D deficit occurs in hepatocellular carcinoma patients [115]. Thus, treatment of severe deficiency of serum concentration of 25(OH)D (below 10 ng/mL) by administration of vitamin D_3 analogs might prevent the development of HCC; they also should inhibit its progression.

Effect of vitamin D_3-hydroxyderivatives on the genes associated with anti--aging activities

Our retrospective analysis of recently reported microarray data on vitamin D_3 (D_3) induced changes in cultured human keratinocytes indicated that D_3-hydroxyderivatives stimulate the expression of genes involved in anti-aging activities [38]. Primary neonatal human epidermal keratinocytes were treated with 10^{-7} M of either $1,25(OH)_2D_3$ (a classical active form of D_3) or $20,23(OH)_2D_3$ (non-calcemic form of D_3) or vehicle. RNA was isolated and submitted for gene expression analysis by Illumina's HumanWG-6 chip/arrays (Tab. 1, 2).

Native vitamin D_3 acts against inflammaging and stimulates circadian clock genes

Cell-autonomous circadian clocks were identified as temporal orchestrators of many biological processes. Disruptions of the circadian clock are one of the causes of aging, as well as inflammation. Some of the types of molecules act through receptors that modulate the expression of circadian clock genes and reduce an inflammaging process. The effect of cholecalciferol (the classic form of vitamin D3) on the inflammatory process was investigated through its influence on the circadian clock and inflammation relief.

Table 1. 24h *in vitro* stimulation of HEKn by vitamin D_3-hydroxyderivatives, microarray data [38]

Gene	$20,23(OH)_2D_3$	$1,25(OH)_2D_3$	Description
S100A9	–2.80	1.10	S100 calcium binding protein A9; Marker of aging
TXNRD1	1.94	2.53	Thioredoxin reductase 1; antioxidative stress enzyme
PSMB7	1.46	1.24	Proteasome 20S subunit beta 7; proteasomes act against accumulation of the disrupted proteins in the process or aging
HMOX1	2.5	1.3	Heme oxygenase 1; an enzyme against oxidative stress
FOXO3	2.3	1.1	Forkhead box P3
KLK6	1.4	25.0	Kallikrein 6; an anti-aging protein involved in ACEI pathway (hypotensive effect)
SIRT1	1.9	–	Sirtuin 1; an anti-aging protein
AKR1C3	–12.12	–	Aldoketo reductase family 1 member C3 (prostaglandin F synthase); strong proinflammatory molecule
KLK13	–	3.1	Kallikrein 13
KLK3	–	2.1	Kallikrein 3
KLK9	–	2.0	Kallikrein 9
KLK5	–	2.0	Kallikrein 5
KLK7	–	1.8	Kallikrein 7
KLK10	–	1.8	Kallikrein 10
NR1D1	1.7	–	Nuclear receptor subfamily 1 group D member 1 (Rev-Erb-Alpha); $20,23(OH)_2D_3$ is an agonist of expression of Rev-Erba
NR1D2	3.0	1.0	Nuclear receptor subfamily 1 group D member 2 (Rev-Erb-Beta); $20,23(OH)_2D_3$ is an agonist of expression of Rev-Erbb

Table 2. 6h *in vitro* stimulation of HEKn by vitamin D3-hydroxyderivatives, microarray data [38]

Gene	20,23(OH)2D3	1,25(OH)2D3	Description
TXNRD1	1.1	3.0	Thioredoxin reductase, cytosolic form; decreases oxidative stress
SERPINB1	1.1	3.7	Inhibition of cathepsin G by SERPINB1 reduces GSDMD-driven inflammation
IL20	1.0	–1.9	Proinflammatory cytokine
IL24	1.1	–1.6	Cytokine contributing in sking inflammation
IL8	–1.4	–1.7	Proinflammatory cytokine
MMP9	–1.3	–2.0	Matrix metalloproteinase 9; proinflammatory molecule degradating extracellular matrix
MMP10	–1.3	–1.9	Matrix metalloproteinase 10; proinflammatory molecule degradating extracellular matrix
IL1F9	1.2	–1.6	Interleukin 1 family, member 9; proinflamamtory factor
CXCR7	–1.1	–1.5	C-X-C chemokine receptor type 7; Proinflammatory molecule
KLK6	1.0	3.0	Kallikrein 6; an anti-aging factor
KLK13	–	1.9	Kallikrein 13
KLK3	–	1.9	Kallikrein 3

The influence of native vitamin D_3 on the gene expression was evaluated by the analysis of the microarray data from blood cloth of the patients after oral administration of cholecalciferol at a dose of 10000 UI for 6 months with no toxic and side effects [39]. In humans, vitamin D_3 caused an increase of the

Table 3. Differentiate expressed genes under influence of native vitamin D_3 after oral administration (microarray data) [39]

Gene	Cholecalciferol change	Description
NR1D2	1.6	Nuclear receptor Subfamily 1 Group D member 2 (Rev-Erb-Beta); cholecalciferol is an agonist of expression of Rev-Erbβ
ALOX5	–1.5	Arachidonate 5-lipoxygenase; ALOX5 – rate-limiting enzyme in the synthesis of leukotrienes, that are family of lipid proinflammatory mediators
COX-2	–1.5	Cyclooxygenase 2 (prostaglandin-endoperoxide synthase 2); proinflammatory enzyme
NLRP 12	–1.6	NLRP12 inflammasome (NLR family pyrin domain containing 12); proinflammatory factor
IL6R	–1.6	Interleukin 6 receptor; (IL 6 is a proinflammatory cytokine)
IL7R	–1.5	Interleukin 7 receptor (IL7 is a proinflammatory chemokine)
IL-1RA	1.7	Interleukin 1 receptor antagonist; interleukin 1 is a proinflammatory cytokine
NR4A2	8.4	Nuclear receptor subfamily 4 group A member 2; a potential target for anti-aging therapy (improve mitochondrial function)
NR4A3	1.7	Nuclear receptor subfamily 4 group A member 3; a potential target for anti-aging therapy by improvement of mitochondrial function
PER1	4.2	Period circadian regulator 1 (genes involved in circadian clock act against aging process)
CRY1	2.0	Cryptochrome circadian regulator 1
TLR1	–1.9	Toll-like receptor 1 (toll-like receptors are proinflammatory agents and contributes to development of age-related diseases like atherosclerosis)
TLR8	–1.8	Toll-like receptor 8
TLR4	–1.8	Toll-like receptor 4
TLR5	–1.6	Toll-like receptor 5
CCR2	–1.6	C-C Motif chemokine receptor 2 (chemokines are proinflammatory agents)
CXCR1	–1.7	C-X-C motif chemokine receptor 1
CXCR2	–1.9	C-X-C motif chemokine receptor 2
CX3CR1	–1.9	C-X3-C motif chemokine receptor 1
CCR3	–1.5	C-C motif chemokine receptor 3
PSMD7	1.9	Proteasome 26S subunit, non-ATPase 7
PSMD12	1.6	Proteasome 26S subunit, non-ATPase 12

expression of NR1D2 gene (1.6 fold), reduced inflammaging by a decrease of the expression of ALOX5 gene (–1.5 fold), cyclooxygenase 2 (COX-2) (–1.5 fold), NLRP 12 inflammasome (–1.6 fold), IL6R (–1.6 fold), an expression increase of the Interleukin 1 Receptor Antagonist (1.7 fold). By upregulation of NR4A2 (8.4-fold) and NR4A3 (1.7-fold), cholecalciferol causes the stimulation of antioxidative activity, DNA repair machinery, and improvement of intrinsic mitochondrial functions. The tested substance induces also genes connected with the circadian clock, such as PER1 (4.2) fold, CRY1 (2.0-fold). A further anti-inflammatory action of vitamin D_3 is connected with influence on the genes involved in inflammatory response: TLR1 (–1.9 fold), TLR8 (–1.8), TLR4 (–1.8), TLR5 (–1.6), CCR2 (–1.6). CXCR1 (–1.7 fold), CXCR2 (–1.9 fold), CX3CR1 (–1.9), CCR3 (–1.5 fold) (Tab. 3).

Agonists of nuclear receptor Rev-Erb α/β may have anti-aging properties

SR-9009, selective REV-ERB (nuclear receptor subfamily 1 group D member 2) agonist administered intraperitoneally to $Bmal1^{flox/flox}$/MHC-$Cre^{-/-}$ mice (a control mice to circadian disruption mice model of heart) at a dose of 100 mg/kg b.w./daily for 8 days, has shown anti-aging properties by exerting influence on gene expression in heart (RNAsequence data from biventricular samples) [40]. SR-9009 acts against oxidative stress through upregulation of Heme oxygenase 1 gene expression (fold change *vs.* vehicle = 3.01) and decreasing the expression of NQO2, and decreasing inflammation by downregulation of CYP26B1 (–14.30 fold). Therefore, it has anti-aging properties and may be useful in preventing CNS dysfunction in Alzheimer's Disease

Table 4. Differentiate expressed genes after treatment intraperitoneally with SR-9009 (RNAseq. data) [40]

Gene	SR-9009 fold change	Description
HMOX1	3.0	Heme oxygenase 1; anti-oxidative enzyme
CYP26B1	–14.3	Cytochrome P450 family 26 subfamily B member 1
NQO2	–1.1	N-ribosyldihydronicotinamide quinone reductase 2
NRGN	1.2	Neurogranin; molecule involved in the prevention of CNS dysfunction in Alzheimer's disease
APBB2	–1.2	Amyloid beta (A4) precursor protein binding family B, member 2; downregulation of this gene prevents against Alazheimer's disease development
HMG-CoA synthase 2	–1.3	3-hydroxy-3-methylglutaryl-coenzyme A synthase 2; downregulation of this gene prevents against development of age-related diseases like atherosclerosis, obesity, atherosclerotic dementia
uPA	1.4	Plasminogen activator, urokinase; prevents against stroke and ischemic gangrene
FGFR1OP2	–1.0	FGFR1 oncogene partner 2
BCL7A	–1.1	B cel CLL/lymphoma 7A
LYZ2	1.3	Lysozyme 2
LYNX1	–1.1	Ly6/neurotoxin 1; Adult *Lynx1*–/– mice shew visual cortex plasticity similar to the plasticity of juveniles, what demonstrates that LYNX1 caused as a break for cortical plasticity [63]. Based on the studies in mice model, LYNX1 is involved in modulatory role in the brain under process of aging [63, 64]. SR9009 might improve visual transduction process in aged visual system by decrese of the expression of LYNX1 gene

by increasing the expression of neurogranin and decreasing the expression of amyloid beta (A4) precursor protein binding family B, member 2. The compound prevents also age-related disorders like atherosclerosis or stroke by the expression decrease of HMG-CoA synthase 2 and an increase for plasminogen activator, urokinase receptor. SR-9009 prevents oncogenesis by decreasing the expression of FGFR1 oncogene partner 2 and B cell CLL/lymphoma 7A. The expression of lysyl oxidase-like 2, a protein involved in the induction of oxidative vascular stress, was decreased after treatment with SR-9009. Some of the engineering approaches to extending lifespan are focused on the intervention into the lysozyme expression, and we noticed that SR-9009 induces the lysozyme 2 gene. The compound upregulated also the circadian clock gene, E4BP4. PPARγ, a strong anti-inflammatory molecule, which exhibits a circadian rhythm of the expression controlled by E4BP4 (Tab. 4) [40].

Free radical scavenging activity of atorvastatin, an inhibitor of HMG-CoA reductase

Atorvastatin, a popular hypolipemic drug may be considered a free radical scavenger and anti-aging therapeutic because it modulates expression of the genes connected with antioxidant function in hepatocellular carcinoma (HepG2) cell line (Tab. 5) [41]. Free Radical Scavenging gene set (-log(p value) = 1.89E-03-1.61E-02) (see some of the involved genes in this pathway in the Tab. 6) belongs to overrepresented Biological Functions in Ingenuity pathway Analysis of RNA sequence data, obtained in HepG2 cells treated with atorvastatin at the concentration of 10 μM for 24 hours. Indirect AMPK activators, metformin, resveratrol, as well as exercise, are widely tested as candidates for anti-aging drugs [42–44]. An increased level of neuregulin is associated with an increased lifespan in rodents [45, 46].

Comparison of the genetic profile of the compounds with metformin, a compound with anti-aging potential

Expression profile of the genes involved in DNA repair pathways, organ regeneration, proteasomal degradation of excessively expressed proteins, and mitochondrial metabolism after metformin treatment of HepG2 cells reveals similarity to the gene profile after incubation of keratinocytes with vitamin D_3-hydroxyderivatives, SR-9009 or atorvastatin (Fig. 2, Tab. 7) [47]. DAVID functional annotation of RNAseq. data from primary human hepatocytes was performed after treatment with metformin [2.5 mM], an anti-diabetic drug, for 8 hours or with 40 μM compound C, an inhibitor of AMPK along with 2.5 mM metformin for 8 hours (Tab. 7) [47].

The study showed a hierarchical gene clustering heatmap containing 1906 differentially expressed genes

Table 5. Canonical pathway analysis (IPA) in HepG2 under *in vitro* treatment with atorvastatin [10 μM] for 24 hours (n = 3), RNAseq. data [41]

Enriched canonical pathway	–log (P-value)	Molecules involed in pathway
AMPK signaling	1,71E+00	PIK3R3 PFKFB4 FASN ACACA HMGCR
Telomerase signaling	2,65E-01	PIK3R3
Neuregulin signaling	3,01E-01	PIK3R3
Neuroprotective role of THOP1 in Alzheimer's disease	5,56E-01	SERPINA3
Ceramide signaling	3,24E-01	PIK3R3
Melanocyte development and pigmentation signaling	3,05E-01	PIK3R3
Circadian rhythm signaling	6,28E-01	BHLHE40
RAR activation	4,00E-01	PIK3R3, RDH11
IL-4 signaling	3,53E-01	PIK3R3
IL-9 signaling	6,17E-01	PIK3R3
p38 MAPK signaling	2,23E-01	PLA2G3
HIF1α signaling	2,54E-01	PIK3R3
VDR/RXR activation	3,28E-01	SULT2A1
Oxidative ethanol degradation III	3,75E+00	ACSL3 ACSS2 ACSL1
Mitochondrial L-carnitine shuttle pathway	3,49E+00	ACSL3 ACSL4 ACSL1
NAD biosynthesis III	1,50E+00	NAMPT
IL-6 signaling	2,13E-01	PIK3R3
IL-15 signaling	4,07E-01	PIK3R3

Table 6. Genes connected with response to oxidative stress in HepG2 cells treated with atorvastatin [10 μM] for 24h (Differentiate expressed genes, RNAseq. data) [41]

Gene	Atorvastatin fold change	Description
ACSS2	2.2	acyl-CoA synthetase short-chain family member 2
MT1E	1.4	Metallothionein 1E; metallothionein scavenges reactive oxygen species [44]
UCP2	1.5	Uncoupling protein 2 (mitochondrial, proton carrier)
DDIT4	1.2	DNA-damage-inducible transcript 4
DHCR24	1.2	24-dehydrocholesterol reductase
SREBF2	1.2	Sterol regulatory element binding transcription factor 2
ACOT1	1.3	Hepatic acyl-CoA thioesterase 1; acot1 knockdown caused increased FA oxidation, reduced PPARα activity, and further increased inflammation and hepatic oxidative stress
ACSL1	1.3	acyl-CoA synthetase long-chain family member 1

after metformin treatment (adjusted $p \leq 0.05$) with segregation into 10 groups [56]. Cluster 1 included 194 genes with an increase of the expression in response to treatment with metformin that remained elevated also after incubation with the compound C (metformin increased, AMPK-independent). Cluster 3 contained 575 genes with an increase of the expression in response to incubation with metformin, which decreased the expression while also under treatment with the compound C (metformin increased, AMPK-dependent).

In the study, the further generation of AMPK-independent and AMPK-dependent clusters was presented by comparing the tested conditions [47]. Since compound C has also off-target effects, only the genes whose expression changed due to metformin response were considered for this experiment. Clusters 2 (containing 134 genes), 3 (with 575 genes), 7 (83 genes), 8 (168 genes) contain molecules whose expression increased after metformin incubation but was reduced under a simultaneous treatment with metformin and

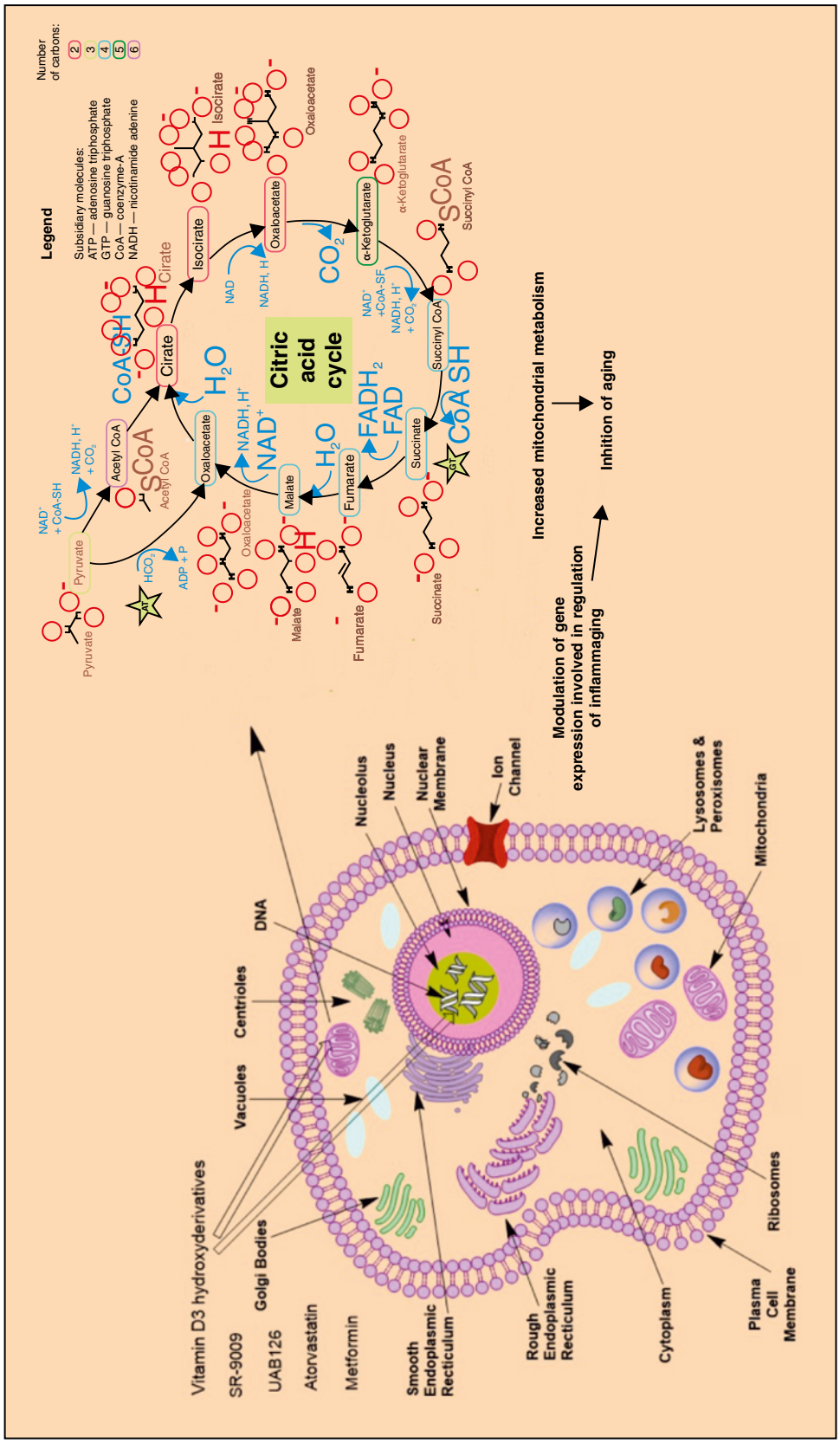

Figure 2. Comparative analysis of the compounds with antiaging properties according their similarity in mechanism of action

Table 7. Results of DAVID functional annotation of RNAseq. Data from HepG2 treated *in vitro* with metformin [2.5 mM] for 8h [47]

David results from RNA-seq cluster 1 (metformin-dependent increased genes, AMPK independent)				
Annotation cluster 8	**Enrichment score: 1.3114286631285683**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0031099~regeneration	5	3.184713376	0.004
GOTERM_BP_FAT	GO:0042246~tissue regeneration	3	1.910828025	0.034
Annotation cluster 17	**Enrichment score: 0.6264708391271191**			
GOTERM_BP_FAT	GO:0048534~hemopoietic or lymphoid organ development	4	2.547770701	0.427
Annotation cluster 20	**Enrichment score: 0.42660630022201657**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0006508~proteolysis	17	10.82802548	0.031
Annotation cluster 23	**Enrichment score: 0.36641602542817736**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	6	3.821656051	0.257
GOTERM_BP_FAT	GO:0033554~cellular response to stress	8	5.095541401	0.262
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	5	3.184713376	0.687
GOTERM_BP_FAT	GO:0006281~DNA repair	3	1.910828025	0.738
David results from RNA-seq cluster 2 (met increased, AMPK dependent)				
Annotation cluster 10	**Enrichment score: 0.8868509634642516**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0043161~proteasomal ubiquitin-dependent protein catabolic process	3	2.4	0.158
GOTERM_BP_FAT	GO:0010498~proteasomal protein catabolic process	3	2.4	0.158
GOTERM_BP_FAT	GO:0006508~proteolysis	7	5.6	0.750
Annotation cluster 17	**Enrichment score: 0.5947274210343121**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0033554~cellular response to stress	8	6.4	0.098
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	5	4	0.261
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	6	4.8	0.275
GOTERM_BP_FAT	GO:0006281~DNA repair	3	2.4	0.590
David results from RNA-seq cluster 3 (met increased, AMPK dependent)				
Annotation cluster 10	**Enrichment score: 1.9018121386666416**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0032436~positive regulation of proteasomal ubiquitin-dependent protein catabolic process	3	0.53	0.0188
GOTERM_BP_FAT	GO:0045862~positive regulation of proteolysis	4	0.71	0.043
GOTERM_BP_FAT	GO:0032434~regulation of proteasomal ubiquitin-dependent protein catabolic process	3	0.53	0.045
Annotation cluster 13	**Enrichment score: 1.6825557240354716**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0046513~ceramide biosynthetic process	4	0.71	0.011
Annotation cluster 33	**Enrichment score: 0.8581528728649036**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0042787~protein ubiquitination during ubiquitin-dependent protein catabolic process	4	0.71	0.015
GOTERM_BP_FAT	GO:0006508~proteolysis	31	5.53	0.751

Table 7 cont. Results of DAVID functional annotation of RNAseq. Data from HepG2 treated *in vitro* with metformin [2.5 mM] for 8h [47]

Annotation cluster 77	**Enrichment score: 0.3243851502337266**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0030097~hemopoiesis	6	1.07	0.870
David results from RNA-seq cluster 4 (met dDecreased, AMPK independent)				
Annotation cluster 6	**Enrichment score: 1.2202185663855596**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_MF_FAT	GO:0016628~oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	3	0.88	0.048
Annotation cluster 13	**Enrichment score: 0.8336371317681409**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_MF_FAT	GO:0015662~ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	4	1.17	0.075
GOTERM_BP_FAT	GO:0006754~ATP biosynthetic process	5	1.47	0.082
GOTERM_BP_FAT	GO:0046034~ATP metabolic process	5	1.47	0.128
GOTERM_MF_FAT	GO:0042626~ATPase activity, coupled to transmembrane movement of substances	5	1.47	0.145
GOTERM_MF_FAT	GO:0043492~ATPase activity, coupled to movement of substances	5	1.47	0.148
GOTERM_MF_FAT	GO:0042625~ATPase activity, coupled to transmembrane movement of ions	4	1.17	0.158
Annotation cluster 41	**Enrichment score: 0.1480055067172435**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0006281~DNA repair	6	1.76	0,602
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	9	2.64	0.719
GOTERM_BP_FAT	GO:0033554~cellular response to stress	10	2.93	0.720
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	6	1.76	0.821
David results from RNA-seq cluster 5 (met decreased, AMPK dependent)				
Annotation cluster 30	**Enrichment score: 0.4088079394382511**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0033554~cellular response to stress	11	5.09	0.108
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	7	3.24	0.512
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	5	2.31	0.612
GOTERM_BP_FAT	GO:0006281~DNA repair	4	1.85	0.627
David results from RNA-seq cluster 6 (met increased, AMPK independent)				
Annotation cluster 7	**Enrichment score: 1.8688950312990442**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0031099~regeneration	3	3.80	0.039
GOTERM_BP_FAT	GO:0031100~organ regeneration	3	3.80	0.006
GOTERM_BP_FAT	GO:0007568~aging	4	5.06	0.013
Annotation Cluster 24	**Enrichment Score: 0.4916356096090675**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0030097~hemopoiesis	3	3.80	0,288
David results from RNA-seq cluster 8 (met increased, AMPK dependent)				

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Table 7 cont. Results of DAVID functional annotation of RNAseq. Data from HepG2 treated *in vitro* with metformin [2.5 mM] for 8h [47]

Annotation cluster 21	**Enrichment score: 0.7644313581632808**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0033554~cellular response to stress	11	6.67	0.046
GOTERM_MF_FAT	GO:0003684~damaged DNA binding	3	1.82	0.092
GOTERM_BP_FAT	GO:0006281~DNA repair	6	3.63	0.138
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	7	4.24	0.150
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	6	3.63	0.539
GOTERM_BP_FAT	GO:0006260~DNA replication	3	1.82	0.548
Annotation cluster 24	**Enrichment score: 0.5976009129847903**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0030097~hemopoiesis	5	3.03	0.192
David results from RNA-seq cluster 9 (met decreased, AMPK independent)				
Annotation cluster 1	**Enrichment score: 4.739401003241343**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0042775~mitochondrial ATP synthesis coupled electron transport	7	14.58	1,34E-09
GOTERM_BP_FAT	GO:0042773~ATP synthesis coupled electron transport	7	14.58	1,34E-09
GOTERM_BP_FAT	GO:0022904~respiratory electron transport chain	7	14.58	3,06E-09
GOTERM_BP_FAT	GO:0015980~energy derivation by oxidation of organic compounds	8	16.67	1,31E-08
GOTERM_BP_FAT	GO:0045333~cellular respiration	7	14.58	3,86E-08
GOTERM_BP_FAT	GO:0006119~oxidative phosphorylation	7	14.58	4,10E-08
GOTERM_BP_FAT	GO:0022900~electron transport chain	7	14.58	1,02E-07
GOTERM_BP_FAT	GO:0006091~generation of precursor metabolites and energy	8	16.67	2,59E-06
GOTERM_BP_FAT	GO:0006120~mitochondrial electron transport, NADH to ubiquinone	4	8.33	8,64E-05
GOTERM_MF_FAT	GO:0008137~NADH dehydrogenase (ubiquinone) activity	4	8.33	1,29E-04
GOTERM_MF_FAT	GO:0003954~NADH dehydrogenase activity	4	8.33	1,29E-04
GOTERM_MF_FAT	GO:0050136~NADH dehydrogenase (quinone) activity	4	8.33	1,29E-04
GOTERM_MF_FAT	GO:0016655~oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	4	8.33	1,91E-04
GOTERM_MF_FAT	GO:0016651~oxidoreductase activity, acting on NADH or NADPH	4	8.33	9,03E-04
GOTERM_BP_FAT	GO:0016310~phosphorylation	8	16.67	9,76E-04

compound C. The genes were defined as metformin increased, AMPK-dependent. The Gene Ontology (GO) analysis found enrichment for DNA repair and response to DNA damage stimulus and several other terms connected with the proteasomal protein catabolic process (connected with an excess of proteins while aging), organ regeneration, response to cellular stress, and pathways with increased mitochondrial metabolism for these clusters. Cluster 5 with 256 genes contained molecules whose expression decreased after incubation with metformin, but after simultaneous treatment with metformin and compound C, the expression returned to the untreated level. This set of the genes was defined as metformin decreased, AMPK-dependent and was enriched also with the response to DNA damage stimulus and DNA repair. What is more, an upstream regulator analysis of IPA showed enrichment for the AMPK signaling canonical pathway, which is related to anti-aging activity of the compound [47].

Some of the AMPK-independent clusters were found [47]. Cluster 1 contained 194 genes, 6 –74 genes, and 10–57 genes showed molecules with the increased expression after incubation with metformin that remained increased after a simultaneous treatment of metformin

and the compound C. The genes were defined as metformin increased, AMPK-independent. These sets of genes were overrepresented for GO terms as tissue regeneration, DNA repair, hemopoiesis, and others. Cluster 4 containing 365 genes, 9 with 20 genes revealed molecules whose expression decreased under incubation with metformin, which remained decreased after the simultaneous incubation with metformin and the compound C. The set of the genes was defined as metformin decreased, AMPK-independent and the enrichment of genes like DNA repair, cellular respiration, oxidative phosphorylation, and other GO terms [47]. Therefore, by increasing mitochondrial metabolism, metformin reveals anti-aging properties.

A comparative analysis of the gene expression affected by the tested substances with resveratrol [48–51] was performed in order to find similarities in the mechanism of anti-aging action. Vitamin D_3-hydroxyderivatives exhibits photoprotective properties and protect against oxidative stress and DNA damage [52–56]. The compounds revealed also anti-inflammatory action [3], which makes them good candidates for drugs against inflammaging [57, 58].

Analog of vitamin D_3, $20,23(OH)_2D_3$, similarly to resveratrol, caused downregulation of S100 Calcium Binding Protein A9 [59–61], a biomarker of aging [62–66].

Native resveratrol, polyphenolic compounds from a natural source, was used in the study as the control for comparison with five structurally modified resveratrol derivatives, such as isobutyrate, butyrate, acetate, palmitoate, as well as diacetate. The aim was to improve functionality and biological activity at 1% concentration of each substance for 24h in the full thickness cultures of the epidermis. To evaluate gene expression connected with inflammaging the gene array and qPCR, mRNA analysis was used [67]. The expression of the sets of the genes connected with anti-aging and aging properties were evaluated, as well as the markers of inflammation, such as interleukin-1A [IL1A], IL6, IL1R2, IL-8, extracellular factors (collagen 1A1, 4A1, 3A1; tissue inhibitor of matrix metalloproteinase 1, elastin, fibrillin 1, matrix metalloproteinase 9, laminin beta1), silent mating type information regulation 2 homolog 1, antioxidants, such as superoxide dismutase, proliferating cell nuclear antigen, metallothionein 1H/2H, catalase and nerve growth factor. The analogs were evaluated according to the gene expression profile ranking, each from highest-to-lowest: butyrate > isobutyrate > diacetate > acetate > palmitoate. The isobutyrate and butyrate analogs have higher biological activity in comparison to resveratrol and might be used in topical applications for an improvement of dermal condition and for other medical purposes [67].

Conclusion

We have found that resveratrol acts in a similar way to vitamin D_3-hydroxyderivatives, cholecalciferol, SR-9009, atorvastatin and metformin through its anti-inflammatory and against-oxidative-stress action and the consequent reduction of inflammaging process [68–73]. Vitamin D_3-hydroxyderivatives improve the proteasome profile gene expression, as well as metformin, and this finding has also anti-aging properties due to the decrease of the number of aberrant proteins (like amyloid beta, which causes Alzheimer's Disease) accumulated during aging. Interestingly, after incubation with atorvastatin, the Neuroprotective Role of THOP1 in Alzheimer's Disease pathway was enhanced and after treatment with SR-9009 (Agonists of Nuclear Receptor Rev-Erb α/β) an amyloid beta (A4) precursor protein binding family B, member 2 expression was decreased, which suggests these compounds have properties against Alzheimer's Disease, which is recognized as a hallmark of aging [74–78]. We postulate that vitamin D_3-hydroxyderivatives have anti-aging potential because of their anti-inflammatory properties and action against oxidative stress and improvement in DNA repair in cells [79–82]. Their mechanism of action is similar to the action of resveratrol, metformin, SR9009, and native cholecalciferol and is connected with modulation of gene expression involved in particular pathways related to the aging process and anti-aging activity.

Metformin seems to be generally a well-tolerated and safe anti-diabetic drug [83, 84]. However, a well-known complication in metformin treatment is lactic acidosis, especially in cases of renal insufficiency or in intentional overdose [83, 84]. The second medication with anti-aging properties, resveratrol is well tolerated and no marked toxicity was reported [85]. Resveratrol is a substance with a natural origin; it occurs in red wine and dark grapes. However, atorvastatin is a synthetic, the most used drug in the world. The complex toxicological study of atorvastatin was performed on dogs with a histopathologic evaluation which revealed multifocal minimal to slight hemorrhages in the submucosa of the gallbladder, and all the findings were reversible [86].

Potential anti-aging properties and advantages of metformin and resveratrol seem to surpass their weak side effects in healthy adults. However, metformin should not be used as a compound which slows the aging process in the case of patients with renal and liver insufficiency. We found also that atorvastatin (Lipitor medication) is the most widely used drug in the world with promising anti-aging properties due to its effect on gene expression involved in the regulation of the aging process. Vitamin D_3 analogs are endogenously produced, they act like hormones, so in physiological concentrations they reveal no toxic effect, and their anti-aging benefits seem to be worthy of further investigation.

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VIA MEDICA

CASE REPORT

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Patient after extensive crushing injury of the lower limb with subacute stent thrombosis

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ABSTRACT

Percutaneous transluminal coronary angioplasty with stent implantation is a basic life-saving treatment of stenotic lesions causing acute coronary syndromes. Stent thrombosis is one of the most serious complications of coronary angioplasty, strongly associated with recurrent myocardial infarction and high mortality. Many factors were identified as increasing the incidence of stent thrombosis including bleeding and inflammation. I am presenting a case of a 54-year-old man after extensive crushing injury of the lower limb with simultaneous stent thrombosis in two recently implanted stents. As a preventive measure for stent thrombosis novel potent antiplatelet agents may be a reasonable choice even for patients with high bleeding risk.

Key words: stent thrombosis, crushing injury, inflammation, bleeding

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Introduction

Percutaneous transluminal coronary angioplasty (PTCA) with new-generation drug-eluting stent (DES) implantation has become the most frequently performed therapeutic procedure in medicine. It is a basic treatment of flow-limiting coronary stenosis both in acute and chronic coronary syndromes. One of the most serious complications of coronary stent implantation is stent thrombosis (ST). ST is usually causing ST-elevation myocardial infarction (MI) associated with a high mortality rate. This detrimental condition may occur any time from immediately after the procedure to several years thereafter. ST categorized as subacute (2–30 days after PTCA) carries the highest mortality risk [1].

Case report

A 54-year-old patient was admitted to the hospital emergency department due to an extensive crushing injury of the right leg as a result of a farm accident. The patient was qualified for immediate surgery. Debridement of the left lower leg wound, tibial stabilization with an external stabilizer, intraoperative application of gentamicin and thrombectomy of the right posterior tibial artery were performed. On admission laboratory tests showed the following deviations: white blood cell count (WBC) — 19.5 x 10^9/L, C-reactive protein (CRP) — 8 mg/L. Postoperatively anaemia with haemoglobin (Hb) concentration 10.5 g/dL and significantly elevated troponin I levels determined by the high sensitive method (hsTPI) - 1853 ng/L were observed. In the next days of hospitalization, elevated WBC (11.7 x 10^9/L), increase in CRP (340 mg/L), further anaemia (Hb 8.9 g/dL) and an increase in hsTPI (4640 ng/L) were observed.

On the second and third day of hospitalization, the patient periodically reported mild resting pressure-type chest pain. Detailed medical history revealed nicotinism, hypertension and exertional chest pain for several months. ECG showed: intermediate axis, sinus rhythm 96 bpm, Q waves in II, III, aVF, ST-segment depression and negative T waves in V4–V6 (Fig. 1). Echocardiography revealed left ventricular hypertrophy, moderate mitral regurgitation, moderate left ventricular systolic dysfunction with regional contractility disorders, and left ventricular ejection fraction of 38%. A diagnosis of non-ST elevation MI was made, and the patient was qualified for coronarography. It revealed advanced three-vessel disease with 100% stenosis of the right coronary artery, 90% stenosis of proximal and 80% stenosis middle segment of left anterior descending artery

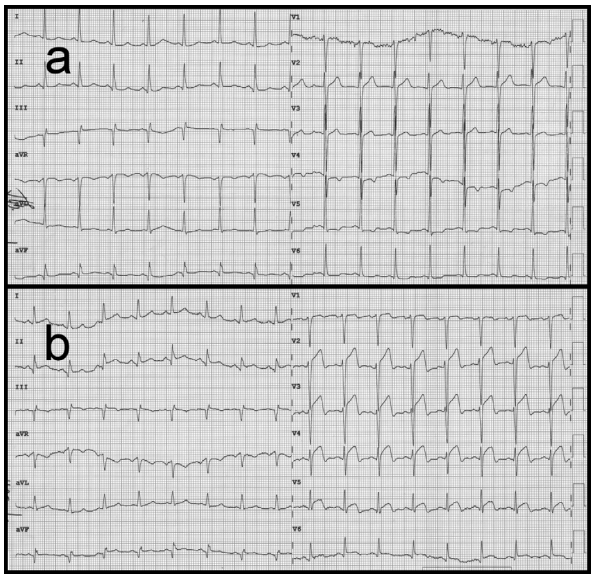

Figure 1. ECG: a — 4th hospitalization day, b — 14th hospitalization day; speed 25mm/s

(LAD), 50% stenosis of the second diagonal branch, 70% ostial stenosis of the first septal branch and 90% stenosis of the second obtuse marginal branch (OM2) (Fig. 2). An immediate cardiac surgery consultation was performed to qualify for coronary artery bypass grafting. After a detailed explanation of treatment options, risks, anticipated benefits and limitations of heart surgery and PTCA the patient refused surgery and chose percutaneous treatment. Accordingly, PTCA OM2 was performed ad hoc by implanting DES Firehawk 2.5 x 29 mm at pressure 14 atm with a good direct result (Fig. 2). Before angioplasty, loading doses of acetylsalicylic acid and clopidogrel were given. Ticagrelor was not used due to recent extensive surgical trauma and a significantly increased risk of bleeding. Two days later, the second stage of PTCA was performed with two DES implantation (Promus Premier 2.5 x 32 mm at 12 atm in middle LAD and Synergy 3.0 x 28 mm at 12 atm in proximal LAD) and post-dilatation with non-compliant (NC) balloons Quantum Apex 3.5 x 15 mm and 4.0 x 12 mm up to 18 atm (Fig. 2). The course of hospitalization at the Cardiology Clinic was without complications. After obtaining microbiological results initial empirical antibiotic therapy was modified. Decreasing hsTPI (893 ng/L) and CRP (193 mg/L), low procalcitonin (PCT) concentration (0.43 ng/mL), stable Hb (9.2 d/dL) and WBC (11.7 x 10^9/L) were observed. After an orthopaedic consultation, the patient was transferred to the Orthopaedics Clinic where surgical drainage of left lower leg wounds was performed. Due to anaemia (7.2 d/dL), four units of packed red blood cells (PRBC) were transfused. Laboratory tests showed stable Hb (10.2 g/dL), WBC (10.6 x 10^9/L), increasing platelet count (PLT) (466 → 787 → 1044 x 10^9/L), low PCT, decrease in CRP (109mg/dL) and hsTPI (165 ng/L).

On the seventh day after being transferred from the Cardiology Clinic, the patient reported severe tearing pain in the chest radiating to the neck, lower jaw and left upper limb. ECG revealed ST-segment elevation in V2–V5 up to 3 mm, with hyperacute T waves (Fig. 1). LAD stent thrombosis was suspected. The patient was transported to the cath lab immediately. Coronary angiography revealed stent thrombosis in both LAD and OM2 (Fig. 2). A loading dose of ticagrelor and a bolus followed by an infusion of abciximab were given. Aggressive dilatations within LAD and OM2 stents with NC balloons Apollo 2.5 x 12 mm and 3.0 x 12 mm at pressures up to 30 atm were carried out. Restoration of flow, good angiographic effect and chest pain resolution were achieved.

In the following days, the local condition of the limb was rapidly deteriorating. A gradual worsening of the general condition was also observed. A decision was made to amputate the limb. Due to the recent acute coronary syndrome (ACS) and ST, the procedure was performed without interrupting antiplatelet therapy. Four PRBC units were transfused during the perioperative period and a transient increase in hsTPI up to 6294 ng/L without new ECG or echocardiography findings was observed (with a perioperative decrease in Hb to 7.4 g/dL).

After amputation further lowering of inflammation parameters and gradual improvement in the patient's condition was observed. Due to negative control cultures, antibiotic therapy was terminated. The patient was discharged home in good general condition 33 days after injury.

Discussion

In recent years, the development of new generation DES, improvement of implantation techniques and introduction in clinical practice novel potent antiplatelet drugs resulted in a significant reduction in the incidence of ST. Recent large-scale registries reported that with contemporary treatment ST occurs in less than 2% of patients [2, 3].

Many clinical, angiographic and procedural factors were proven to be related to an increased risk of ST [4]. Clinical factors with higher ST risk include premature antiplatelet treatment discontinuation, diabetes, renal failure, anaemia, impaired LVEF, malignancy, smoking, advanced age, antiplatelet drugs resistance. Angiographic and device-related factors predisposing to ST include ACS, complex lesion morphology, multiple lesions, stent under expansion, residual stenosis, vessel dissection, stenting in small vessels, suboptimal

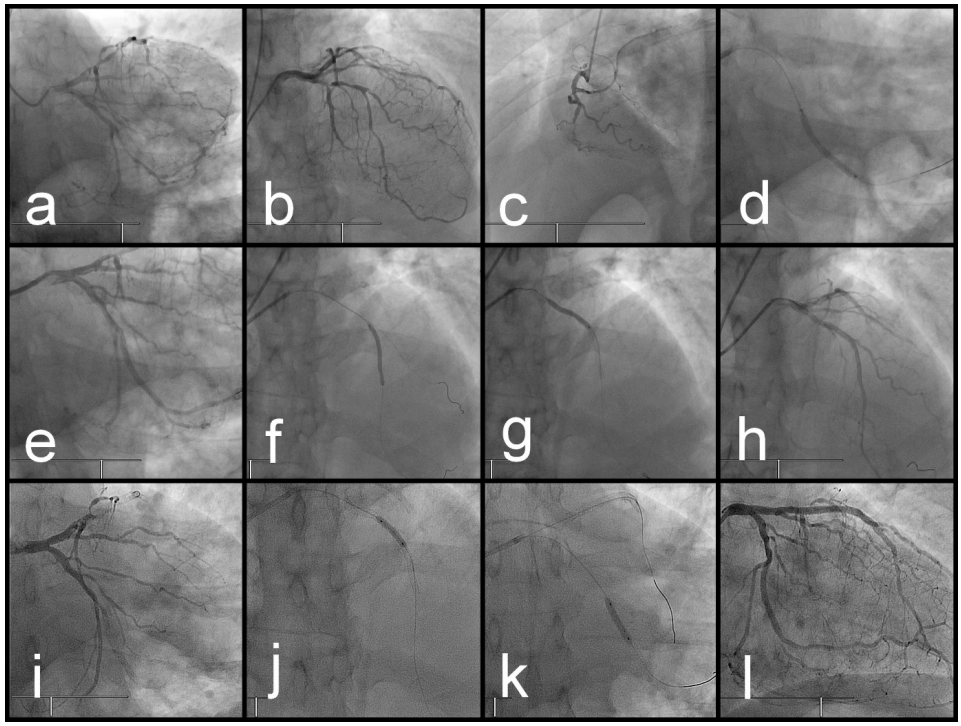

Figure 2. Coronary angiograms: a — pre-PTCA AP caudal view, b — pre-PTCA AP cranial view, c — pre-PTCA left anterior oblique view — RCA, d — OM2 stent implantation, e — post-PTCA OM2 AP caudal view, f — middle LAD stent implantation, g — proximal LAD stent implantation, h — post-PTCA LAD AP cranial view, i — LAD and OM2 ST AP caudal view, j — LAD ST baloon angioplasty AP cranial view, k — OM2 ST baloon angioplasty AP caudal view, l — final result after ST angioplasty right anterior oblique caudal view; AP — anterio-posterior, LAD — left anterior descending artery, OM2 — second obtuse marginal branch, PTCA — percutaneous transluminal coronary angioplasty, RCA — right coronary artery, ST — stent thrombosis

stenting result with the impaired coronary flow. The risk of ST depends also on the type of implanted stent. New-generation DES with enhanced biocompatibility exclusively sirolimus-analogue active drugs and thin struts have a significantly lower risk of stent thrombosis in comparison to both early-generation DES and BMS [5]. Accordingly, new-generation DES should be the default stent type for PTCA regardless of clinical presentation, lesion subtype, concomitant therapies, or comorbidities [6].

Several studies have reported an association between inflammatory cytokines concentrations and stent thrombogenicity [7–9]. Park et al reported that pre-procedural CRP was significantly associated with increased risks of ST, death, and MI [7]. Hwang et al. [8] reported a positive association between elevated levels of interleukin 6 and DES thrombosis. Katayama et al. have shown that in patients with MI who are treated with primary coronary stenting, inflammation indicators such as CRP and serum amyloid-A protein may be closely related to ST [9]. Some previous reports have shown that major bleeding may also be correlated with the development of ST [1]. This case report presented the patient with both high-grade inflammation and recurrent anemization.

In general, as a preventive measure against ST novel more potent P2Y12 inhibitors, namely ticagrelor and prasugrel, may be used. These drugs achieve a faster, greater and more consistent degree of P2Y12 inhibition as compared to clopidogrel. They are more effective in preventing early and late ST in patients with ACS [10, 11]. However, this comes at the cost of higher bleeding liability and previous intracranial haemorrhage or ongoing bleeds are contraindications for ticagrelor and prasugrel [10, 11]. Due to recent trauma with peripheral vessel injury, the presented patient was initially assessed as ineligible for ticagrelor or prasugrel treatment.

Observational studies have shown a high risk of ST recurrence after the first episode [12, 13]. Armstrong et al reported the cumulative hazard of angiographic definite recurrent ST 11% at 1 year and 20% at 5 years. The cumulative hazard of definite or probable recurrent ST was 16% at 1 year and 24% at 5 years. According to this registry, the risk of recurrence is highest in the first few months after the first event [12]. Both prasugrel and ticagrelor are associated with a significant reduction

of first and recurrent ST as compared to clopidogrel [10, 13]. Based on this data after ST occurrence patients previously on clopidogrel should be switched to ticagrelor or prasugrel if not contraindicated [6]. Bolus and infusion of glycoprotein IIb/IIIa receptor antagonist (abciximab, tirofiban or eptifibatide), potent antiplatelet drug and aggressive high-pressure balloon dilations are standard ST treatments [6]. In most cases, satisfactory results are obtained with balloon dilation and repeated stenting may be avoided. However, a new stent may be required to overcome edge-related dissections and adjacent lesions, or to optimize final results [6, 14]. Despite the high bleeding risk, in the face of ST in stents implanted both in LAD and OM2, the presented patient was given abciximab and was switched to ticagrelor. Subsequent leg amputation was carried out successfully on full dual antiplatelet therapy with reasonable blood loss.

In conclusion, ST is an important serious complication following angioplasty which prevention and treatment often require difficult clinical decisions with risk-benefit assessment. Novel potent antiplatelet agents may be a reasonable choice even for patients with high bleeding risk.

Conclusions

ST is an important serious complication following angioplasty which prevention and treatment often require difficult clinical decisions with risk-benefit assessment. As a preventive measure novel potent antiplatelet agents may be a reasonable choice even for patients with high bleeding risk.

Conflict of interest: *None.*

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VIA MEDICA

CASE REPORT

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Horner's syndrome in the course of COVID-19: a case report

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ABSTRACT

In December 2019, in China appeared a new infectious disease — coronavirus disease-2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Immediately it has spread worldwide. The disease manifests itself in different ways. It may be asymptomatic. It can also cause various, non-specific symptoms such as cough, fever, sore throat, rhinitis, malaise, headache, muscle pain, diarrhea, loss of smell and taste, or rash. Sometimes, the infection leads to severe pneumonia, which may cause respiratory failure and death. But there are also less frequent manifestations of the disease. For example, increasing numbers of studies reported neurological complications, such as cerebrovascular events, seizures, meningoencephalitis, encephalopathies, acute myelitis, acute facial nerve palsy, or Guillain-Barré syndrome. In our knowledge, up to now, only a few cases of Horner's Syndrome due to COVID-19 were described. Thus, in this article, we present the case of a patient with COVID-19 pneumonia complicated by Horner's Syndrome.

Key words: Horner's syndrome, COVID-19

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Case presentation

A 38-year-old, right-handed male patient was referred to the Emergency Department due to ptosis, general weakness, fever, and mild headache. Co-workers of the patient noticed ptosis and urged him to go to the hospital as those "may be the symptoms of a stroke." He denied asymmetric muscle weakness, sensory loss, or speech abnormalities. He did not complain of dyspnea, cough, or any other upper-respiratory tract symptoms. Findings from the neurologic exam, at admission, included: left ptosis, slight left pupil constriction in the dim light, and left endophtalmus. There were no other cranial nerves abnormalities, no motor deficits were present, muscle tone was normal, reflexes were symmetric, meningeal signs were negative. The ptosis did not change during the day, no apokamnosis was present. Based on the findings from the neurologic examination, left-sided Horner's syndrome was stated as an initial diagnosis. Head computed tomography (CT) and ANGIO-CT of the cervical and intracranial arteries did not reveal abnormalities. No artery dissection was present. Due to lung inflammation noticed at the periphery of the ANGIO-CT scan, a full chest CT examination was performed and revealed ground-glass changes suggesting COVID-19 inflammation (Fig. 1). The real-time reverse transcription polymerase chain reaction (rt-RT PCR) test was done. Then the patient did not agree to further examination and hospitalization, and he was sent home to self-isolate before obtaining the SARS-CoV-2 test result.

The next day, the patient changed his mind and returned to the Emergency Department. He already had dyspnea and felt very weak. In addition, he reported a loss of smell and taste. Polymerase chain reaction for SARS-CoV-2 was positive, and the patient was diagnosed with COVID-19. He presented a SpO_2 93% on room air.

The patient was admitted to the COVID-19 ward. He received low-flow oxygen therapy with a flow velocity of 5 L/min through the nasal cannula. Dexamethasone 6 mg was administered by intravenous drip infusion every 24 h. The patient was also treated with a unit of convalescent plasma. Enoxaparin 40 mg was added by

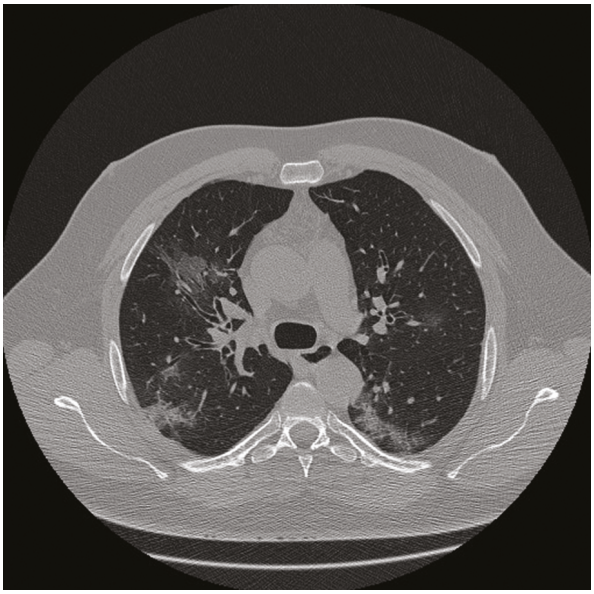

Figure 1. Chest CT-scan shows some areas of ground glass with widening of the vessels and consolidation in the posterior parts of the lobe

subcutaneous injection every 24 h. The treatment resulted in symptoms relief. After 10 days of hospitalization patient, in good condition, was discharged.

A control neurological examination showed a significant reduction of all symptoms of Horner syndrome. Magnetic resonance imaging (MRI) of the head with gadolinium enhancement showed no pathologies. Ultrasonography examination of the neck showed enlarged lymph nodes, possibly of reactive etiology.

Discussion

The potential pathophysiology, in this case, is debatable. We excluded the most common causes of Horner's syndrome: brain stem lesions (MRI), carotid artery dissection (ANGIO-CT), mass lesions in the cervical region (USG, CT) having found no pathology.

Potential causes of Horner's syndrome include a mass in the lung's apex destroying the sympathetic stem – the so-called Pancoast's syndrome — most commonly observed in patients with pulmonary adenocarcinoma. A question arises if other abnormities in the apex of the lung, such as pneumonia, may produce similar symptoms. According to the literature, the prevalence of Horner's syndrome in pneumonia is very rare. However, a few cases exist. Knyazer et al. [1] described a 7-month-old girl with complicated pneumonia and Horner's syndrome. In our knowledge, up to now, only a few of cases of Horner's Syndrome due to COVID-19 were described [2, 3]. The cause of this syndrome in the course of the disease is unclear. In our case, the ground-glass opacities reached the superior portion of the lungs. However, another possible explanation of the case may be the reactive enlargement of the cervical lymph nodes. SARS-CoV-2 is also known as a neurotropic virus, and many neurological manifestations have been described [4, 5]. One cannot exclude a possibility of direct virus action against the sympathetic tract that resolved during treatment.

Further studies are needed to determine the possible underlying mechanisms and more cases might confirm our observations.

Conclusions

Horner's syndrome can be one of the symptoms of COVID-19. The pathomechanism of this syndrome is debatable and involves the inflammatory process reaching the upper part of the lungs. Another explanation could be a reactive enlargement of the cervical lymph nodes or a direct virus effect on the nervous system.

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