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Editorial Office
ul. Marcinkowskiego 2–6
50-368 Wrocław, Poland
Tel.: +48 71 784 11 36
E-mail: redakcja@umw.edu.pl

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Contents

SGLT2 inhibitors in non-diabetic kidney disease

Vladimír Tesař[C–F]

Department of Nephrology, Charles University in Prague, Czech Republic

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Address for correspondence
Vladimír Tesař
E-mail: vladimir.tesar@vfn.cz

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Abstract

There is an accumulating evidence demonstrating renoprotective and cardioprotective role of sodium-glucose cotransporter 2 (SGLT2) inhibitors in early to advanced diabetic kidney disease. Data from recently published Dapagliflozin and Prevention of Adverse Outcomes in the Chronic Kidney Disease (DAPA-CKD) trial clearly show that dapagliflozin is similarly renoprotective in non-diabetic chronic kidney disease in a wide range of estimated glomerular filtration rate (eGFR) of 25–75 mL/min/1.73 m^2 (0.42–1.25 mL/s/1.73 m^2) and albumin/creatinine ratio 200–5000 mg/g (approx. 20–500 mg/mmol). Patients with type 1 diabetes, autosomal dominant polycystic kidney disease, antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis and lupus nephritis were excluded from the study, but, on the other hand, prespecified subanalysis demonstrated that dapagliflozin should be renoprotective also in patients with immunoglobulin A (IgA) nephropathy. The renoprotective effect of SGLT2 inhibitors is additive to the renoprotection conferred with blockers of renin–angiotensin system, including both inhibitors of angiotensin converting enzyme (ACEI), or angiotensin receptor blocker (ARB). These promising data will be hopefully confirmed by the ongoing the Study of Heart and Kidney Protection With Empagliflozin (EMPA-KIDNEY) trial, the results of which are expected later in 2022.

Key words: SGLT2 inhibitor, dapagliflozin, chronic kidney disease

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Introduction

Inhibitors of sodium-glucose cotransporter 2 (SGLT2) in the proximal tubule of the kidney were shown to slow down the progression of diabetic kidney disease (DKD) and to lower in patients with DKD the cardiovascular morbidity and mortality.

Dapagliflozin, the SGLT2 inhibitor tested in the Dapagliflozin and Prevention of Adverse Outcomes in the Chronic Kidney Disease (DAPA-CKD) study,[1] was already demonstrated to reduce renal risk in the Dapagliflozin Effect on CardiovascuLAR Events (DECLARE-TIMI) study with primary cardiovascular endpoints in patients with low renal risk.[2] Renoprotective effect of another SGLT2 inhibitor – canagliflozin – was recently demonstrated in the Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation (CREDENCE) study with primary renal endpoints in patients with type 2 diabetes and chronic kidney disease (CKD) with high renal risk.[3] As SGLT2 inhibitors do not induce hypoglycemia in non-diabetic patients and their renoprotective effect seems to be predominantly hemodynamically mediated (decrease of glomerular pressure due to constriction of the pathologically dilated afferent arteriole), they were supposed to be renoprotective also in non-diabetic patients.

The DAPA-CKD study

The DAPA-CKD study recruited 4304 patients with CKD and either type 2 diabetes or without type 2 diabetes, with estimated glomerular filtration rate (eGFR) of 25–75 mL/min/1.73 m^2 (0.42–1.25 mL/s/1.73 m^2) and albumin/creatinine ratio of 200–5000 mg/g (approx. 20–500 mg/mmol) on stable dose of either inhibitor of angiotensin converting enzyme (ACEI) or angiotensin receptor blocker (ARB), or not tolerating any of them. Patients were randomized in a ratio 1:1 to either SGLT2 inhibitor dapagliflozin (at a dose of 10 mg daily) or placebo. Patients with type 1 diabetes, autosomal dominant polycystic kidney disease, antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, and lupus nephritis were excluded from the study. Mean age of recruited patients was 61.8 ±12.1 years, mean eGFR was 43.1 ±12.4 mL/min/1.73 m^2 and mean albumin/creatinine ratio was 949 mg/g. Patients with diabetes represented 67.5% of patients. The study was preliminarily stopped after 2.4 years because of a clearly positive result.

Primary endpoint defined as the first occurrence of either decrease of GFR by 50%, end-stage renal disease or cardiovascular or renal death occurred in 9.2% of patients treated with dapagliflozin and 14.5% of patients in the placebo limb (relative risk reduction 39%, $p < 0.001$). Dapagliflozin reduced the risk of all individual components of the primary endpoint, and the effect was consistent in all predefined subgroups. The risk of primary endpoint was reduced by 36% in patients with type 2 diabetes and by 50% in non-diabetic patients. Dapagliflozin also decreased the risk of composite renal endpoint by 44% and composite cardiovascular endpoint (cardiovascular mortality or hospitalization for heart failure) by 29%. All-cause mortality was decreased by dapagliflozin by 31%.

Dapagliflozin was very well tolerated and the occurrence of serious adverse events was similar in patients treated with dapagliflozin and placebo. There was no reported case of diabetic ketoacidosis in patients with type 2 diabetes and no severe hypoglycemia in non-diabetic patients with CKD.

The lower limit of eGFR in the CREDENCE study was 30 mL/min/1.73 m^2. In the DAPA-CKD study, 14.5% of patients had eGFR lower than 30 mL/min/1.73 m^2, so the DAPA-CKD study provided first data on the renoprotectivity of SGLT2 inhibitors in this population of patients with CKD and low eGFR. Moreover, the renoprotective (and cardioprotective) effect of SGLT2 inhibitors was extended from patients with type 2 diabetes to non-diabetic patients with CKD.

Importantly, the renoprotective effect of dapagliflozin observed in the DAPA-CKD study was consistent across the whole spectrum of recruited patients: it was comparable in patients with albumin/creatinine ratio higher and lower than 1000 mg/kg and in patients with eGFR both higher and lower than 45 mL/min/1.73 m^2, although the patients with higher albuminuria and lower eGFR may benefit most.[4] In a prespecified analysis, the effect of dapagliflozin on the rate of loss of eGFR was more expressed in patients with type 2 diabetes compared to non-diabetic patients, and in patients with higher glycated hemoglobin and albuminuria.[5] In an interesting subanalysis, in 270 patients with immunoglobulin A (IgA) nephropathy recruited to the DAPA-CKD study, dapagliflozin reduced the primary outcome by 71%, suggesting that dapagliflozin could have a great potential to improve renal outcome in the patients with most frequent glomerulonephritis.[6]

Other recent and ongoing studies in DKD and non-diabetic CKD

There is a great unmet need in non-diabetic CKD patients. In patients with type 2 diabetes, we currently have the evidence for renoprotective effect of not only the inhibition of the renin–angiotensin system (e.g. The Irbesartan Diabetic Nephropathy Trial (IDNT) study[7]), but also endothelin type A (ETA) receptor antagonist atrasentan (Study Of Diabetic Nephropathy With Atrasentan (SONAR) study[8]) and non-steroidal selective inhibitor of mineralocorticoid receptor finerenone (FIDELIO-DKD study[9]).

In non-diabetic patients with CKD, there is an evidence for renoprotective effect of the renin–angiotensin system (RAS) blockade only.[10] It is important to stress than in the DAPA-CKD study, most patients were treated with RAS inhibitors, so the renoprotective effect of dapagliflozin is additive to the effect of the RAS blockade.

Since patients with CKD (and especially patients with DKD) have high cardiovascular morbidity and mortality, any renoprotective drug should be also cardioprotective, or at least without increased cardiovascular risk. Some previous studies in patients with DKD were prematurely discontinued, e.g., studies with sequential inhibition of the RAS either with the combination of lisinopril and losartan[11] or aliskiren and losartan,[12] because of an increased rate of cardiovascular events partly related to hyperkalemia. It is important to emphasize that in the DAPA-CKD study, dapaglifozin also decreased all-cause mortality and cardiovascular morbidity (hospitalization for heart failure and mortality).

Based on the data from CREDENCE and DAPA-CKD studies, the SGLT2 inhibitors should be used in all patients with DKD and eGFR > 30 (or even 25) mL/min/1.73 m^2.[13] Main contribution of DAPA-CKD is, however, the evidence for renoprotective effect of dapagliflozin also in patients with non-diabetic CKD, as for these patients for the last 20 years the only option is the inhibition of the renin–angiotensin system. Therefore, the results of the DAPA-CKD study are of utmost importance for all patients with CKD. Similar study, the Study of Heart and Kidney Protection With Empagliflozin (EMPA-KIDNEY), recruiting patients with type 2 diabetes and CKD and non-diabetic patients with CKD to either empagliflozin or placebo group, is currently ongoing and the results should be available later in 2022.[13]

Hopefully, based on the data from the DAPA-CKD study, the treatment with dapagliflozin should be also available for non-diabetic patients with CKD at risk of progression to end-stage kidney disease.

ORCID iDs

Vladimír Tesař https://orcid.org/0000-0001-6982-0689

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Impact of sacubitril/valsartan on systolic heart failure: Right heart location and clustering analysis

Yanis Bouali[1,A–D,F], Elena Galli[1,2,E,F], Elise Paven[1,2,E,F], Clement Laurin[1,2,B–D,F], Hubert Arnaud[1,2,B–D,F], Emmanuel Oger[2,A,C,E,F], Erwan Donal[2,A–F]

[1] Department of Cardiology, Rennes University Hospital, LTSI, INSERM 1099, University of Rennes, France
[2] EA Reperes, CHU Rennes, University of Rennes, France

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Address for correspondence
Erwan Donal
E-mail: Erwan.DONAL@chu-rennes.fr

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Abstract

Background. Heart failure with reduced ejection fraction (HFrEF) is a heterogeneous syndrome. In heart failure (HF) classifications, right ventricle (RV) function was for a long time unrecognized in favor of left ventricular ejection fraction (LVEF). The response to sacubitril/valsartan might differ according to phenotypes and the impact of right ventricular characteristics on this response remains controversial.

Objectives. First, we applied clustering analysis in a HFrEF population undergoing sacubitril/valsartan treatment according to guidelines, to identify phenotypes and their associated clinical outcomes. Secondly, we evaluated RV-remodeling.

Materials and methods. It is a prospective, observational, single-center study conducted on 108 symptomatic patients (mean age 66 ±12.8 years, 22.2% women). First, the clustering analysis was applied in a HFrEF population undergoing sacubitril/valsartan treatment, according to the guidelines, in order to identify phenotypes and clinical outcomes associated with them. Secondly, we evaluated RV-remodeling.

Results. Two distinct clusters were identified. Among the differences between phenotypes, RV (tricuspid annular plane systolic excursion (TAPSE) 16 ±4 mm compared to 19 ±4 mm, $p < 0.001$; RV free wall strain −19 ±5% compared to −21 ±4%, $p = 0.046$; RV fraction area change (FAC) 31 ±9% compared to 38 ±9%, $p < 0.001$), LV-filling pressure (E-wave deceleration time 138 (median: 41) ms compared to 180 (median: 94) ms, $p < 0.001$; E/e' 16.7 (median: 8.0) ms compared to 13.0 (median: 9.7) ms, $p = 0.02$) and creatinine level (106 ±34 µmol/L compared to 90 ±19 µmol/L, $p = 0.002$) were substantially different at the initiation of therapy. Major adverse cardiac events (MACEs) or death occurred in 38 out of 107 patients: 51.1% in cluster 1 compared to 24.2% in cluster 2 ($p = 0.0074$). A significant improvement in RV-functional parameters was observed under treatment. The TAPSE improved and correlated with the change in left ventricular (LV) function. Yet, it did not correlate with systolic pulmonary artery pressure (sPAP) and LV end-diastolic diameter.

Conclusions. The HFrEF phenotype characterized by more severe RV dysfunction has a worse prognosis during sacubitril/valsartan therapy. Both RV- and LV functions significantly improve when the patient is treated with sacubitril/valsartan.

Key words: remodeling, heart failure, clustering, right heart, sacubitril/valsartan

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Background

Chronic heart failure (CHF) is a complex and heterogeneous syndrome.[1] Despite a good understanding of its physiopathology, the classifications used in clinical practice continue to rely on imperfect parameters, such as left ventricular ejection fraction (LVEF)[2] or the New York Heart Association (NYHA) classification.[3] However, the evidence in favor of a continuum of LV systolic failure exists.[2] For instance, the DANISH trial perfectly demonstrated the limitations of LVEF in the stratification of arrhythmic risk and therefore the selection of patients for treatment.[4] Right ventricle (RV) function remains absent in latest guidelines despite its pivotal place in the heart. Clustering analysis is an exploratory and hypothesis-generating approach that has been shown to play an important role in identifying subtypes of complex diseases.[5] Using this approach, Ahmad et al. identified 4 phenotypically distinct and clinically meaningful CHF types responding differently to exercise programmes.[6] These findings demonstrate the heterogeneity within heart failure with reduced ejection fraction (HFrEF) patients, and the existing need for the improved phenotyping, instead of focusing on few parameters, in order to enhance the therapeutic efficacy through a more personalized management. In their analysis, LVEF was the only echocardiographic parameter and was not statistically different among the clusters.[6]

Sacubitril/valsartan, a neprilysin inhibitor combined to an angiotensin II receptor antagonist, has been shown to improve HFrEF patient prognosis.[7] This prognostic effect is associated with a striking improvement in LV-function and reverse remodeling.[8] Despite these impressive results, the response in terms of prognosis is heterogeneous across baseline LVEF-spectrum.[9] Unfortunately, RV failure was not included in PARADIGM-HF subgroup analysis, its prognosis value was not established and RV-remodeling remains poorly studied.[10–13]

Objectives

Therefore, first, we applied a clustering analysis in the HFrEF population undergoing sacubitril/valsartan treatment, according to the current recommendations,[1] in order to identify phenotypes using clinical but also echocardiographic parameters at the inclusion. Then, we studied the clinical outcome across clusters using a composite primary endpoint including major adverse cardiac events and overall death. Secondly, we evaluated remodeling of the right heart.

Materials and methods

Patients

This was a prospective, observational, single-center study conducted from November 2015 to January 2018. A total of 108 symptomatic patients with HFrEF and an indication to receive sacubitril/valsartan according to current recommendations[1] were prospectively and consecutively enrolled. They were followed in our heart failure (HF) program because they were severe and symptomatic. These were CHF patients without any acute event over the past 3 months and without any change in their treatment over the 6 weeks preceding the introduction of sacubitril/valsartan. The patients had more than 6 months of follow-up before their inclusion in the study. Clinical data, including age, sex, NYHA functional class, congestive signs, systolic and diastolic blood pressure (SBP and DBP), medical therapy, creatinine level and sinus rhythm, were assessed for each patient before the sacubitril/valsartan administration (baseline), and at the 12-month follow-ups. Coronary artery disease (CAD) was defined as a history of myocardial infarction (MI) or coronary revascularization or angiographic evidence of multiple-vessel disease or single-vessel disease with ≥75% stenosis of the left main or left proximal anterior descending artery. Transthoracic echocardiography was performed at baseline and at the 12-month follow-up. Vital status, hospitalization and ventricular arrhythmic events were extracted from hospital medical records or by interviewing the physicians in charge of the patients. The presence of ventricular arrhythmias was defined by the occurrence of sustained ventricular tachycardia demanding hospitalization or implantable cardioverter-defibrillator (ICD) appropriate shock. Major adverse cardiac events (MACEs) were defined as the composite of overall death, HF-related hospitalization and sustained ventricular arrhythmias. A previous report about this cohort has been published.[14]

The study was conducted in accordance with "Good Clinical Practice" Guidelines of the Declaration of Helsinki. All patients provided written informed consent for participation in the study. The study protocol was approved by the Comité de Protection des Personnes (CPP) Sud-Ouest et Outre Mer (CCP Sud Est V, No. ID RCB: 2017-A02217-46).

Sacubitril/valsartan therapy

All patients received the maximum tolerated HF treatment before the sacubitril/valsartan initiation. The 1st dose of 24/26 mg or 49/51 mg was administered twice daily, according to the blood pressure, age and biological parameters. In patients switching from an angiotensin-converting enzyme (ACE) inhibitor, a washout period of 36 h was implemented before the initiation of the treatment. The uptitration of the treatment was performed at the discretion of the physicians up to a target dose of 97/103 mg twice daily, if well tolerated.

Echocardiographic analysis

Before the initiation of sacubitril/valsartan and at the 12-month follow-up, all patients underwent

transthoracic 2D echocardiography using standard equipment (Vivid 9 or 95; GE Healthcare, Horten, Norway), supplied with a 3S or M5S 3.5-mHz transducer, with the same imaging protocol. Bidimensional, colour Doppler, pulsed wave, and continuous wave Doppler data were stored on a dedicated work station for offline analysis (EchoPAC; GE Healthcare).

Cardiac dimensions and functions were measured according to the current recommendations.[15]

The RV function was described using tricuspid annular plane systolic excursion (TAPSE), Doppler S velocity, RV free wall strain in apical RV-dedicated 4-chamber view, RV fraction area change (FAC) and Tei index (myocardial performance index (MPI)).

Diastolic function and LV filling pressure were quantified according to the recommendations.[16] In patients with detectable tricuspid regurgitation, systolic pulmonary artery pressure (sPAP) was estimated. The RV-arterial coupling was estimated with the TAPSE/sPAP ratio.[17]

Statistical analysis

Data are presented as mean ± standard deviation (SD), median (interquartile range (IQR)) and frequency/percentage depending on the nature of variables.

Prior to the analysis, missing data were imputed using the singular value decomposition (SVD) impute function within the impute package in R software (The R Project for Statistical Computing, Vienna, Austria). The percentage of missing values ranged from 0% to 50% (for TAPSE/sPAP ratio).

Before the cluster analysis was performed, the Gower dissimilarity was used to measure the closeness between each observation. This measure implies standardization, which is set to range for interval and ordinal variables. A hierarchical cluster analysis was conducted in PROC CLUSTER (SAS v. 9.4; SAS Institute Inc., Cary, USA), using Ward's minimum variance method with standardization. All clustering was blinded to the clinical outcome data. To better define the relevant number of clusters, we looked for a consensus among 3 statistics – local peaks of the cubic clustering criterion (CCC) and pseudo-F statistic combined with a small value of the pseudo t^2 statistic and a larger pseudo t^2 for the next cluster fusion. Once clusters were defined, they were compared in regard to demographic, clinical and echocardiographic characteristics.

For outcome analyses, Fisher's exact test (main outcome) or Kruskal–Wallis test (secondary outcomes) was used to test the independent associations between the clusters and the outcomes. Freedom from MACEs or death was plotted for both clusters using Kaplan–Meier curves, and between-cluster differences in freedom from events were tested using the log-rank test, Wilcoxon test and the –2 log likelihood-ratio (LR) test.

The comparison of changes from baseline to the 12 months follow-up was based on the signed-rank statistic test. For the null hypothesis, the mean change was equal to 0.

Correlations between relative variation of TAPSE and relative variation of LVEF, global longitudinal strain (GLS), left ventricular end-diastolic diameter (LVEDD) and sPAP in overall population were estimated with Pearson's correlation coefficient.

Results

Patient characteristics

The characteristics of patients with missing values at baseline are depicted in Table 1.

The patients' mean age was 66 ±12.8 years. Twenty-four (22.2%) patients were female, and coronary artery disease (CAD) was observed in 52 (48.1%) patients. Congestive signs at enrollment were present in 41 (39%) patients. The mean creatinine level was 97 ±31 µmol/L. The mean N-terminal prohormone of brain natriuretic peptide (NT-proBNP) level was 1624 (IQR 897–2761) pg/mL. Seventy-eight (75%) patients were in sinus rhythm at enrollment.

Echocardiographic parameters

Echocardiographic characteristics and missing values of patients at baseline are depicted in Table 2.

Our population had significantly reduced mean LVEF, mean cardiac index and LV dilatation at baseline. The median E/e' was in favor of elevated LV-filling pressures. The left atrium (LA) was dilated. The sPAP was elevated considering the mean tricuspid regurgitation peak velocity (TR Vmax). The RV function, characterized by mean TAPSE, free wall strain, Doppler S velocity, Tei index (MPI) and FAC, was slightly impaired. The right heart was dilated based on the mean tricuspid annulus diameter.

Cluster analyses: patient and echocardiographic characteristics

Cluster analysis identified 2 clusters. Patient characteristics across clusters are shown in Table 3, and echocardiographic characteristics are shown in Table 4.

Cluster 1 was smaller (n = 45) than cluster 2 (n = 63). In the 1st cluster, patients had more comorbid conditions, with higher creatinine levels and higher rates of atrial fibrillation (AF). This cluster seemed to have more advanced HF pathology, with more patients in stage III or IV of the NYHA classification, the presence of congestive signs, and higher NT-proBNP levels. Regarding the treatment, 100% of patients used β blockers. Loop diuretics were more often used in this cluster as aldosterone receptor antagonists.

Regarding echocardiographic parameters, cluster 1 included patients with lower LVEF (27 ±11% compared to 30 ±8%, p = 0.045), and LV diastolic function parameters

Table 1. Baseline and 12-month follow-up population characteristics

Characteristics	Values	Baseline missing values n (%)	Baseline values* n (%)	12-month FU values* n (%)
Female, n (%)	–	0	24 (22.2)	–
Age [years]	–	0	66.0 ±12.8	–
BMI [kg/m^2]	–	0	27.8 ±5.10	–
Creatinine [µmol/L]	–	25 (23.1)	97 ±31	106 ±41
SBP [mm Hg]	–	18 (16.7)	122 ±19	123 ±19
NYHA class	I	0	4 (3.70)	31 (39.7)
	II	–	78 (72.2)	38 (48.7)
	III	–	21 (19.4)	9 (11.5)
	IV	–	5 (4.63)	0 (0)
Congestive HF	–	3 (2.78)	41 (39.0)	15 (18.7)
NT-proBNP [ng/L]	–	27 (25.0)	1624 [897–2761] [min–max]	1014 [412–2306] [min–max]
CAD	MI	0	51 (47.2)	–
	PCI	–	1 (0.93)	–
COPD	–	0	16 (14.8)	–
Sinus rhythm	–	4 (3.70)	78 (75.0)	–
β-blockers	–	7 (6.48)	96 (95.0)	79 (98.7)
Loop diuretic	no treatment	–	20 (21.3)	23 (30.3)
	low dose (≤80 mg)	14 (13.0)	57 (60.6)	–
	medium (120 mg)	–	8 (8.51)	–
	high dose (>125 mg)	–	9 (9.57)	–
Aldosterone receptor antagonists	–	13 (12.0)	57 (60.0)	49 (64.5)

BMI – body mass index; HF – heart failure; CAD – coronary artery disease; COPD – chronic obstructive pulmonary disease; NYHA – New York Heart Association; SBP – systolic blood pressure; NT-proBNP – N-terminal prohormone of brain natriuretic peptide; MI – myocardial infarction; PCI – percutaneous coronary intervention; FU – follow-up; * mean ± standard deviation (SD), median (Q1–Q3) or frequency (%).

were more altered in regard to E-wave deceleration time (138 (median: 41) ms compared to 180 (median: 94) ms, $p < 0.001$), E/e' (16.7 (median: 8.0) ms compared to 13.0 (median: 9.7) ms, $p = 0.022$) and left atrium volume index (LAVI) (55 ±21 mL/m^2 compared to 42 ±17 mL/m^2, $p < 0.001$). The TR Vmax was higher (3.10 ±0.44 m/s compared to 2.92 ±0.45 m/s, $p = 0.037$). The RV function was more impaired in terms of TAPSE, RV free wall strain and FAC (16 ±4 mm compared to 19 ±4 mm, $p < 0.001$; –19 ±5% compared to –21 ±4%, $p = 0.046$; 31 ±9% compared to 38 ±9%, $p < 0.001$, respectively). Right cavity remodeling was also significantly greater for the tricuspid annulus and right atrium (RA) (40 ±7 mm compared to 37 ±7 mm, $p = 0.010$; 78 (median: 45) mL compared to 51 (median: 36) mL, $p = 0.001$, respectively). The TAPSE/sPAP ratio was more impaired (0.37 ±0.15 mm/mm Hg compared to 0.52 ±0.25 mm/mm Hg, $p = 0.031$).

Follow-up

The median clinical follow-up was 359 (median: 133) days. The MACEs or death occurred in 38 patients: 51.1% in cluster 1 as compared to 24.2% in cluster 2 ($p = 0.0074$) (Fig. 1). The Kaplan–Meier survival curves (Fig. 1A) and stacked bar graphs (Fig. 1B) show that cluster 1 was at significantly higher risk for the primary endpoint of MACEs or overall death than cluster 2.

Right ventricular function improvement

Functional parameters improvement was observed in the right heart, from baseline to 12-month follow-up, regarding TAPSE, TAPSE/sPAP ratio, RV free wall strain, and FAC (Fig. 2). The TAPSE, FAC and RV free wall strain were also significantly improved in each cluster.

Moreover, tricuspid annulus diameter was significantly decreased at 12-month follow-up ($p < 0.001$).

A significant LV reverse remodeling was observed from baseline to 12-month follow-up regarding LVEF ($p < 0.0001$), GLS ($p < 0.0001$) and LVEDD ($p < 0.0001$). The LV diastolic function parameters, including E/A, E/e' and TR Vmax, were particularly improved from baseline to the 12-month follow-up. The LAVI did not significantly change (Fig. 3).

Table 2. Baseline and 12-month follow-up echocardiographic characteristics

Characteristics	Baseline missing values n (%)	Baseline values*	12-month FU values*	p-value
LV end-diastolic diameter [mm]	2 (1.85)	64 ±9	60 ±10	<0.01
LV end-systolic diameter [mm]	6 (5.55)	54 ±11	50 ±11	<0.01
LVEF (%)	2 (1.85)	29 ±9	39 ±12	<0.01
Cardiac output [L/min]	3 (2.78)	2.01 ±0.57	2.36 ±0.74	<0.01
GLS (%)	22 (20.4)	−9 (−11; −7)	−13 (−16; −9)	<0.01
Peak systolic dispersion [ms]	22 (20.4)	67 (57; 82)	63 (51; 74)	<0.01
E/A	26 (24.1)	1.3 (0.8; 2.6)	0.9 (0.6; 1.5)	<0.01
DTE [ms]	6 (5.55)	155 (129; 195)	190 (148; 261)	<0.01
E/e'	18 (16.7)	14 (10; 21)	11 (9; 15)	<0.01
LAVI [mL/m^2]	2 (1.85)	48 ±20	47 ±20	0.045
RA volume [mL/m^2]	6 (5.55)	62 (41; 88)	56 (35; 73)	<0.01
TAPSE [mm]	4 (3.70)	18 ±5	21 ±5	<0.01
RV free wall strain (%)	28 (25.9)	−20 ±6	−22 ±9	<0.01
S' tricuspid [cm/s]	9 (8.33)	0.10 ±0.02	0.11 ±0.02	0.07
Tei-index TDI	21 (19.4)	0.61 ±0.16	0.53 ±0.13	<0.01
FAC (%)	15 (13.9)	35 ±10	39 ±10	<0.01
Tricuspid annulus [mm]	8 (7.41)	38 ±7	33 ±8	<0.01
TR Vmax [m/s]	39 (36.1)	2.99 ±0.57	2.72 ±0.61	<0.01
TAPSE/sPAP [mm/mm Hg]	54 (50)	0.46 ±0.23	0.62 ±0.31	<0.01

DTE – E-wave deceleration time; FAC – fractional area change; GLS – global longitudinal strain; LAVI – left atrial volume index; LVEF – left ventricular ejection fraction; RA – right atrium; RV – right ventricle; sPAP – systolic pulmonary artery pressure; TAPSE – tricuspid annular plane systolic excursion; TDI – tissue doppler imaging; TR Vmax – tricuspid regurgitation peak velocity; FU – follow-up. * mean ± standard deviation (SD), median (Q1–Q3).

Table 3. Baseline population characteristics across clusters

Variables	Value	Total 1 (n = 45)	Cluster (1 n = 45)	Cluster 2 (n = 63)	p-value
Male sex	–	84	84.4 (38)	73.0 (46)	0.159
Age [years]	–	–	66.6 ±14.2	65.6 ±11.9	0.715
BMI [kg/m^2]	–	–	28.6 ±5.42	27.1 ±4.74	0.134
Creatinine [µmol/L]	–	–	106 ±34	90 ±19	0.002
SBP [mm Hg]	–	–	119 ±20	123 ±14	0.189
NYHA	I–II	82	53.3 (24)	92.1 (58)	<0.001
	III–IV	26	46.7 (21)	7.90 (5)	–
Congestive HF	–	41	73.3 (33)	12.7 (8)	<0.001
NT-proBNP	–	–	2144 (1894)	1646 (1510)	0.003
CAD-MI	–	51	42.2 (19)	50.8 (32)	0.379
COPD	1	16	13.3 (6)	15.9 (10)	0.714
Sinus rhythm	1	82	53.3 (24)	92.1 (58)	<0.001
β-blockers	1	103	100 (45)	92.1 (58)	0.054
Aldosterone receptor antagonists	1	70	75.6 (34)	57.1 (36)	0.049
Loop diuretic	no treatment	20	4.44 (2)	28.6 (18)	–
	low dose (≤80 mg)	71	62.2 (28)	68.2 (43)	<0.001
	high dose (>80 mg)	17	33.3 (15)	3.17 (2)	–

BMI – body mass index; HF – heart failure; CAD-MI – coronary artery disease-myocardial infarction; COPD – chronic obstructive pulmonary disease; NYHA – New York Heart Association; NT-proBNP – N-terminal prohormone of brain natriuretic peptide; SBP – systolic blood pressure. Data are presented as mean ± standard deviation (SD) or median (interquartile range (IQR)).

Table 4. Baseline echocardiographic characteristics across clusters

Variable	Cluster 1 (n = 45)	Cluster 2 (n = 63)	p-value
LV end-diastolic diameter [mm]	66 ±10	62 ±8	0.068
LVEF (%)	27 ±11	30 ±8	0.045
Cardiac output [mL/m^2]	1.91 ±0.55	2.08 ±0.57	0.138
GLS (%)	−9 (2)	−9 (3)	0.187
Peak systolic dispersion [ms]	73.9 (12.0)	68.0 (22.0)	0.396
E/A	2.21 (0.33)	1.27 (1.44)	0.627
DTE [ms]	138 (41)	180 (94)	<0.001
E/e'	16.7 (8.0)	13.0 (9.7)	0.022
LAVI [mL/m^2]	55 ±21	42 ±17	<0.001
RA volume [mL/m^2]	78 (45)	51 (36)	<0.001
TAPSE [mm]	16 ±4	19 ±4	<0.001
Strain lat. RV (%)	−19 ±5	−21 ±4	0.046
Tei-index TDI	0.62 ±0.15	0.59 ±0.13	0.245
FAC (%)	31 ±9	38 ±9	<0.001
Tricuspid annulus [mm]	40 ±7	37 ±7	0.010
TR Vmax [m/s]	3.10 ±0.44	2.92 ±0.45	0.037
TAPSE/sPAP [mm/mm Hg]	0.37 ±0.15	0.52 ±0.25	0.031

DTE – E-wave deceleration time; GLS – global longitudinal strain; LAVI – left atrial volume index; LVEF – left ventricular ejection fraction; RA – right atrium; RV – right ventricle; sPAP – systolic pulmonary artery pressure; TAPSE – tricuspid annular plane systolic excursion; TDI – tissue doppler imaging; TR Vmax – tricuspid regurgitation peak velocity; FAC – fractional area change. Data are presented as mean ± standard deviation (SD) or median (interquartile range (IQR)).

Correlation of right ventricular function improvement

Correlation between TAPSE improvement and LVEF, GLS, LV end-diastolic diameter and sPAP are presented in Fig. 4. The TAPSE improvement was significantly associated with LVEF and GLS improvement, whereas sPAP and LV end-diastolic diameter were not.

Discussion

The main findings of the study are as follows: 2 patient phenotypes were identified based on the study inclusion criteria (at the initiation time of sacubitril/valsartan), and the RV function seems to be a major factor distinguishing these subsets. Cluster 1 included significantly more symptomatic and congestive patients with higher NT-proBNP levels, where RV-functional parameters were more impaired concomitantly to LVEF, LV diastolic function and renal function abnormalities. Patients included in cluster 1 had a worse prognosis regarding the primary outcome of MACEs or death. The RV function under sacubitril/valsartan was significantly improved, and TAPSE improvement significantly correlated with change in LVEF and GLS, but not with sPAP and LVEDD.

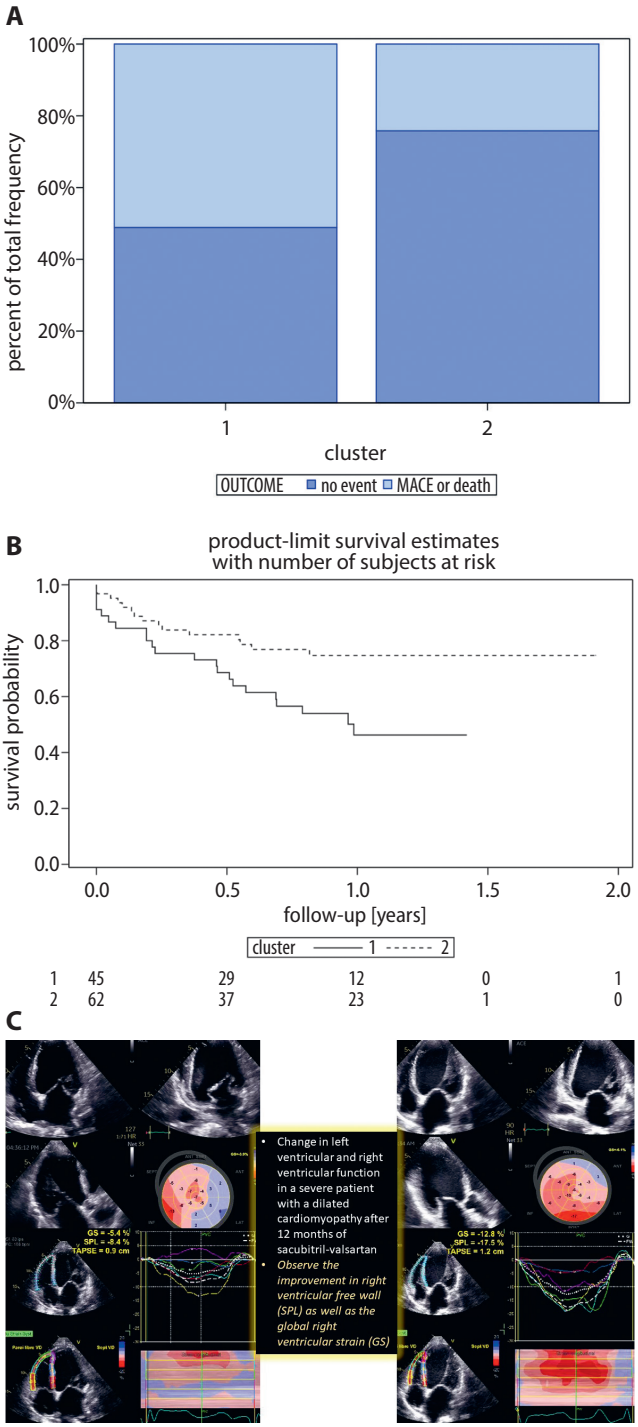

Fig. 1. A. Stacked bar graph of outcome by cluster; B. Kaplan–Meier survival curve for time to major adverse cardiac event (MACE) or overall death. Thirty-eight MACEs or overall deaths occurred among 107 patients (1 lost to follow-up); 51.1% (23 MACEs or deaths out of 45 patients) compared to 24.2% (15 MACEs or deaths out of 62 patients), p = 0.0074; C. The example of left ventricular (LV) and right ventricular function at inclusion and 1 year later

Prognosis role of baseline RV failure in HFrEF using clustering analysis

The cluster analysis allowed for the identification of 2 distinct meaningful demographic, clinical and echocardiographic phenotypes in a population of patients with

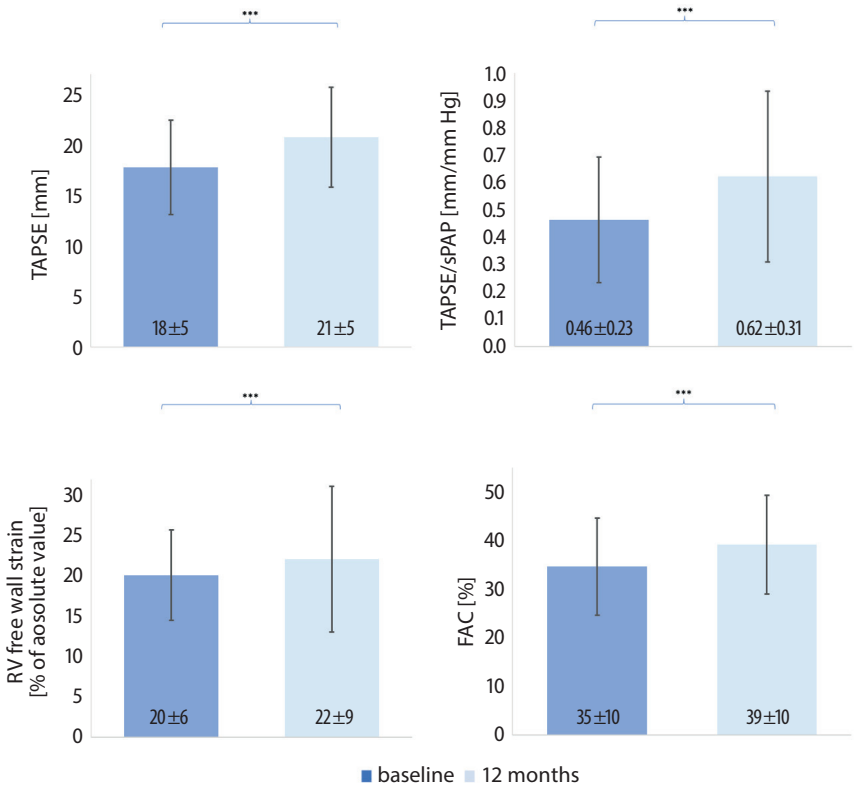

Fig. 2. Evolution of right ventricle (RV) function parameters under sacubitril/valsartan in overall population. Tricuspid annular plane systolic excursion (TAPSE), RV fraction area change (FAC) and RV free wall strain significantly improved in each cluster ($p < 0.05$). Mean ± standard deviation (SD)

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; sPAP – systolic pulmonary artery pressure.

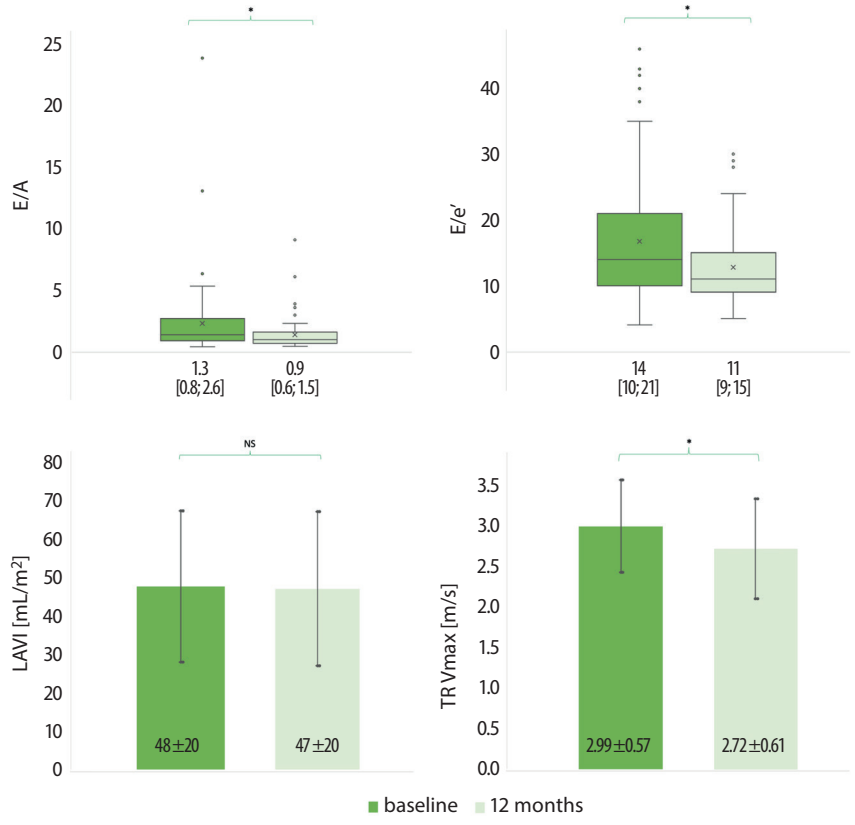

Fig. 3. Evolution of left ventricular (LV) diastolic function parameters under sacubitril/valsartan in the whole study population. Mean ± standard deviation (SD) or median (Q1–Q3)

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; LAVI – left atrial volume index; TR Vmax – tricuspid regurgitation peak velocity.

chronic HFrEF receiving sacubitril/valsartan treatment. All clustering was blinded to the clinical outcome data. Yet, we demonstrate that cluster 1, representing a more advanced stage of HFrEF, has a significant dismal prognosis.

Our results seem to be in accordance with existing data. In the ESCAPE trial, renal dysfunction and NT-proBNP level, as well as HF-ACTION population were associated with a higher risk of clinical outcome in a cluster

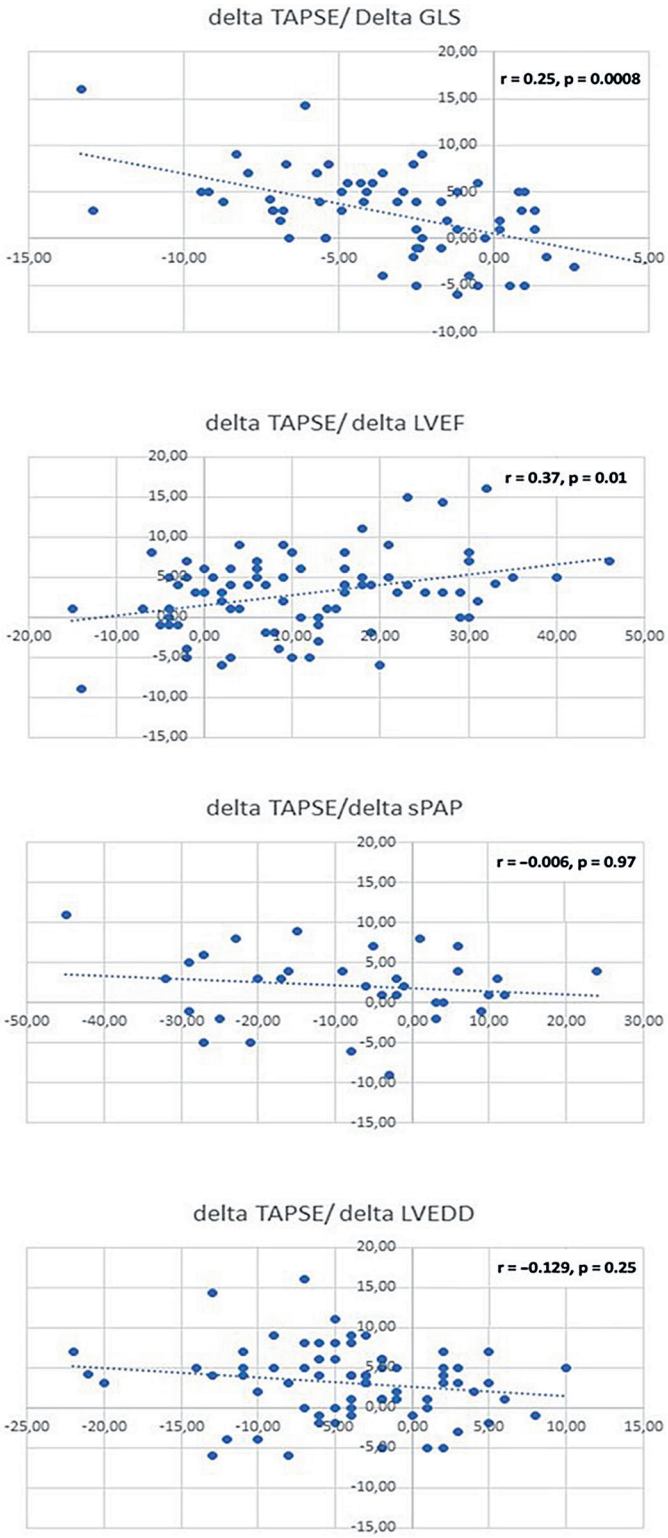

Fig. 4. Correlation between tricuspid annular plane systolic excursion (TAPSE) relative variation and left ventricular ejection fraction (LVEF), global longitudinal strain (GLS), systolic pulmonary artery pressure (sPAP), and left ventricular end-diastolic diameter (LVEDD) relative variation at 12-month follow-up in overall population

consisting mostly of older patients with ischemic cardiomyopathy, AF and diuretics.[17] In a Swedish population of HF patients, the cluster that had the worst prognosis consisted of patients with the most advanced age, more important adverse symptoms, lowest creatinine clearance, and the highest NT-proBNP levels despite diuretic use, AF and MI rate (24% of patients with HF had preserved ejection fraction (EF) in this registry).[18] Curiously, no differences were observed in terms of age or frequency of ischemic etiology in our cohort.

Cluster 1, exhibiting a more severe clinical and echocardiographic profile with documented overload and activation of neurohormones (which are one of the main therapeutic targets of sacubitril/valsartan[7]), had the most unfavorable prognosis. This result might seem obvious but was never demonstrated until now. Thus, it could have an important clinical impact. Indeed, sacubitril/valsartan has not been first introduced in this study. It has been used in patients already uptitrated with ACE-inhibitors, like in the PARADIGM-HF trial.[7] Nevertheless, our results encourage a much earlier therapy introduction, especially in the youngest patients. The main risk of this therapy in patient expected to have a dismal prognosis would be wasting time and removing the patient from a group with indication for transplant. Heterogenous response across LVEF spectrum was observed.[9] Sacubitril/valsartan is superior to enalapril. Neverthelesss, the examination of the LV only cannot help in predicting the response expected at the introduction of the therapy.[1] Our study has the advantage of providing other parameters, such as RV function parameters, which were not included in cluster analysis of HF ACTION, ESCAPE and the Swedish registry, despite the prognosis burden of the RV failure.[19] Indeed, a major result of our study is a significant baseline RV failure regarding TAPSE, FAC and RV-free wall strain in cluster 1, as compared to cluster 2. Considering the TAPSE/sPAP ratio, RV pulmonary arterial coupling seemed to be impaired in cluster 1, indicating a lack of RV-adaptation to an increased afterload, and therefore an altered contractile reserve.[16] Bosch et al. demonstrated the independent prognostic value of abnormal RV-pulmonary arterial coupling in such patients.[19]

Given the significant improvement of each parameter in both clusters, baseline RV failure seems to be involved in the prognosis more than RV function improvement. The patients from cluster 1 might have been switched to sacubitril/valsartan too late (once RV failure was too advanced). Prospective randomized trials are necessary to compare the introduction of sacubitril/valsartan before and after RV dysfunction related to the occurrence of HFrEF. For the most severe cases, the switch to sacubitril/valsartan could be proposed without delaying the decision-making process for more invasive therapeutic strategies, such as ventricular assist devices or transplantation.

RV failure within the pathological process of HFrEF

Cluster 1 requires higher doses of loop diuretics and mineralocorticoid receptor antagonists in patients

suffering from a cardiorenal syndrome (type II).[20] Despite an important prevalence of renal insufficiency in HF patients, the physiopathology of this syndrome remains largely open to discussion. In the context of HF, the association of renal impairment, even moderate, with poor prognosis is well known.[21] The NT-proBNP, a major prognostic factor in HF,[22] is known to be higher in cases of renal failure, underlying the coherence of the results we observed with our cohort. The association between higher creatinine and NT-proBNP levels and RV dysfunction (and right cavities remodeling), highlighted by our findings, is consistent with existing data.[20] The RV function, more than LV function, seems to have a major influence on cardiorenal syndrome with the elevated central venous pressures leading to renal venous hypertension and to the reduction of intrarenal blood flow.[23] The independence of the association between elevated central venous pressures and renal dysfunction was demonstrated, and right atrial pressure was shown to be a predictor of all-cause mortality.[24] Although RV failure was never directly correlated to renal failure, our findings suggest a possible RV role in the genesis of elevated central venous pressures and in type II cardiorenal syndrome. The cause–effect relationship remains to be elucidated, and further studies are needed.

The concomitant presence of atrial arrhythmias and RV failure in cluster 1 is consistent. In our analysis, 92% of patients in cluster 2 were in sinus rhythm, compared with only 53% in cluster 1, in accordance with the difference in right atrial and LA volumes between clusters. Indeed, atrial arrhythmias were identified as an independent prognostic factor of RV dysfunction in left-sided HF patients.[19] The atrial arrhythmias were also largely described as a major prognostic factor in HFrEF.[25]

Improvement of RV-functional parameters with sacubitril/valsartan

One of the main results of this study was the capability of the right heart to improve with sacubitril/valsartan. This is important as RV recovery in HFrEF patients is associated with an improved prognosis.[26] According to the previous studies, although it is demonstrated and observed in practice that sacubitril/valsartan decreases pulmonary pressures, Correale et al.[12] were the only researchers to have recently prospectively shown the associated improvement in RV function in regard to TAPSE.[10,11,13,27] Yenerçağ et al.[28] retrospectively showed the significant increase of TAPSE, FAC and MPI at 6-month follow-up. Supposedly, studies that did not show any significant RV improvement did not have follow-up long enough. Our cohort has the advantage to be the first to allow for a multiparametric assessment of RV function. It brings strength to the results despite the limited size of the cohort. Several parameters that could be required for the best assessment of RV function and its change over time were used.[14,29] Tricuspid annulus diameter was used as a surrogate marker of RV remodeling. It significantly decreased in size over time, confirming the right heart reverse remodeling that could happen over time, especially in cluster 2.

Correlations of RV function improvement under sacubitril/valsartan

In this study, it was demonstrated that switching from an ACE inhibitor to sacubitril/valsartan positively impacts loading conditions and diastolic filling patterns, as well as TASPE/sPAP ratio and RV function parameters. Therefore, the RV afterload decrease could be a possible mechanism of RV function improvement. However, TAPSE improvement was not significantly correlated with sPAP but with LVEF and GLS. It confirms the key role of systolic RV-LV interactions involved in the pathological process of HFrEF.[19] This correlation was already described under optimal medical treatment. The RV FAC normalization was significantly associated with subsequent LV-reverse remodeling, but only at 24 months, and sacubitril/valsartan was not introduced in this study.[30] A recent publication suggested a possible association between LV- and RV-functional reverse remodeling, but no correlation analyses were performed.[31] In the field of cardiac resynchronization, a post hoc analysis of MADIT-CRT trial demonstrated that significant RV function improvement was correlated with change in LVEF and LV end-diastolic volume but not in sPAP.[32] The LV contribution to RV ejection was previously described[33] and this interdependence is anatomically supported by shared myocardial fibers through the septum or free wall epicardial layer. According to some authors, RV longitudinal deformation holds the major role in RV ejection,[34,35] despite the scarcity of RV longitudinal fibers. The interventricular septum shortening might play a crucial role in the interaction between right and left ventricles. In dilated cardiomyopathy, LV spherization leads to an alteration of septal fibres' helical orientation, turning to a more transversal pattern, and decreasing RV longitudinal deformation mediated by the septum.[34] It results in lower mechanical efficiency and afterload adaptation, responsible for RV dysfunction followed by dilation. Consequently, TR worsens the vicious circle of HF. Therefore, we can formulate a hypothesis that LV reverse remodeling, already demonstrated under sacubitril/valsartan,[8] corrects fibres' orientation and their contribution to longitudinal RV systolic function. In our study, the importance of longitudinal deformation in RV ejection could explain the stronger correlation of TAPSE improvement with GLS, rather than LVEF. The downregulation of fibrosis signaling mediated by sacubitril/valsartan[36] could be involved, enhancing LV fibers contractility and their contribution to RV ejection. The direct effect of sacubitril/valsartan on RV cannot be ruled out.

Limitations

Several limitations of this study must be considered. First, the cohort was prospective, but limited in size and monocentric. Second, it is challenging to extrapolate the prognostic impact of the severity of the disease and the impact of sacubitril/valsartan introduction. It could have been interesting to have a group of patients in which sacubitril/valsartan would have been introduced before, as recommended by the American guidelines,[37] in order to make a comparison in terms of a prognosis impact possible.

Conclusion

According to the clustering analysis, 2 phenotypes of HFrEF patients were generated, where RV failure, apart from AF, LV dysfunction and renal dysfunction at the initiation of therapy appears to be an important prognostic determinant during sacubitril/valsartan therapy. The RV function, similarly to LV function, significantly improves under sacubitril/valsartan, as indicated by TAPSE, FAC and RV free wall strain.

ORCID iDs

Yanis Bouali https://orcid.org/0000-0001-9923-6547
Elena Galli https://orcid.org/0000-0001-6692-9101
Elise Paven https://orcid.org/0000-0003-4543-2141
Clement Laurin https://orcid.org/0000-0002-1697-2504
Hubert Arnaud https://orcid.org/0000-0002-4994-1165
Emmanuel Oger https://orcid.org/0000-0001-9837-2977
Erwan Donal https://orcid.org/0000-0003-2677-3389

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Effect of sodium hypochlorite, isopropyl alcohol and chlorhexidine on the epoxy sealant penetration into the dentinal tubules

Wojciech Wilkoński[1,A–D], Lidia Jamróz-Wilkońska[1,A,C,D], Mariusz Kępczyński[2,B,C], Szczepan Zapotoczny[2,B,C], Urszula Maziarz[2,B,C], Janusz Opiła[3,B,C], Jolanta Pytko-Polończyk[4,A,E,F]

[1] Research Department of the Polish Endodontic Society, Kielce, Poland
[2] Department of Physical Chemistry and Electrochemistry, Faculty of Chemistry, Jagiellonian University, Kraków, Poland
[3] Department of Applied Computer Science, Faculty of Management, AGH University of Science and Technology, Kraków, Poland
[4] Department of Integrated Dentistry, Dental Institute, Faculty of Medicine, Jagiellonian University Medical College, Kraków, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Wojciech Wilkoński
E-mail: wilkonski@onet.eu

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Abstract

Background. The sealer penetration into the dentinal tubules might be beneficial, especially in necrotic endodontic cases, as it provides the obstruction of the contaminated tubules.

Objectives. To determine the effect of 3 final irrigants (sodium hypochlorite (NaOCl), alcohol and chlorhexidine (CHX)) on the penetration of an epoxy sealer into the dentinal tubules.

Materials and methods. The study was carried out on 60 single-canal human teeth with straight roots. The root canals were prepared to the ISO 40/04 size, using the Reciproc® instruments. The teeth were divided into 4 groups (n = 15). The canals in each group were irrigated according to the following scheme: group 1 (control) – 5.25% NaOCl; group 2 – smear layer removal (40% citric acid (CA) and 5.25% NaOCl) and 5.25% NaOCl; group 3 – smear layer removal (as in group 2), and 40% CA, water and 98% isopropyl alcohol; and group 4 – smear layer removal (as in group 2), and 40% CA, water and 2% CHX. The root canals were filled using the vertical condensation technique with gutta-percha and the porphyrin-labeled AH Plus™ sealer. After 3 days, 1-milimeter-thick cross-section slices were cut from the roots at a distance of 2 mm, 5 mm and 8 mm from the apex. The sections were imaged under a confocal microscope and the sealant penetration depth into the dentinal tubules was measured.

Results. The longest resin tags in all parts of the roots were found in group 4 (CHX), and the shortest in group 1 (control). The mean depth of the sealer penetration (in micrometers) was as follows: 21, 22 and 23 (group 1); 201, 231 and 374 (group 2); 170, 232 and 280 (group 3); and 330, 408 and 638 (group 4) in the apical, middle and coronal parts, respectively.

Conclusions. The final irrigation with CHX resulted in the deepest penetration of the epoxy sealer into the tubules. Isopropyl alcohol had the most negative impact on the sealer penetration into the tubules.

Key words: obturation, chlorhexidine, AH Plus™, confocal laser scanning microscopy, dentinal tubules penetration

Background

The primary factors that affect the success of endodontic treatment are the decontamination of the root canal system, and its tight filling and protection against the oral cavity environment. Due to the complexity of the endodontic system, it is impossible to completely eliminate impurities and microorganisms through mechanical preparation only.[1] In addition, during the root canal instrumentation, a smear layer is formed, which closes the dentinal tubules.[2,3] In the case of endodontic system infections, pathogens occur not only within the root canal and on the surface of its walls, but also penetrate deep into the dentinal tubules.[2,3] The intensive irrigation of the root canals and the dentinal tubules before filling is therefore necessary to remove mechanical processing remnants, the leftovers of the smear layer, and also to effectively decontaminate the whole endodontic space.[2–8] After the smear layer removal, the dentinal tubules are opened, which allows antiseptic irrigants to penetrate and decontaminate the dentin more effectively.[2] Additionally, during the gutta-percha condensation, the sealer is inserted into the open dentinal tubules to provide a potential additional barrier, with the sealing and micromechanical anchoring of the material in the canal.[3–9] The depth of the sealer penetration into the dentinal tubules is influenced by the completeness of the smear layer removal, the obturation method, the type and properties of the sealer, and its interaction with the dentin and the residual remnants of the final irrigant used during irrigation.[3–6,8] For the root canal irrigation protocol, a variety of final irrigants can be used, such as distilled water, saline, sodium hypochlorite (NaOCl), alcohol, or chlorhexidine (CHX). These liquids are characterized by different properties, such as hydrophilicity, the contact angle, surface tension, and osmotic pressure.[10] Sodium hypochlorite decomposes into a hyperosmotic sodium chloride solution, the residue of which can theoretically act as an osmotic barrier against the deeper penetration of the sealer.[11] On the other hand, NaOCl has some lubricating properties, as it can reduce torsional stress during the instrumentation of the root canal.[12] Isopropyl alcohol has a low surface tension, dissolves many hydrophobic substances and displaces water, improves the bond strength of the sealer, and thus has a potentially beneficial effect on the penetration of the hydrophobic sealer into the dentinal tubules.[13,14] In contrast, a CHX aqueous solution is a typical wetting agent with a low surface tension.[15] However, its effect on the sealer penetration is not known.

The null hypothesis of this study is the assumption that the use of alcohol as a final irrigant will increase the depth of the epoxy sealer penetration into the dentinal tubules.

Objectives

The aim of the study was to determine the effect of 3 irrigants (NaOCl, alcohol and CHX), on the penetration of the AH Plus™ epoxy sealer into the dentinal tubules.

Materials and methods

Tooth selection and preparation

The study material consisted of 60 extracted human upper incisors with straight roots and single root canals. The teeth were removed for periodontal reasons. Following the extraction, the teeth were stored in a 1% chloramine solution. After cleaning, the roots were cut at the cementoenamel junction, using a diamond-coated, double-sided separator with continuous water-air cooling.

Root canal preparation

The root canals were opened using ISO 10 size C files (VDW, Munich, Germany). The working length was established by obtaining an anatomical foramen and subtracting 1 mm. The canals were prepared using the crown-down technique with the Reciproc® 25 instruments (VDW). A Silver Reciproc micromotor (VDW) was used at the "Reciproc all" settings, according to the manufacturer's guidelines (a reciprocating motion: 120 degrees counterclockwise and 30 degrees clockwise; 300 cycles/min). Then, the canals were widened with the Reciproc 40 instruments (VDW) and calibrated with ISO 40 size K files (VDW). Before preparation, a small amount of a FileCare® lubricant (VDW) was applied to each instrument, and after using each instrument, the root canals were rinsed with 1 mL of 5.25% NaOCl. During the flaring of the coronal and middle parts of the root canals (with the use of the Reciproc 25 instruments), pecking and brushing motions were performed. The apical parts of the root canals were instrumented with the Reciproc instruments (25 and 40) using a pecking motion. All the preparation of the root canals was carried out by a highly trained operator (W.W.).

Dividing into groups and root canal irrigation

Following the mechanical preparation, the roots were randomly divided into 4 groups (n = 15) and the apical foramina were closed with sticky wax to simulate the natural closed system of the root apex and the periapical tissues.

The root canals in group 1 were rinsed only with 5.25% NaOCl. Thus, this was the control group with no smear layer removal. In groups 2, 3 and 4, the smear layer was removed by double rinsing with 40% citric acid (CA) and 5.25% NaOCl. In group 2, 5.25% NaOCl was used in the final irrigating protocol. In groups 3 and 4, after the smear

layer removal, NaOCl was reduced by brief irrigation with CA and immediate intensive irrigation with distilled water to prevent dentin demineralization. In group 3, the irrigation protocol was completed with 98% isopropyl alcohol, and in group 4, the irrigation protocol was completed with 2% CHX. A detailed list of the applied irrigation protocols is presented in Table 1.

The irrigating solutions were introduced into the canals in small portions (0.5 mL), using a 0.4 × 19 mm oblique needle inserted into the canals with an up-and-down motion, to a depth of 1 mm shorter than the working length. During the irrigation steps marked with an asterisk in Table 1, passive ultrasound activation was applied for 5 s to each irrigant portion (0.5 mL). The activation was made using an ISO 35 spreader (VDW) connected to the E1 tip of a Smart Piezo scaler (Mectron, Carasco, Italy). Each irrigation–activation cycle lasted 15 s, which means that 2, 4 and 8 irrigation–activation cycles lasted 30 s, 60 s and 120 s, respectively. The spreader was used with an up-and-down motion, having a 2–3 mm shorter reach than the working length.

Table 1. Irrigation protocols (consecutive steps listed in top-down order) in 4 experimental groups

Group	Irrigation protocol
Group 1	5.25% NaOCl: 120 s – 4 mL*
Group 2	40% CA: 30 s – 1 mL* 5.25% NaOCl: 30 s – 1 mL* 40% CA: 30 s – 1 mL* 5.25% NaOCl: 60 s – 2 mL*
Group 3	40% CA: 30 s – 1 mL* 5.25% NaOCl: 30 s – 1 mL* 40% CA: 30 s – 1 mL* 5.25% NaOCl: 60 s – 2 mL* 40% CA: 2 s – 0.1 mL distilled water: 60 s – 2 mL* 98% isopropyl alcohol: 60 s – 2 mL
Group 4	40% CA: 30 s – 1 mL* 5.25% NaOCl: 30 s – 1 mL* 40% CA: 30 s – 1 mL* 5.25% NaOCl: 60 s – 2 mL* 40% CA: 2 s – 0.1 mL distilled water: 60 s – 2 mL* 2% CHX: 60 s – 2 mL

NaOCl – sodium hypochlorite; CA – citric acid; CHX – chlorhexidine; * passive ultrasound activation.

Root canal obturation

After the irrigation protocols were completed, the root canals were dried with paper points (VDW), and then filled with gutta-percha and the AH Plus sealer (Dentsply Sirona, Bensheim, Germany) by means of the thermoplastic vertical condensation technique, using a SuperEndo™ device (B&L Biotech, Seoul, South Korea). The sealer was modified with the addition of a saturated alcoholic porphyrin solution to obtain a concentration of 0.1%. The excess alcohol was evaporated from the sealer by blow-drying with room temperature air.

Sample preparation and microscopic assessment

After filling the canals, the roots were left for 3 days in a humid environment at 37°C. Then, with an IsoMet® 1000 precision saw (Buehler, Lake Bluff, USA), three 1-millimeter-thick cross-section slices were cut from each root at a distance of 2 mm (apical), 5 mm (middle) and 8 mm (coronal) from the root apex. The samples were coded and analyzed using a Nikon Ti-E inverted microscope with a Nikon A1 confocal system (Nikon, Tokyo, Japan). Magnifications of ×10 and ×20, and wavelengths of 488 nm and 561 nm were used. Each sample was imaged clockwise in 4 quadrants at an imaging thickness of 25 µm. Digital images were acquired by averaging 50 stacks made every 0.5 µm, starting from 10 µm from the sample surface down to 35 µm. The images were acquired at a resolution of 1024 × 1024 pixels, which gave 0.62 µm/pixel. The images were encoded and the resin tag length measurements were made using the ImageJ 1.45s software (National Institutes of Health, Bethesda, USA) in all 4 quadrants, for each section. A series of 32 measurements was made for each image, at fixed fields arranged radially in relation to the root canal axis every 2.8 degrees (Fig. 1). The measurement result for each field was the average value of the length of the resin tags in that field. After the measurements, the software made the export data with 10 average measurements per sample's quadrant, resulting in 40 average values per sample on each level. The summary data was 600 measurements per group on each level (n = 15 x 40 averaged measurements).

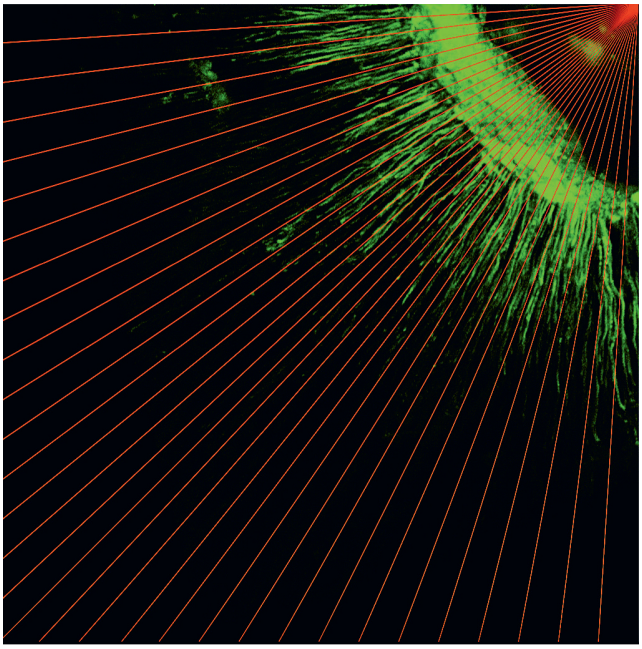

Fig. 1. Photograph of the sample with auxiliary lines. The measurements were carried out at fixed fields arranged radially in relation to the root canal axis

Data collection and statistical analyses

The collected and relevantly encoded data were statistically analyzed using both parametric and nonparametric tests. Since the assumptions for the commonly used in such cases one-way analysis of variance (ANOVA), concluded by Tukey's honestly significant difference (HSD) test at the a posteriori analysis stage turned out to be unfulfilled even after the Box–Cox transformation (the normality Kolmogorov–Smirnov test, Shapiro–Wilk test, Lilliefors test as well as the variance homogeneity tests – Levene's, Hartley's, Fisher's and Bartlett's – failed), the authors decided to perform the analysis using the nonparametric Kruskal–Wallis test followed by relevant nonparametric multiple comparison tests. Although the assumptions for parametric tests were not met, the numerical results were in full accordance with the results of nonparametric tests. This may be assigned to a very large sample size. The obtained results are in line with the additional check based on the reversed decision tree technique. In this case, the usual roles of dependent and independent variables (predictors) are exchanged, namely the irrigation protocol was selected as a dependent variable and the depth of penetration was one of predictors.

Due to the intrinsic characteristic of the data, i.e., the qualitative data ordered on 3 integer levels (the number of the canal sections), the correlation analysis based on Pearson's r parametric test would lead to wrong statements. Thus, for the correlation analysis, Kendall's tau nonparametric test was performed.

In all tests, a significance level of $p \leq 0.05$ was assumed. For the sake of accuracy, the p-values are given with 4 digits following the decimal point.

Results

The values of the depth of the sealers penetration into the dentinal tubules in the studied groups are presented in Table 2. As shown in Table 3 and Table 4, statistically significant differences were found between all pairs of groups ($p < 0.0001$) in the apical and coronal parts of the canals.

Table 2. Depth of the sealer penetration into the dentinal tubules [µm]

Group	Level of 2 mm		Level of 5 mm		Level of 8 mm	
	M ±SD	Me	M ±SD	Me	M ±SD	Me
Group 1	21.0 ±11.2	19	22.0 ±11.6	20	23.0 ±11.9	22
Group 2	201.2 ±35.6	198	230.9 ±64.3	237	374.0 ±87.9	376
Group 3	169.5 ± 52.5	162	231.5 ±66.5	235	280.0 ±103.0	260
Group 4	330.0 ±80.0	325.5	408.0 ±106.5	403	638.0 ±178.0	629.5

M – mean; SD – standard deviation; Me – median.

Table 3. Raw results of the Kruskal–Wallis analysis of variance (ANOVA) rank tests

Section	Number of groups	Number of samples per group	p-value
Apical (2 mm)	4	600	<0.0001*
Middle (5 mm)	4	600	<0.0001*
Coronal (8 mm)	4	600	<0.0001*

* statistically significant.

Table 4. Raw results of the Kruskal–Wallis rank multiple comparison analysis

Section	Group	p-value		
		group 2	group 3	group 4
Apical (2 mm)	group 1	<0.0001*	<0.0001*	<0.0001*
	group 2	–	<0.0001*	<0.0001*
	group 3	–	–	<0.0001*
Middle (5 mm)	group 1	<0.0001*	1.0000	<0.0001*
	group 2	–	<0.0001*	<0.0001*
	group 3	–	–	<0.0001*
Coronal (8 mm)	group 1	<0.0001*	<0.0001*	<0.0001*
	group 2	–	<0.0001*	<0.0001*
	group 3	–	–	<0.0001*

* statistically significant.

In the middle part of the canals, statistically significant differences were found between group 4 and all the other groups ($p < 0.0001$), between group 2 and group 3, and between group 2 and group 1 ($p < 0.0001$ in both cases). However, no statistically significant difference in this section was found between group 1 and group 3 ($p = 1.0000$). For all groups, a statistically significant correlation was found between the root part and the sealer penetration depth ($p < 0.0500$), employing Kendall's tau correlation coefficient (Table 5). However, while the tau correlation coefficient indicates that for groups 1, 2 and 4 there is a positive dependency between the section of the canal (i.e., the distance from the root apex) and the depth of the sealant penetration, for group 3, the depth of penetration does not depend on the part of the canal. This might result from the fact that the sealant penetration in group 3 was the worst, and thus it was equally bad along the whole canal.

Table 5. Kendall's tau correlation coefficients regarding the sealer penetration in relation to the canal section for all experimental groups

Group	Tau correlation coefficient	p-value
Group 1	0.5921	<0.0500*
Group 2	0.0494	<0.0500*
Group 3	0.3995	<0.0500*
Group 4	0.5614	<0.0500*

* statistically significant.

The representative images of the experimental groups are shown in Fig. 2–5.

Discussion

Decontamination and a tight seal on the root system are fundamental to the success of endodontic treatment. During chemical and mechanical preparation, the surface of the canal walls is modified by the activity of chelating agents (demineralization) and NaOCl (deproteination).[2,3] Even after drying the root canals, the open dentinal tubules constitute a potential micro-reservoir for the final irrigant that was used at the end of irrigation.[3,4,6] Thus, the physicochemical properties of the final irrigant can influence the interaction between the sealer and the dentin, and the sealer distribution to the dentinal tubules.

In this study, group 1 served as the control group, since the root canals were irrigated only with NaOCl. The lowest values of the sealer penetration into the dentinal tubules were recorded in this group. Most likely, the smear layer that was not removed acted as a mechanical barrier to the sealer. In the remaining groups, the smear layer was removed by double irrigation with CA and NaOCl, using passive ultrasound activation. A previous study conducted by the authors shows that this protocol is highly effective in removing the smear layer.[16] After removing the smear layer, the canals were irrigated with NaOCl to dissolve the exposed (due to demineralization) protein structures. For the final irrigation in group 2, NaOCl was used; NaOCl spontaneously decomposes into a hyperosmotic sodium chloride solution after a few minutes. Despite the high surface tension and osmotic pressure of the irrigant, a relatively high penetration of the sealer into the dentinal tubules was obtained. This may be related to the abilities of NaOCl to reduce torsional stress and act as a lubricant.[12] In group 3, 98% isopropyl alcohol was used for the final irrigation, and in group 4, 2% chlorhexidine gluconate aqueous solution was used. Alcohols such as ethanol or isopropanol are commonly used in endodontics.[13,14,17] They are very good solvents and can even help remove the propylene glycol-based calcium hydroxide paste dressing from the root canal.[17] Alcohols enable dentin dehydration and have a low surface tension, so theoretically, their use should have a positive effect on the penetration of the sealer into the dentinal tubules. In the present study, a high concentration of isopropyl alcohol was used in order to optimize the dehydration of the dentin, as the authors assumed that dry dentin should be better penetrated by a hydrophobic epoxy sealer. Despite this assumption, the null hypothesis was rejected, as the results of this study showed that using both NaOCl and CHX for the final irrigation provided better effects as compared to alcohol. A study by Nagas et al. demonstrated that leaving the dentin slightly moist rather than dry was more beneficial in terms of the interaction with the sealer.[18]

In this study, the epoxy AH Plus sealer was used, as it is one of the most commonly used sealers. The epoxy AH Plus sealer is characterized by high chemical stability and a relatively good, fluid-proof seal.[5,9] However, the greatest disadvantage of this type of sealer with regard to its interaction with dentin is its hydrophobicity. The dentine is a hydrated tissue, and thus it may have a low affinity to hydrophobic sealers. The authors assumed that dehydration with alcohol would have a beneficial effect on the penetration of the sealer into the dentinal tubules. However, the results of the experiment demonstrated something completely opposite, i.e., the hydrated dentine with residual NaOCl or CHX led to better effects. Perhaps the beneficial results of CHX were due to its surface properties, as CHX performed significantly better when compared to NaOCl. This might be interpreted as the penetration of an epoxy sealer into the dentinal tubules through capillary action.[5] The use of CHX may result in lowering of the surface tension, and thus optimizing the sealer penetration into the tubules. Alcohol may also have adversely affected the sealers penetration by possibly disrupting the binding of the sealer. This is even more likely, as in everyday practice, alcohols are commonly used to remove the excess sealer from the pulp chamber, due to their ability to dissolve the unbound sealer.

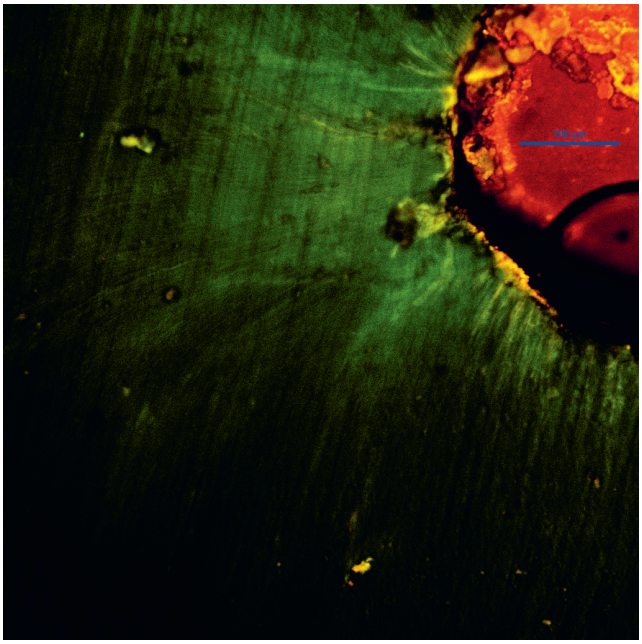

Fig. 2. Representative image for group 1

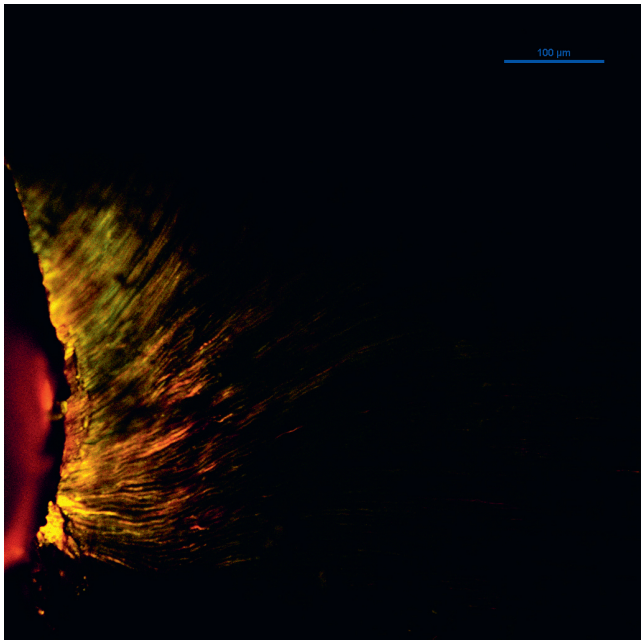

Fig. 3. Representative image for group 2

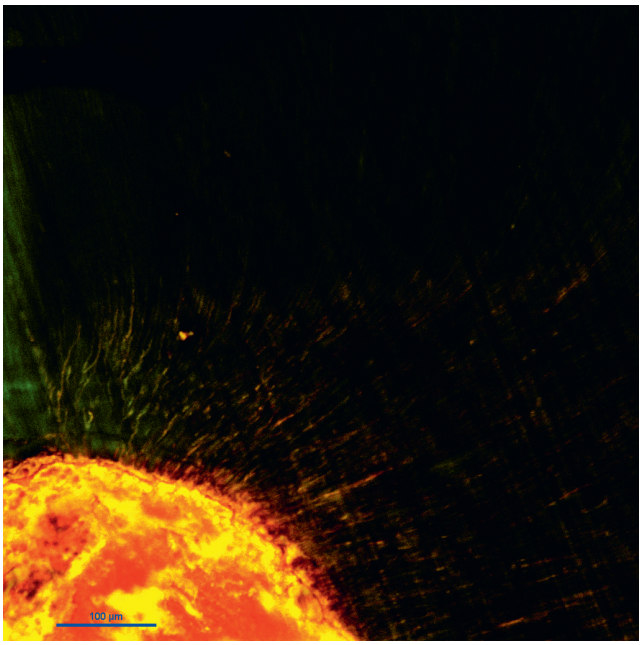

Fig. 4. Representative image for group 3

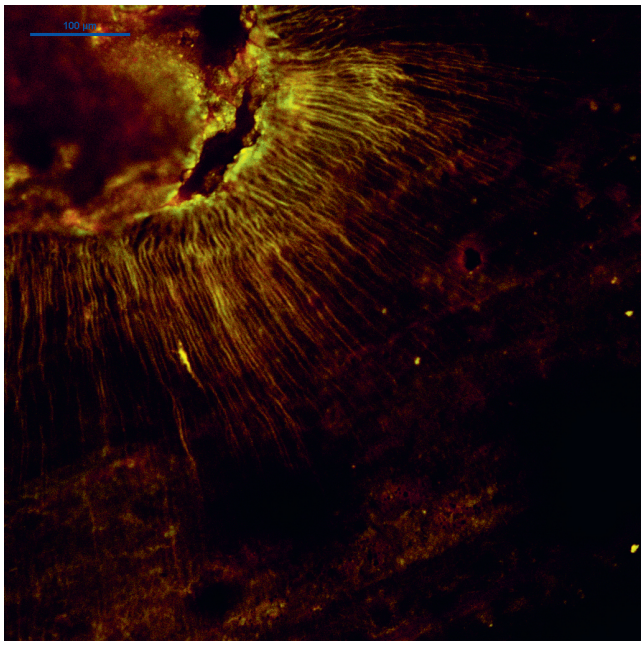

Fig. 5. Representative image for group 4

Limitations

It is important to acknowledge several limitations of this study. Physical properties of the sealer mixed with the marker, such as viscosity and flow, can be different as compared to the normal sealer. Thus, its penetration into the dentinal tubules could in theory be more pronounced in the real clinical scenario. The proper assessment of the sealer penetration may be altered by distribution or sampling errors. The study gives an insight of the tubule penetration only in 3 selected regions, not in the entire space of the root canal. Only straight root canals were selected and used in this study. The penetration of the sealer into the dentinal tubules in curved root canals could be different than in this study. The values of the measured sealer penetration should not be treated as precise data that represent the clinical outcomes. They should be perceived as the reflection of the phenomena in the sealer–dentin interaction after applying different irrigation protocols.

Conclusions

Based on this study and considering its limitations, it can be concluded that:

– the use of CHX as the final irrigant resulted in the deepest penetration of the epoxy sealer into the dentinal tubules;

– isopropyl alcohol had the most negative impact on the sealer penetration into the dentinal tubules;

– the final rinse with NaOCl led to better results as compared to the use of alcohol, but worse as compared to the use of CHX;

– in some kinds of treatment, the sealer penetration may depend on the canal section; the nature of this phenomenon requires future in-depth research.

Further research using different methodologies should be carried out to fully explain the nature of the sealer–dentin interaction.

ORCID iDs

Wojciech Wilkoński https://orcid.org/0000-0001-7205-5586
Lidia Jamróz-Wilkońska https://orcid.org/0000-0003-0637-1104
Mariusz Kępczyński https://orcid.org/0000-0002-7304-6881
Szczepan Zapotoczny https://orcid.org/0000-0001-6662-7621
Urszula Maziarz https://orcid.org/0000-0002-3564-6907
Janusz Opiła https://orcid.org/0000-0003-1179-1920
Jolanta Pytko-Polończyk https://orcid.org/0000-0002-5700-2387

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Effect of thiamine pyrophosphate on cyclophosphamide-induced oxidative ovarian damage and reproductive dysfunction in female rats

Mehmet Ozer[1,A], Sefa Ince[2,A], Betul Gundogdu[3,C], Mehmet Aktas[4,C], Kemine Uzel[5,A], Cebrail Gursul[6,C], Halis Suleyman[7,D,F], Zeynep Suleyman[8,B,F]

[1] Department of Obstetrics and Gynecology, Tepecik Regional Training Research Hospital, Izmir, Turkey
[2] Department of Obstetrics and Gynecology, Regional Training Research Hospital, Erzurum, Turkey
[3] Department of Pathology, Faculty of Medicine, Atatürk University, Erzurum, Turkey
[4] Department of Biochemistry, Faculty of Medicine, Erzincan Binali Yıldırım University, Turkey
[5] Department of Obstetrics and Gynecology, Faculty of Medicine, Erzincan Binali Yıldırım University, Turkey
[6] Department of Physiology, Faculty of Medicine, Erzincan Binali Yıldırım University, Turkey
[7] Department of Pharmacology, Faculty of Medicine, Erzincan Binali Yıldırım University, Turkey
[8] Department of Nursing, Faculty of Health Sciences, Erzincan Binali Yıldırım University, Turkey

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Zeynep Suleyman
E-mail: zeynep1105@gmail.com

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Abstract

Background. Cyclophosphamide is a drug used in various types of cancer. It can cause oxidative and inflammatory ovarian damage and infertility. Thiamine pyrophosphate (TPP) to be investigated for its effect on cyclophosphamide-induced ovarian damage and reproductive dysfunction in the present study is the active metabolite of thiamine. It has been shown that TPP protects organs and tissues from oxidative stress and proinflammatory cytokine damage.

Objectives. To investigate the effect of TPP against the ovarian damage and reproductive dysfunction caused by cyclophosphamide in rats.

Materials and methods. Albino Wistar type female rats were divided into healthy control (HG), cyclophosphamide (CYC) and TPP + cyclophosphamide (TPPC) groups (for each group, n = 12). Thiamine pyrophosphate at a dose of 25 mg/kg was injected intraperitoneally (ip.) in the TPPC group, and 0.9% NaCl solution was injected ip. in the CYC and HG groups. One hour after the injection, 75 mg/kg of cyclophosphamide was administered ip. in the TPPC and CYC groups. This procedure was repeated once a day for 30 days. At the end of this period, 6 rats from each group were euthanized with a high dose of anesthetic (50 mg/kg of sodium thiopental). Biochemical and histopathological examinations were performed on the extracted ovarian tissue. The remaining animals were kept in the laboratory with mature male rats for 2 months for reproduction.

Results. Thiamine pyrophosphate significantly decreased the cyclophosphamide-induced increase in the levels of the oxidant parameter malondialdehyde (MDA), proinflammatory nuclear factor kappa B (NF-κB), tumor necrosis factor alpha (TNF-α), and interleukin 1 beta (IL-1β). In addition, TPP decreased the severe histopathological damage associated with cyclophosphamide in the ovarian tissue and prevented infertility.

Conclusions. Our experimental results have suggested that TPP could be beneficial in the treatment of cyclophosphamide-induced ovarian injury and infertility.

Key words: infertility, rat, cyclophosphamide, ovarian

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Background

The Food and Drug Administration (FDA) has approved the use of cyclophosphamide in the treatment of lymphomas, multiple myeloma, breast cancer, diffuse neuroblastomas, retinoblastoma, pediatric minimal change nephrotic syndrome, and ovarian adenocarcinoma.[1] Cyclophosphamide is also used in the treatment of autoimmune diseases, such as multiple sclerosis, and in the prevention of transplant rejection.[2,3] In the clinic, cyclophosphamide is initiated intravenously (iv.) at doses of 40–50 mg/kg, depending on tolerance.[4] However, serious adverse reactions of cyclophosphamide during the treatment are considered. The common adverse reactions reported in clinical studies include hemorrhagic cystitis, amenorrhea, myelosuppression, alopecia, nausea, and vomiting.[5,6] Renal tubular necrosis, pulmonary fibrosis, cardiotoxicity, and infertility have been shown among other toxic effects associated with cyclophosphamide.[7] It is stated that cyclophosphamide-induced infertility is caused by ovarian failure.[8] Infertility due to ovarian failure is the most common and serious side effect of cyclophosphamide and occurs in 30–70% of women receiving the treatment.[9,10] It has been reported that cyclophosphamide causes ovarian failure through oxidative and inflammatory damage.[11] Nair et al. showed that cyclophosphamide-induced damage in the ovarian tissue was caused by a decrease in the endogenous antioxidant glutathione (GSH) and an increase in proinflammatory tumor necrosis factor alpha (TNF-α).[12] In a study by Khedr, it was emphasized that cyclophosphamide-induced infertility was associated with an increase in malondialdehyde (MDA) – a lipid peroxidation (LPO) product in the ovarian tissue.[13] The information obtained from the literature suggests that antioxidant and anti-inflammatory drugs can be beneficial in the prevention or treatment of cyclophosphamide-induced infertility.

Thiamine pyrophosphate (TPP) to be investigated in this study for its effect on cyclophosphamide-induced ovarian damage and reproductive dysfunction is the active metabolite of thiamine. It is also the cofactor of enzymes playing a role in maintaining the cell redox state by synthesizing nicotinamide adenine dinucleotide phosphate (NADPH) and GSH.[14] Thiamine pyrophosphate has been reported to prevent the LPO and oxidative DNA damage caused by ischemia/reperfusion damage.[15] Moreover, there are findings in the literature showing that TPP prevents the oxidative damage and infertility caused by cisplatin in rats.[16] It has also been noted in another study that TPP suppresses the overproduction of TNF-α, interleukin 1 beta (IL-1β) and other proinflammatory cytokines.[17] The data indicate that TPP can be beneficial in the treatment of cyclophosphamide-induced ovarian injury and infertility.

Objectives

To investigate the effect of TPP against the possible ovarian damage and reproductive dysfunction induced by cyclophosphamide in female rats, and to examine the ovarian tissue biochemically and histopathologically.

Materials and methods

Experimental animals

In our study, a total of 36 albino Wistar type female and 6 male rats weighing 235–250 g and aged 3.5–4 months were obtained from the Atatürk University Medical Experimental Application and Research Center (Erzurum, Turkey). The animals were kept at normal room temperature (22°C) in the laboratory of the same center and fed with standard animal feed. The animal experiments were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. This study was specifically approved by the local Animal Experimentation Ethics Committee (meeting No. 77040475-641.04-E.2000115834, as of May 4, 2020).

Chemicals

Cyclophosphamide used in the experiments was supplied by Eczacıbaşı Pharmaceuticals Marketing Co. (EIP) (Istanbul, Turkey) (a vial containing 1 g of solution powder to be used for infusion), sodium thiopental was supplied by E. Ulagay İlaç Sanayii Türk A.Ş (Istanbul, Turkey) and TPP (50 mg of solution powder) was obtained from Bio-Pharma (Moscow, Russia).

Experimental groups

The rats to be used in the experiment were divided into cyclophosphamide (CYC), TPP + cyclophosphamide (TPPC) and healthy control (HG) groups (for each group, n = 12).

Drug preparation

Preparation of cyclophosphamide solution

A total of 1 g of cyclophosphamide solution powder was dissolved in the vial with 10 mL of normal saline (0.9% NaCl). The cyclophosphamide solution was taken into injectors at a dose of 75 mg/kg. This dose resulted in 18.2 mg given to an animal with an average weight of 242.5 g. A total of 0.18 mL of cyclophosphamide solution was taken into each injector for this dose to be administered to each animal.

Preparation of thiamine pyrophosphate solution

A total of 50 mg of TPP solution powder was diluted with 2 mL of 0.9% NaCl. This solution was taken into injectors at a dose of 25 mg/kg. The dose was calculated to be 6 mg for an animal with an average weight of 242.5 g. In order to apply this dose to each animal, 0.24 mL of TPP solution was taken into each injector.

Experimental procedure

For the experiment, TPP was injected intraperitoneally (ip.) into the anterior part of the abdomen in the TPPC group at a dose of 25 mg/kg. This dose of TPP has been reported to protect the tissue from oxidative damage in previous studies.[18] Normal saline (0.9% NaCl) was used as a solvent for the CYC and HG groups. One hour after the injection, 75 mg/kg of cyclophosphamide was administered to the anterior part of the abdomen in the TPPC and CYC groups once a week. Previous studies have shown that this dose of cyclophosphamide causes ovarian damage.[12] This procedure was performed once a day for 30 days. At the end of this period, 6 rats from each group were euthanized with a high dose of anesthetic (50 mg/kg of sodium thiopental), and biochemical and histopathological examinations were performed on the extracted ovarian tissue. The remaining animals (6 female rats from each group) were kept in the laboratory with mature male rats for 2 months for reproduction. The rats which became pregnant during this period were taken to separate cages and kept alone in a suitable environment. The rats which did not get pregnant and give birth within 2 months were considered infertile. In addition, maternity duration was calculated by subtracting the standard duration of gestation (21 days) from the period between the day the female rats met the male rats until the time of birth (A) (maternity period = A – 21). All biochemical, histopathological and reproductive test results obtained in the TPPC and HG groups were compared with those obtained in the CYC group.

Biochemical analyses

Prior to dissection, all tissues were rinsed with phosphate-buffered saline (PBS) solution. The ovarian tissues were homogenized in ice-cold phosphate buffers (50 mM, pH 7.4) that were appropriate for the variable to be measured.[19] The tissue homogenates were centrifuged at 5000 rpm for 20 min at 4°C, and the supernatants were extracted to analyze MDA, total glutathione (tGSH), total antioxidant system (TAS), total oxidant system (TOS), and protein concentration levels. The protein concentration of the supernatant was measured using the method described by Bradford.[20] The drug concentrations in all the tissues were expressed per 1 g of protein.[20] All spectrophotometric measurements were recorded using a microplate reader (BioTek, Winooski, USA).

MDA analysis

The MDA measurement was based on the spectrophotometric measurement (at 532 nm) of the absorbance of the pink-colored complex formed by thiobarbituric acid and MDA at high temperature (95°C).[21]

tGSH analysis

The 5,5'-Dithiobis (2-nitrobenzoic acid) in the measurement medium is a disulfide chromogen, and is readily reduced by compounds with sulfhydryl groups. The resulting yellow color was measured spectrophotometrically at 412 nm.[22]

NF-κB, TNF-α and IL-1β analysis

The rat-specific sandwich enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of NF-κB, TNF-α and IL-1β in the tissue homogenates: rat NF-κB ELISA kits (Cat. No. 201-11-0288; Shanghai Sunred Biological Technology Co., Ltd., Shanghai, China); rat TNF-α and rat IL-1β ELISA kits (Cat No. YHB1098Ra; Shanghai LZ Biotech Co., Ltd., Shanghai, China).

Histopathological examination

After a routine tissue follow-up, five-micrometer sections were obtained for histopathological evaluation. These sections were stained with hematoxylin and eosin (H&E), and the ovarian tissues were evaluated with a light microscope (Olympus BX 51; Olympus Corp., Tokyo, Japan) by a pathologist who was blinded to the treatment protocol; the photographs were taken with a digital camera (Olympus DP 71; Olympus Corp.). The histopathological damage severity in each ovarian tissue section was scored as grades 0–3 (0 – normal; 1 – mild damage; 2 – moderate damage; and 3 – severe damage).

Statistical analyses

The results obtained in the experiments were expressed as mean and standard deviation (M ±SD) or median (minimum–maximum) (Me (min–max)). The normality of variables was checked with the Shapiro–Wilk normality test. The homogeneity of variances was evaluated with Levene's test. The significance of the difference between groups was determined using the one-way analysis of variance (ANOVA), followed by Tukey's post hoc tests. While comparing groups, the Kruskal–Wallis test and the Dunn's post hoc test were used, and adjusted p-values were presented. All statistical procedures were performed using the IBM SPSS Statistics for Windows software, v. 20.0 (IBM Corp., Armonk, USA), and a p-value <0.05 was considered significant.

Results

Biochemical results

MDA and tGSH analysis results

As can be seen in Fig. 1, the amount of MDA in the ovarian tissue from the CYC group was found to be significantly higher as compared to the HG and TPPC groups ($p < 0.0001$). The amount of MDA in the TPPC group was higher than in the HG group ($p < 0.0001$). However, the difference in the MDA level between the TPPC group and the HG group was statistically nonsignificant. Cyclophosphamide caused a decrease in the amount of tGSH in the ovarian tissue. Thiamine pyrophosphate significantly prevented the reduction of tGSH by cyclophosphamide ($p < 0.0001$). The difference in the tGSH level between the TPPC group and the HG group was statistically nonsignificant (Table 1,2).

NF-κB, TNF-α and IL-1β analysis results

Cyclophosphamide caused an increase in the NF-κB, TNF-α and IL-1β levels in the ovarian tissues of animals. The NF-κB, TNF-α and IL-1β levels in the ovarian tissues of the animals treated with cyclophosphamide (the CYC group) were significantly higher, as compared to the HG and TPPC groups ($p < 0.0001$) (Table 1). Thiamine pyrophosphate prevented the increase in the levels of NF-κB, TNF-α and IL-1β induced by cyclophosphamide ($p < 0.0001$). The differences in the levels of these proinflammatory cytokines between the TPPC and HG groups were found to be statistically nonsignificant (Fig. 2, Table 2).

Histopathological results

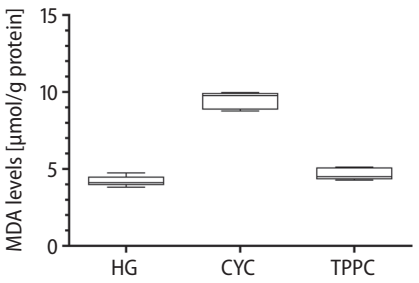
As seen in Fig. 3, there were no histopathological findings in the ovarian tissue of the HG group, which was evaluated as grade 0, except mild fluid accumulation with cystic changes in the lumen of follicule structures.

In the ovarian tissue of the CYC group, grade 3 vacuolized and degenerated follicle cells, a cystic change with fluid accumulation in the lumen of the follicle structure, congestion, and hemorrhages were observed (Fig. 4).

Vacuolized and degenerated follicle cells in the developing follicle structures were not detected in the ovarian tissue of the TPPC group (grade 0). However, other histopathological findings, such as fluid accumulation with cystic changes in the lumen of follicle structures, congestion and hemorrhages in the corpus luteum and other areas, were observed in the TPPC group and evaluated as grade 1 (Fig. 5) (Table 3,4).

Reproductive test results

As can be seen in Table 5, all 6 animals in the HG group gave birth at around 25–28 days. In the TPPC group, 4 out of 6 animals gave birth on day 35–37, but 2 animals in this group did not give birth within the waiting period (2 months). In the CYC group, none of the 6 animals taken for breeding gave birth within 2 months.



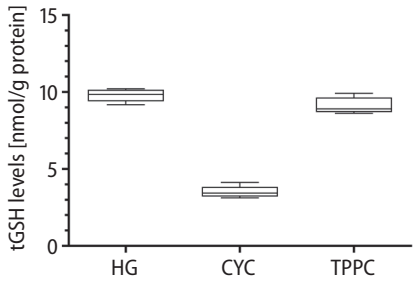
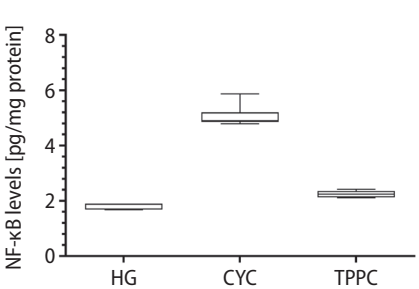
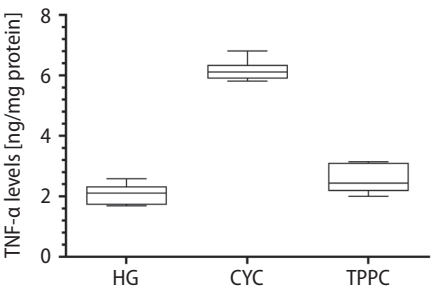


Fig. 1. The malondialdehyde (MDA) and total glutathione (tGSH) levels in the ovarian tissues of the study groups

HG – healthy control group; CYC – cyclophosphamide group; TPPC – thiamine pyrophosphate (TPP) + cyclophosphamide group; * $p < 0.0001$ for CYC compared to HG and TPPC ($n = 6$).





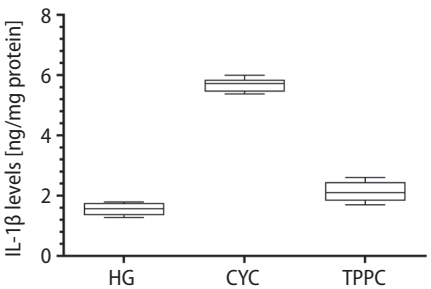


Fig. 2. The nuclear factor kappa B (NF-κB), tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) levels in the ovarian tissues of the study groups

HG – healthy control group; CYC – cyclophosphamide group; TPPC – thiamine pyrophosphate (TPP) + cyclophosphamide group; * $p < 0.0001$ for CYC compared to HG and TPPC ($n = 6$).

Table 1. Malondialdehyde (MDA), total glutathione (tGSH), nuclear factor kappa B (NF-κB), tumor necrosis factor alpha (TNF-α), and interleukin 1 beta (IL-1β) levels in the ovarian tissues of the study groups

Variable	HG	CYC	TPPC	F	p-value
MDA [μmol/g protein]	4.20 ±0.33	9.52 ±0.54	4.64 ±0.37	285.677	<0.0001
tGSH [nmol/g protein]	9.78 ±0.43	3.51 ±0.37	9.10 ±0.52	357.326	<0.0001
NF-κB [pg/mg protein]	1.81 ±0.10	5.05 ±0.40	2.24 ±0.12	292.425	<0.0001
TNF-α [ng/mg protein]	2.07 ±0.34	6.16 ±0.34	2.56 ±0.47	190.872	<0.0001
IL-1β [ng/mg protein]	1.55 ±0.21	5.68 ±0.22	2.13 ±0.33	427.250	<0.0001

HG – healthy control group; CYC – cyclophosphamide group; TPPC – thiamine pyrophosphate (TPP) + cyclophosphamide group. Data are presented as mean ± standard deviation (M ±SD). The degrees of freedom (df) are the same for all groups – F (2, 15). The Kruskal–Wallis test was used for MDA and NF-κB, and the analysis of variance (ANOVA) was used for tGSH, TNF-α and IL-1β.

Table 2. The p-values of post hoc comparisons for variables between the study groups

Variable	p-value		
	HG vs CYC	HG vs TPPC	CYC vs TTPC
MDA	<0.001*	0.210	<0.001*
tGSH	<0.001*	0.047*	<0.001*
NF-κB	<0.001*	0.025*	<0.001*
TNF-α	<0.001*	0.119	<0.001*
IL-1β	<0.001*	0.005*	<0.001*

HG – healthy control group; CYC – cyclophosphamide group; TPPC – thiamine pyrophosphate (TPP) + cyclophosphamide group; MDA – malondialdehyde; tGSH – total glutathione; NF-κB – nuclear factor kappa B; TNF-α – tumor necrosis factor alpha; IL-1β – interleukin 1 beta; * statistically significant. The Kruskal–Wallis test and the Dunn's post hoc test were used.

Discussion

In this study, the protective effect of TPP against possible cyclophosphamide-induced ovarian damage and reproductive dysfunction in female rats was investigated. In addition, it was evaluated whether reproductive dysfunction was associated with the severity of ovarian damage. The ovarian damage induced by cyclophosphamide was determined by measuring the MDA, tGSH, NF-κB, TNF-α, and IL-1β levels in the ovarian tissues, and the histopathological examination of 6 out of 12 animals treated with cyclophosphamide. Our experimental results showed that cyclophosphamide administered at a dose of 75 mg/kg once a week (4 doses in total) increased the oxidant and proinflammatory cytokine levels, and decreased

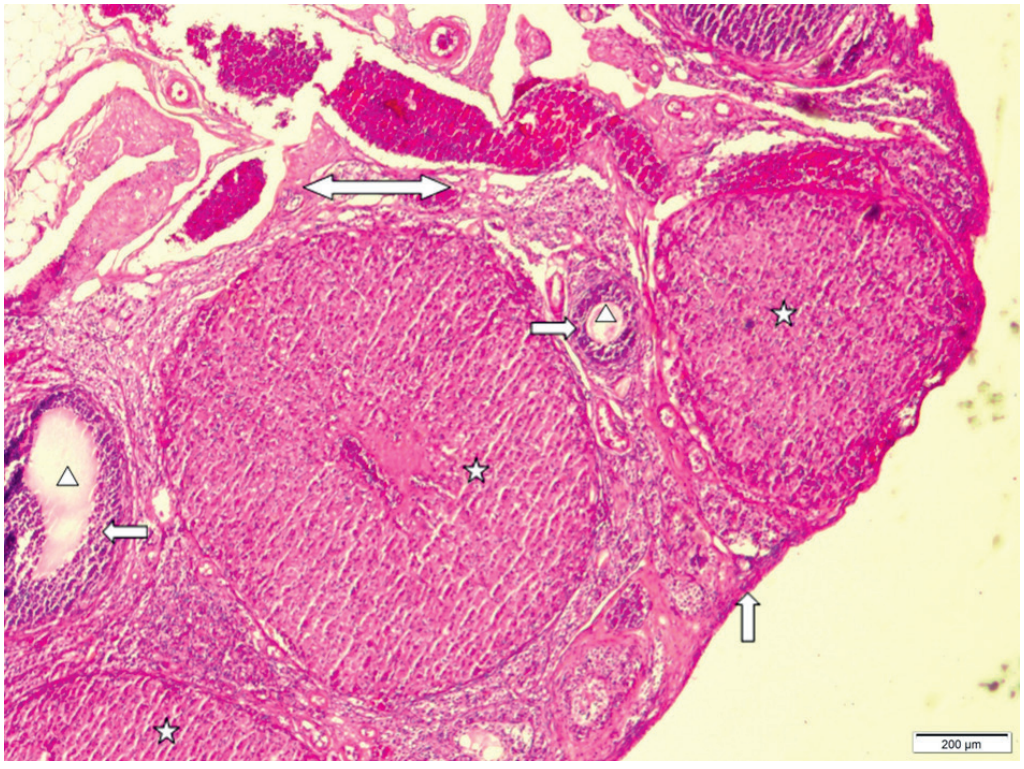


Fig. 3. Tissue section showing the corpus luteum (asterisks), developing follicle structures (horizontal white arrows), mild cystic change with fluid accumulation in the lumen of the follicle structure (triangle), cortex (vertical white arrow), and medulla (horizontal bidirectional arrow) in the ovary of the healthy control (HG) group. Hematoxylin and eosin (H&E) staining, ×40 magnification

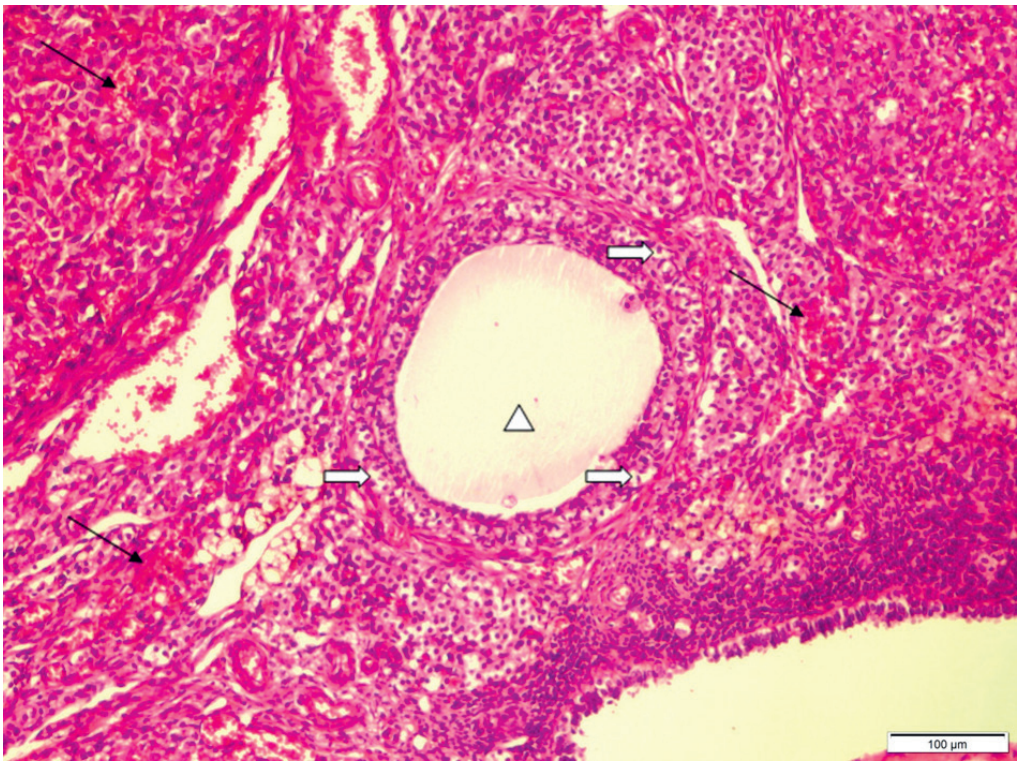


Fig. 4. Tissue section showing vacuolized and degenerated follicle cells (horizontal white arrows), a cystic change with fluid accumulation in the lumen of the follicle structure (triangle), congestion (vertical white arrows) and hemorrhages (black cross-arrows) in the ovarian tissue of the cyclophosphamide (CYC) group. Hematoxylin and eosin (H&E) staining, ×100 magnification

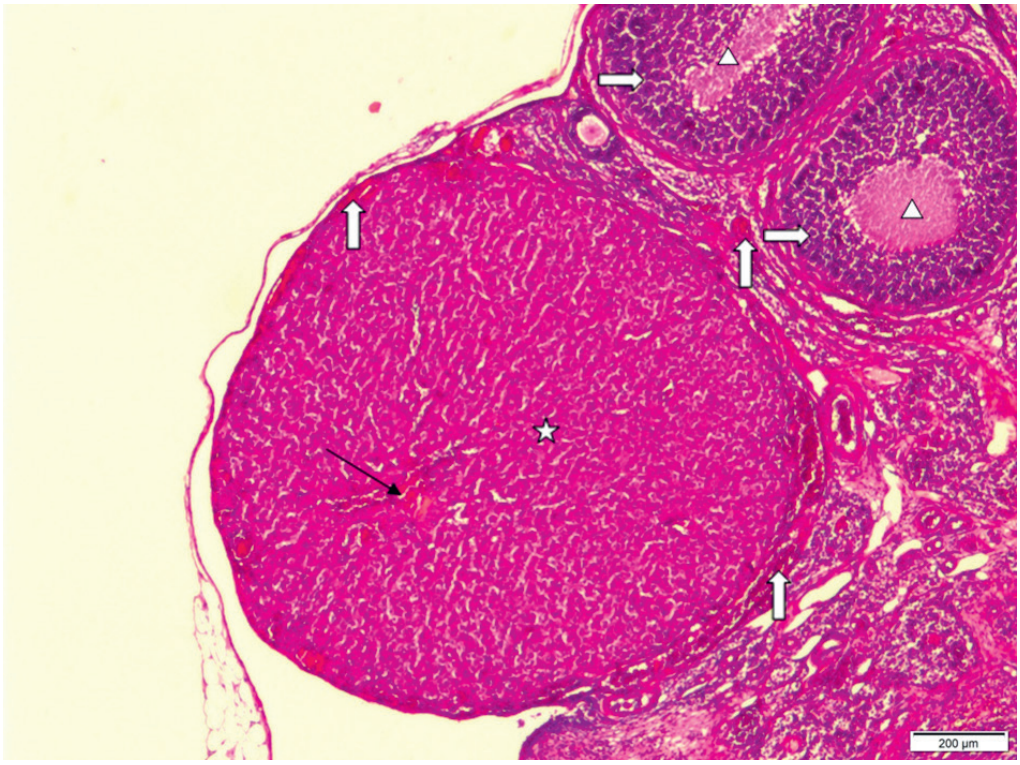


Fig. 5. Ovarian tissue section showing a mild hemorrhage in the corpus luteum (asterisk) and an area of a partially reduced hemorrhage (a black cross-arrow), developing follicle structures without significant vacuolized and degenerated follicle cells (horizontal white arrows), a mild fluid accumulation with cystic changes in the lumen of follicule structures (triangle), and a mild congestion (vertical white arrows) in the thiamine pyrophosphate (TPP) + cyclophosphamide (TPPC) group. Hematoxylin and eosin (H&E) staining, ×40 magnification

the antioxidant level in the ovarian tissue as compared to the healthy and TPP-treated groups. In addition, reproductive dysfunction was observed in the CYC group with high oxidant and cytokine levels, together with a low antioxidant level. In a previous study, cyclophosphamide was used at a higher dose (200 mg/kg) to create toxic effects on the ovarian tissue in rats.[23] There is evidence in the literature supporting our biochemical findings related to cyclophosphamide.[11–13] As it is known, MDA is a toxic product formed as a result of the oxidation of cell membrane lipids by reactive oxygen species (ROS).[24] Hamzeh et al. histopathologically demonstrated that the increased ROS and MDA production induced by cyclophosphamide was associated with ovarian damage.[25]

Table 3. Statistical analysis of histopathological findings

Group	Ovarian follicle			Ovarian tissue		
	vacuolization	degeneration	fluid accumulation in the follicular lumen	cystic change	congestion	hemorrhage
HG	0.00 ±0.00 0 (0–0)	0.00 ±0.00 0 (0–0)	0.00 ±0.00 0 (0–0)	0.00 ±0.00 0 (0–0)	0.00 ±0.00 0 (0–0)	0.00 ±0.00 0 (0–0)
CYC	2.83 ±0.39 3 (2–3)	3.00 ±0.00 3 (3–3)	2.67 ±0.44 3 (2–3)	2.67 ±0.44 3 (2–3)	3.00 ±0.00 3 (3–3)	3.00 ±0.00 3 (3–3)
TPPC	0.00 ±0.00 0 (0–0)	1.00 ±0.33 1 (0–2)	0.00 ±0.00 0 (0–0)	0.00 ±0.00 0 (0–0)	0.83 ±0.28 1 (0–1)	0.83 ±0.28 1 (0–1)

HG – healthy control group; CYC – cyclophosphamide group; TPPC – thiamine pyrophosphate (TPP) + cyclophosphamide group. Data are presented as mean ± standard deviation (M ±SD) and as median (minimum–maximum) (Me (min–max)). The Kruskal–Wallis test and the Dunn's post hoc test were used. The values explain histopathological grades: 0 – normal; 1 – mild damage; 2 – moderate damage; and 3 – severe damage.

Table 4. The p-values of post hoc comparisons for variables between the study groups in terms of histopathological evaluation

Variable	p-value		
	HG vs CYC	HG vs TPPC	CYC vs TTPC
Vacuolization	<0.0001*	1.0000	<0.0001*
Degeneration	<0.0001*	0.0870	0.0260*
Fluid accumulation in the follicular lumen	<0.0001*	1.0000	<0.0001*
Cystic change	<0.0001*	1.0000	<0.0001*
Congestion	<0.0001*	0.0850	0.0250*
Hemorrhage	<0.0001*	0.0850	0.0250*

HG – healthy control group; CYC – cyclophosphamide group; TPPC – thiamine pyrophosphate (TPP) + cyclophosphamide group;* statistically significant. The Kruskal–Wallis test and the Dunn's post hoc test were used.

Table 5. Reproductive test results

Group	Number of animals taken for breeding	Breeding animals n (%)	A [days]	Maternity period (A – 21) [days]
HG	6	6 (100.0)	26.50	5.50
CYC	6	0 (0.0)	–	–
TPPC	6	6 (66.7)	33.25	12.25

HG – healthy control group; CYC – cyclophosphamide group; TPPC – thiamine pyrophosphate (TPP) + cyclophosphamide group; A – period between the day the female rats met the male rats until the time of birth; maternity period was calculated by subtracting the standard duration of gestation (21 days) from A.

It has also been reported that this oxidative stress is due to phosphoramide mustard, one of the metabolites of cyclophosphamide.[23]

In previous studies, cyclophosphamide was shown to cause an increase in MDA and a decrease in GSH levels in the ovarian tissue of animals.[26] The literature data being consistent with our experimental results show that cyclophosphamide disturbs the oxidant/antioxidant balance in the ovarian tissue in favor of oxidants. As it is known, the oxidant/antioxidant balance is provided by the dominance of antioxidants in physiological conditions. The disruption of this balance in favor of oxidants causes tissue damage. This condition is defined as oxidative stress.[24] Our biochemical findings revealed that the amount of tGSH in the ovarian tissue of the CYC group significantly decreased as compared to that of the HG and TPPC groups. In the literature, a decrease in GSH is explained by the inadequacy of antioxidants in neutralizing oxidants.[27] Having an important role in the antioxidant defense system in living organisms, GSH directly reacts with ROS to oxidize thiol groups and preserve cell integrity.[28,29]

Furthermore, cyclophosphamide was found to increase the levels of proinflammatory cytokines, such as NF-κB, TNF-α and IL-1β, in the ovarian tissue in our study. In a recent study, it was emphasized that these proinflammatory cytokines played a role in the pathogenesis of cyclophosphamide toxicity.[30] Elkady et al. reported that cyclophosphamide caused an increase in NF-κB in the ovarian tissue.[31] In a study by Nair et al., it was stated that proinflammatory TNF-α as well as oxidative stress were responsible for cyclophosphamide-induced ovarian damage.[12] In another study, the role of IL-1β in ovarian injury was presented, and it was also argued that the suppression of the increase in the IL-1β production caused a decrease in ovarian tissue damage.[23] In our study, the levels of NF-κB, TNF-α and IL-1β increased in the CYC group, where MDA, a ROS product, has also increased. There is evidence in the literature reporting

that ROS increase the NF-κB production.[32] In addition, it has been shown that NF-κB induces the secretion of inflammatory mediators, such as IL-1β and TNF-α.[33,34]

In our study, the biochemical and histopathological findings regarding the ovarian tissue of the entire animal group were found to be consistent with the reproductive test results. The CYC group presented with severe histopathological damage in the ovarian follicles and tissue, and none of the animals in this group gave birth. In previous studies, animals that were kept in a breeding environment and did not give birth within 2 months were considered infertile.[35] Supporting our experimental results, Saleh et al. reported that cyclophosphamide caused infertility in animals.[23] It was argued that this infertility due to cyclophosphamide was caused by oxidative stress and inflammation in the ovaries; in addition, it was reported that ovarian damage increased with antioxidant and anti-inflammatory therapy.[33] Moreover, in a study on cyclophosphamide supporting our histopathological results, it was reported that degenerative damage developed in the ovarian follicles at decreased antioxidant levels and increased cytokine (TNF-α) levels.[12] In another study, cyclophosphamide-associated stromal and follicle degeneration, edema, vacuolization, and the severity of vascular congestion decreased with the elimination of inflammation and oxidative stress.[25] As can be seen from our experimental results, the attenuation of the cyclophosphamide-induced oxidant and the proinflammatory cytokine increase with TPP resulted in a decrease in histopathological damage and the number of infertile animals. It is known from previous studies that TPP prevents infertility caused by ovarian ischemia/reperfusion damage.[36] It has been stated in the literature that the protective effect of TPP on the ovary is due to an increased LPO reaction and the inhibition of DNA oxidation.[15] However, no information has been found in the literature stating that TPP inhibits the proinflammatory cytokine production in the ovarian tissue. However, in a recent experimental study, it was reported that TPP suppressed the ethanol-induced increase in TNF-α and IL-1β in the optic nerve tissue.[17]

Limitations

This study has some limitations. Firstly, more studies are needed to clarify the mechanism of action of TPP in the ovarian damage caused by cyclophosphonamide. Secondly, the estrogen and progesterone levels can also be measured in relation to infertility. Finally, the pathology can be measured at the molecular level.

Conclusions

Cyclophosphamide caused an increase in the oxidant and proinflammatory parameters, and a decrease in antioxidants in the ovarian tissue of animals. It was observed that the ovarian tissue damage caused by cyclophosphamide resulted in infertility. Decreasing the severity of the cyclophosphamide-associated oxidative and inflammatory ovarian injury with TPP resulted in a decrease in the number of infertile animals. Our experimental results suggest that TPP can be beneficial in the treatment of cyclophosphamide-induced ovarian injury and infertility.

ORCID iDs

Mehmet Ozer https://orcid.org/0000-0003-0683-0710
Sefa Ince https://orcid.org/0000-0002-9170-1704
Betul Gundogdu https://orcid.org/0000-0002-3786-3286
Mehmet Aktas https://orcid.org/0000-0003-1931-8353
Kemine Uzel https://orcid.org/0000-0002-4615-5601
Cebrail Gursul https://orcid.org/0000-0001-6521-6169
Halis Suleyman https://orcid.org/0000-0002-9239-4099
Zeynep Suleyman https://orcid.org/0000-0003-0128-7990

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Overexpression of *GSTP1* promotes colorectal cancer cell proliferation, invasion and metastasis by upregulating *STAT3*

Feifei Wang[1,2,B–D,F], Ceng Zhang[1,2,B,C,F], Xiaohui Zhu[1,2,B,C,F], Dan Zhang[1,2,B,F], Zhaowen Zhang[1,2,C,F], Shunjie Ni[3,C,F], Zhizhi Wang[4,C,F], Shuyi Xu[5,C,F], Xiaoliang Lan[6,C,F], Yanqing Ding[1,2,C,F], Li Liang[1,2,A,E,F]

[1] Department of Pathology, Nanfang Hospital, Southern Medical University, Guangzhou, China
[2] Guangdong Province Key Laboratory of Molecular Tumor Pathology, Basic Medical College, Southern Medical University, Guangzhou, China
[3] Department of Neurology, Nanfang Hospital, Southern Medical University, Guangzhou, China
[4] Department of Thoracic Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, China
[5] Basic Medical College, Southern Medical University, Guangzhou, China
[6] Department of General Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Li Liang
E-mail: lli@fimmu.com

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Abstract

Background. The abnormal expression of glutathione S-transferase P1 (*GSTP1*) is associated with the progression of several tumor types. However, its role and molecular mechanism in the progression of colorectal cancer (CRC) are largely unknown.

Objectives. To examine the effect of *GSTP1* in CRC and determine its possible mechanisms.

Materials and methods. In the present study, immunohistochemistry (IHC) and the quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis were used to detect the expression of *GSTP1* and signal transducer and activator of transcription 3 (*STAT3*) in CRC tissues. Western blotting was applied to detect the expression of GSTP1 and proteins of the Janus kinase (JAK)-STAT3 pathway in different CRC cell lines. The interaction and co-localization of GSTP1 and STAT3 were detected using co-immunoprecipitation (co-IP) and immunofluorescence (IF) in the SW620 cell line.

Results. A positive correlation was identified between the expression of *GSTP1* and *STAT3* in human CRC tissues. The overexpression of *GSTP1* promoted the proliferation, invasion and metastasis of CRC cells by upregulating *STAT3*. The *GSTP1* and *STAT3* can directly bind to and regulate each other. The interaction between them is regulated by the upstream gene called F-box only protein 8 (*FBX8*).

Conclusions. The present study demonstrated that *GSTP1* could enhance the expression of *STAT3* to promote the proliferation, invasion and metastasis of CRC cells, which provides a potential therapeutic target for the clinical treatment of CRC.

Key words: metastasis, proliferation, colorectal cancer, glutathione S-transferase P1, signal transducer and activator of transcription 3

Background

Following lung and breast cancer, colorectal cancer (CRC) is the 3rd most common cancer worldwide.[1] In many cases, primary CRC can be treated with surgery, chemotherapy and radiotherapy. However, it is limited in the restriction of the development of metastatic disease.[2] Therefore, there is an urgent need to further clarify the molecular mechanism of CRC tumorigenesis and pathogenesis.

Glutathione S-transferases (GSTs) (the Enzyme Commission (EC) number: EC 2.5.1.18) are phase II metabolic enzymes,[3] which play essential roles in xenobiotic biotransformation,[4] drug metabolism,[5] protection against oxidative stress, and regulating cell proliferation and signaling pathways.[6,7] As an isoform of GST, glutathione S-transferase P1 (*GSTP1*) is a significant regulator of cell signaling in response to stress, hypoxia, growth factors, and other stimuli.[8] The *GSTP1* is overexpressed in various human cancers, including gastric cancer, pancreatic cancer and bladder cancer.[9,10] The *GSTP1* is also involved in the proliferation and invasion of tumor cells; the overexpression of *GSTP1* promotes the proliferation and inhibits the apoptosis in head and neck squamous cell carcinoma (HNSCC),[11] whereas it inhibits the proliferation of T24 cells in bladder cancer.[12] A recent study suggested that *GSTP1* might be applied as an essential biomarker of liquid biopsy.[13]

The signal transducer and activator of transcription (*STAT*) family proteins are phosphorylated via Janus kinases (*JAKs*) in response to the binding of growth factors or cytokines to their corresponding receptors.[14–16] These factors are known to stimulate the activation of intracellular STAT proteins, which are phosphorylated and dimerized, and subsequently translocated to the nucleus for the transactivation of several genes involved in numerous cellular processes.[17] The persistent activation of *STAT3* has been observed in multiple human malignancies, including various CRC stages.[18–20] Furthermore, a high expression of *STAT3* alters the cell cycle[21,22] and inhibits the apoptosis by upregulating anti-apoptotic signaling[23,24] in inflammation-associated CRC and other human cancers.[25] Besides, *GSTP1* negatively regulates *STAT3* activation in epidermal growth factor (EGF) signaling, and is also a regulator of the cell cycle via EGF signaling in human hepatocellular carcinoma (HCC).[8] However, the regulatory mechanisms between *GSTP1* and *STAT3* in the progression of CRC remain unknown.

Our previous studies identified that the loss of F-box only protein 8 (*FBX8*) was associated with patients' poor survival in HCC, gastric cancer and CRC.[26–28] The *FBX8* is a metastatic suppressor downstream of miR-223 and targets the degradation of mammalian target of rapamycin (mTOR) in CRC.[27] We also found that *FBX8* inhibited the proliferation, invasion and metastasis of CRC by promoting the degradation of *GSTP1*. Simultaneously, we confirmed that *GSTP1* could be used as an effective marker to predict the prognosis of CRC.[29]

Objectives

Our present study revealed that the overexpression of *GSTP1* could promote the proliferation, invasion and metastasis of CRC cells by upregulating *STAT3*, and that *FBX8* could regulate this function.

Materials and methods

Samples collection and ethical approval

A total of 20 paraffin-embedded CRC primary tumor samples were obtained from the Department of Pathology of Nanfang Hospital, Southern Medical University, Guangzhou, China. Each sample was diagnosed with primary CRC: tubular adenocarcinoma (in 14 samples); papillary adenocarcinoma (1); mucinous adenocarcinoma (3); and signet-ring cell carcinoma (2). Fresh CRC tissues were collected immediately after resection from 8 patients who underwent CRC resection without prior radiotherapy and chemotherapy at the Department of General Surgery of Nanfang Hospital in 2019. All aspects of the study complied with the principles of the Declaration of Helsinki and the study was approved by the Nanfang Hospital Research Ethics Committee. No informed consent was required, as the data were analyzed anonymously. Paraffin-embedded mice CRC tumor samples used in the study were prepared in our previous experiments.[29] The research related to the use of animals complied with all relevant national regulations and institutional policies for the care and use of laboratory animals.

Immunohistochemistry

The CRC human and animal tissue samples, which were formalin-fixed and paraffin-embedded, were dewaxed and rehydrated, and 3% hydrogen peroxide (H_2O_2; ZSGB-Bio, Beijing, China) was used to eliminate endogenous peroxidase. The antigens on the tissue slides were retrieved with 0.01 M sodium citrate buffer (pH 6.0) by microwave oven boiling for 5 min. After blocking the nonspecific antigens of the tissues with 5% goat serum (ZSGB-Bio) for 1 h at room temperature, the anti-GSTP1 (1:200; Abcam, Cambridge, UK) or anti-STAT3 (1:100; Abcam) antibody was diluted in an appropriate proportion and incubated overnight at 4°C. The tissue slides were incubated with the horseradish egg protein rabbit secondary antibody (ZSGB-Bio) or the murine secondary antibody (ZSGB-Bio) for 90 min at room temperature. Streptavidin was labeled with appropriate horseradish peroxidase (HRP; ZSGB-Bio), and incubated for 30 min at room temperature. The 3,3′-diaminobenzidine (DAB; ZSGB-Bio) color developer was needed for microscopic observations.

Staining was scored in a double-blind manner by 2 researchers with a score of 0 (representative negative), 1 (weak),

2 (medium), and 3 (strong). Depending on the percentage of the stained area relative to the total cancerous tissue area or blood vessel area, the staining range was divided as follows: 0 points (0%); 1 point (1–25%); 2 points (26–50%); 3 points (51–75%); and 4 points (76–100%). The sum of the dyeing strength and range was taken as the final dyeing value (0–7): (–) total score <3 points; (+) total score 3 points; (++) total score 4 points; and (+++) total score ≥5 points, in which (–) or (+) form the low-expression groups, whereas (++) and (+++) are the high-expression groups.

Co-immunoprecipitation

In brief, the extracts of SW620 cells were blocked with immunoglobulin G (IgG) or protein A/G agarose (BioWorld, Visalia, USA) for 2 h at 4°C to remove nonspecific protein binding, and then incubated with anti-FBX8 (Abcam, Cambridge, UK) or anti-GSTP1 overnight at 4°C. Protein A/G Agarose was separated by centrifugation (2500 rpm) at 4°C. After blocking with 5% skim milk for 1 h at room temperature, polyvinylidene difluoride (PVDF) membranes (Roche Applied Science, Penzberg, Germany) were incubated with anti-GSTP1 (1:200) and anti-STAT3 (1:100) overnight at 4°C. The protein bands were visualized by using an enhanced chemiluminescence (ECL) HRP kit (fluorescence detection (FD) bio-Femto (Yeasen, Shanghai, China).

Immunofluorescence

A total of 0.5 × 10^4 cells were seeded on confocal NEST® glass-bottom Petri dishes (NEST, Wuxi, China) for a 24-hour incubation. Then, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.02% Triton-X-100/1 × phosphate-buffered saline (PBS) and blocked in a blocking fluid (1 × PBS + 10% fetal bovine serum (FBS) and 1% bovine serum albumin (BSA)) (Thermo Fisher Scientific, Waltham, USA). The Petri dishes were incubated with anti-GSTP1 (1:200) and anti-STAT3 (1:100) overnight at 4°C. The secondary antibodies conjugated to Alexa Fluor® 488 or 594 (Invitrogen, Waltham, USA) were added to the incubated cells for 2 h at room temperature. The 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the cell nucleus. Confocal images were taken with the use of an inverted fluorescence microscope (Olympus Corp., Tokyo, Japan) and outputted by means of the PV10-ASW viewer software v. 1.7 (Olympus Corp.).

Glutathione S-transferase pull-down assay

We used GST-mediated pull-down assays (Thermo Fisher Scientific, Rockford, USA) to detect the interaction between the truncated GSTP1 and STAT3 in HCT116 and SW620 cell lines. Firstly, recombinant GST-STAT3-CCD (coiled-coil domain) (218-400), GST-STAT3-DBD (DNA-binding domain) (401-564), GST-STAT3-linker (565-663), and GST-STAT3-SH2 (Src homology 2 domain) (664-768) proteins were expressed and purified. Secondly, the purified GST-STAT3-CCD (218-400), GST-STAT3-DBD (401-564), GST-STAT3-linker (565-663), and GST-STAT3-SH2 (664-768) fragments were bound to glutathione resin as a GST-fusion protein and incubated with anti-GSTP1 for 2 h at 4°C. Thirdly, the complex was washed with the assay buffer and eluted with 5 mM reduced glutathione, and then the bound protein complexes were disrupted. Finally, the proteins were separated by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and detected with a western blotting assay.

Plasmid and siRNA transfection

The cells were transfected with plasmids or siRNA for *STAT3* or *GSTP1*, respectively, with Lipofectamine® 3000, as per the manufacturer's instructions (Thermo Fisher Scientific, Waltham, USA).

Cell proliferation assay (CCK-8)

The medium in a 96-well plate was replaced with 100 µL of the complete medium and Cell Counting Kit-8 (CCK-8) reagent mixture (9:1), and the plate was incubated in 5% CO_2 for 2–4 h at 37°C. The optical density (OD) value at 450 nm for each well was detected with an enzyme labeling instrument (BioTek, Winooski, USA), and the OD value was adjusted to zero based on the blank control group. The cell proliferation curve was plotted with the mean value of 5 multiple holes in each group.

Cell invasion assays in vitro

Cells invasion assays in vitro were performed to assess the cell invasion ability. In brief, 200 µL of a serum-free medium containing 5 × 10^4 cells was added to the upper chamber with the Corning® BioCoat™ Matrigel (Corning Life Sciences, New York, USA) and allowed to invade toward the lower chamber with 10% FBS. After incubation for an appropriate period of time at 37°C, the cells were fixed with methanol for 30 min and stained with crystal violet solution for 20 min. The migrated cells were photographed and counted in 6 random regions with an inverted microscope (Olympus Corp.).

Western blotting

During immunoblotting, the proteins were extracted with a lysis buffer, and then quantified by means of a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Equivalent amounts of cell lysates were separated using SDS–PAGE and transferred to PVDF membranes. The membranes were immunoblotted overnight at 4°C with anti-β-actin antibodies (1:1000; Proteintech, Chicago,

USA), GSTP1 (1:200; Abcam, London, England), STAT3 (1:200; Abcam), GST (1:1000; Abcam), followed by appropriate second antibodies. The bands were visualized using the Pierce™ ECL western blotting substrate (Thermo Fisher Scientific). The immunoblotting image density was determined with gel densitometry (Bio-Rad Laboratories, Hercules, USA).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

The RNA of the cultured cells and the CRC tissues were extracted using the TRIzol® reagent (Invitrogen). The RNA expression level of *GSTP1* was detected with the use of ABI PRISM 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). The relative mRNA levels were calculated using the comparative Ct method (ΔΔCt). The primer sequences for qRT-PCR were as follows:

– *GSTP1*: Fwd: CGG GGT ACC ATG CCG CCC TAC ACC GT; Rev: CCG CTC GAG TCA CTG TTT CCC GTT GCC ATT;

– *STAT3*: Fwd: CAG CAG CTT GAC ACA CGG TA; Rev: AAA CAC CAA AGT GGC ATG TGA.

Cell culture and treatment

All cell lines were obtained from American Type Culture Collection (ATCC; Manassas, USA). The human CRC cell lines (SW480, SW620, HCT116, and LOVO) were cultured in the Roswell Park Memorial Institute medium (RPMI) 1640 (high glucose) (Gibco Laboratories, Gaithersburg, USA) supplemented with 10% FBS (Gibco Laboratories). The medium was supplemented with 100 µg/mL streptomycin and 100 U/mL penicillin (Gibco™, Thermo Fisher Scientific, Waltham, USA), and the abovementioned cells were incubated at 37°C in a humidified chamber containing 5% CO_2. The stable knocking down *GSTP1* cancer cells (SW620/shGSTP1 and HCT116/shGSTP1 cell lines) were from the previous study.

Statistical analysis

All statistical analyses were performed by means of GraphPad Prism v. 6.02 (GraphPad Software, San Diego, USA). The results are presented as mean (M) and standard deviation (SD) or median (Me) and 95% confidence interval (95% CI). Pearson's correlation coefficient was used to analyze the correlation between *GSTP1* and *STAT3*. The one-way analysis of variance (ANOVA) was used to compare differences in the invasion ability of the 3 groups in invasion assays, and the two-way ANOVA to analyze differences in the proliferation ability of CRC cells at different time points between different groups. Before ANOVA, Levene's test for the equality of variances was used. A two-tailed $p < 0.05$ was considered statistically significant in all tests.

Results

The expression levels of *GSTP1* and *STAT3* in human CRC tissues are positively correlated

The *STAT3*, widely recognized as a cancer gene, is typically associated with a poor prognosis for various human malignancies, as it promotes cancer progression or metastasis.[30–33] The direct interaction between *GSTP1* and *STAT3* can promote HCC progression,[8] and our previous study found that *GSTP1* could be ubiquitinated by *FBX8*, thus inhibiting its function in promoting CRC proliferation, invasion and metastasis.[29] Therefore, immunohistochemistry (IHC) was used to detect the expression of *GSTP1* and *STAT3* in 20 human CRC tissue samples. The results demonstrated that the expression levels of *GSTP1* and *STAT3* in human CRC tissues were positively correlated (Fig. 1A). Western blotting and qRT-PCR were used to detect the expression levels of *GSTP1* and *STAT3* in 8 paired fresh CRC tissue samples (Fig. 1B,C), and the results of these 2 experiments showed that the expression patterns of *GSTP1* and *STAT3* were highly correlated. After statistical analysis, it turned out that the expression of *GSTP1* was positively correlated with the expression of *STAT3* in the paired fresh CRC tissue samples, as shown in Fig. 1D (Pearson's correlation analysis, two-tailed test, $r^2 = 0.8781$, $p = 0.0006$).

The overexpression of *GSTP1* promotes the proliferation, invasion and metastasis of CRC cells depending on *STAT3*

The expression of *GSTP1* was positively correlated with the expression of *STAT3*. Taking into account previous studies, it was predicted by authors that *GSTP1* might play a role in CRC progression by regulating *STAT3*. Therefore, the SW620 and HCT116 cell lines which stably knockdown *GSTP1* were constructed (Fig. 2A). Next, STAT3 was overexpressed in the above 2 cell lines; the results showed that the overexpression of STAT3 was successful (Fig. 2A). Therefore, the SW620/shGSTP1/*STAT3* and HCT116/shGSTP1/*STAT3* cell lines were used to perform relevant recovery experiments. The results showed that the overexpression of *STAT3* could significantly promote the invasion (Fig. 2B) and proliferation (Fig. 2C) of cells in the *GSTP1*-knockdown group in vitro. Besides, the expression levels of STAT3 and GSTP1 were detected in subcutaneous tumors (Fig. 3A), in situ implants (Fig. 3B) and liver metastases (Fig. 3C) of CRC in the nude mouse tissues obtained from a previous study. The results demonstrated that in these 3 tumor tissues, the expression of STAT3 was significantly upregulated in the *GSTP1* overexpressed group, which indicated that the expression levels of *GSTP1* and *STAT3* in the mouse tissue samples were consistent.

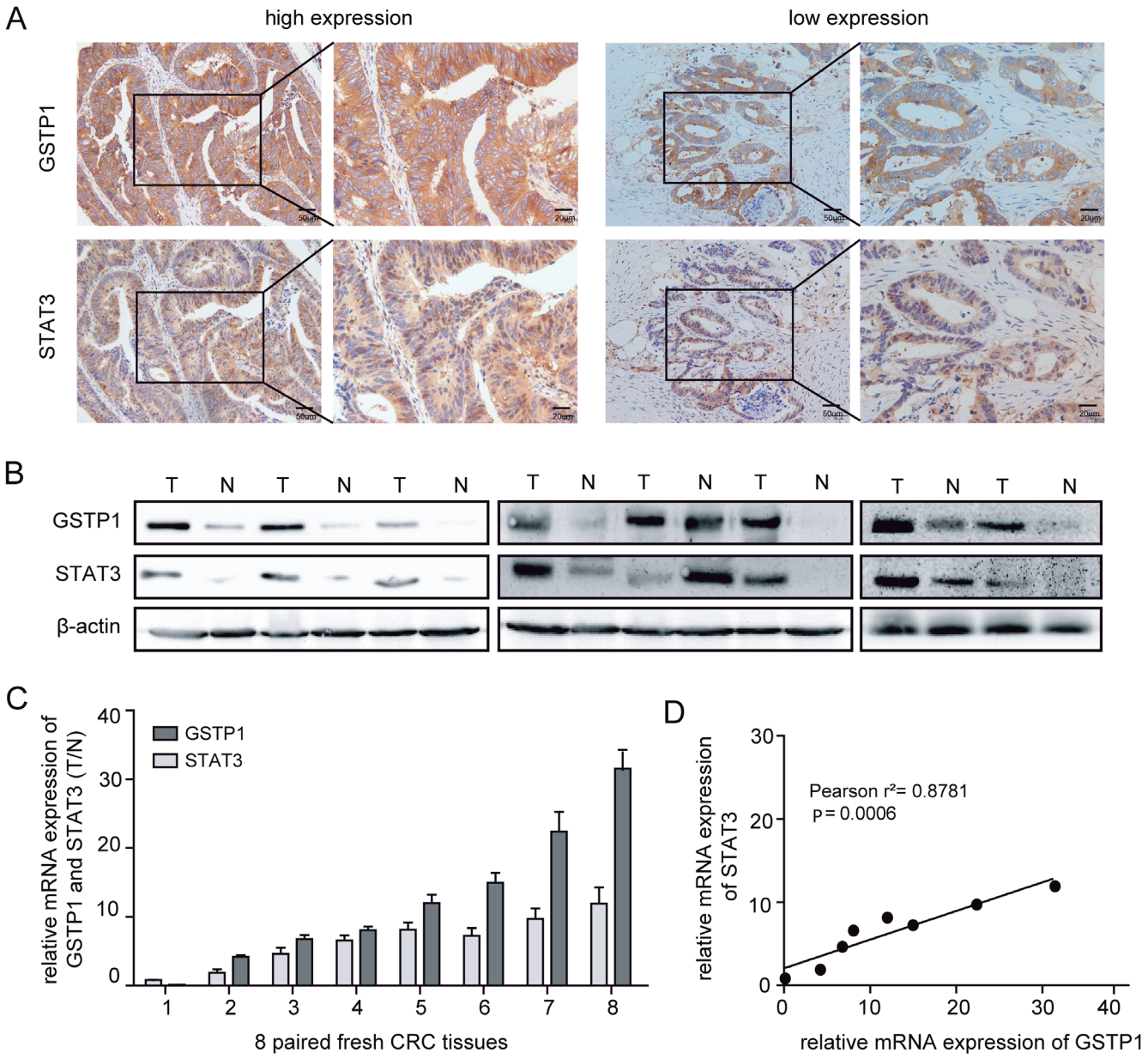

Fig. 1. Expression levels of glutathione S-transferase P1 (GSTP1) and signal transducer and activator of transcription 3 (STAT3) in human colorectal cancer (CRC) tissues are positively correlated

A. The expression levels of GSTP1 and STAT3 in CRC tissues were detected by means of immunohistochemistry (IHC). Scale bars represent 50 μm (left) and 20 μm (right); B. The western blot analysis was performed to detect the expression levels of GSTP1 and STAT3 in 8 paired fresh tissue samples of human CRC; C. The relative mRNA expression levels of GSTP1 and STAT3 in 8 paired fresh tissue samples of human CRC were detected by means of the quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. All samples were tested in triplicate. Bars represent mean ± standard deviation (M ±SD); D. The analysis of correlation between GSTP1 and STAT3 was performed using Pearson's correlation coefficient (two-tailed test).

GSTP1 and *STAT3* can directly bind to and regulate each other

It was further hypothesized that *GSTP1* could interact with *STAT3* in CRC cells. Therefore, the co-immunoprecipitation (co-IP) and immunofluorescence (IF) analyses were performed to identify the interaction between the 2 proteins. As expected, the existence of GSTP1 in the immunoprecipitates obtained with an antibody against STAT3 was successfully detected (Fig. 4A). The IF results demonstrated that GSTP1 and STAT3 exhibited co-localization in the cytoplasm of the SW620 cells (Fig. 4B). The present study cloned 4 truncated constructs of STAT3: CCD (218-400); DBD (401-564); linker (565-663); and SH2 (664-768) (Fig. 4C), and then identified an interaction between the CCD domain of STAT3 and GSTP1 by means of a GST pull-down assay (Fig. 4C). It turned out that the CCD domain of STAT3 was essential for the interaction with GSTP1.

Thus, it was examined whether *GSTP1* could activate the *STAT3* signaling pathway in CRC cells, and then it was

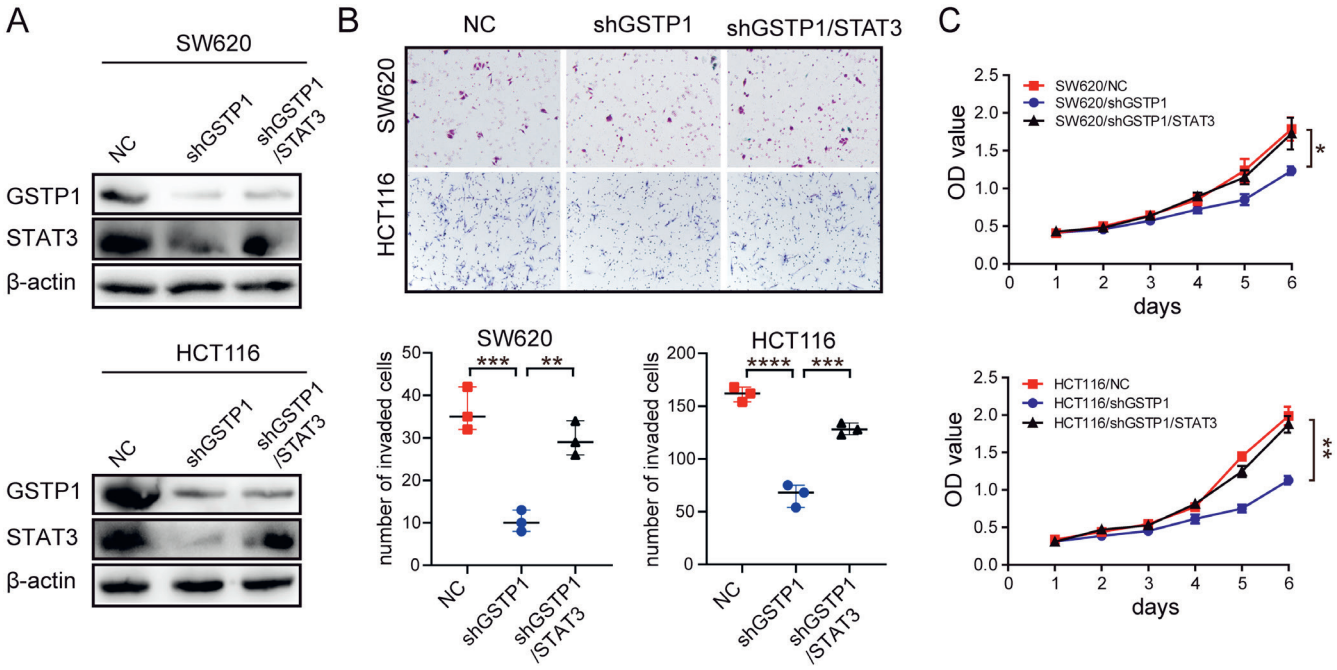

Fig. 2. Overexpression of signal transducer and activator of transcription 3 (STAT3) can reverse the inhibition of proliferation and invasion induced by downregulating glutathione S-transferase P1 (GSTP1)

A. The western blot analysis was performed to detect the effects of the overexpression of STAT3 on SW620/shGSTP1 and HCT116/shGSTP1 cells; B. The effect of the overexpression of STAT3 on SW620/shGSTP1 and HCT116/shGSTP1 cell invasion was assessed with an invasion assay. Scale bars represent 50 μm. All samples were tested in triplicate. The statistical analysis was performed with the one-way analysis of variance (ANOVA). Point charts represent median (Me) and 95% confidence interval (95% CI); **p < 0.005; *** p < 0.0005; **** p < 0.0001; C. The effect of the overexpression of STAT3 on SW620/shGSTP1 and HCT116/shGSTP1 cell proliferation was detected with a cell counting-8 (CCK-8) assay. All samples were tested in triplicate. The statistical analysis was performed with the two-way ANOVA. Bars represent mean ± standard deviation (SD); OD – optical density; NC – negative control; *p < 0.05; **p < 0.005.

identified that the exogenous expression of *GSTP1* further increased the protein expression of phosphorylated (p)-STAT3, STAT3 and downstream STAT3 targets (cyclin D1 and CDC25A) in the SW480 cells (Fig. 4D). However, there was no appreciable effect on the upstream components of the STAT3 signaling pathway, such as JAK2 and p-JAK2 (Fig. 4D). By contrast, the depletion of *GSTP1* in the SW620 cells decreased the levels of p-STAT3, STAT3, cyclin D1, and CDC25A (Fig. 4D). Notably, this regulation was not one-way; the ectopic expression of STAT3 could also up-regulate the protein level of GSTP1 and induce higher levels of downstream STAT3 targets (cyclin D1 and CDC25A). Simultaneously, silencing STAT3 could decrease the expression levels of GSTP1, cyclin D1 and CDC25A, but not the expression of p-JAK2 (Fig. 4E). Also, when AG490 (100 μM) was used to block the JAK2-*STAT3* pathway, a significant decrease was observed in the expression of JAK2, p-JAK2, STAT3, p-STAT3, and GSTP1 24 h after treating the LoVo cells (Fig. 4F).

The interaction between GSTP1 and STAT3 is regulated by FBX8

As a downstream target of *FBX8*, *GSTP1* can interact with *STAT3*. Therefore, it was predicted that *FBX8* could regulate the interaction between *GSTP1* and *STAT3*. The co-IP analysis demonstrated that there was an interaction between GSTP1 and STAT3 in the SW620 cell line, with a high endogenous expression of *FBX8* (Fig. 5A). However, after adding AG490, a specific inhibitor of JAK2, the signal transduction of the JAK2 pathway was inhibited, resulting in a significant decrease in the expression of STAT3 in the SW620 cell line. In comparison with the group without AG490, the STAT3 that GSTP1 could bind to was also significantly reduced (Fig. 5B). Simultaneously, in the SW480 cell line with the overexpression of *FBX8*, the expression of STAT3 detected with a co-IP assay was significantly reduced as compared to the control group (Fig. 5C). These results indicated that *FBX8* was a suppressive factor for the combination of *GSTP1* and STAT3.

Figure 6 is the schematic diagram of the role of *GSTP1*, *STAT3* and *FBX8* in CRC.

Discussion

Previously, we identified *GSTP1* as a downstream target of *FBX8* using the co-IP and mass spectrometry analyses, and confirmed that GSTP1 could promote the proliferation, invasion and metastasis of CRC.[29] Besides, it has been found that *GSTP1* could regulate *STAT3* to affect the development of HCC.[8] Therefore, we hypothesized that *GSTP1* might be involved in the progression of CRC by regulating *STAT3*.

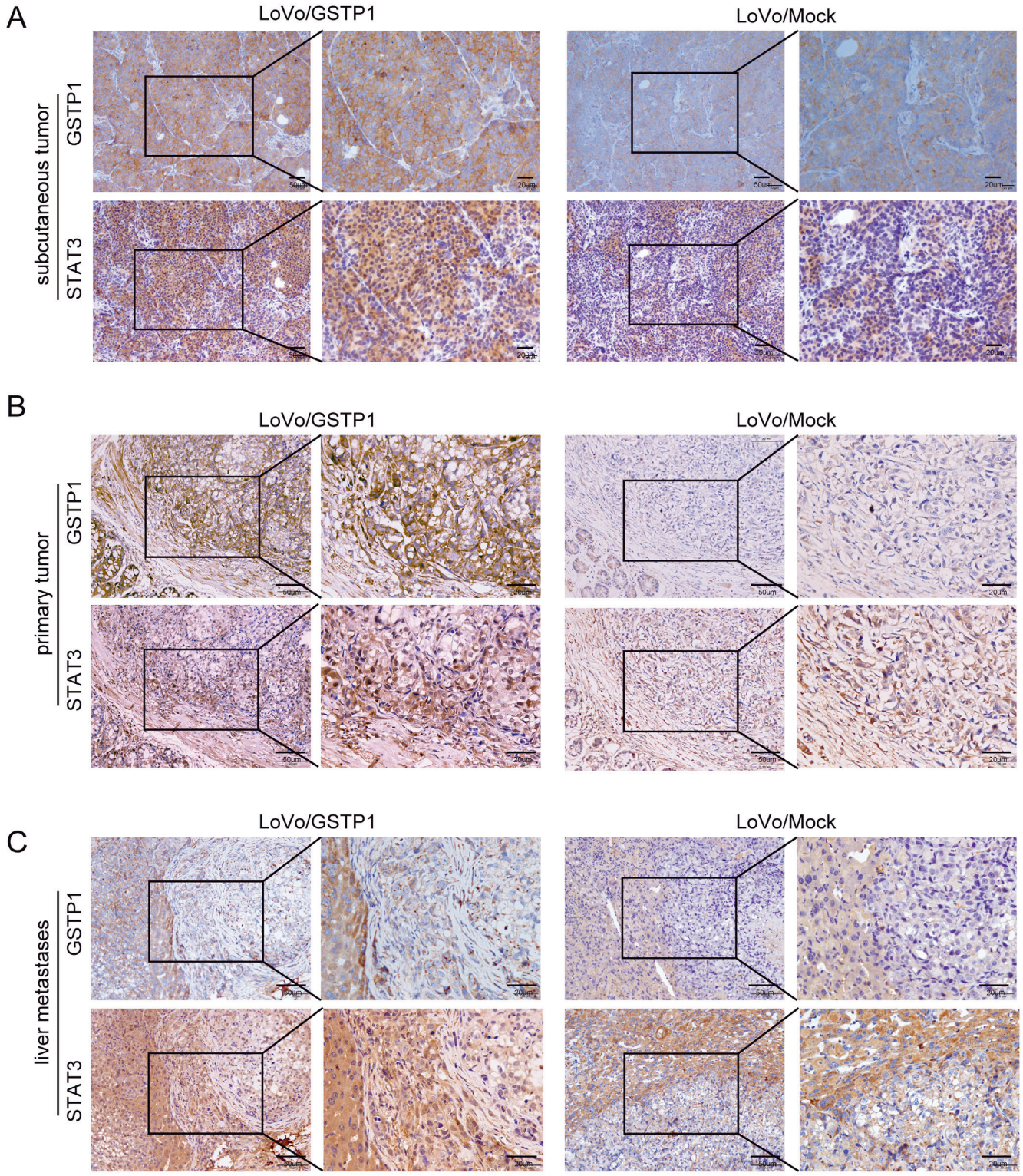

Fig. 3. Overexpression of glutathione S-transferase P1 (GSTP1) can upregulate the expression of signal transducer and activator of transcription 3 (STAT3) in vivo

A. The expression of GSTP1 and STAT3 in subcutaneous tumors in nude mice was detected by means of immunohistochemistry (IHC). Scale bars represent 50 μm (left) and 20 μm (right); B. The expression of GSTP1 and STAT3 in the orthotopic implantation of colorectal cancer (CRC) in nude mice was detected by means of IHC. Scale bars represent 50 μm (left) and 20 μm (right); C. The expression of GSTP1 and STAT3 in liver metastases of CRC in nude mice was detected by means of IHC. Scale bars represent 50 μm (left) and 20 μm (right).

The present study detected *GSTP1* and *STAT3* in human CRC tissue samples and found that the expression of *GSTP1* was positively correlated with the expression of *STAT3*. This result revealed that *GSTP1* might be able to regulate the expression of *STAT3*, and thus plays a role in the progression of CRC. Recent evidence suggests that *GSTP1* is involved in tumor cell proliferation and invasion; the overexpression of GSTP1 increases cell proliferation

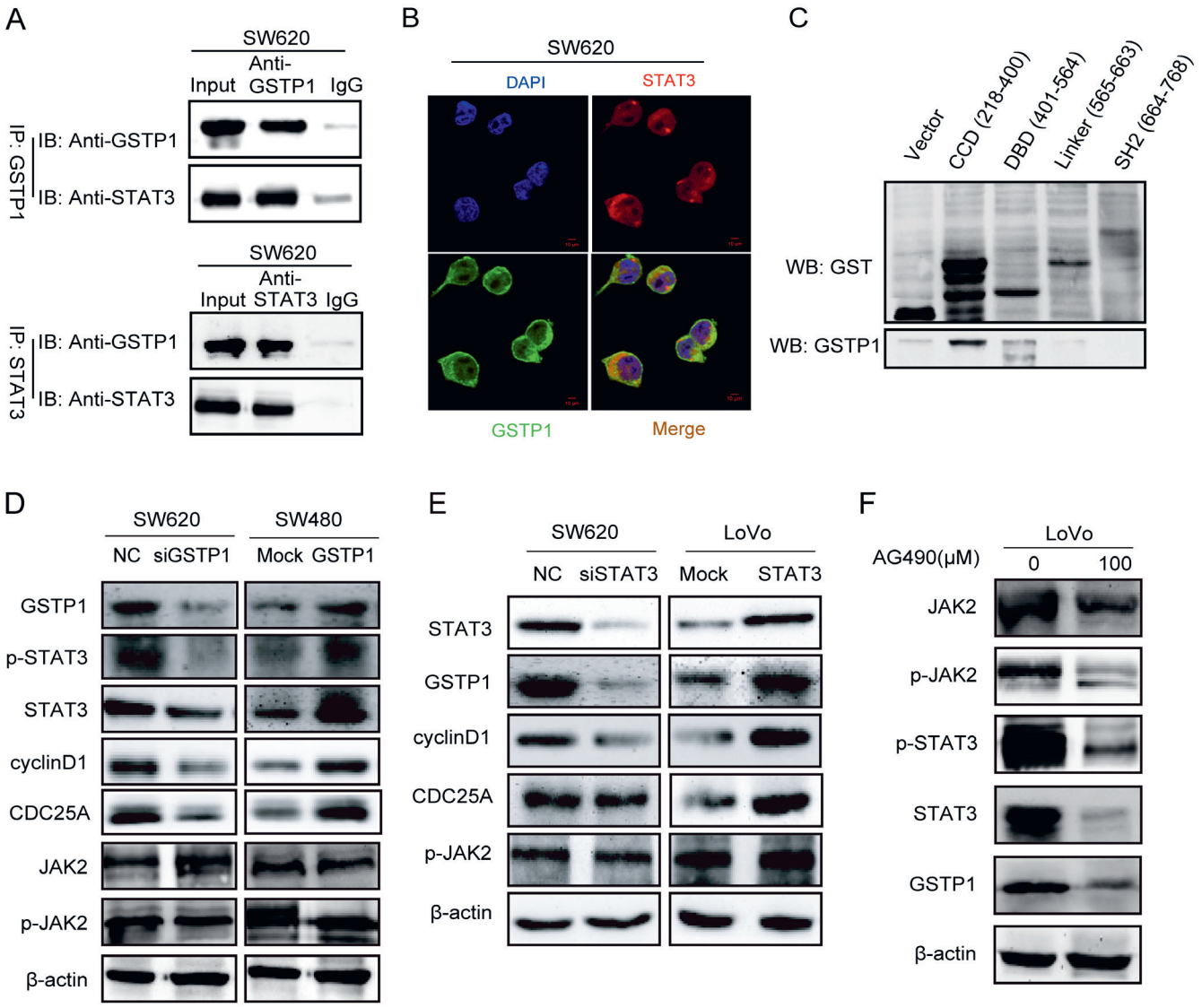

Fig. 4. Glutathione S-transferase P1 (GSTP1) and signal transducer and activator of transcription 3 (STAT3) can interact with each other

A. The interaction between GSTP1 and STAT3 was detected by means of a co-immunoprecipitation (co-IP) assay with the SW620 cell line; IB – immunoblotting; IgG – immunoglobulin G; B. The co-localization between GSTP1 and STAT3 was detected by means of an immunofluorescence (IF) assay with the SW620 cell line. Scale bars represent 10 μm; DAPI – 4′,6-diamidino-2-phenylindole; C. The direct interaction site between GSTP1 and STAT3 was detected by means of a GST pull-down assay; WB – western blotting; D. The western blot analysis was performed to detect the expression of Janus kinase (JAK)/STAT3 signaling pathway-related proteins in the SW620/siGSTP1 and SW480/GSTP1 cell lines; p-STAT3 – phosphorylated STAT3; p-JAK2 – phosphorylated JAK2; E. The western blot analysis was performed to detect the expression of JAK/STAT3 signaling pathway-related proteins in the SW620/siSTAT3 and LoVo/STAT3 cell lines; F. The western blot analysis was performed to detect the expression of GSTP1 and JAK/STAT3 signaling pathway-related proteins following the AG490 treatment of the LoVo cells.

in HNSCC.[11] By comparison, *GSTP1* can arrest tumor cells in the G0/G1 phase and upregulate the expression of p21 in bladder cancer.[12] The present study investigated the effect of *GSTP1* on the proliferation and invasion of CRC cells in vitro by recovery experiments. The results demonstrated that the overexpression of *STAT3* could significantly promote the proliferation and invasion of CRC cells after GSTP1 downregulation. This indicated that GSTP1 enabled the proliferation and invasion of CRC cells depending on STAT3. Besides, the IHC results of subcutaneous tumors, in situ implanted tumors and liver metastases of CRC in mice also confirmed the conclusion mentioned above.

In terms of mechanism, this study demonstrated that GSTP1 and STAT3 could form a complex, and GSTP1 upregulation leads to the activation of the STAT3 pathway. Meanwhile, STAT3 can positively regulate the protein expression of GSTP1. The STAT3 is phosphorylated via JAK, then dimerized and subsequently translocated to the nucleus for the transactivation of several genes involved in numerous cellular processes.[14–17] Besides, the overexpression of *STAT3* can affect the cell cycle[21,22] or inhibit the apoptosis by enhancing anti-apoptotic signaling[23,24] in CRC.

Therefore, identifying the association between *GSTP1* and the *STAT3* pathway is a meaningful way to illustrate the molecular mechanisms of *GSTP1* in CRC. This study confirmed the interaction between GSTP1 and STAT3 and

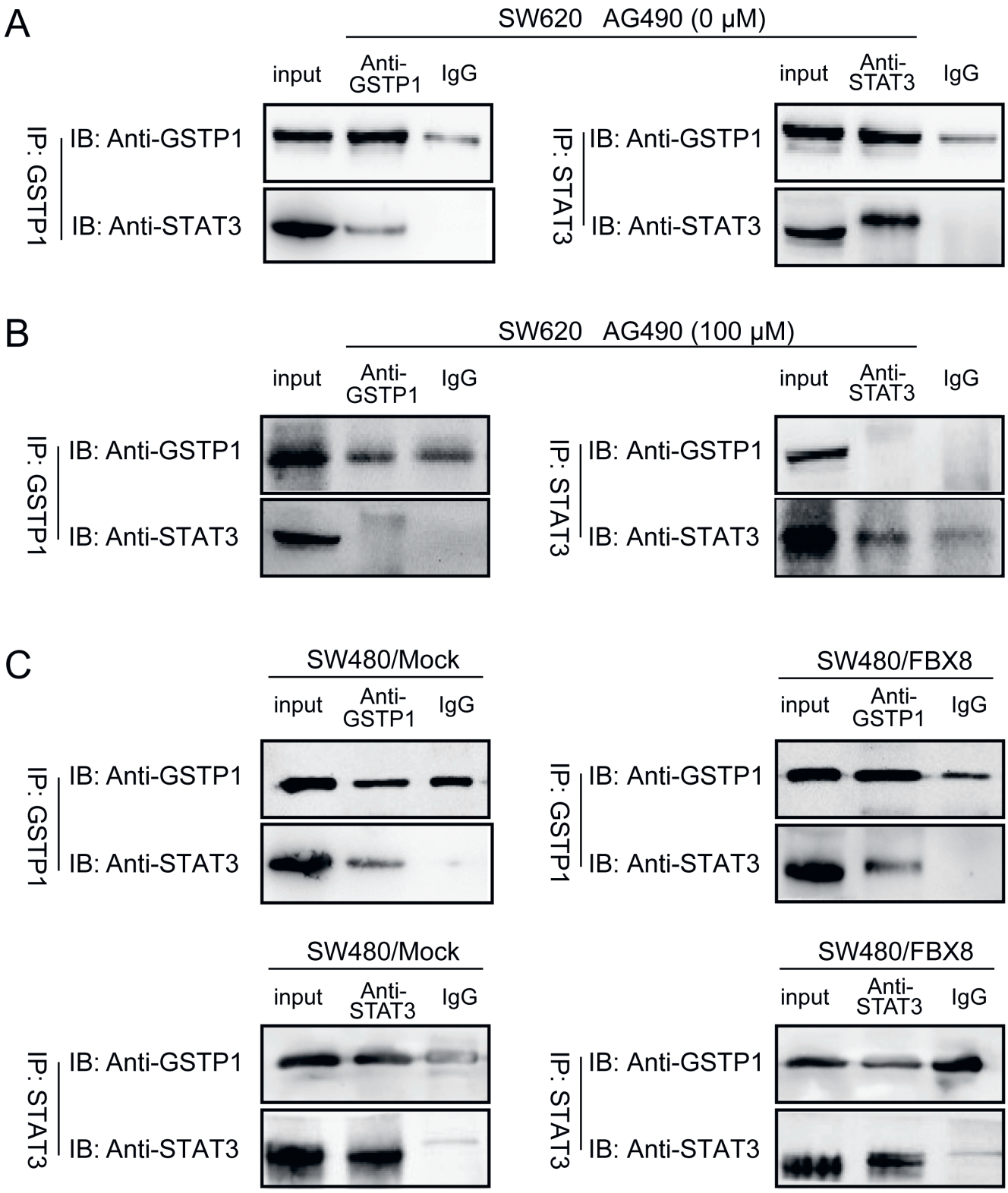

Fig. 5. Interaction between glutathione S-transferase P1 (GSTP1) and signal transducer and activator of transcription 3 (STAT3) is regulated by F-box only protein 8 (FBX8)

A. The interaction between GSTP1 and STAT3 was detected by means of a co-immunoprecipitation (co-IP) assay in the SW620 cell line; B. The interaction between GSTP1 and STAT3 was detected by means of a co-IP assay in the SW620 cell line after AG490 treatment; C. The interaction between GSTP1 and STAT3 was detected by means of a co-IP assay in the SW480/FBX8 cells.

proved that *GSTP1* positively regulates the *STAT3* signaling pathway, leading to changes in target genes, such as p-STAT3 and STAT3, cyclin D1, and CDC25A. The STAT3 siRNA significantly abolished increases in *STAT3*, cyclin D1 and CDC25A and decreased the protein expression of GSTP1, but there was no change in p-JAK2. Exogenous

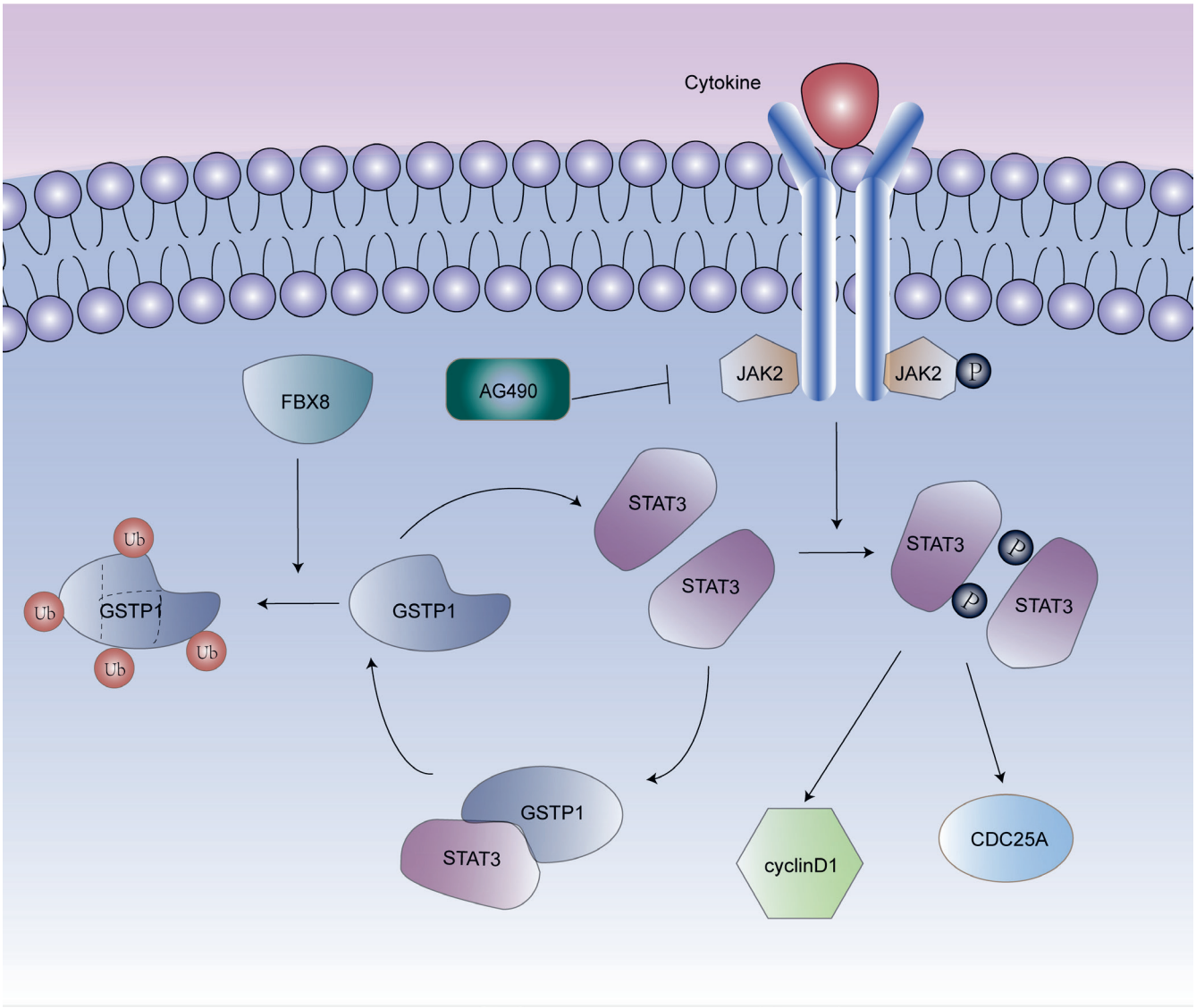

Fig. 6. Schematic diagram of the role of glutathione S-transferase P1 (GSTP1) and signal transducer and activator of transcription 3 (STAT3) in colorectal cancer (CRC)

STAT3 exhibited adverse results. Besides, western blotting revealed a concentration-dependent decrease in the level of JAK2, p-JAK2, STAT3, p-STAT3, and GSTP1 after 24 h of treating LoVo cells with the specific inhibitor of JAK2 (AG490). These results demonstrated that GSTP1 interacted with STAT3 without the involvement of JAK2. With regard to the previous research showing that FBX8 can degrade the expression of GSTP1,[29] it was speculated that FBX8 could affect the association of GSTP1 and STAT3. Subsequent experiments confirmed that *FBX8* was a restraining factor for the combination of *GSTP1* and *STAT3*.

Limitations

In this study, the analysis of the relationship between *GSTP1* and *STAT3* was performed using limited cohorts. Therefore, the relationship needs to be further verified in clinical samples. Besides, we showed a region that is responsible for the interaction. However, we haven't studied this region much for further phenotypic and genetic changes.

Conclusions

In summary, *GSTP1*, as a downstream effector of *FBX8*, was identified as an essential promoter and a useful prognostic marker for CRC. The *GSTP1* could interact with *STAT3* and upregulate the expression of *STAT3* as well as of its related downstream molecules[34] to promote the proliferation, invasion and metastasis of CRC. Therefore, the present study provided a potential new molecular target for the treatment of CRC metastasis.

ORCID iDs

Feifei Wang https://orcid.org/0000-0002-5962-3136
Ceng Zhang https://orcid.org/0000-0002-1335-6618
Xiaohui Zhu https://orcid.org/0000-0002-9031-3283
Dan Zhang https://orcid.org/0000-0001-5921-1898
Zhaowen Zhang https://orcid.org/0000-0001-8121-5086
Shunjie Ni https://orcid.org/0000-0003-1193-8574
Zhizhi Wang https://orcid.org/0000-0002-1213-479X
Shuyi Xu https://orcid.org/0000-0002-8664-5429
Xiaoliang Lan https://orcid.org/0000-0002-0582-7942
Yanqing Ding https://orcid.org/0000-0003-1775-6923
Li Liang https://orcid.org/0000-0001-5302-2754

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1,25-$(OH)_2D_3$ participates and modulates airway remodeling by reducing MGP and TGF-β1 expression in TNF-α-induced airway smooth muscle cells

Yan-Min Xing[B–F], Pei-Shan Li[B,C,F], Ying Liu[A–F]

Department of Pediatrics, Tianjin Union Medicine Centre, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Ying Liu
E-mail: kkkejinge@sina.com

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Abstract

Background. Asthma has been proven to be a respiratory disorder that is characterized by the airway remodeling, airway inflammation and reversible airway obstruction. The 1,25-hydroxyvitamin D_3 (1,25-$(OH)_2D_3$) plays critical roles in delaying remodeling.

Objectives. To investigate the effects of 1,25-$(OH)_2D_3$ on the airway remodeling in tumor necrosis factor α (TNF-α)-induced airway smooth muscle cells (ASMCs).

Materials and methods. The human ASMCs were divided into a blank control group (without treatment), a TNF-α group (treated with 10 ng/mL TNF-α) and a 1,25-$(OH)_2D_3$+TNF-α group (pre-treated with 10^{-7} M 1,25-$(OH)_2D_3$, then with 10 ng/mL TNF-α). The MTT assay was used to evaluate cell proliferation. Matrix Gla protein (MGP) and transforming growth factor β1 (TGF-β1) were examined using western blot assay.

Results. The TNF-α treatment significantly increased ASMCs proliferation and enhanced MGP and TGF-β1 expression compared to a blank control group ($p < 0.05$). The 1,25-$(OH)_2D_3$ treatment (1,25-$(OH)_2D_3$+TNF-α group) significantly inhibited cell viability (0.83 ±0.01), compared to that in the TNF-α group (0.92 ±0.01) ($p < 0.05$). The 1,25-$(OH)_2D_3$ treatment significantly downregulated MGP expression (0.61 ±0.02), compared to that of the TNF-α group (1.51 ±0.35) ($p < 0.05$). The 1,25-$(OH)_2D_3$ treatment significantly reduced TGF-β1 expression (0.69 ±0.17), compared to that of the TNF-α group (1.6 ±0.18) ($p < 0.05$).

Conclusions. The 1,25-$(OH)_2D_3$ could participate and modulate airway remodeling by reducing MGP and TGF-β1 expression in TNF-α-induced ASMCs. This study provided therapeutic insight and theoretical basis for clinical research.

Key words: TGF-β1, TNF-α, 25-hydroxyvitamin D_3, airway smooth muscle cells

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Background

Asthma has been proven to be a respiratory disorder that is characterized by airway remodeling, airway inflammation and reversible airway obstruction.[1,2] The airway obstruction and airway remodeling demonstrates many characteristics, including the enhancement of vascular permeability, the promotion of angiogenesis, and hypertrophy and hyperplasia of the airway smooth muscle cells (ASMCs).[3,4] In the occurrence and development processes of asthma, the ASMCs convert from the contractile type to synthetic type. Meanwhile, the ASMCs could synthesize and release the cytokines and inflammatory factors, and accelerate the airway remodeling in the asthma processes.[5] The tumor necrosis factor α (TNF-α), a kind of pro-inflammatory cytokine, could induce the formation of synthetic ASMCs.[6] Therefore, in this study, the TNF-α was administered to ASMCs to establish a synthetic ASMCs model of asthma.

The 1,25-hydroxyvitamin D_3(1,25-$(OH)_2D_3$) could assist the balance of calcium and phosphate levels, promote the calcium absorption, and – ultimately – keep the bone health.[7] Studies by Hall and Agrawal and Chen et al. reported that deficiency of (1,25-$(OH)_2D_3$) is positively correlated with the enhanced morbidity of asthma.[8,9] A few recent studies also discovered that (1,25-$(OH)_2D_3$) could directly inhibit the proliferation and migration of ASMCs in vitro.[10,11] The matrix Gla protein (MGP) is a specific biomarker for the synthetic smooth muscle cells, and could reflect the status and the amounts of the ASMCs.[12] Recently, plenty of cytokines or growth factors secreted by the inflammatory cells have been proven to be associated with the proliferation of ASMCs – especially for the transforming growth factor β1 (TGF-β1), which participates in the airway remodeling and the asthma-associated inflammation.[13,14]

Objectives

In the present study, we administered TNF-α to ASMCs to mimic the inflammatory condition of the airways injured in asthma, and aimed to evaluate the effects of (1,25-$(OH)_2D_3$) on proliferation of ASMCs as well as explore the associated mechanisms.

Materials and methods

Cell culture and trial grouping

The human ASMCs were purchased from Shanghai BioLeaf BioTech. Co. Ltd. (Cat. No. SX-C1160; Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL. Co. Ltd., Grand Island, USA), containing 10% fetal bovine serum (FBS; Gibco BRL. Co. Ltd.) at 37°C and 5% CO_2. After passaging the cells for 3–6 generations, ASMCs were digested and prepared to the single-cell suspension, and seeded into 96-well plates (Corning-Costar, Corning, USA). The ASMCs were then divided into 3 groups, including a blank control group (without any treatment), a TNF-α group (ASMCs treated with TNF-α at final concentration of 10 ng/mL) and a 1,25-$(OH)_2D_3$+TNF-α group (ASMCs pre-treated with 10^{-7} M 1,25-$(OH)_2D_3$ for 1 h, and then treated with TNF-α at a final concentration of 10 ng/mL). The TNF-α was purchased from the PeproTech Co. Ltd. (Rocky Hill, USA). The 1,25-$(OH)_2D_3$ was purchased from Sigma-Aldrich (St. Louis, USA).

This study has been approved by the Ethical Committee of Tianjin Union Medicine Centre (Tianjin, China).

MTT assay

Cell proliferation of ASMCs was determined using MTT detection kit (Cat. No. M1020; SolarBio, Beijing, China), according to the manufacturer's protocol. In brief, the ASMCs (at a density of 3×10^3 cells/well) were cultured in 96-well plates and treated as previously described for 24 h. The MTT was added into the 96-well plates (20 μL per well) and incubated for 4 h at 37°C. Then, 150 μL dimethyl sulfoxide (DMSO; Cat. No. D8371; SolarBio) was added to each well to dissolve the formed crystal in the wells. The absorbance for each well at the wavelength of 490 nm was measured by employing the microplate reader.

Western blot assay

The ASMCs were lysed using Lysis Buffer (Cat. No. P0013C; Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instruction. Lysates were separated using 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; Amresco, Inc., Solon, USA) and electro-transferred onto polyvinylidene difluoride (PVDF; Millipore, Boston, USA). Then, PVDF membranes were blocked in phosphate-buffered saline (PBS) using 5% defatted milk supplemented with 0.05% Tween-20 (Beyotime Biotechnology). The PVDF membranes were then treated with rabbit antihuman MGP polyclonal antibody (1:3000, Cat. No. ab86233; Abcam, Cambridge, USA), rabbit antihuman TGF-β1 polyclonal antibody (1:3000, Ca. No. ab92486; Abcam) and rabbit antihuman β-actin polyclonal antibody (1:2000, Cat. No. ab82275; Abcam) at 4°C overnight. Subsequently, PVDF membranes were incubated using 1:2000 horseradish peroxidase (HRP)-conjugated goat antirabbit immunoglobulin G (IgG) (Cat. No. ab6721; Abcam) at room temperature for 2 h. The western blot bands were visualized with BeyoECL Plus kit (Cat. No. P0018S; Beyotime Biotechnology). Finally, the images of western blot bands were captured and analyzed with Image Pro Plus v. 6.0 software (Media Cybernetics, Inc., Bethesda, USA).

Statistical analysis

The data were analyzed using GraphPad Prism software v. 6.0 (GraphPad Software, San Diego, USA). The continuous variables were represented as mean ± standard deviation (SD) and analyzed using analysis of variance (ANOVA) test validated with Tukey's post hoc test. The assumption for the normality and the homogeneity of variances necessary for the comparisons of means were analyzed and tested with Kolmogorov–Smirnov test and Levene's test, respectively (all with normality and homogeneity in this study). The statistical significance was defined when $p < 0.05$. All experiments and tests were conducted at least 6 times.

Results

1,25-$(OH)_2D_3$ inhibited the TNF-α-induced ASMCs proliferation

The results showed that the cell viability for TNF-α-treated ASMCs (0.92 ±0.01) was significantly higher compared to the blank control group (0.69 ±0.02) (Fig. 1, $p < 0.05$). However, the 1,25-$(OH)_2D_3$ treatment (1,25-$(OH)_2D_3$+TNF-α group) markedly inhibited the cell viability (0.83 ±0.01), as compared to the TNF-α group (0.92 ±0.01) (Fig. 1, $p < 0.05$).

1,25-$(OH)_2D_3$ downregulated MGP expression in TNF-α stimulated ASMCs

Western blot analysis was conducted to evaluate the expression of MGP (Fig. 2A). The results indicated that MGP expression in the TNF-α group (1.51 ±0.35) was significantly higher compared to the blank control group (0.17 ±0.04) (Fig. 2B, $p < 0.05$). However, the 1,25-$(OH)_2D_3$ treatment (1,25-$(OH)_2D_3$+TNF-α group) markedly downregulated the MGP expression (0.61 ±0.02) compared the TNF-α group (1.51 ±0.35) (Fig. 2B, $p < 0.05$).

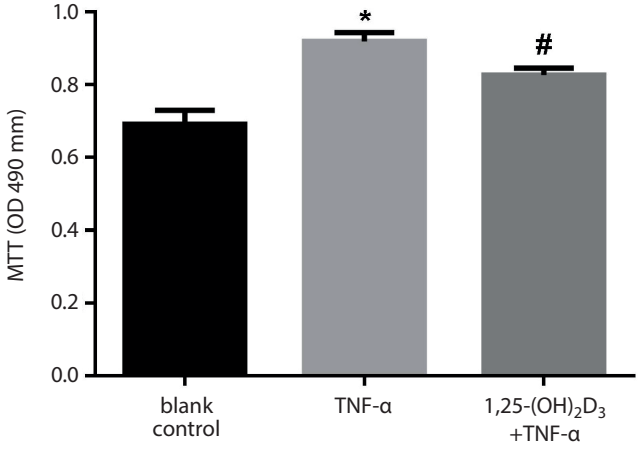

Fig. 1. Evaluation for the effects of 1,25-$(OH)_2D_3$ on the cell proliferation of airway smooth muscle cells (ASMCs) (n = 6 for all groups)

* $p < 0.05$ compared to blank control group; # $p < 0.05$ compared to tumor necrosis factor α (TNF-α) group.

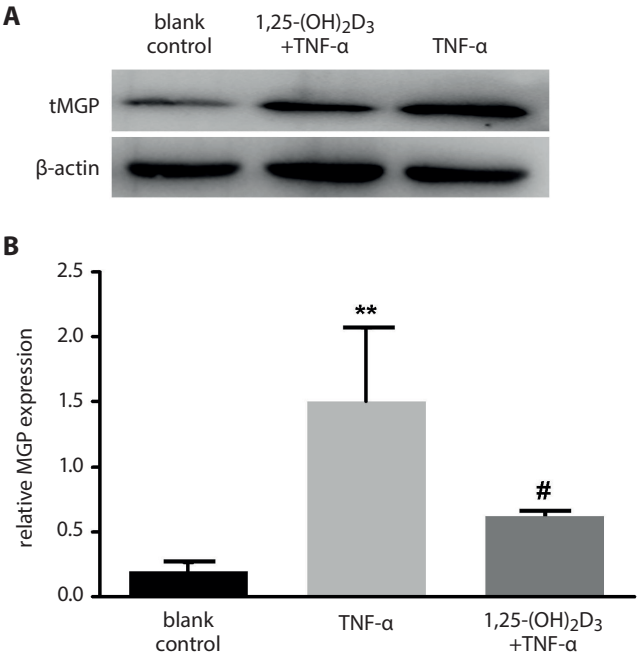

Fig. 2. Determination for the matrix Gla protein (MGP) expression in tumor necrosis factor α (TNF-α)-induced airway smooth muscle cells (ASMCs), using western blot analysis (n = 6 for all groups). A. Western blot images; B. Statistical analysis for the MGP expression

** $p < 0.05$ compared to blank control group; # $p < 0.05$ compared to TNF-α group; tMGP – total MGP.

1,25-$(OH)_2D_3$ reduced the TGF-β1 expression in TNF-α-stimulated ASMCs

The TGF-β1 expression was also determined using the western blot analysis (Fig. 3A). The findings showed that TGF-β1 expression was significantly higher in TNF-α group (1.6 ±0.18) compared to the blank control group (0.59 ±0.06) (Fig. 3B, $p < 0.05$). Meanwhile, the 1,25-$(OH)_2D_3$ treatment (1,25-$(OH)_2D_3$+TNF-α group) markedly reduced the TGF-β1 expression (0.69 ±0.17) compared to the TNF-α group (1.6 ±0.18) (Fig. 3B, $p < 0.05$).

Discussion

According to the report published by Fanta in 2009, approx. 300 million people worldwide suffer from asthma.[15] Although some advances in research on asthma have been made, the present therapeutic strategy is limited to controlling the symptom, without complete cure. The airway remodeling in the asthma patients is thoroughly studied, and the ASMCs hyperplasia and phenotype transformation are the basic characteristics of the airway remodeling.[16] The ASMCs are present as the contractile type in normal lung tissue, while presenting as the synthetic

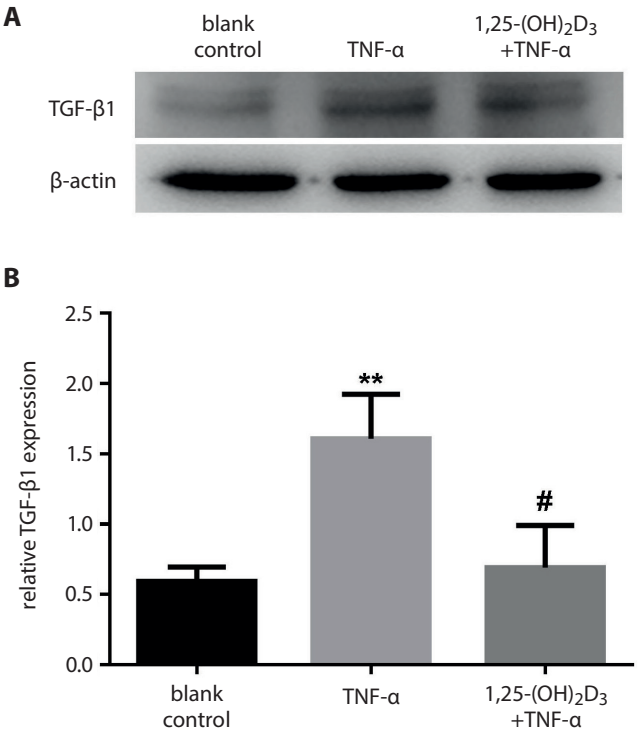

Fig. 3. Effects of 1,25-$(OH)_2D_3$ treatment on the transforming growth factor β1 (TGF-β1) expression determined with western blot analysis (n = 6 for all groups). A. Western blot images for TGF-β1. B. Statistical analysis for the TGF-β1 expression

* $p < 0.05$ compared to blank control group. # $p < 0.05$ compared to TNF-α group; TNF-α – tumor necrosis factor α

type or as contractile-synthetic transformation type in the asthma lung tissue.[17] The synthetic-type ASMCs can synthesize and release the immunoregulatory factors and chemical substances, and are characterized by higher proliferative activity.[18] Therefore, inhibiting the transformation of ASMCs from the contractile type into synthetic type is the key strategy for preventing and treating the asthma.

The 1,25-$(OH)_2D_3$ plays a critical role in counteracting the airway inflammation and regulating the immune response in vivo.[19,20] In recent years, 1,25-$(OH)_2D_3$ has been proven to play a critical role in modulating airway remodeling; however, only a few studies were conducted on the effects of 1,25-$(OH)_2D_3$ on the contractile-synthetic transformation of ASMCs.[1,21] Other studies also demonstrated that the administration of 1,25-$(OH)_2D_3$ could inhibit the airway hyperresponsiveness, lower the immunoglobulin E (IgE) levels and alleviate airway eosinophilia, all of which are mediated by cytokines and growth factors such as MGP and TGF-β1.[19,22]

Therefore, in this study, we evaluated the expression of MGP and TGF-β1 in TNF-α-stimulated ASMCs to verify the effects of 1,25-$(OH)_2D_3$ on the airway remodeling. The MGP is a vitamin K-dependent molecule, with higher expression in the lung tissue.[23] Our findings showed that under the TNF-α treatment, the ASMCs were transformed into the synthetic type, which is consistent with the study by Dogan et al.[24] However, the 1,25-$(OH)_2D_3$ treatment significantly reduced the TGF-β1 expression in TNF-α-stimulated ASMCs, which suggests that 1,25-$(OH)_2D_3$ could inhibit the transformation of ASMCs from contractile type into the synthetic type. The TGF-β1 is a cytokine characterized by powerful biological activity and powerful functions.[25] Studies by Yeganeh et al. and Chen et al. also reported that TGF-β1 is a critical risk factor for the abnormality of airway smooth muscle structures and dysfunction, and is overexpressed in the development and progression of asthma.[26,27] In the present study, we discovered that 1,25-$(OH)_2D_3$ treatment could significantly reduce the expression of TGF-β1 in the TNF-α-stimulated ASMCs. This result suggests that the 1,25-$(OH)_2D_3$ could suppress the release of TGF-β1 and inhibit the airway remodeling.

Limitations

Some limitations of the study should be mentioned. Firstly, the correlation between the two 1,25-$(OH)_2D_3$-associated cytokines, MGP and TGF-β1, has not been clearly elucidated. Secondly, the effects of 1,25-$(OH)_2D_3$ on the airway remodeling have not been verified and the associated mechanisms have not been fully clarified. In an upcoming study, the correlation between MGP and TGF-β1 should be determined using Pearson's coefficient analysis, and the effects of 1,25-$(OH)_2D_3$ on the airway remodeling should be investigated further.

Conclusions

The 1,25-$(OH)_2D_3$ could participate in and modulate the airway remodeling in the TNF-α-treated ASMCs. It not only inhibited the cytokines-induced phenotypic transformation of ASMCs, but also reduced the secretion of inflammatory factors, both of which are the potential strategy for alleviating the airway remodeling. This study provided the therapeutic insight and theoretical basis for the clinical research.

ORCID iDs

Yan-Min Xing https://orcid.org/0000-0001-6102-1559
Pei-Shan Li https://orcid.org/0000-0001-7125-1294
Ying Liu https://orcid.org/0000-0002-0872-2193

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Various aspects of transition of care for adolescents with urological conditions

Barbara Dobrowolska-Glazar[1,A,D,F], Rafał Chrzan[1,A,E,F], Maciej Bagłaj[2,E,F]

[1] Department of Pediatric Urology, Faculty of Medicine, Jagiellonian University Medical College, Cracow, Poland
[2] Division of Pediatric Propedeutics and Rare Disorders, Department of Pediatrics, Wroclaw Medical University, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Barbara Dobrowolska-Glazar
E-mail: barbara.dobrowolska-glazar@uj.edu.pl

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Abstract

Transition into adulthood is a common issue in many disciplines. However, urology faces additional difficulties due to different models of care and training as well as a wide diversity of pathologies. The goal of this paper is to discuss various aspects of the transition of urological care.

This review provides some examples of pathologies that might require special attention of specialists. Most patients with rare diseases must be closely followed up in the long term. However, high-volume conditions may also have a huge impact on the well-being and quality of life in adulthood. Children who are cured due to oncological conditions will probably need additional attention in adulthood. The urological care during childhood is provided by a pediatric urologist, a pediatric surgeon or a urologist, depending on the local regulations and the organization of care. All patients are subsequently referred to a general urologist. Nowadays, a multidisciplinary approach is recommended in many cases, with a pediatric urologist as one of the team members.

The patient, caregivers and healthcare professionals must be fully involved and focused on close cooperation to make the transition process smooth and successful.

Key words: transition, urology, congenital genitourinary conditions

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Introduction

Transition into adulthood is a common issue in many disciplines. Urology may face additional difficulties due to possessing different models of care and professional training as well as to a wide diversity of pathologies.

More and more children with severe congenital and acquired diseases of the urogenital system enter adulthood as medicine is developing. Congenital anomalies of the kidney and urinary tract (CAKUT) are the common cause of end-stage renal disease (ESRD) in the pediatric population. Maintaining good renal function is the main goal in patients with CAKUT. Proper care from the neonatal period has a huge impact on the survival rate and the quality of life in patients with serious congenital anomalies.[1,2] For the most severe cases, delaying the necessity of renal replacement therapy (RRT) is also of great importance. Nowadays, kidney transplantation can be applied in children who have had major urological interventions, and the survival rate at 10 years is similar as in the case of kidney transplantation performed at presence of the native and healthy bladder.[3] There is also no significant difference in mortality after transplantation in patients with spina bifida.[4] However, many patients with stable chronic kidney disease (CKD) during childhood progress to ESRD requiring RRT during adulthood. The median age at the start of RRT is significantly lower in the CAKUT cohort (31 years) than in the non-CAKUT cohort (61 years).[5]

Due to well-organized care, newborns can survive the most critical period and proper management can be provided through childhood. Puberty is a critical moment for the adolescents who need a long-term follow-up, as some are less cooperative. During this period of life, these adolescents should also be trained on how to take care of themselves to make the transition into adulthood as smooth as possible, minimizing the risk of failure, and to ensure a good quality of life. Therefore, improving management during transition to postpone the need for RRT should be the goal of the healthcare providers in the future.

Objectives

The aim of this paper was to discuss various aspects of the transition of urological care as well as provide examples of pathologies that might require special attention of healthcare professionals.

Methodology

A PubMed search was performed in October 2020. Articles published in English between 2000 and 2020, and related to the transition of patients with selected urological conditions (key words: 'neuropathic bladder'; 'posterior urethral valve'; 'exstrophy-epispadias complex'; 'disorders of sexual development'; 'anorectal malformations'; 'hypospadias'; 'urinary incontinence'; and 'pediatric oncology') were selected. Most of the articles were reviews and expert opinions. The authors assessed the content of the abstracts, and then the articles were subjected to further evaluation. Finally, 43 articles were approved by all authors to discuss the epidemiology, clinical course, prognostic factors, and long-term follow-up of the selected conditions. There are hardly any original studies focusing on this topic, which obviously might have influenced the selection process.

Transition into adulthood

Long-term follow-up: By whom?

Pediatric urology is a subspecialty of both urology and pediatric surgery, depending on the local regulations. This may impact the transition of care. Professionals with a pediatric surgical background are familiar with a wide range of different congenital anomalies involving other organs/systems, such as anorectal malformations (ARMs). Those with urology as a background are better prepared to deal with the common problems in adulthood, such as tumors. Szymanski et al. stated that urologists could provide the best care for patients who require a long-term follow-up.[6] According to Hsieh et al., the knowledge of congenital genitourinary conditions (CGCs) among general urologists is limited and they need additional training.[7] There is no literature on this topic referring to pediatric surgeons who deal with CGCs. They are, however, mostly involved in the transition of patients with congenital diseases of the gastrointestinal tract (e.g., Hirschsprung disease, ARMs).[8]

There is a recent tendency for the centralization of care in order to improve its quality. High-volume centers are thought to be the best solution to achieve good outcomes with acceptable complication rates. At the same time, proper medical training for healthcare professionals must be maintained. In some countries, children with rare urological diseases (e.g., exstrophy-epispadias complex (EEC), disorders of sexual development (DSD), tumors) are treated only in a limited number of dedicated units. An important implication of this model is a limited exposure of the trainees to the whole spectrum of pediatric urological cases outside those centers. The centralization of cases in one place means less experience in other locations. In terms of transition of care, this can have an adverse effect on a proper follow-up, as only few professionals can acquire adequate knowledge in this field.

Many patients with rare anomalies require a multidisciplinary approach during childhood. Depending on the condition, a pediatric urologist is part of the team, together with an endocrinologist, a nephrologist, a neurosurgeon,

a pediatrician, and a psychologist. Before entering adulthood, a clear summary of the medical history must be provided to the patient. Nevertheless, the question arises as to who should follow up the adult patient 20 years after feminizing surgery – a gynecologist or a urologist? Is a multidisciplinary approach required, and if so, who should be in charge – an endocrinologist or a gynecologist? What if the patient is not fully prepared mentally to take care of themselves?

Long-term follow-up: For whom?

There is no doubt that some individuals with congenital anomalies or acquired conditions will need a long-term follow-up. These individuals may present with rare and common diseases. Some may be at risk of impairment during their lifetime, but for some, the clinical course is not really known. Taking all the above into consideration, several different groups of patients can be defined.

Low-volume and rare anomalies with long-term consequences

Neuropathic bladder

Neurogenic lower urinary tract dysfunction (NLUTD) in patients with spinal dysraphism is the most frequently described model of transition in pediatric urology. Spina bifida is a common neurologic abnormality, with worldwide incidence estimated at 0.3–4.5 per 1000 births. The main goal of the urological management is to protect the upper urinary tract (UUT) and maintain good renal function by means of a proper control of the bladder function. Another important factor for obtaining optimal quality of life is the independence with respect to the bladder and bowel management and sexuality.[9] Some problems that urologists will have to tackle in an adult patient with NLUTD are the consequences of surgical interventions in childhood, such as bladder augmentation or urinary diversion. Due to the underlying pathology, other problems are common – urinary tract infections (UTIs), urinary incontinence (UI), urinary stone disease, and sexual and reproductive issues. These patients might also suffer from the side effects of the chronic use of medicines (e.g., antimuscarinics); however, the literature on this issue is scarce.[10] A regular control allows the identification of risk factors before irreversible changes in the lower urinary tract (LUT) and UUT occur. As Averbeck and Madersbacher recommend,[11] the European Association of Urology (EAU) Guidelines on Neuro-Urology should be followed in patients with congenital NLUTD (Table 1).[12] The adult patient may need help from other specialists, such as a gynecologist, a neurosurgeon, an orthopedist, a gastroenterologist, a psychologist, a physical therapist, or a urotherapist. The interdisciplinary collaboration is necessary not only on the medical but also on social level to enable the patient a full participation in social life.[7,13]

Table 1. Recommendations for the follow-up, according to the European Association of Urology (EAU) Guidelines on Neuro-Urology 2016[12]

Recommendations
Evaluation of the upper urinary tract at regular intervals in high-risk patients.
Physical examination and urine laboratory tests every year in high-risk patients.
Any significant clinical changes should initiate specialized investigation.
Urodynamic investigation as a baseline diagnostic intervention in high-risk patients at regular intervals.

Posterior urethral valve

Posterior urethral valve (PUV) is the most common congenital obstructive uropathy that can lead to ESRD. The incidence of PUV ranges from 1:5000 to 1:8000 births. The severe consequences of the outlet obstruction with regard to the developing bladder include poor compliance, detrusor overactivity and reduced functional volume. Renal hypo/dysplasia can already be diagnosed in utero. However, an accurate prognosis regarding the postnatal bladder and kidney function based on the findings from the prenatal screening is not possible.[14] To avoid or postpone renal impairment, all boys require a close follow-up. While PUV can be successfully managed in children, there are no guidelines on how to follow up adult patients. Holmdahl and Sillén stated that 32% of adult men aged 31–44, treated for PUV in childhood, were uremic, 21% had moderate renal failure and 47% had not been checked since adolescence; there were also signs of bladder dysfunction in 40% of patients.[15] That is why the kidney and bladder function follow-up must be an essential part of the transition. However, an interdisciplinary supervision of the adult "valve" patient is usually not necessary. Nevertheless, regular checks for serum creatinine, blood pressure and the bladder function will help to avoid irreversible changes. If needed, a nephrologist should be involved in the long term.

Exstrophy-epispadias complex

Exstrophy-epispadias complex is a spectrum of genitourinary malformations, ranging from mild epispadias, throughout the classical bladder exstrophy, to the exstrophy of the cloaca. The overall incidence of the EEC spectrum can be estimated at 1 in 10,000 births, with higher occurrence in males as compared to females.[16] Urinary continence and the voiding pattern are mostly evaluated as the end point of the treatment of bladder exstrophy. Woodhouse at el. showed that continence can be achieved in up to 80% of children in highly specialized centers, but only 40% of adults are dry.[17] Furthermore, about 84% of children are able to void, but this function is lost with time in 70% of patients.[17] These are not the only reasons why the EEC patients need attention in adulthood. Those who had surgical bladder augmentation are at risk of metabolic disorders, urolithiasis and

malignancies. The sexual function and fertility are additional issues in adults to be dealt with by urologists and gynecologists. Little is known about the sexual dysfunctions in these populations.[18] In females, fertility is usually maintained (delivery should be by cesarean section), but only 50% of male patients are able to become a father.[19] Due to the anatomical abnormalities of the pelvic ring, the EEC patients may also require the orthopedic care. Patients with the cloacal exstrophy should be treated as NLUTD patients in terms of the bladder management, and they may also need help because of fecal incontinence.[13] Due to the urinary diversion, oncological surveillance is necessary. During the follow-up, the probability of developing a malignant tumor after augmentation cystoplasty is in the range of 0–5.5%.[20] Neoplasia at the anastomosis of the ureters and the colon occurs in about 24% of patients at 20 years of follow-up.[21]

Disorders of sexual development

Patients with DSD are another group requiring a multidisciplinary approach, in many cases starting at birth. Pediatric and adult urologists should provide lifelong, continuous care for the DSD patient in the aspects of reconstruction, sexual function, fertility, and possible lower urinary tract dysfunction (LUTD). In adulthood, female patients are usually supervised by a gynecologist. The gynecological care should cover the newly diagnosed patients (phenotypically female), those who did not undergo reconstruction in childhood and may have complications after feminizing genitoplasty, as well as those who are potentially fertile (limited to congenital adrenal hyperplasia patients).[22] Some DSD patients are at risk of developing gonadal tumors, but the absolute risk of malignancy is unknown.[23] The cooperation of an endocrinologist, a urologist and a gynecologist is crucial in terms of treatment optimization. The psychosexual outcome is important, because the patient's well-being in the long term could be one of the factors determining the decisions regarding indications and timing for the surgical treatment.[24] Urologists and gynecologists are predisposed to offer help and therapy in this area.

Anorectal malformations

Anorectal malformations include a wide spectrum of diseases that affect the anus, the rectum and the urogenital system, i.a., CAKUT. The incidence of ARMs is approx. 1 per 5000 live births. One of the major problems experienced by these patients is fecal incontinence, which requires a follow-up and functional training. This issue should be covered by the surgical team. In some cases, urethral stricture may occur as a result of fistula closure. Furthermore, a number of these patients are at risk of NLUTD. The reason for this is not really known, but these patients require lifelong urological and nephrological supervision. Female patients need gynecological support due to additional abnormalities of the genital tract and possible trouble during pregnancy. For many reasons, they also require concern and help from a psychologist as well as psychosexual assistance.[25,26]

High-volume anomalies with a possible risk for the urogenital tract

Hypospadias

Hypospadias is one of the most common congenital genitourinary conditions (CGCs), affecting 1 per 250 males at birth. This disorder comprises a heterogeneous group of patients. In approx. 70% of them, the urethral meatus is located distally and the defect is not severe, but there are also proximal, more complex cases. The most common urinary complications in patients who have undergone hypospadias repair include meatal stenosis, fistula, urethral stenosis, ventral curvature, UTIs, or lichen sclerosis; these complications may occur many years after the initial surgery. A portion of these patients will present with lower urinary tract symptoms (LUTS), but some might be asymptomatic for a long time.[27,28] The patient requires physical examination and uroflowmetry with postvoid residual measurements. After proximal hypospadias repair, each patient should be examined after puberty, and again as a sexually active man. Unfortunately, there are no standardized questionnaires for the evaluation of the psychosexual function after hypospadias repair for adult patients with a mild hypospadias correction. [27]

High-volume conditions affecting the quality of life

Urinary incontinence

Urinary incontinence is one of the most bothersome signs of LUTD.[29] Approximately 5–10% of schoolchildren suffer from UI, and a low percentage of them do not outgrow it. The majority of patients can be cured by means of standard urotherapy, and the bowel management is often needed as well. Some patients require specific urotherapy (physical therapy, neuromodulation) and pharmacotherapy. Effective therapy depends on a good cooperation between a urotherapist and a urologist.[30,31] Although controversial, an endoscopic intervention is needed in some boys to eliminate intravesical obstruction. The long-term consequences of the invasive treatment are not known, and these patients might be at risk of urethral stenosis and require a proper follow-up.[32] There is a lack of literature on UI in adolescents and on the transition of these patients. Von Gontard et al. hold the view that incontinence in adolescents is a neglected research topic, and that an organized transition process is recommended to improve care in this respect.[33]

Oncology

Adult tumors in children

Renal cell carcinoma (RCC), MiT family translocation renal cell carcinoma (tRCC)[34] and urothelial bladder

cancer[35] are extremely rare in the pediatric population. There is hardly any literature on how to follow up those patients in childhood. Usually, the recommendations from the adult group are used, but the clinical course of those diseases in prepubertal children and adolescents can differ significantly from that observed in the adult population.

Typical pediatric tumors

In recent decades, there has been an increase in the overall survival rate for childhood malignancies. Two out of 3 childhood cancer survivors develop at least 1 late-onset therapy-related complication; in 25% of these patients, the complication is severe or even life-threatening.[36] Based on the Children's Oncology Group guidelines, Bhatia et al. recommended a "shared-care model" that involves both a primary care provider and an oncologist to facilitate the best long-term follow-up.[37] The coordinating physician should select the type and the frequency of visits in cooperation with the oncologist, along with psychological support. Urological tumors may require additional urological and nephrological control (Table 2).[37]

Table 2. Potential late effects of selected therapeutic interventions for childhood cancer by organ/system[37]

Organ system	Potential late effects
Renal	glomerular toxicity tubular dysfunction renal insufficiency hypertension
Bladder	hemorrhagic cystitis bladder fibrosis dysfunctional voiding neurogenic bladder bladder malignancy
Sexual/reproductive (males)	hemorrhagic cystitis bladder fibrosis dysfunctional voiding neurogenic bladder bladder malignancy

Oncofertility

Oncofertility is a field regarding the reproductive future of cancer survivors. The American Society of Clinical Oncology (ASCO) first published guidelines on the preservation of fertility for children and adolescents, and recommended semen cryopreservation for post-pubertal boys before the initiation of therapy. The cryopreservation of the testicular tissue from prepubertal patients and hormonal suppression to preserve the gonadal tissue are still under investigation.[38] Closely cooperating units responsible for particular stages of the process must be created in order to follow the recommendations for the newly diagnosed cancer in a male child or adolescent.

Limitations

The main limitation of the present review is a small number of original and prospective studies related to the topic. Most of the included articles are reviews and expert opinions. Transition into adulthood is becoming an important part of pediatric specialties, which arises from the need to take care of patients whose prolonged life expectancy is a consequence of the development of medicine. For the time being, there are hardly any objective data on this subject in the literature.

Furthermore, only a few urological conditions have been discussed in the review for obvious reasons. Nevertheless, patients with other abnormalities, e.g., undescended testis, primary megaureters and urinary stone disease, might also need a long-term follow-up.

Vesicoureteral reflux (VUR) is a perfect example of an urological problem with a wide spectrum of therapeutic options, from active surveillance, through continuous antibiotic prophylaxis (CAP) and endoscopic procedures, to the most invasive one – ureteral reimplantation. Taking into account the risk factors, the management of VUR should be individualized. Nevertheless, the diagnosis and treatment of VUR are the source of a never-ending debate among pediatric urologists and nephrologists, with the goal remaining the same, i.e., the prevention of febrile UTIs, and thus scarring of the kidneys.[39,40] Although this condition can have severe consequences in the long term, there are no publications on the transition process for those patients.[41]

Conclusions

Children with chronic diseases require a long-term follow-up. Transition of care into adulthood is a challenging issue in all disciplines. The patient, caregivers and healthcare professionals must be all involved in this process. The pitfalls regarding urogenital conditions in terms of transition are as follows:

– there is a wide spectrum of congenital as well as acquired pathologies;

– children are treated in different locations (pediatric urology units, pediatric surgery units, urology units) and all adult patients are referred to urology departments;

– there are different policies/protocols, especially in the case of rare diseases;

– there is a limited number of common teaching activities for pediatric and adult specialists.

Perspectives

The knowledge of transition or rather adolescent medicine should be an additional competence. Recently, it has been gaining more attention, as there is an urgent need to take care of patients who enter adulthood having been

cured from severe diseases. A significant improvement of the healthcare may increase the length and quality of life of such patients. Even in the best-organized centers, a proper transition is successful for only 40% of patients.[42] Transition carries many pitfalls, as mentioned above. Special attention should be paid to cooperation and efficient coordination between healthcare professionals. Multidisciplinary meetings and congresses are needed, but also evaluation and feedback from patients are necessary. In a multidisciplinary approach, medical, social and environmental aspects are taken into consideration. Following the World Health Organization's International Classification of Function, Disability and Health (ICF) model, high-quality care encompasses not only health-related outcomes, but also activities, social participation and environmental factors to address an individual's need to fully function in the society.[43]

Adequate theoretical and practical training of adult urologists interested in taking care of patients with "pediatric" genitourinary conditions must be ensured. The pediatric urologist, in turn, should compile the patient's medical history recapitulation, including in detail all surgical procedures, ongoing health problems, suggestions on the necessary specialist visits, and diagnostic tests. It must be noted that a transition requires a careful approach during a challenging moment of a human life – puberty. All parties must be fully involved to make the whole process smooth and successful.

ORCID iDs

Barbara Dobrowolska-Glazar https://orcid.org/0000-0002-0192-9263
Rafał Chrzan https://orcid.org/0000-0001-8620-1898
Maciej Bagłaj https://orcid.org/0000-0002-6291-1577

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Anticoagulant-related nephropathy: Focus on novel agents. A review

Izabela Zakrocka[A–F], Wojciech Załuska[E,F]

Department of Nephrology, Medical University of Lublin, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Izabela Zakrocka
E-mail: izabela.zakrocka@umlub.pl

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Abstract

Anticoagulant-related nephropathy (ARN) is a novel and not well-studied cause of acute kidney injury (AKI). The prevalence of ARN varies significantly between studies and is estimated at 20% in patients treated with warfarin. Patients with ARN have a significantly higher mortality risk and an increased risk of chronic kidney disease (CKD). Unexplained AKI with hematuria are clinical manifestations of ARN. In most cases, ARN is diagnosed within the first 2 months of anticoagulant therapy, but later ARN occurrence is possible. Among the studied anticoagulants, most data concern warfarin toxicity, whereas cases of ARN caused by direct oral anticoagulants (DOACs) have also been presented. Tubular obstruction by red blood cell casts or hemoglobin and iron tubular toxicity are the postulated mechanisms of ARN. On the molecular level, the inhibition of thrombin and protease-activated receptor-1 (PAR-1), leading to endothelial susceptibility to damage or abnormal protein C endothelial signaling, is suggested to contribute to ARN. Older age, impaired kidney function, hypertension, and diabetes mellitus are the main risk factors for ARN, but their significance may differ between anticoagulants. From therapeutic options, the withdrawal of the anticoagulant and the administration of its antidote, as well as corticosteroids or N-acetylcysteine, are proposed. Since the number of patients with kidney diseases on anticoagulants increases, and DOACs are starting to be more useful in this group of patients, we aim to summarize the pathogenesis, clinical picture and possible ways of treatment of DOAC-induced ARN.

Key words: acute kidney injury, warfarin, anticoagulants, dabigatran, rivaroxaban

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Introduction

Anticoagulants are one of the most commonly prescribed drugs worldwide and their administration may increase in the following years due to their suboptimal use.[1] Direct oral anticoagulants (DOACs), previously called novel oral anticoagulants (NOACs), have been repeatedly shown to be not inferior in comparison with vitamin K antagonists (VKAs), in the prevention and treatment of venous thromboembolic disease, as well as in the prevention of ischemic stroke in patients with non-valvular atrial fibrillation, without increasing the risk of life-threatening bleeding.[2] People with chronic kidney disease (CKD) are a special group of patients, with many comorbidities on the one hand, and with different pharmacokinetics of most drugs on the other.[3] In an animal model of CKD, a lower transcription and a lower concentration of cytochrome P-450 enzymes were demonstrated, which can be responsible for a lower drug metabolism and an increased risk of overdose.[4] The inhibitory effect of uremic toxins on cytochrome P-450 activity was also postulated.[5] In a recently published meta-analysis by Randhawa et al. regarding patients with end-stage renal disease (ESRD), warfarin was reported not to increase the risk of ischemic stroke, major bleeding and overall mortality, contrary to a higher risk of hemorrhagic stroke, showing an increased risk of complications after the use of VKAs in the CKD population.[6] Unfortunately, the lack of results of head-to-head trials comparing anticoagulants efficacy and safety, as well as the lack of data from randomized controlled trials (RCTs) on patients with estimated glomerular filtration rate (eGFR) lower than 30 mL/min taking DOACs, limit our knowledge about the appropriate anticoagulation in CKD patients. To the well-known limitations of anticoagulants, like drug and food interactions, changes in their pharmacokinetic profile according to the kidney function and the risk of vascular calcification, should be added another, recently described complication, called anticoagulant related nephropathy (ARN), primarily observed after the administration of VKAs. Since ARN can affect patients with both normal and impaired kidney function, it can possibly be caused by every anticoagulant and may lead to ESRD, this novel nephropathy will gain increasing significance.

Objectives

In this narrative review, we aimed to summarize our knowledge about DOAC-related ARN – its prevalence, risk factors, clinical manifestation, the methods of treatment, and its impact on renal outcome and survival.

Definition

Primarily, ARN was defined as an unexplained kidney injury observed within 1 week of international normalized ratio (INR) greater than 3.0 in patients on anticoagulants.[7] After longer observation, the definition of ARN was modified as acute kidney injury (AKI) of unknown origin (a rise in serum creatinine level by more than 0.3 mg/dL) in a patient receiving anticoagulants, even without any signs of coagulopathy and active bleeding.[8] What is interesting, except from oral anticoagulants, some researchers add antiplatelet drugs (dipyridamole or drugs used in dual antiplatelet therapy) and other anticoagulants, like low-molecular-weight heparin or unfractionated heparin, to the list of drugs potentially involved in the pathogenesis of ARN.[9–11]

Anticoagulant-related nephropathy was first described by Brodsky et al. in 9 patients with AKI, hematuria and warfarin overdose, so the primary name of this phenomenon was warfarin-related nephropathy.[12] Each analyzed patient had an elevated INR on admission, with a mean value of 4.4 ±0.7, and a high serum creatinine level (4.3 ±0.8 mg/dL). Kidney biopsy results revealed acute tubular injury and glomerular hemorrhage, with red blood cells in Bowman's space and plenty of red blood cell casts occluding the tubules.[12] Unfortunately, only 3 out of 9 patients recovered from AKI, giving the first evidence of irreversibility of ARN in some patients.[12] Similarly, Mikič et al. showed that dabigatran-related ARN could be characterized by occlusive intratubular red blood cell casts, diffuse acute tubular injury and diffuse interstitial edema.[10] Interestingly, the red blood casts obstructing the renal tubules did not contain Tamm–Horsfall protein and were present more often in the distal tubules, and therefore in the renal medulla.[10] It should be noted that kidney biopsy results in the suspicion of ARN are scarce, since coagulation abnormalities significantly limit the number of performed biopsies. However, kidney biopsy may reveal crucial information about the severity of kidney damage, as well as other pathologies related to abnormal kidney function, like immunoglobulin A (IgA) nephropathy or focal segmental glomerulosclerosis. Based on the provided results, a decision can also be made to withdraw particular anticoagulant or switch to another drug, potentially less related with ARN occurrence. The role of kidney biopsy in the diagnosis and prognosis of ARN is still underestimated and needs to be evaluated in future studies.

Epidemiology

Although the exact frequency of ARN is not known due to the novelty of the disease and the lack of kidney biopsy results, Brodsky et al. in a retrospective analysis of 103 patients estimated the prevalence of ARN after the administration of warfarin at 37%.[13] In a further retrospective study in 15,258 patients taking warfarin, it was reported that ARN occurred in 20.5% of the entire cohort, 33% of the CKD cohort and 16.5% of the non-CKD cohort.[14] Data about DOAC-related ARN prevalence are often controversial, indicating both nephroprotective and toxic effects of DOACs. However, in a large study by Marcelino et al. analyzing

data from 134 national registries, dabigatran was associated with 4.6% of renal side effects, whereas other DOACs – rivaroxaban, apixaban and edoxaban – were shown to be related with 3.5%, 2.0% and 1.7% of renal side effects, respectively.[15] What is interesting, the Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation RCT (ARISTOTLE; https://clinicaltrials.gov/, NCT00412984) showed much higher incidence of kidney damage after apixaban, reaching 13.6% of the analyzed population, despite lower incidence of major bleeding and lower mortality.[16] Due to the lack of results from other clinical trials (Renal Hemodialysis Patients Allocated Apixaban versus Warfarin in Atrial Fibrillation trial (RENAL-AF; https://clinicaltrials.gov/, NCT02942407) was prematurely terminated because of no funding and inconclusive results)[6] and possibly much higher incidence of ARN than expected, new ongoing trials regarding the safety of dabigatran (Dabigatran vs Warfarin in AF Patients with T2DM and CKD (RE-ELECT; https://clinicaltrials.gov/, NCT03789695)) and apixaban (Strategies for the Management of Atrial Fibrillation in Patients Receiving Dialysis (SAFE-D; https://clinicaltrials.gov/, NCT03987711), Compare Apixaban and Vitamin-K Antagonists in Patients with Atrial Fibrillation (AF) and End-Stage Kidney Disease (ESKD) (AXADIA; https://clinicaltrials.gov/, NCT02933697)) in CKD patients hopefully will provide reliable data about the incidence of ARN after the administration of DOACs.

Pathogenesis

The mechanism of ARN is not well understood (Fig. 1), since renal dysfunction cannot be explicitly treated as a result of the blockade of the renal tubules by red blood cells.[17] Brodsky raised concerns about predicting kidney damage in ARN patients, in whom the risk of AKI cannot be directly related to INR and warfarin overdose is not sufficient to cause ARN.[9] Ware et al. reported in 5/6 nephrectomy rats receiving warfarin increased oxidative stress in the kidney as compared to control, with the lack of iron in tubular epithelial cells and no beneficial effect of an iron chelator, deferoxamine, on the kidney function.[18] Free hemoglobin was suggested to affect tubular epithelial cells through the production of reactive oxygen species (ROS) and lipid peroxidation,[19] whereas after intracellular incorporation – to activate caspases and apoptosis.[20] Later, it was postulated that heme released from hemoglobin caused further cellular damage by activating pro-inflammatory pathways.[21] The activation of endothelial cells by heme to express adhesion molecules and complement the receptors, promoting the activation of the alternative complement pathway, was also suggested.[22] Mitochondrial dysfunction after the intracellular incorporation of heme was postulated as well.[21] Of note, patients with CKD are known to have lower activity of antioxidant enzymes, like glutathione peroxidase and catalase, which makes them especially vulnerable to warfarin toxicity.[8]

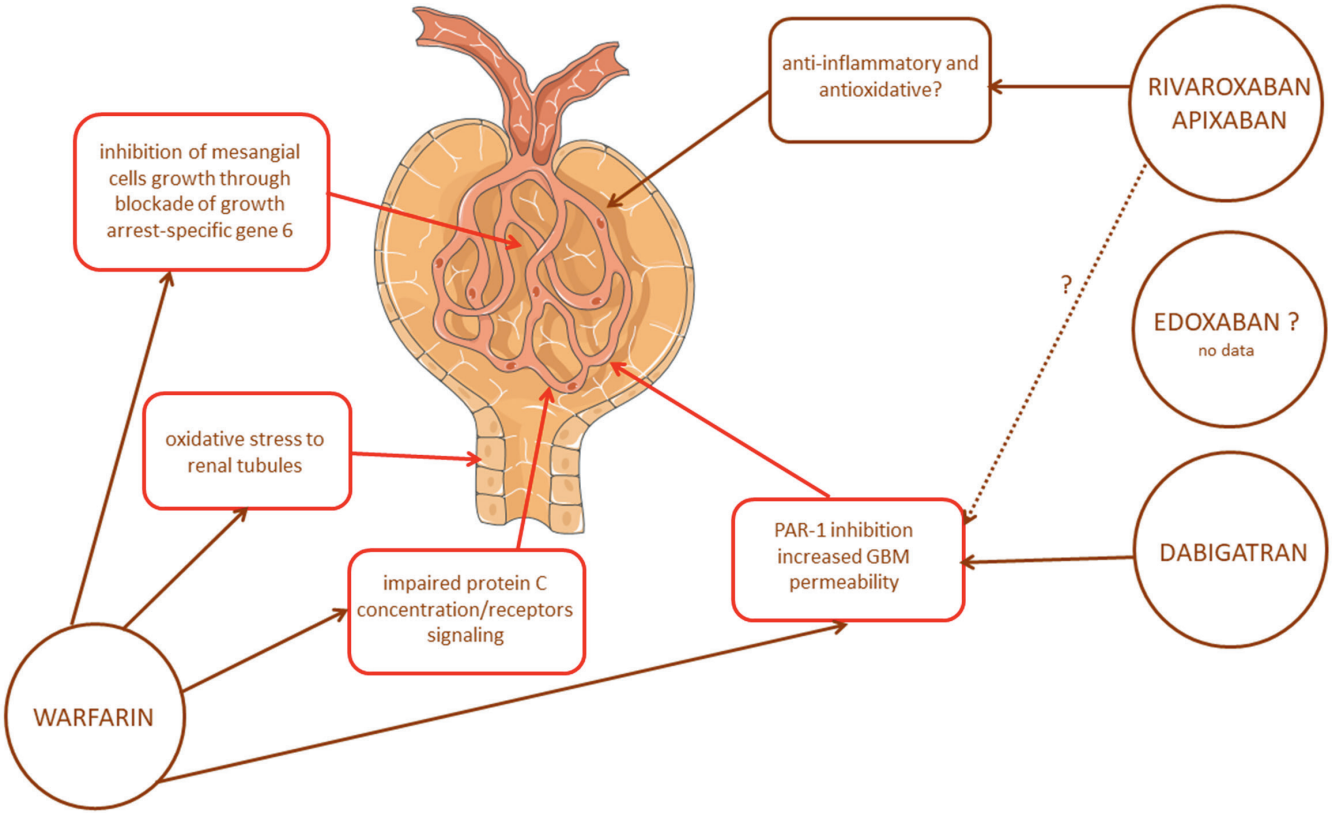

Fig. 1. Mechanisms of anticoagulant-related nephropathy, adapted from Reference 17. Red boxes indicate toxic effect, brown box indicates potentially protective effect

PAR-1 – protease-activated receptor-1; GBM – glomerular basement membrane.

Other potential mechanisms are suspected in the pathogenesis of ARN, like atheroembolism, allergic interstitial nephritis, the apoptosis of glomerular endothelial cells, and the direct toxic effect of warfarin on the glomeruli.[8,23] It was suggested that warfarin might affect glomerular hemodynamics and the proliferation of mesangial cells through interfering with the process of activation of growth arrest-specific gene 6, responsible for the production of vitamin K-dependent autocrine growth factor for mesangial cells.[24] Interestingly, the results of animal studies shed light on the pathogenesis of ARN, postulating that ARN is due to a diminished thrombin activity. Protease-activated receptor-1 (PAR-1) is known to take part in controlling endothelial cell functions, vascular permeability and leukocyte migration and adhesion.[25,26] The activation of PAR-1 has been shown to control endothelial monolayer integrity.[27] In an animal model of CKD, Brodsky observed that the selective inhibition of PAR-1 by SCH79797 resulted in the elevation of serum creatinine, hematuria and the formation of red blood cell casts, effects that were more pronounced in 5/6 nephrectomy rats.[9] This observation confirmed the theory of kidney damage caused by the direct inhibition of thrombin. A reduction in protein C concentration and impaired endothelial protein C receptor signaling are other possible mechanisms related to the pathogenesis of ARN.[28,29]

Since its introduction onto the market in 2010, dabigatran has been the most extensively studied DOAC in relation to ARN.[30] However, the available data remain inconclusive and do not clearly state whether dabigatran leads to kidney function impairment. As dabigatran in its unchanged form in over 80% is excreted through the kidneys, patients with impaired kidney function are already in danger of hemorrhagic complications and further kidney injury.[30] Similar to animal studies with VKAs, Medipally et al. observed acute tubular epithelial cell injury, red blood cell casts in the tubules and an increase in blood pressure in 5/6 nephrectomy rats treated with dabigatran.[31] Additionally, the modification of the eGFR by angiotensin-converting enzyme inhibitor, enalapril, seemed not to have any effect on dabigatran-induced ARN in that study.[31] Importantly, Ryan et al. observed ARN not only in 5/6 nephrectomy rats, but also in control animals receiving dabigatran in a dose-dependent manner,[32] differently than in animal studies with VKAs. Despite histopathological changes comparable to those observed in 5/6 nephrectomy rats after the administration of VKAs, the effect of a PAR-1 antagonist, SCH79797, was lesser than that of dabigatran, indicating that the inhibition of PAR-1 only partially explains the mechanism of ARN observed after this DOAC (Fig. 1).

Adding to previous reports, more studies are needed to analyze the benefits and risks related with the inhibition of PAR-1 by dabigatran. In an anti-glomerular basement membrane (GBM) glomerulonephritis animal model, PAR-$1^{-/-}$ mice presented less kidney damage and fewer crescents, fibrin deposits and macrophage infiltrates.[33] In a recently published study, dabigatran, through the inhibition of PAR-1, was shown to ameliorate tubulointerstitial fibrosis in unilateral ureteral obstruction-induced renal injury.[34] The antiplatelet effect of dabigatran has also been postulated based on the effect on PAR-1 receptors,[35] which in turn may increase the risk of bleeding and ARN. Interestingly, in an RCT performed by Correa et al., a PAR-1 inhibitor, vorapaxar, was shown to be safe for CKD patients.[36]

What is worth mentioning, the anti-inflammatory effect of rivaroxaban postulated by Terry et al.[37] can be partly responsible for a lower incidence of ARN after this DOAC. It was demonstrated that rivaroxaban reduced cell proliferation, plasma monocyte chemotactic protein-1 and matrix metalloproteinase-9 (MMP-9) levels, which led to the improvement in the patency of central venous catheters in mice.[37] Similarly, in preliminary studies, it was recognized that another factor X inhibitor, apixaban, reduced oxidative stress in mesangial cells through the reduction of expression and transcription of monocyte chemotactic protein-1 and intercellular adhesion molecule-1,[38] which in turn might be related to a lower kidney injury level. Further studies in humans are needed to confirm this observation.

Additionally, Ware et al. reported an increase in blood pressure in control and 5/6 nephrectomy rats after the administration of warfarin and dabigatran.[39] Both anticoagulants raised systolic blood pressure in the animals in a dose-dependent manner. Vitamin K prevented a blood pressure increase in warfarin-treated animals, whereas N-acetylcysteine only delayed it. A PAR-1 inhibitor, SHC79797, had similar effects to those of anticoagulants. What should be emphasized, the rise in blood pressure was independent of the kidney function, suggesting a direct drug effect.[39]

Risk factors

Based on clinical observations, it was shown that ARN occurred more often in patients with preexisting GBM abnormalities, like thin GBM disease[40] or thick GBM in a patient with inactive systemic lupus erythematosus.[41] In a cohort of Slovenian patients, Mikič et al. showed that most patients with ARN had underlying IgA nephropathy; however, the degree of histopathological disarrangement was not proportional to the level of kidney damage.[10] Other diseases, like diabetic nephropathy, focal segmental glomerulosclerosis, postinfectious nephritis, chronic interstitial nephritis, nephrosclerosis, and vasculitis were also related to a higher risk of ARN.[10] Brodsky et al., after analyzing the data of 4006 patients, reported that the strongest ARN risk factors were CKD, diabetes mellitus, heart failure, and hypertension.[42] The presence of an arteriovenous fistula in kidney allograft recipients was also suggested to increase the susceptibility to ARN, due to local hemodynamic changes.[43] Among the available medications, acetylsalicylic acid and drugs resulting in higher intraglomerular pressure, like directly acting

smooth muscle relaxants and dihydropyridine calcium channel blockers, were recognized to increase the risk of ARN, indicating that glomerular hemorrhage and tubular obstruction are the 2 crucial mechanisms involved in kidney damage.[14] Mitsuboshi et al. suggested that risk factors for ARN may differ between anticoagulants, with body weight higher than 80 kg and the use of dabigatran as the strongest predictors of kidney damage after DOACs, but they should be clarified in future studies.[44]

Clinical course

In most cases, ARN is presented as AKI in patients taking anticoagulants, after excluding other possible causes of kidney function impairment. It has been recognized that the highest risk of ARN is within the first 2 months after starting anticoagulant therapy[45]; however, it may vary between 1 week to 1 year after introducing DOAC (Table 1). In some cases of ARN, kidney replacement therapy may

Table 1. Reported anticoagulant-related nephropathy (ARN) cases related to direct oral anticoagulants (DOACs) exposure

Drug name	Patient's characteristics	Kidney replacement therapy due to ARN	Kidney function improvement after ARN	Time to ARN onset	Reference
Dabigatran	69-year-old male with atrial fibrillation, arterial hypertension, diabetes mellitus type 2, and liver cirrhosis	no	yes	2 months	10
Dabigatran	82-year-old female with atrial fibrillation, arterial hypertension and diabetes mellitus type 2	yes	yes	18 days	10
Dabigatran	81-year-old male with chronic heart failure and chronic Budd–Chiari syndrome, after ischemic stroke	no	no	3 weeks	10
Dabigatran	78-year-old female with atrial fibrillation and hypertension	no	yes	1 year	46
Dabigatran	67-year-old male with atrial fibrillation and coronary ischemic heart disease	no	yes	1 week	47
Dabigatran	69-year-old female with atrial fibrillation and hypertension	yes	yes	2 weeks	48
Dabigatran	81-year-old female with atrial fibrillation, diabetes mellitus, hypertension, asymptomatic chronic lymphocytic leukemia, and hypothyroidism	yes	no	2 years	49
Dabigatran	61-year-old male with atrial fibrillation and liver cirrhosis	yes	yes	1 year	50
Dabigatran	67-year-old female with deep vein thrombosis, hypertension and diabetes mellitus	no	yes	10 days	51
Dabigatran	82-year-old man with atrial fibrillation, hypertension, anemia, prostate cancer, and CKD	yes	yes	not known	52
Dabigatran	78-year-old man with atrial fibrillation, mitral valve prolapse and fusion of lower lumbar vertebral bodies	no	yes	not known	53
Apixaban/ rivaroxaban	82-year-old female with atrial fibrillation, arterial hypertension and ischemic heart disease	no	yes	1 week	10
Apixaban	82-year-old female with hypertension, chronic obstructive pulmonary disease, coronary artery disease, heart failure, and CKD	yes	no	10 days	54
Apixaban/dabigatran/ rivaroxaban	67-year-old male with atrial fibrillation and IgA vasculitis with skin involvement	no	yes	2.5 months	10
Rivaroxaban	87-year-old male with atrial fibrillation, hypertension, dyslipidemia, heart failure, carotid artery stenosis, and arteriopathy of the lower limbs, after ischemic stroke	no	yes	2 days	55
Rivaroxaban	82-year-old male with atrial fibrillation, hypertension, pacemaker due to third-degree atrioventricular block, and CKD	no	yes	3 weeks	56
Rivaroxaban	82-year-old female with CKD	yes	no	2 months	57
Rivaroxaban	45-year-old male with asthma	no	no	7 days	58
Rivaroxaban	75-year-old male with atrial fibrillation, hypertension, diabetes mellitus, and IgA nephropathy, after stroke	yes	no	3 years	59

CKD – chronic kidney disease; IgA – immunoglobulin A.

be needed due to severe kidney damage, whereas restoring the kidney function is not always observed.

Besides kidney damage, a hypertensive effect of anticoagulants should be mentioned. Human studies are often inconclusive, linking an increase in blood pressure with vascular calcification after the administration of warfarin,[60] but this direct hypertensive effect should be taken into account while administering anticoagulants due to the possible increase of the cardiovascular risk.[61]

Therapeutic approach

The management of ARN depends on the anticoagulant responsible for kidney damage. The treatment of VKA-related ARN includes careful drug dosing, the regular monitoring of coagulation parameters, avoiding drug interactions, and the discontinuation of the use of VKAs in severe cases. The beneficial effect of N-acetylcysteine, prednisolone[62] and vitamin K[63] on kidney function improvement was observed, but it needs to be confirmed in future studies. Lowering the anticoagulation level or the temporal discontinuation of DOACs seem to be reasonable in patients with mild clinical manifestations of ARN, but may be insufficient in severe cases. It was shown that both hemodialysis procedures and the administration of methylprednisolone can be useful in the treatment of dabigatran-induced ARN.[10] Interestingly, Alsamarrai et al. reported a case of ARN treatment with idarucizumab, a humanized monoclonal antibody, reversing the anticoagulant effect of dabigatran.[53] Before the introduction of idarucizumab in 2015, hemodialysis was the only available specific therapy in dabigatran overload due to a low molecular weight of the drug and weak protein binding.[64] In a recently published study, Galassi et al. proposed an algorithm of dabigatran overdose management in patients with eGFR lower than 30 mL/min.[52] According to this study, idarucizumab should be considered in the case of major bleeding, surgical urgencies or in patients with a high risk of complications during hemodialysis catheter implantation, whereas hemodialysis can be performed in the presence of severe AKI and related complications.[52] However, patients need to be carefully monitored due to the possible rebound increase in dabigatran level in AKI.[65] Data about the specific treatment of ARN caused by rivaroxaban, apixaban and edoxaban are not available.

Effect on renal outcome and survival

Contrary to studies by Brodsky et al., confirming the impact of VKAs on irreversible kidney damage,[12–14] the data concerning the effect of DOACs on patients' outcome is scarce, since people receiving DOACs require less monitoring, fewer kidney biopsies are performed in patients with abnormal coagulation, and impaired kidney function limits the use of this group of anticoagulants. Additionally, the results of the available studies are often ambiguous, limiting our knowledge about DOAC-related ARN.

In a retrospective study, patients receiving dabigatran were shown to have ARN less frequently when compared to patients on VKAs.[66] A similar effect was observed in patients with normal kidney function (hazard ratio (HR) 0.62; 95% confidence interval (95% CI): [0.49; 0.77]; $p < 0.001$), as well as in patients with CKD (HR 0.56; 95% CI: [0.46; 0.69]; $p < 0.001$), independently of the dose of dabigatran.[66] In a nationwide retrospective cohort study by Chan et al., dabigatran again was associated with a lower risk of ARN in comparison with warfarin (HR 0.68; 95% CI: [0.64; 0.74] in the normal kidney function group, and HR 0.54, 95% CI: [0.49; 0.59] in the CKD group).[66,67] Unfortunately, since dabigatran is a thrombin inhibitor, it also blocks PAR-1[26] and may cause ARN in selected patients according to the available case reports (Table 1). Mikič et al. presented a case of an 82-year-old woman with a history of arterial hypertension, diabetes mellitus type 2, hypothyroidism, cognitive decline, and CKD, who developed AKI after the introduction of dabigatran.[10] Due to severe kidney function impairment, hemodialysis procedures were started, and ARN together with IgA nephropathy were confirmed through kidney biopsy. Interestingly, the authors reported that the kidney function improved and hemodialysis treatment was stopped after the administration of methylprednisolone.[10]

Based on the data obtained by Mikič et al., it was surprisingly suggested that the renal outcome was slightly better in patients treated with VKAs than in those on DOACs (dabigatran or rivaroxaban).[10] Similar observations were made by Marcelino et al., who analyzed data from the World Health Organization (WHO) pharmacovigilance program in the VigiAccess™ database.[15] The authors showed that the annual risk of renal side effects was about 2 times higher for DOAC-treated patients as compared to patients receiving VKAs, and AKI was the most commonly reported clinical finding.[15] In the available studies, most patients with ARN did not have a CKD history, had variable renal outcomes, and quite often required hemodialysis procedures; however, in most cases, kidney function improvement was observed (Table 1).

Rivaroxaban, a factor X inhibitor, is a relatively novel anticoagulant, so data about the effect of rivaroxaban on the kidney function are very limited and inconclusive. In a study by Chan et al., rivaroxaban compared to warfarin was reported to decrease the incidence of AKI in patients with normal kidney function (HR 0.73; 95% CI: [0.68; 0.79]), as well as in CKD patients (HR 0.53; 95% CI: [0.49; 0.58]).[67] Adding to that, Zhang et al., in a pooled analysis of RCTs and observational studies, showed that rivaroxaban was associated with a low risk of kidney injury (HR 0.66; 95% CI: [0.55; 0.77]).[68] The same conclusions were presented by Mitsuboshi et al. after the analysis of the Japanese adverse drug event report database of kidney injury cases.[44]

On the other hand, Marcelino et al. showed a 3.5% annual risk of renal side effects in patients receiving rivaroxaban.[15] Since ARN after rivaroxaban was presented in few case reports with variable kidney outcomes (Table 1), more clinical studies should be performed to evaluate the exact role of rivaroxaban in the pathogenesis of ARN.

The same conflicting results about apixaban in relation to ARN came from other available studies. In a retrospective cohort study, apixaban was shown to decrease the risk of AKI when compared to warfarin in patients with preserved (HR 0.65; 95% CI: [0.60; 0.72]) and impaired kidney function (HR 0.50; 95% CI: [0.45; 0.56]).[67]

However, data from the ARISTOTLE RCT showed the worsening of GFR by more than 20% in 13.6% of patients receiving apixaban, which was associated with older age and cardiovascular comorbidities.[16] As more ARN cases after the administration of apixaban were reported (Table 1), a possible impact of apixaban on the kidney function should be taken into account.

Edoxaban as the newest DOAC on the market was not well studied with regard to ARN. In recently published studies, Mitsuboshi et al.,[44] as well as Zhang et al.[68] did not show any harmful effect of edoxaban on the renal function, but more data are needed to draw any conclusions.

Conclusions

Anticoagulant-related nephropathy is a novel and dangerous complication in the prevention and treatment of thromboembolic episodes. Due to a large number of patients receiving anticoagulants and the lack of appropriate monitoring and histopathological testing, the prevalence of ARN is still underestimated. Scarce clinical trials on patients with impaired kidney function taking anticoagulants significantly limits our knowledge about ARN. Special awareness is needed in reference to patients taking anticoagulants, with kidney injury of unknown origin. Based on the available data, VKAs are responsible for even 37% of ARN cases, whereas DOACs for about 5–14% of them. The inhibition of thrombin and PAR-1 are the main mechanisms related to ARN. The anti-inflammatory and antioxidative effects of novel DOACs, rivaroxaban and apixaban, are interesting findings, explaining to some extent a lower incidence of ARN after these DOACs. Despite the fact that CKD is one of the strongest risk factors for ARN, anticoagulants may cause ARN even in previously healthy patients. Since in patients with ARN the kidney function may not be restored, this specific kind of kidney injury should be taken into consideration as a cause of CKD in a large group of patients.

ORCID iDs

Izabela Zakrocka https://orcid.org/0000-0001-5251-7763
Wojciech Załuska https://orcid.org/0000-0001-6043-3236

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Updates for the diagnosis and management of cardiac amyloidosis

Piotr Gościniak[1,2,A–F], Tomasz Baron[3,4,A–F], Sławomir Milczarek[5,E,F], Magdalena Kostkiewicz[6,7,D–F], Bogusław Machaliński[5,A,F]

[1] Laboratory for Non-Invasive Cardiac Imaging, Tomasz Sokolowski Public Hospital, Pomeranian Medical University in Szczecin, Poland
[2] Department of Endocrinology, Metabolic and Internal Diseases, Pomeranian Medical University in Szczecin, Poland
[3] Uppsala Clinical Research Center, Department of Medical Sciences, Cardiology, Uppsala University, Sweden
[4] Centre for Medical Imaging, Uppsala University Hospital, Sweden
[5] Department of Bone Marrow Transplantation, Hematology and Transplantology Clinic, Tomasz Sokolowski Public Hospital, Pomeranian Medical University in Szczecin, Poland
[6] Department of Cardiovascular Diseases, Jagiellonian University Medical College, John Paul II Hospital, Cracow, Poland
[7] Department of Nuclear Medicine, Jagiellonian University Medical College, John Paul II Hospital, Cracow, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Address for correspondence
Piotr Gościniak
E-mail: piotrgosciniak1@gmail.com

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Abstract

A substantial increase in the interest in transthyretin cardiac amyloidosis (ATTR-CA) is a result of the constantly growing number of patients, the use of clear diagnostic protocols and the availability of the first selective drug for these patients. This has also raised the awareness of the disease among physicians of all specialties. The topic is particularly relevant to cardiologists, who use non-invasive multimodal imaging in their daily practice.

The differential diagnosis of the causes of myocardial hypertrophy includes arterial hypertension, hypertrophic cardiomyopathy, aortic stenosis (AS), athletic heart syndrome, Fabry disease, and cardiac amyloidosis (CA). It turns out that in patients with myocardial hypertrophy >15 mm, amyloidosis is the most common cause of left ventricular (LV) hypertrophy. In parallel, CA is one of the most common infiltrative diseases leading to a clinical picture that may mimic heart failure with preserved ejection fraction (HFpEF).

The accumulation of amyloid in the extracellular space impairs the diastolic function of the myocardium, which is observed as the restrictive cardiomyopathy phenotype. In advanced cases, the LV systolic function is also impaired. Moreover, protein deposits contribute to the disturbances of calcium metabolism and cell metabolism as well as to cardiotoxicity, leading to edema and damage to cardiomyocytes.

Key words: cardiomyopathy, amyloidosis, multimodal imaging, tafamidis, apical sparing

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Introduction

The emergence of new therapies and guidelines for the management of patients with cardiac amyloidosis (CA) has increased the interest in this disease and brought a new approach to the diagnosis of the causes of myocardial hypertrophy.[1–3] The position statement of the European Society of Cardiology (ESC) Working Group on Myocardial and Pericardial Diseases recommends screening for cardiac involvement if left ventricular (LV) hypertrophy exceeds 12 mm and coexists with specific red flags or a clinical scenario.[3] An invasive diagnostic pathway, referring to all forms of CA, comprises solely cardiac biopsy positive for amyloid or a combination of an extracardiac biopsy positive for amyloid, and a presence of characteristic for CA findings on echocardiography or cardiac magnetic resonance. So far, there has been great awareness of storage diseases, particularly among echocardiographers, and the diagnosis of patients has often been limited to the exclusion or confirmation of light-chain cardiac amyloidosis (AL-CA), the treatment of which remains the domain of hematologists. If transthyretin cardiac amyloidosis (ATTR-CA) is suspected, a non-invasive diagnostic pathway is recommended, including scintigraphy, haematologic tests and echo/cardiac magnetic resonance (CMR).[3]

Cardiac amyloidosis manifests itself as a heart failure (HF) with initially preserved and – in more advanced stages – reduced ejection fraction, commonly with concomitant LV hypertrophy.[4] Since amyloid deposits were found postmortem in 25% of the unselected elderly population[5] and in 13–19% of those with a history of HF with preserved ejection fraction (HFpEF),[6] CA tends to be an underdiagnosed condition. Seferović et al., in a position paper from the Heart Failure Association of the ESC, draws attention to the possible change in the clinical phenotype of amyloidosis.[7] In the natural history of the disease, depending on the timing of the diagnosis, the phenotype can be hypertrophic cardiomyopathy (frequently asymptomatic), restrictive cardiomyopathy (mildly symptomatic) or end-stage cardiomyopathy with severe LV contractile dysfunction and advanced HF symptoms.

The development of cardiological diagnostics enabled the non-invasive differentiation of the causes of myocardial hypertrophy, which proved to be particularly valuable in patients with HFpEF. This differential diagnosis includes arterial hypertension, hypertrophic cardiomyopathy, aortic stenosis (AS), athletic heart syndrome, Fabry disease, and CA. It turns out that in patients with myocardial hypertrophy >15 mm, amyloidosis is the most common cause of LV hypertrophy.[8]

Various heterogeneous pathophysiological processes are responsible for HFpEF, including a systemic inflammatory reaction, the dysfunction of large and small vessels as well as the fibrosis and remodeling of the affected tissues.[9] There are clinical differences between amyloid cardiomyopathy and inflammatory metabolic HFpEF. Cardiomyopathy associated with ATTR-CA primarily affects elderly men with normal or low blood pressure, and the hypertrophy of the heart wall muscle is significant and affects all chambers. The LV filling pressure is high, the LV end-diastolic volume is reduced and a disproportionate increase in the natriuretic peptide concentrations is observed. On the other hand, HFpEF caused by other factors more frequently affects women, usually middle-aged, often with metabolic syndrome and other chronic inflammatory diseases or hormonal disorders. The LV walls are usually slightly thickened and the LV end-diastolic volume is normal or slightly increased. Other parameters that distinguish the remaining forms of HFpEF from CA are usually only slightly elevated natriuretic peptides, significantly increased systolic blood pressure (SBP) and increased inflammatory parameters.[10]

Transthyretin cardiac amyloidosis is also considered a common pathology accompanying AS (Fig. 1).[11,12] The coexistence of CA with moderate or significant AS is estimated in up to 16% of patients over 80 years of age.[13,14] In several studies, the coexistence of amyloidosis and AS was assessed at 14–16% in elderly patients undergoing transcatheter aortic valve implantation (TAVI).[13–15]

The clinical and echocardiographic profile of patients with ATTR-CA and AS is quite similar. It seems that it particularly often affects elderly men with low-flow, low-gradient AS with preserved ejection fraction and reduced ejection volume.[13] Chacko et al. showed that the coexistence of ATTR-CA and AS was associated with a poor prognosis and a significantly shorter survival time (22 months compared to 53 months; $p = 0.001$).[16] These authors also noted statistically significantly longer survival for patients who underwent TAVI as compared to those who did not receive any treatment for severe AS ($p = 0.012$).[16]

Establishing the relationship and coexistence of AS and amyloidosis seems important, because an improvement after surgical valve replacement in these patients may be limited due to both, a higher risk of complications and death and the lack of clinical improvement caused by the presence of amyloid deposits in the heart. Therefore, it is recommended that the diagnosis of patients with a characteristic echocardiography image confirming the coexistence of significant LV hypertrophy and AS should be extended to include radioisotope testing, in order to exclude or confirm the presence of ATTR-CA.

Scully et al. showed that the TAVI procedure seemed to be promising for patients with ATTR-CA and AS.[17] Periprocedual complications ($p = 0.780$) and deaths ($p = 0.710$) in the groups with and without amyloidosis did not differ statistically, and TAVI turned out to be more favorable than conservative treatment ($p = 0.030$).[17] Recently published data showed that TAVI should not be delayed; post-TAVI survival in patients with CA and AS was no different than in lone AS.[18]

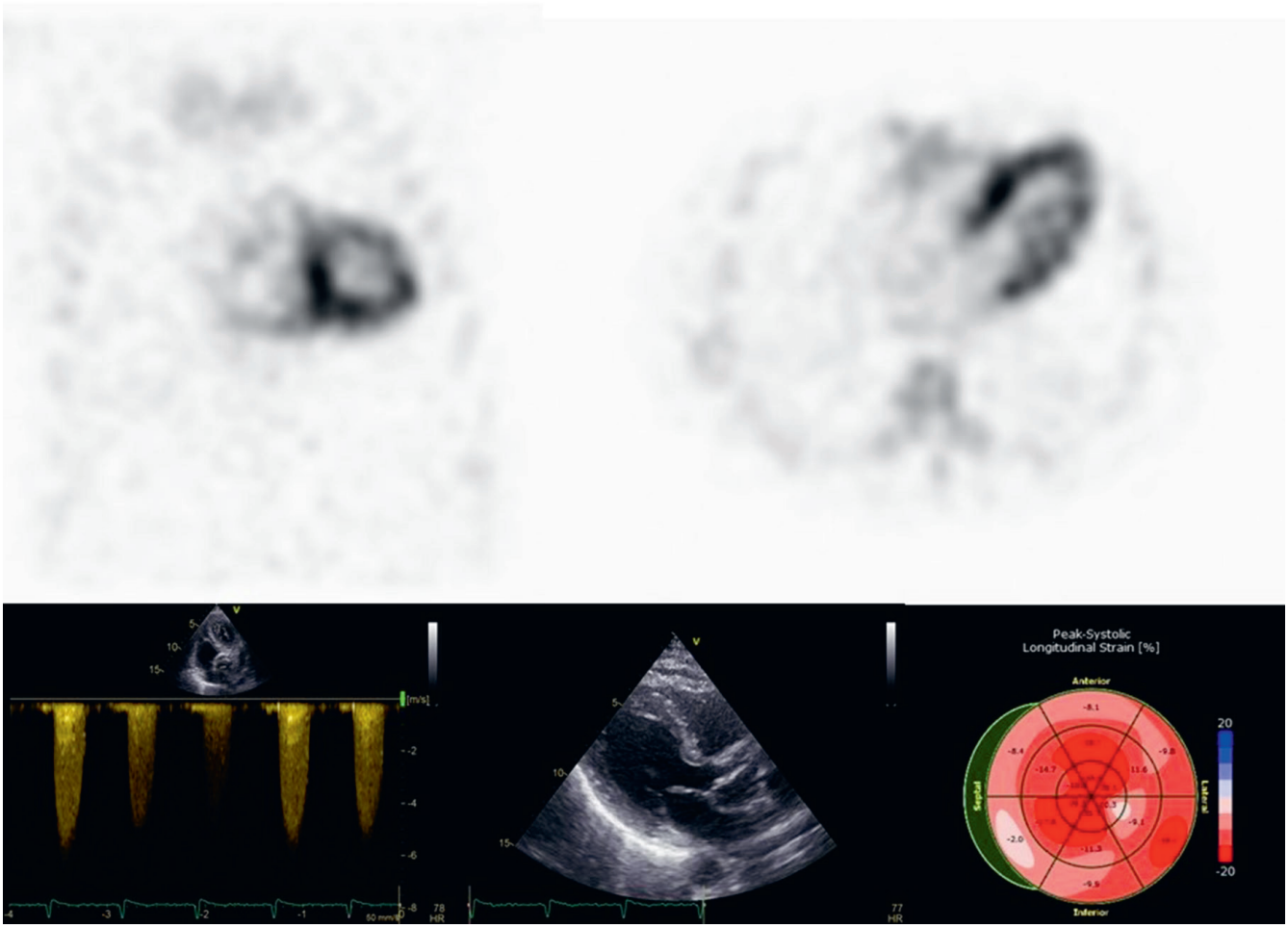

Fig. 1. Transthyretin cardiac amyloidosis (ATTR-CA) accompanying aortic stenosis (AS). In the single-photon emission computed tomography (SPECT) test – grade 3 according to the Perugini scale, i.e., strong cardiac uptake and no skeletal uptake of the tracer – a picture typical of ATTR-CA. The echocardiogram shows concentric myocardial hypertrophy, morphological valve changes with a significant mean transvalvular gradient (89 mm Hg), and the impaired deformation of the basal and middle segments of the myocardium

Pathogenesis – forms of amyloidosis

Light-chain cardiac amyloidosis is the most common form of CA, accounting for approx. 70–80% of all forms of the disease.[19] What is characteristic of AL-CA are the deposits of monoclonal light chains that are produced by clonal bone marrow plasmacytes and, as a result of dissociation, accumulate in tissues and organs in the form of misfolded and insoluble proteins, called amyloids. The basis for the diagnosis of AL-CA is the detection of amyloid in a given organ or tissue during the histopathological examination (the presence of amyloid when staining the tissue material with Congo red), the assessment of the concentrations of kappa and lambda light chains and their ratio in serum free light chains (sFLCs) as well as the assessment of immunofixation (monoclonal protein) in blood serum and urine. The reference technique is the analysis of the amyloid composition using mass spectrometry, but this method is still not widely available.[20] The use of chemotherapy or autologous hematopoietic stem cell transplantation (auto-HSCT) in the treatment of AL-CA depends, among other things, on how much various organs are affected. The Mayo Amyloidosis Staging System uses biomarkers to assess heart involvement.[21] In untreated AL-CA, the survival is estimated at less than 6 months from the diagnosis. For this reason, early detection is very important, because in low-risk patients treated with auto-HSCT, the 2-year survival in good centers is 94%, and in medium-risk patients treated with classic chemotherapy, it is about 40–50%.[22]

Transthyretin cardiac amyloidosis is the 2nd most common type of CA. Together, both forms of amyloidosis (AL-CA and ATTR-CA) account for approx. 95% of cases of the disease. Other unique types of amyloidosis are associated with amyloid A and apolipoprotein A.

Transthyretin (TTR) is a physiological protein produced in the liver that transports the thyroid hormone thyroxine (T4) and vitamin A. The essence of ATTR-CA is the instability of the tetramer of which TTR consists.[23,24] The breakdown of the protein molecule causes the formation of misfolded monomers that form

insoluble amyloid proteins, which accumulate in the form of deposits in tissues and organs, including the heart.[25] The abnormal protein breakdown may be caused by both the mutated *TTR* gene[5] and the spontaneous tetramer breakdown characteristic of wild-type ATTR-CA.[25] It has been established in autopsy tests that ATTR-CA may occur in as much as 25% of the elderly population over 85 years of age.[5]

The accumulation of amyloid in the extracellular space impairs the diastolic function of the myocardium, which is observed as the restrictive cardiomyopathy phenotype. In advanced cases, LV ejection fraction (LVEF) is also impaired. Moreover, amyloid protein deposits disturb the transport of calcium and cell metabolism, and contribute to cardiotoxicity, leading to cellular edema and damage to cardiomyocytes.[26]

Clinical presentation

Unfortunately, the clinical symptoms of both forms of amyloidosis (AL-CA and ATTR-CA) are not characteristic of this disease, and an early diagnosis is therefore difficult, requires consultations with many specialists and takes a long time,[27] often until echocardiography is performed by an experienced cardiologist.[28] The most common signs and symptoms of all forms of amyloidosis are listed in Table 1.

Diagnostic procedure

The abnormalities found in CA in non-invasive cardiological diagnostics are presented in Table 2.

Cardiac biomarkers

In everyday cardiological practice, the diagnostic procedure should include the assessment of cardiac markers. Brain natriuretic peptide (BNP), N-terminal prohormone of BNP (NT-proBNP) and troponin T (TnT) are the most widely researched biomarkers of cardiac involvement for the diagnosis of CA.[29] The combination of these biomarkers allows also the prognostic stratification. It has been shown that the levels of BNP/NT-proBNP are disproportionately high in CA and are very useful in a prognostic assessment.[29,30] In addition, TnT has a very good prognostic value.[31]

Electrocardiogram

The analysis of the electrocardiogram (ECG) waveform with a characteristic image of low-voltage QRS complexes and pseudo-myocardial infarction is not a sufficiently sensitive method.[32] Low QRS voltage in a patient with echocardiographic myocardial hypertrophy is characteristic of amyloid cardiomyopathy, but is estimated to occur

Table 1. Symptoms and clinical manifestations of amyloidosis

Symptoms and clinical manifestations of amyloidosis			Amyloidosis type
Symptoms		fatigue and weakness	*AL, ATTR*
		shortness of breath with activity or when lying down	*AL, ATTR*
		tingling, pain, altered sensation in hands or feet	*AL, ATTR*
		difficulty in walking	*AL, ATTR*
Clinical manifestations	cardiac	conduction disturbances	*AL, ATTR*
		supraventricular tachyarrhythmias	*AL, ATTR*
		fainting and syncope	*AL, ATTR*
		hypotension	*AL, ATTR*
	sensory-motor neuropathy	bilateral carpal tunnel syndrome	*ATTR*
		polyneuropathy	*AL, ATTR*
	autonomic neuropathy	orthostatic hypotension	*AL, ATTR*
		vomiting, nausea	*AL, ATTR*
		diarrhea, constipation	*AL, ATTR*
		weight loss	*AL, ATTR*
	renal	proteinuria, nephrotic syndrome	*AL, ATTR*
	other	macroglossia, hepato- and splenomegaly	*AL*
		stenosis of the lumbar spine	*ATTR*
		rupture of the biceps tendon	*ATTR*
		intolerance to common cardiac drugs	*AL, ATTR*
		stroke, ataxia, convulsions, headaches	*AL, ATTR*
		eye symptoms, bruising around the eyes	*AL*
		skin bruising	*AL*

Table 2. Non-invasive cardiological diagnosis of amyloidosis

Diagnostic tests	Abnormalities
Cardiac biomarkers	elevated BNP or NT-proBNP
	elevated TnT
ECG	low-voltage QRS complexes
	no R-wave progression/pseudo-infarction in precordial leads
	atrial fibrillation
	conduction disturbances
Echocardiography	granular sparkling of the myocardium
	hypertrophy of the myocardium and the atrial septum
	thickening of the valve apparatus
	atrial enlargement
	pericardial fluid
	elevated LV filling pressure
	intracardiac thrombus
	apical sparing in GLS
MRI	apical sparing in GLS
	cardiac morphology as in echocardiography
	generalized or diffuse subendocardial or transmural LGE lesions
	LGE lesions in the walls of the enlarged atria and the atrial septum
	enlarged ECV and native T1
Tc-99m-DPD/PYP/MDP scintigraphy with SPECT	semi-quantitative assessment of the cardiac uptake on the Perugini scale (0–3): 0 – no uptake 1 – lesser than the rib uptake 2 – similar to the rib uptake 3 – greater that the rib uptake
	SPECT/CT perfusion assessment
	H/CL >1.5 after 1 h or >1.3 after 3 h
^{11}C-PiB PET/CT	– uptake degree >1.4: AL-CA – uptake degree 1–1.4: ATTR-CA

ECG – electrocardiogram; MRI – magnetic resonance imaging; Tc-99m – technetium-99m; DPD – 3,3-diphosphono-1,2 propanodicarboxylic acid; PYP – pyrophosphate; MDP – methylenediphosphonic acid; SPECT – single-photon emission computed tomography; ^{11}C-PiB – ^{11}C-labeled Pittsburgh compound-B; PET – positron emission tomography; CT – computed tomography; BNP – brain natriuretic peptide; NT-proBNP – N-terminal prohormone of BNP; TnT – troponin T; LV – left ventricular; GLS – global longitudinal strain; LGE – late gadolinium enhancement; ECV – extracellular volume; T1 – longitudinal relaxation time; H/CL – heart/contralateral lung index; ATTR-CA – transthyretin cardiac amyloidosis; AL-CA – light-chain cardiac amyloidosis.

in less than 50% of patients. In addition, atrial fibrillation (AF) and conduction disturbances may also be present.

Echocardiogram

This section refers to Fig. 2. The early diagnosis of amyloidosis is greatly facilitated by modern imaging techniques, in particular echocardiography, using the deformation analysis by means of the automatic tracking of myocardial acoustic markers (speckle tracking echocardiography – STE). Global longitudinal strain (GLS) is a non-invasive method to assess the shortening of cardiomyocytes regionally and globally. The technique detects discrete changes in the heart function that cannot be detected with classical echocardiography. In the deformation analysis in patients with amyloidosis, the impaired deformation is observed in the basal and middle segments of the left ventricle as compared to the apical segments. This so-called apical sparing is common in patients with amyloidosis and has a high diagnostic and prognostic value.[33,34] Barros-Gomes et al. showed that GLS most accurately provided additional prognostic information for all-cause mortality as compared to other clinical, echocardiographic and serological indicators.[35] Apical sparing is observed both in patients with ATTR-CA and those with AL-CA. The classic manifestations of echocardiographic heart involvement in amyloidosis are shown in Table 2.

Other potentially useful echocardiographic tools for the early detection of abnormalities[36] include the LV end-systolic elastance, the LV relaxation time constant – tau, and the assessment of the LV stiffness and the peak left atrial longitudinal strain.[37] Moreover, Aimo et al. proposed a simple echocardiographic score to rule out CA.[38] The AMYLoidosis Index (AMYLI: relative wall thickness (RWT) × the early mitral inflow velocity (E) to mitral

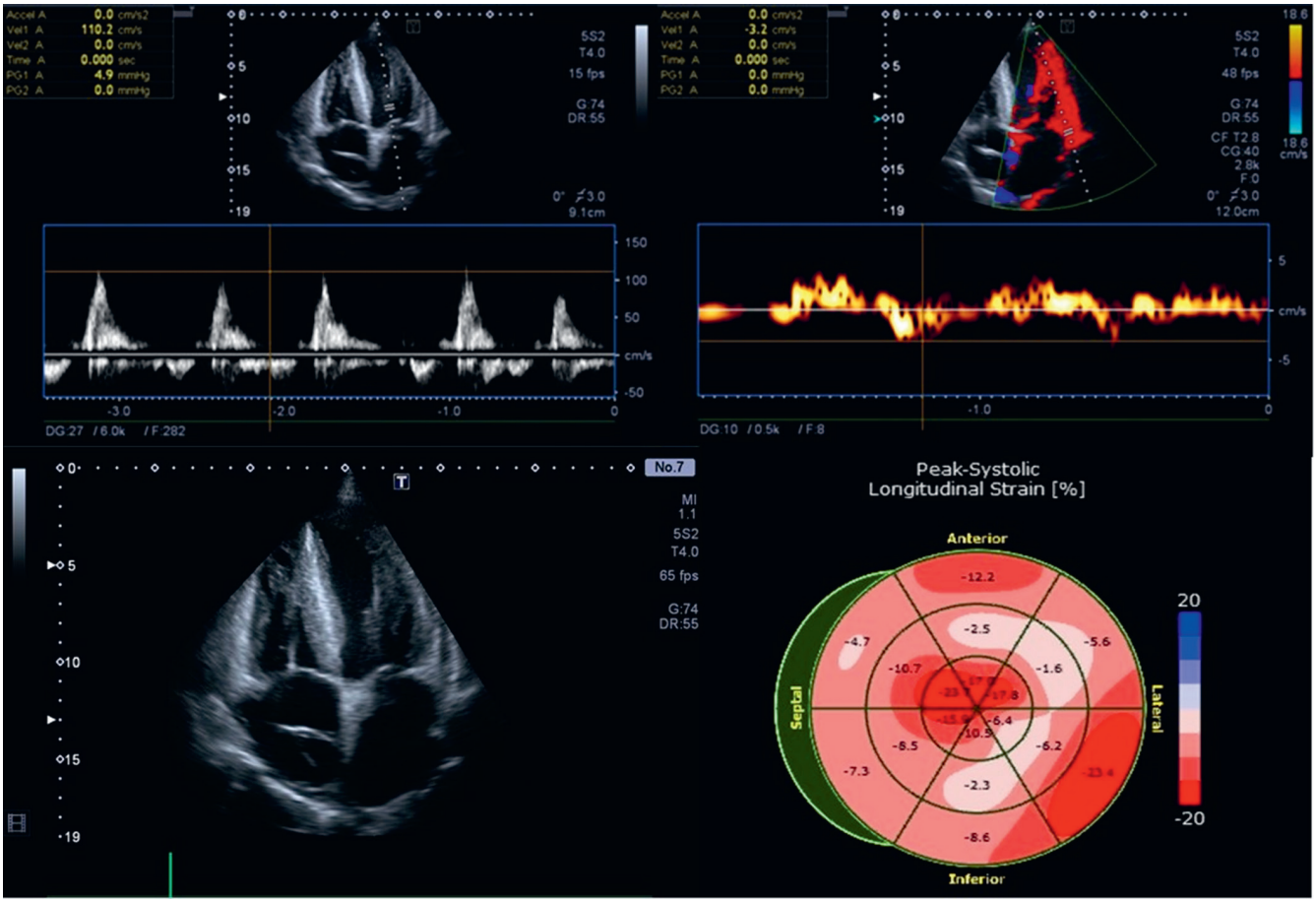

Fig. 2. Echocardiogram of a 57-year-old patient with multiple myeloma and light-chain cardiac amyloidosis (AL-CA): concentric myocardial hypertrophy; the thickening of the valve apparatus; atrial enlargement; pericardial fluid; an increased filling pressure (E/e' = 30); and apical sparing

annular early diastolic velocity (e') ratio (E/e')) <2.22 excluded heart involvement in that research.[38] Other models reported by Boldrini et al. include RWT, the E/e' ratio, longitudinal strain, and tricuspid annular plane excursion.[39] The accuracy of those models was very good, but the results still require external validation.[39]

Magnetic resonance imaging of the heart

This section refers to Fig. 3. The cardiac morphology and apical sparing specific to echocardiography are also visible in CMR images, the major advantage of which, however, is that they enable tissue characterization of the myocardium. The early deposition of amyloid fibers in the myocardium causes an increase in the extracellular volume (ECV), as can be observed from the presence and location of late gadolinium enhancement (LGE). In the case of amyloidosis, the most common observations are the presence of generalized subendocardial LGE foci, alternatively diffuse subendocardial or even full-thickness lesions.[40,41] There are relatively frequent pseudo-technical difficulties in the so-called complete extinction of the myocardial signal due to the diffuse presence of amyloid. Gadolinium contrast can also be seen in the walls of the enlarged atria and the often thickened atrial septum. New quantitative mapping techniques involve evaluating the native longitudinal relaxation time (T1) and the contrast-enhanced T1, as well as ECV calculated based on them. Similarly to ECV, the native T1 is significantly increased in CA.[42]

Radioisotope techniques

This section refers to Fig. 4,5. Radioisotope techniques are currently the basic method for diagnosing ATTR-CA. The techniques employ the technetium (Tc)-99m radioisotope and the tracers classically used in the skeletal examination: 3,3-diphosphono-1,2 propanodicarboxylic acid (DPD); pyrophosphate (PYP); and methylenediphosphonic acid (MDP).[43] However, scintigraphy is often available only in reference centers. The simplicity of imaging and a high specificity of nearly 100% (in the absence of light chains and monoclonal protein) for ATTR-CA are the advantages of scintigraphy emphasized by many authors.[44–46] Currently, a semi-quantitative visual assessment is applied using the Perugini 4-point scale (0–3).[44] The degree of isotope accumulation is compared to the flat sections of the ribs. What is characteristic of ATTR-CA is the diffuse uptake of the radioisotope in the myocardium. A complete

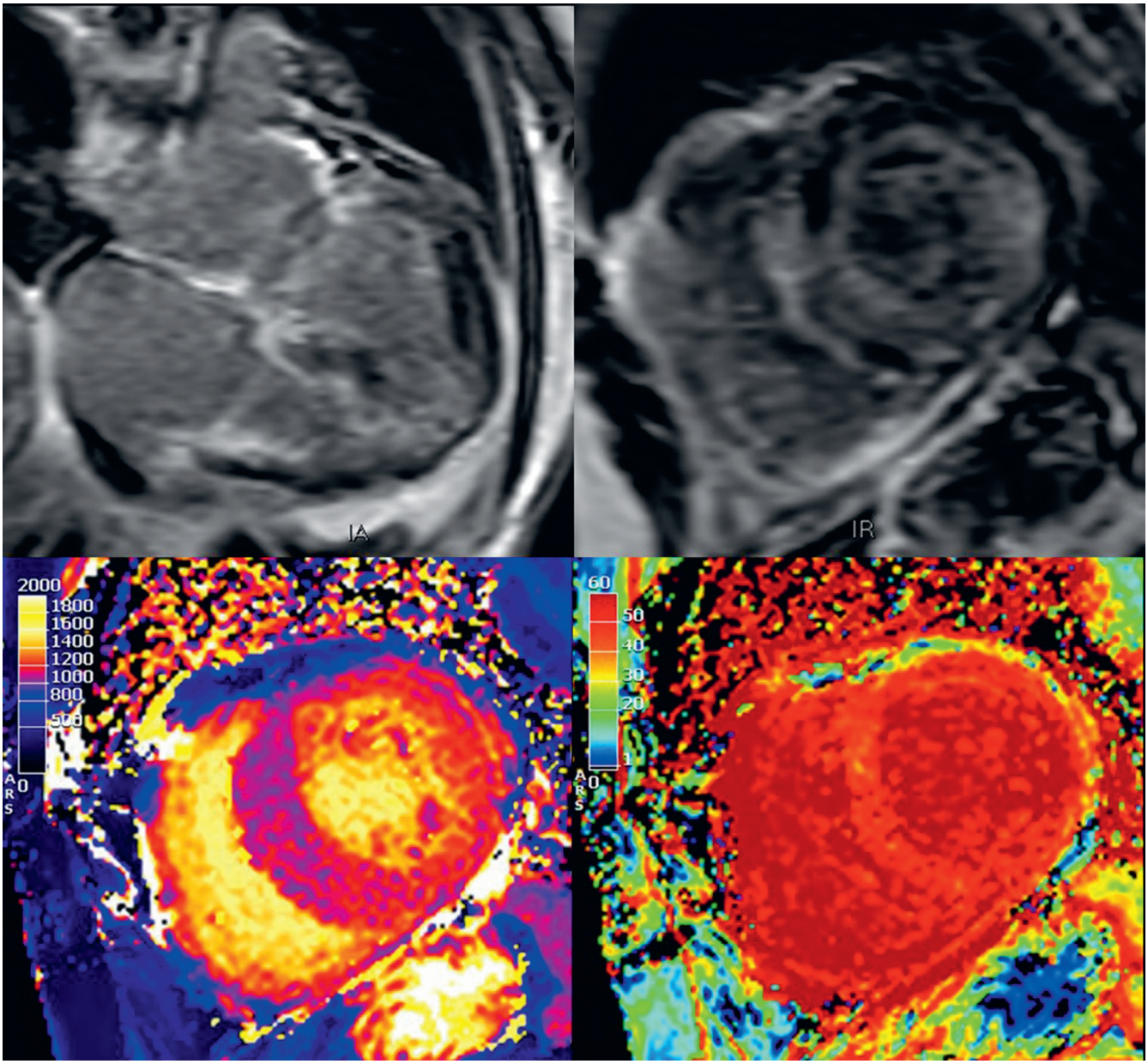

Fig. 3. Magnetic resonance imaging (MRI) of the heart of a 73-year-old patient with cardiac amyloidosis (CA): concentric left ventricular (LV) hypertrophy with generalized diffuse and confluent foci of late post-contrast enhancement; late post-contrast enhancement visible also in the walls of the enlarged atria and the free wall of the right ventricle; thickened atrial septum; trace of fluid in the pericardial sac; native longitudinal relaxation time (T1) of 1110 ms (norm: 950–1040 ms); extracellular volume (ECV) of 45% (norm: 20–30%)

scintigraphy analysis of the heart may also include the interpretation of the single-photon emission computed tomography (SPECT) perfusion as well as the calculation of the heart/contralateral lung (H/CL) index. This index is a quantitative comparison of the region of interest (ROI) ratio in the heart and the contralateral lung tested after 1 h (>1.5) or 3 h (>1.3).[47] The observations of Gillmore et al. confirmed the considerable usefulness of scintigraphy with the abovementioned bone tracers.[48] In the absence of monoclonal protein in serum or urine, and with the grade 2 or 3 uptake of the radioisotope, the specificity of the ATTR-CA diagnosis with this method was 100% in that publication. The authors concluded that it was unnecessary to perform an endomyocardial biopsy.[48] Furthermore, Marume et al. assessed a combination of parameters to increase the pretest probability of scintigraphy.[49]

Positron emission tomography (PET) using the Pittsburgh compound-B (PiB) radiotracer is another recommended method of examination that uses radiotracers. Pittsburgh compound-B is a radioactive ^{11}C-labeled analog of thioflavin T, originally used for imaging beta-amyloid plaques in the brain tissue in the diagnosis of Alzheimer's disease. The PiB radiotracer binds directly to the amyloid protein in both ATTR-CA and AL-CA. The myocardial uptake is quantified in relation to the blood activity of the heart cavities, adopting different breakpoints for the 2 types of amyloidosis. The normal myocardial/blood uptake ratio is approx. 1.[50]

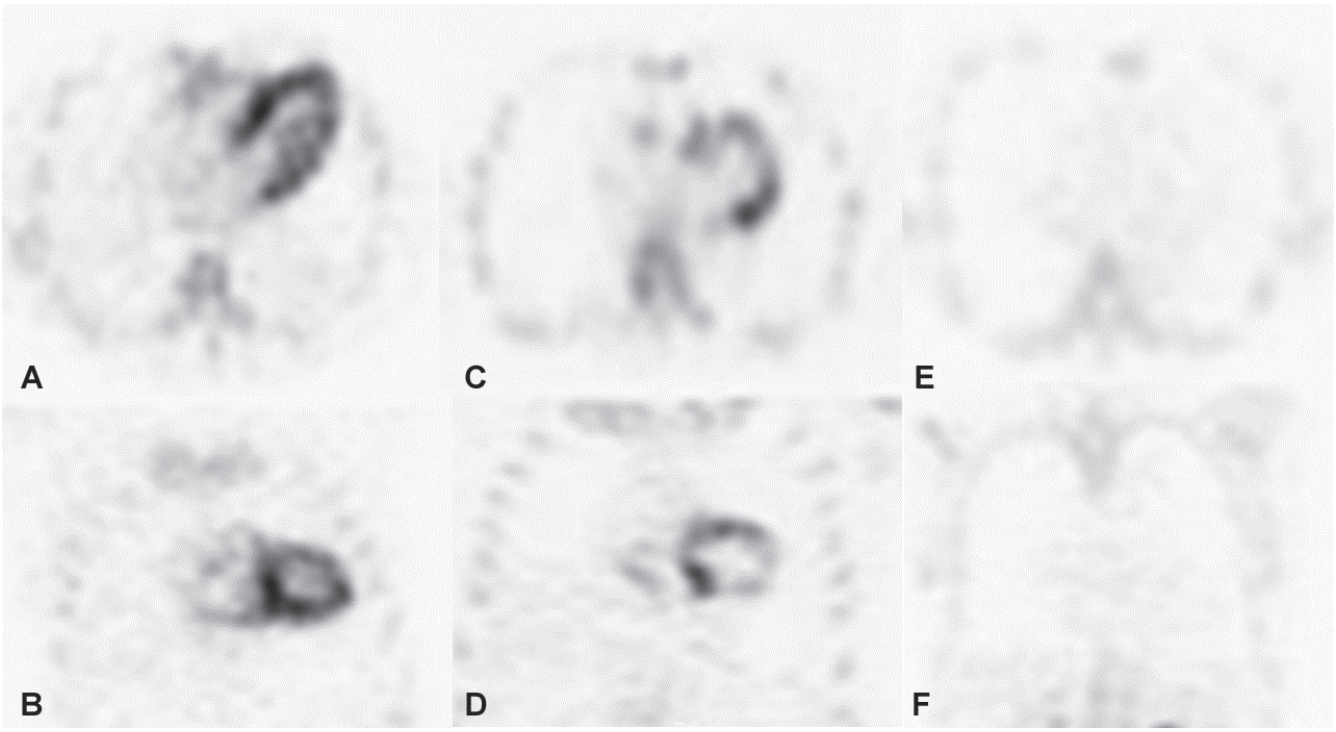

Fig. 4. 3,3-diphosphono-1,2 propanodicarboxylic acid (DPD)-single-photon emission computed tomography (SPECT)

A,B – grade 3 uptake – strong uptake in both ventricles, weak/no skeletal uptake – a picture typical of transthyretin cardiac amyloidosis (ATTR-CA); C,D – grade 2 uptake – moderate myocardial uptake, greater than the skeletal uptake – a picture typical of ATTR-CA; E,F – no myocardial uptake – the image allows the exclusion of ATTR-CA, but further diagnostics is necessary to exclude light-chain cardiac amyloidosis (AL-CA).

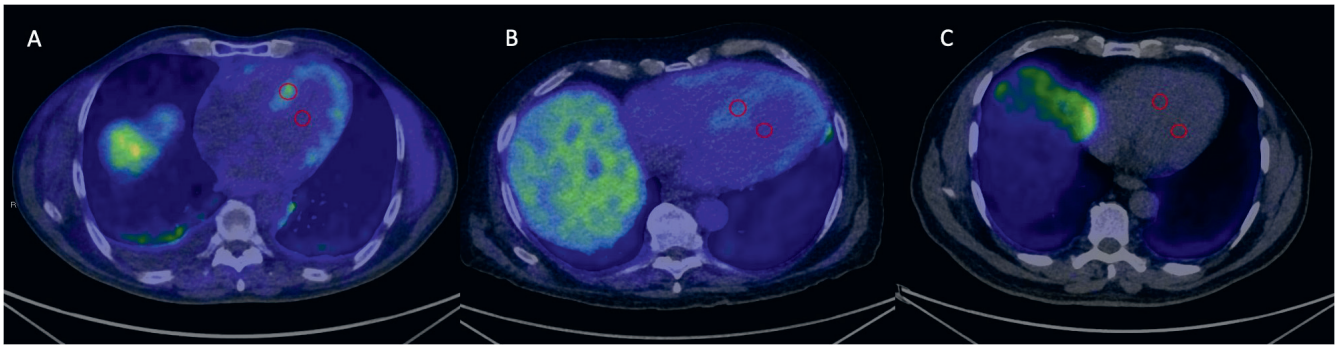

Fig. 5. ^{11}C-labeled Pittsburgh compound-B (^{11}C-PiB) positron emission tomography/computed tomography (PET/CT)

A – isotope uptake seen in the left ventricular (LV) and right ventricular (RV) myocardium, the myocardial/blood uptake ratio of about 2.2, diagnostic for light-chain cardiac amyloidosis (AL-CA); B – visually diffuse moderate LV and RV myocardial uptake, the myocardial/blood uptake ratio of about 1.3, diagnostic for transthyretin cardiac amyloidosis (ATTR-CA); C – no visible myocardial Pittsburgh compound-B (PiB) uptake, the myocardial/blood uptake ratio of about 0.9, myocardial amyloidosis can be excluded.

Machine learning models

The application of artificial intelligence for screening CA has been reported recently.[51,52] The authors showed that machine learning models well identified patients with CA.

Therapeutic procedure

There are 3 distinct areas of treatment for amyloidosis.

The 1st area of treatment is the management of cardiac complications due to the accumulation of abnormal protein throughout the heart, causing the symptoms of HF and impairing the exercise capacity, but also potentially leading to atrioventricular conduction disorders or supraventricular tachyarrhythmias.

Angiotensin receptor antagonists in low doses may be profitable, as they act as afterload-reducing agents, and improve the forward cardiac flow and renal perfusion. Similarly, low doses of β-blockers are helpful as rate-controlling agents. However, the use of medicines typical for HF, such as angiotensin-converting enzyme inhibitors (ACEIs), β-blockers or angiotensin receptor–neprilysin inhibitors (ARNIs), is not recommended in advanced heart involvement. On this level,

the adverse effects of these agents (hypotension, kidney failure, conduction disorders) overwhelm benefits.[52] The use of calcium antagonists, particularly non-dihydropyridine calcium channel blocker and digoxin is also avoided.[53]

The 2nd area of treatment is reserved for hematologists and concerns AL-CA. Depending on the patient's classification to the appropriate risk group, the most commonly used regimens are immunochemotherapy based on bortezomib, cyclophosphamide and melphalan. Selected patients undergo high-dose therapy supported with auto-HSCT.[54]

The 3rd area of therapy concerns patients with ATTR-CA. The treatment of this specific pathology involves 3 management strategies. These are as follows: the inhibition of protein production in the liver; the stabilization of the tetramer; and the disruption of the permanent bonding of amyloid fibers.

The 1st strategy of ATTR-CA management, which involves impairing the production of abnormal protein in the liver, associated with the genetically determined form of ATTR-CA, can now be performed with the use of gene therapy approved by the U.S. Food and Drug Administration (FDA), applying an RNA interference therapeutic agent – patisiran[55] or an antisense oligonucleotide – inotersen.[56] Both drugs are registered for amyloid polyneuropathy, but not for ATTR-CA. The estimated annual cost of such therapy in the USA is currently around $300,000. Liver transplantation is also performed less and less often for these indications.

The stabilization of the TTR tetramer is possible with the drug tafamidis, which binds selectively to the 4-part TTR oligomer. The Transthyretin Amyloidosis Cardiomyopathy Clinical Trial (ATTR-ACT) showed a significant decrease in the number of hospitalizations and mortality of patients treated with tafamidis.[57] In 2019, the drug was also approved in the USA for ATTR-CA. Another TTR tetramer stabilizer, diflunisal, is a non-steroidal anti-inflammatory drug, but its use was not associated with an improved prognosis in patients with cardiac involvement in randomized studies and was accompanied by numerous complications, primarily from the gastrointestinal tract.[58–60]

Another therapeutic strategy in ATTR-CA is an attempt to amyloid degradation with doxycycline or tauroursodeoxycholic acid. However, the effects of this procedure require a further assessment. There are also clinical trials with the use of a specific monoclonal antibody PRX 004 affecting amyloid degradation.[61] In extreme cases, heart and liver transplants are performed.

Conclusions

ATTR-CA 'timeline'

An interesting timeline for the progression of ATTR-CA was proposed by the authors from the Mayo Clinic.[62] The presence of bilateral carpal tunnel syndrome, the stenosis of the lumbar spine or the rupture of the biceps tendon, which may appear many years before the disease manifestation, are considered to be extremely early clinical symptoms ("red flags") of the disease. After some time,

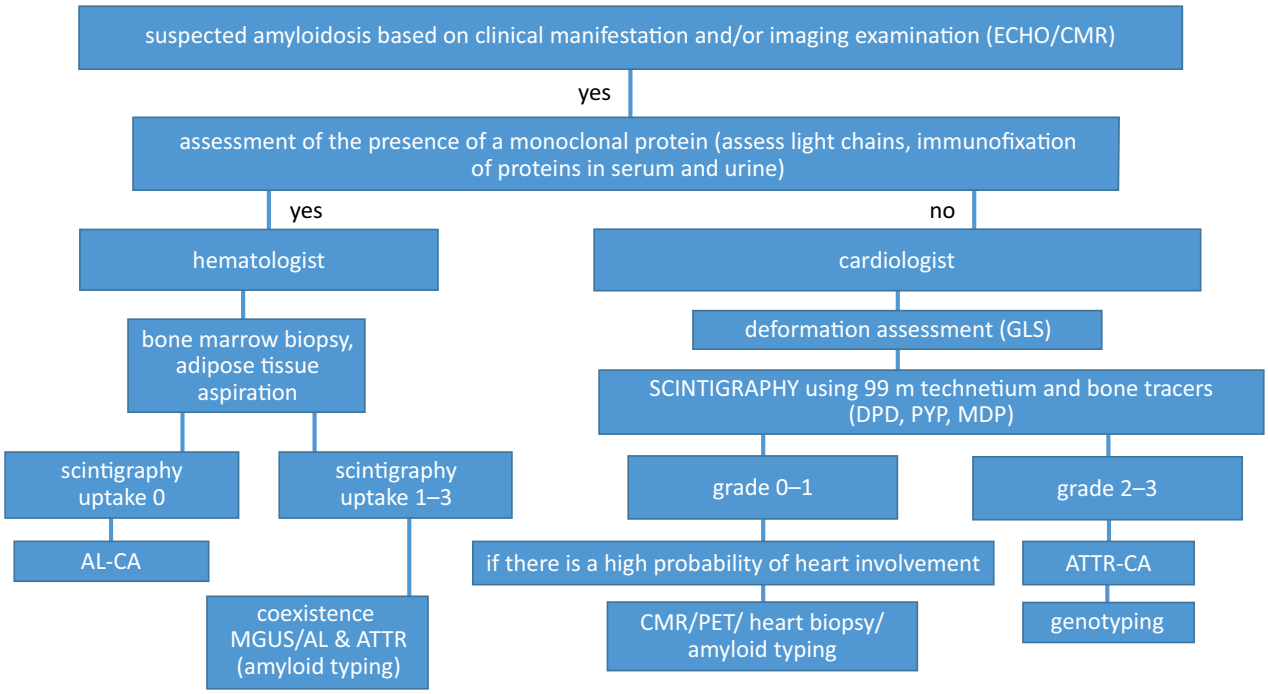

Fig. 6. Modified algorithm[3,34,62] for the management of suspected cardiac amyloidosis (CA) used by the authors

ECHO – echocardiography; CMR – cardiac magnetic resonance; MGUS – monoclonal gammopathy of undetermined significance; PET – positron emission tomography; GLS – global longitudinal strain; DPD – 3,3-diphosphono-1,2 propanodicarboxylic acid; PYP – pyrophosphate; MDP – methylenediphosphonic acid; ATTR-CA –transthyretin cardiac amyloidosis; AL-CA – light-chain cardiac amyloidosis

there may be abnormalities in PET/CT, SPECT/CT and CMR imaging tests with normal cardiac biomarkers and no morphological changes in echocardiography. On the other hand, typical changes in the echocardiographic examination and other clinical symptoms indicate that the disease is highly advanced and they clearly manifest the full-blown disease. It has been noticed that ECG abnormalities become apparent only at a very late stage.

Algorithm for assessing a patient with suspected amyloidosis

The authors of this study use a modified algorithm for assessing a patient with suspected amyloidosis (Fig. 6).[3,48,62] It primarily includes the echocardiographic or CMR assessment using GLS as well as the laboratory assessment of concentrations and the ratio of free light chains (FLCs) and immunofixation, in both serum and urine, to exclude AL-CA.

If there is 1 or more of these 3 protein laboratory abnormalities, then the patient should be referred for further hematological evaluation. A typical picture of bone marrow biopsy changes or Congo red-stained amyloid confirm the diagnosis of AL-CA with high probability. If scintigraphy shows in the patient the cardiac uptake (grade 1–3), monoclonal gammopathy of undetermined significance (MGUS) or AL-CA co-occurrence with ATTR-CA should be considered.[3]

If the abovementioned proteins are not detected, the SPECT/CT examination is performed with the use of DPD or PYP labeled with technetium-99m (Tc-99m). Grade 2 or 3 uptake in the absence of light chains and monoclonal protein allows the diagnosis of ATTR-CA. In doubtful cases (scintigraphy grade 0–1), when both AL-CA and ATTR-CA are highly suspected, patients should be additionally referred for CMR or PET/CT. A myocardial biopsy and the analysis of amyloid protein composition using mass spectrometry should also be considered.

ORCID iDs

Piotr Gościniak https://orcid.org/0000-0003-1290-0391
Tomasz Baron https://orcid.org/0000-0002-8290-557X
Sławomir Milczarek https://orcid.org/0000-0003-4445-6192
Magdalena Kostkiewicz https://orcid.org/0000-0001-9094-3202
Bogusław Machaliński https://orcid.org/0000-0002-6013-0419

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Impaired fertility in women and men with chronic kidney disease

Piotr Kuczera[A,B,D,F], Andrzej Więcek[E,F], Marcin Adamczak[A,D–F]

Department of Nephrology, Transplantation and Internal Medicine, Medical University of Silesia, Katowice, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Piotr Kuczera
E-mail: pkuczera@sum.edu.pl

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Abstract

Chronic kidney disease (CKD) is accompanied by a great number of comorbidities. One of the most clinically important, present in women as well as in men, is infertility. In this review paper, the entire issue of impaired fertility in women and men with CKD is discussed. In both genders, impaired fertility is caused by the interconnection of several factors. In women, these are as follows: the accumulation of uremic toxins; endocrine disorders (e.g., reduced renal clearance of different hormones, disturbed activity of the pituitary–gonadal axis); the impairment of the ovarian function; a reduced ovarian reserve; sexual function disorders; and depression. In men, quite similarly: the accumulation of uremic toxins; endocrine disorders; the impairment of spermatogenesis; direct testicular damage; erectile dysfunction (ED); and depression. The prevalence of impaired fertility increases with the degree of kidney function deterioration in women and men. The highest prevalence of these disturbances is observed in patients with CKD stage 5. Successful kidney transplantation (KTx) in women reduces the accumulation of uremic toxins, restores the function of the endocrine system and improves, but does not normalize, fertility. Similarly in men, KTx restores the function of the endocrine system and improves fertility up to a point, but cannot fully reverse the morphological damage already done to the gonads by the uremia itself. Infertility is one of the important, yet sometimes depreciated complications in women and men with CKD. The etiology and pathogenesis of infertility in CKD is complicated. Kidney transplantation alleviates, but does not fully reverse fertility impairment in CKD patients.

Key words: infertility, chronic kidney disease, kidney transplantation

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Introduction

Fertility disorders occur commonly in patients with chronic kidney disease (CKD). These disorders affect both women and men with comparable severity, but in a different manner. Disturbances concerning sexual life and procreation are important clinical problems in these patients, and may lead to the worsening of the quality of their lives or the development of depression. It seems, though, that they are quite often overlooked by the clinicians dealing with CKD patients. The aim of this review paper is to describe the entirety of fertility abnormalities in women and men with CKD and after kidney transplantation (KTx), in a detailed yet concise manner, and thus support physicians in making adequate decisions concerning patients with renal diseases.

Infertility in women with chronic kidney disease

Impaired fertility is one of the important, yet sometimes depreciated comorbidities in women with advanced CKD. It is most common in women with CKD stage 5. In women younger than 40 years of age requiring chronic hemodialysis, primary ovarian insufficiency is more prevalent than in the general population.[1] Menopause occurs in these women around 4.5 years earlier as compared to healthy females.[2,3] Pregnancy in women on chronic hemodialysis is rare – its prevalence is 0.3/100 females/year, which is about 40 times less than in the general population.[4,5] Interestingly, pregnancy is more likely to occur in women receiving renal replacement treatment (RRT) with preserved residual renal function.[6]

Infertility is defined as a failure to become pregnant after 1 year of regular sexual intercourse without using any contraception.[6] In the general population, the prevalence of infertility varies between 8% and 12%, with the female factor of infertility contributing to more than 72% of cases in developed countries.[7,8] The prevalence of infertility in women with CKD stage 5 has been reported as exceeding 90%.[1]

Impaired fertility in women with CKD may be caused by the accumulation of uremic toxins, sexual function disorders, decreased renal catabolism of different hormones, and other abnormalities of the endocrine system. Moreover, the function of the ovaries is deteriorated, which is manifested by the reduction of the ovarian reserve (Table 1). The aforementioned complications may lead to irregular, usually anovulatory menstrual cycles and significant difficulties in successful pregnancies in female CKD patients.[9–11]

Table 1. Causes of fertility disturbances in women with chronic kidney disease (CKD)

Fertility disturbances	Causes
Sexual function disorders	– decrease in libido (HSDD) – depression and adverse effects of antidepressant medications – peripheral neuropathy – anemia – cardiovascular disorders
Endocrine abnormalities leading to irregular menstrual cycles	– hyperprolactinemia – disturbed pulsatile secretion of GnRH, LH and FSH – low estradiol concentration
Impaired ovarian function (reduced ovarian reserve)	– uremic toxin-related damage – low AMH concentration

HSDD – hypoactive sexual desire disorder; GnRH – gonadotropin-releasing hormone; LH – luteinizing hormone; FSH – follicle-stimulating hormone; AMH – anti-Müllerian hormone.

Sexual function disorders in women with chronic kidney disease

In CKD women, the disorders of the sexual function are frequent and may arise from psychological as well as organic factors. Among chronically hemodialyzed women, 60–70% suffer from sexual dysfunction.

Over 50% of these women complain of a significant libido decrease and a concomitant decline in the ability to reach orgasm. This causes a marked reduction in the frequency and quality of sexual intercourse,[2,11] and falls into a definition of hypoactive sexual desire disorder (HSDD), which is described as a persistent absence of sexual thoughts and/or desire for and receptivity to sexual activity, and is regarded as a cause of personal distress.[2,3,12] Another important psychological factor leading to sexual dysfunction in women with CKD is the coexistence of depression.[13,14]

Amid the organic causes of sexual dysfunction in CKD, a variety of conditions may be listed. The uremic milieu per se, peripheral neuropathy, anemia, cardiovascular diseases (CVDs), the disturbances of the endocrine system affecting the pituitary–gonadal axis, as well as the adverse effects of antidepressant medications can all contribute to the sexual derangements in these women.[11,12]

All of the aforementioned conditions may lead to a significant impairment of the sexual function in women with CKD. In self-reported questionnaire analyses, the magnitude of sexual dysfunction in CKD women[14] was comparable to that of "post-menopausal women with sexual dysfunction",[15] and was markedly higher than in women with diabetes.[16] Even more significant, over 55% of the participants in a study by Peng et al. had no sexual life at all during the course of the study, so they were not counted as patients with sexual dysfunction.[14] Of note, dialysis adequacy (Kt/V >1.2) seems not to have a beneficial influence on the magnitude of sexual dysfunction in dialyzed women.[14]

Endocrine disorders in women with chronic kidney disease

Severe alterations of the endocrine gland function (i.a., the pituitary gland and the ovaries) are a frequent finding in women with CKD. They have a complex nature and may be due to improper synthesis, secretion or metabolism of different hormones, which is mostly caused by the deterioration of the kidney function. The most severe consequences of the aforementioned disturbances are anovulatory menstrual cycles and infertility. The disturbances of the hypothalamic–pituitary–ovarian axis occur early in CKD and tend to progress with the course of disease, as well as after the initiation of RRT.[9,17]

Prolactin

Serum prolactin (PRL) concentration is increased in 30–65% of women with CKD. The basal serum PRL concentration is usually elevated and improper diurnal rhythm of PRL secretion can be observed. Additionally, the sleep-induced bursts of PRL secretion are usually absent, although episodic secretion of PRL in the daytime has been noted.

The most prominent factor in the pathogenesis of hyperprolactinemia in women with CKD is reduced renal clearance of PRL. Also, an increase in PRL secretion in the pituitary gland, caused by inadequate dopaminergic inhibition,[17,18] contributes to hyperprolactinemia in these patients. Additionally, an increase in serum PRL concentration after stimulation by thyrotropin-releasing hormone (TRH) administration is usually blunted.

In CKD women with hyperprolactinemia, amenorrhea is a frequent finding.[9] An increased serum PRL concentration is one of the major factors contributing to the dysregulation of the hypothalamic–pituitary–ovarian axis. This inhibits gonadotropin secretion, leading to hypogonadotropic hypogonadism.

In a small clinical study conducted on CKD patients with hyperprolactinemia, bromocriptine treatment led to the reduction of blood pressure and left ventricular hypertrophy, along with the normalization of prolactinemia.[19] Moreover, sustained treatment with erythropoietin-stimulating agents (ESAs) may also decrease serum PRL concentration in patients treated with chronic hemodialysis.[20]

Luteinizing hormone and follicle-stimulating hormone

In apparently healthy premenopausal females, luteinizing hormone (LH) is secreted in a pulsatile manner. Women with CKD are characterized by the loss of pulsatile gonadotropin-releasing hormone (GnRH) secretion in the hypothalamus, which leads to the lack of normal pulsatile LH release by the pituitary gland.

Premenopausal CKD patients are characterized by a normal or mildly elevated basal serum LH concentration, but the pre-ovulatory peak of serum LH concentration is absent.[9] In healthy women, estradiol blunts the amplitude of LH pulses. In women with CKD, estradiol does not influence the serum LH concentration surge. This suggests the impairment of the physiological feedback loop and contributes to the impairment of ovulation. In consequence, the loss of normal pulsatile LH secretion in the pituitary gland in CKD leads to infertility.[21]

The loss of pulsatile GnRH release in the hypothalamus also affects the pituitary follicle-stimulating hormone (FSH) secretion. Nevertheless, in contrast to LH, serum FSH concentration usually is normal or just slightly elevated in most premenopausal females with CKD. This causes a decrease in the FSH/LH ratio, which is also the reflection of a significant hypothalamic–hypophyseal dysregulation in women with CKD.[11]

Estrogens and progesterone

In female CKD patients, serum estradiol concentration may be normal, but more often decreased, and is consistently lower in women with CKD and concomitant hyperprolactinemia. In the luteal phase of the menstrual cycle, serum progesterone concentration is decreased as a result of defective luteinization of the follicles.[9]

One of the major clinical consequences of a decreased serum estrogen concentration are bone disorders.[22] Patients with amenorrhea present not only low serum estrogen concentration, but also lower bone mineral density (BMD), as compared to women requiring RRT that do not have menstruation disorders. The results of small clinical interventional studies suggest that treatment with transdermal estradiol (with the cyclic addition of progestin – norethisterone acetate) or treatment with selective estrogen receptor modulators (SERMs, e.g., raloxifene) may increase the BMD of the lumbar spine in postmenopausal women on chronic hemodialysis.[23] Still, taking into consideration the potential adverse cardiovascular effects of hormonal replacement therapy (HRT), it has to be underlined that currently no conclusive results of long-term clinical studies on SERMs and HRT in women with CKD are available.

Anti-Müllerian hormone

Besides the aforementioned sexual function disorders and alterations of the hypothalamic–pituitary–gonadal axis, also impaired ovarian function per se is a prominent cause of fecundity disorders in CKD women.[3] One of the indicators of ovarian impairment is a reduced ovarian reserve. The ovaries are susceptible to injury from a variety of factors, which is due to the limited number of germinal cells present in the ovaries and their inability to regenerate. The term “ovarian reserve” refers to the remaining endowment of resting and primary ovarian follicles. It is used to define the quality and quantity of follicles that are present in the ovaries at a given time.[24]

One of the best markers of the ovarian reserve in women with preserved renal function is the serum concentration of anti-Müllerian hormone (AMH). Anti-Müllerian hormone is a 140 kDa glycoprotein that circulates as a dimer. It is mostly synthesized by granulosa cells that surround each oocyte in primary, preantral and small antral follicles until they reach a diameter of 6–8 mm.[25,26] The abrupt decrease of AMH expression is observed in larger follicles, as the follicle becomes dominant. The most important physiological function of AMH is the inhibition of the excessive recruitment of primordial follicles and the inhibition of the growth of preantral and antral follicles, which is mediated through the reduction of their sensitivity to FSH stimulation. This takes place in the follicular phase of the menstrual cycle and leads to the selection of a dominant follicle. The quantity of resting primordial follicles that remain intact in the ovaries determines the ovarian reserve.[26–28]

Physiological monthly serum sex hormone concentration fluctuations do not influence serum AMH concentration in women. It tends to be constant during the entire menstrual cycle.[28,29] Serum AMH concentration reflects the number of growing follicles and is proportional to the pool of primordial follicles. That is why serum AMH concentration is considered to be one of the best markers of the ovarian reserve. Physiologically, the highest serum AMH concentration is observed after puberty (in women around 25 years of age). Then, serum AMH concentration decreases with age at a rate of about 6% per year until circulating AMH is virtually undetectable in postmenopausal woman. This decrease in serum AMH concentration is tightly connected to a decrease in the number of growing follicles.[29,30]

The diminishing serum AMH concentration may be an indicator of either physiological or premature aging of the gonads.[29,31] The results of our clinical study showed significantly lower serum AMH concentration in hemodialyzed women with regular menstrual cycles than in age-matched regularly menstruating females from the control group.[10] This seems to suggest that a decrease in AMH secretion by the damaged granulosa cells and the reduction of the ovarian reserve are the most pronounced causes of diminished fertility in women with CKD. The results seem to imply that infertility in CKD women is at least partially caused by the damage of the ovaries due to uremic toxins.[10] Later studies regarding this topic showed similar results,[31,32] with an additional finding that serum AMH concentration seems not to depend on the presence of proteinuria.[31]

Impaired fertility in women after kidney transplantation

The results of various clinical studies suggest that successful KTx tends to improve, but not normalize, female fertility. Pregnancy is 4 times more common in women with well-functioning kidney allografts than those requiring chronic hemodialysis. Still, it is about 10 times less probable than in the general population. Kidney transplantation usually restores the sexual function and decreases menstrual cycle disturbances. It also tends to normalize the secretion of sex hormones. It was demonstrated that the serum concentrations of LH, FSH and PRL in women after successful KTx are similar to those in healthy controls. It seems that the restoration from the uremic milieu to normal endocrine environment leads to normal maturation of the Graafian follicles, normal ovulation and luteinization.[11,33,34]

However, it is important to stress that women after successful KTx are characterized by significantly lower serum AMH concentration as compared to healthy females. This gives a rationale for an assumption that although KTx leads to the alleviation of the hypothalamus–pituitary–ovarian axis derangements, it does not restore the ovarian reserve. Therefore, it is likely that the disturbances of fertility, which are to some extent still present after KTx, are caused by the impairment of the ovarian function. This impairment of the ovaries seems to arise from the irreversible damage caused by uremic toxicity present before the transplantation procedure.[10]

Anti-Müllerian hormone is a protein hormone, thus it is most likely metabolized by the kidneys, like other proteins. This may explain the observed decrease in serum AMH concentration accompanying the improvement of the kidney function after a uccessful KTx. However, this hypothesis needs to be confirmed in cross-sectional studies amidst patients with different stages of CKD and in experimental animal studies.

Infertility in men with chronic kidney disease

As mentioned before, infertility is defined as the inability to conceive after a 1-year period of regular intercourse. In the general population, approx. 30–50% of infertility cases can be attributed to male factors.[35] Little is known about the prevalence and exact pathogenesis of fertility disorders in men with CKD. It seems, though, that they stem from the interconnection of 3 major causes: erectile dysfunction (ED); endocrine abnormalities; and testicular morphology alterations/impaired spermatogenesis (Table 2).

Erectile dysfunction

The prevalence of ED in CKD patients reaches 70–80%, which is much more than in the general population. Erectile dysfunction is most commonly observed in men requiring RRT.[36,37] The nature of this alteration is complex, as physiological, psychological and organic factors are involved in its pathogenesis. The most important causes of ED are cardiovascular and nervous system alterations.

Table 2. Causes of fertility disturbances in men with chronic kidney disease (CKD)

Fertility disturbances	Causes
ED	– cardiovascular disorders – decrease in the NO synthesis – autonomous nervous system dysfunction – depression and adverse effects of antidepressant medications – peripheral neuropathy – anemia – zinc deficiency – adverse effects of antihypertensive medications – obesity – smoking
Endocrine abnormalities	– low TT and FT concentrations – elevated LH and FSH concentrations – hyperprolactinemia
Testicular damage/impaired spermatogenesis	– decreased number and motility of sperm cells – Sertoli cell atrophy – testicular fibrosis – low AMH concentration – morphological extra-testicular disturbances

ED – erectile dysfunction; NO – nitric oxide; TT – total testosterone; FT – free testosterone; LH – luteinizing hormone; FSH – follicle-stimulating hormone; AMH – anti-Müllerian hormone.

Other important contributing conditions are anemia, erythropoietin (EPO) and zinc deficiency, as well as co-existing diseases and their pharmacotherapy.

Chronic kidney disease is often accompanied by CVDs. One of the critical components of CVD is endothelial dysfunction, in which the production of nitric oxide (NO) in endothelial cells is severely compromised. Nitric oxide is a crucial player in the vasodilatation of cavernous arteries, which is necessary for the accumulation of blood in the cavernous bodies, required for the initiation and maintenance of erection.[38,39] Thus, a diminished NO synthesis in the endothelium leads to the development of erectile dysfunction in this group of patients.

The amount of blood flow through the cavernous arteries is strictly orchestrated by the autonomous nervous system. In the penis flaccid state, the sympathetic nervous system is predominant and after sexual stimulation, the parasympathetic nervous system activation starts to dominate. This leads to vasodilatation and the increase of blood flow through the cavernous bodies. So unsurprisingly, autonomous nervous system neuropathy, frequently observed in uremia, contributes to the development of ED.[40]

Patients with CKD have a tendency to have a decreased blood hemoglobin concentration, which is most pronounced in CKD stage 5. Erythropoietin-stimulating agents are currently commonly used in these patients in order to restore the optimal hemoglobin concentration. Treatment with ESAs is also reported to have a beneficial influence on ED in CKD men.[41,42] At first, this effect was attributed to the serum PRL concentration decrease caused by the treatment with ESAs; still, later studies yielded conflicting results concerning this matter.[41,43] Recently, it has been proposed that ESAs may contribute to an increase in serum testosterone concentration and the regeneration of the cavernous nerve as well as antiapoptotic activity in the cavernous bodies.[44–46] These factors may lead to the decreased magnitude of ED in CKD patients treated with ESAs.

Zinc deficiency has been lately regarded as one of possible causes of EPO-resistant anemia in CKD patients.[47] This phenomenon is caused by the reduced absorption in the gastrointestinal duct and direct removal during each hemodialysis session. The results of studies conducted so far demonstrate a beneficial influence of oral zinc supplementation on the reticulocyte count, but also on an increase in serum testosterone concentration and the alleviation of ED in this group of patients.[47,48]

The prevalence of depression among CKD patients may reach 20–30%.[49,50] Depression can be another factor leading to the development of ED in this population.[51] On the other hand, the treatment of depression with tricyclic antidepressants can exacerbate the course of ED in CKD patients.[50]

Chronic kidney disease is accompanied by many comorbidities. A vast proportion of these diseases can also be linked to the pathogenesis of ED. Globally, the most prevalent cause of kidney disease is diabetes mellitus. It is one of the most prominent risk factors for ED, as it causes autonomic neuropathy and vasculopathy as well as endothelial damage.[52,53]

Hypertension leads to the aggravation of atherosclerosis and endothelial dysfunction, so it can be considered as another risk factor for the development of ED. Despite strong evidence of such a relationship in the general population,[54,55] the results of studies conducted on men with CKD are not fully conclusive.[52,55] Medications commonly used in the treatment of hypertension, such as thiazide/thiazide-like diuretics, most of beta-adrenergic receptor antagonists (β-blockers) and alfa-2-adrenegic receptor antagonists (α_2-blockers) may contribute to the aggravation of ED in CKD men.[56,57] On the other hand, the treatment with angiotensin-converting enzyme (ACE) inhibitors or angiotensin II type 1 receptor (AT1)-blockers seems to have some beneficial influence on the prevalence of ED in men on maintenance hemodialysis.[53]

Other risk factors for ED in CKD patients are obesity, especially with the body mass index (BMI) exceeding 28 kg/m^2, and dyslipidemia.[58,59] Another important modifiable risk factor for atherosclerosis, and thus ED, is cigarette smoking. The results of studies conducted so far seem to imply that smokers are characterized by higher prevalence as well as magnitude of ED as compared to non-smokers.[56,58]

Endocrine disorders in men with chronic kidney disease

Men with CKD are characterized by several sex hormone derangements. Usually, the serum concentrations of both total testosterone (TT) and free testosterone (FT)

are decreased, while the binding capacity and the serum sex hormone-binding globulin (SHBG) level remain normal.[9] Additionally, serum LH concentration is elevated in uremic men, so hypergonadotropic hypogonadism can be diagnosed. It seems, though, that the main factor responsible for the low serum TT and FT concentrations is the uremia-related injury of the testes. On the other hand, the administration of human chorionic gonadotropin (HCG) in uremic subjects results in a blunted response in testosterone release. Human chorionic gonadotropin acts through a similar mechanism as LH, so hyporesponsiveness to LH in the uremic milieu can be diagnosed, which could suggest that it is rather the resistance of Sertoli cells to LH that causes low testosterone release. Interestingly, a compound blocking luteinizing hormone receptor (LHR) was found in uremic serum, yet so far only in in vitro conditions.[60]

Low serum testosterone concentrations in CKD may lead to diminished libido, but testosterone is also crucial in the correct functioning and morphology of the penis. Testosterone deficiency leads to the replacement of smooth muscle cells in the cavernous bodies with collagen fibers.[61] This phenomenon directly links endocrine disturbances with ED, described in the previous paragraph. Interestingly, Fugl-Meyer et al. found no association between the CKD stage and/or serum TT concentration and the magnitude of sexual dysfunction in men, despite the fact that serum TT concentration decreased along with the stages of CKD.[62]

Besides the aforementioned elevation of serum LH concentration, also serum FSH concentration is increased in CKD males. This seems to be primarily caused by low serum inhibin concentration. Inhibin, which negatively regulates FSH release in the pituitary gland, is produced in Sertoli cells in the testes.[63] Another hormone produced by Sertoli cells in males is AMH. Its serum concentration in men with terminal renal failure is decreased, as it was recently shown by Eckersten et al.[64] Similarly to women, an increased serum concentration of PRL is often found in men with CKD. It is caused by increased production and decreased renal catabolism.[9]

It is important to stress that also the pharmacotherapy routinely used in men with CKD may contribute to changes in hormone concentrations and to the aggravation of fertility disorders. One of the potential examples is cinacalcet, widely used in the treatment of secondary hyperparathyroidism in CKD patients. Male patients treated with cinacalcet develop a decrease in the already low serum TT and FT concentrations.[65] Moreover, it seems that cinacalcet treatment may further decrease the already abnormally low serum AMH concentration in this group of patients (data not yet published). On the other hand, the results of recent studies seem to suggest that transdermal testosterone replacement therapy (TRT) might be safe and effective in reversing the symptoms of testosterone deficiency, and improve quality of life in men with CKD.[66]

Impaired spermatogenesis

Chronic kidney disease is usually linked to the impairment of spermatogenesis. The analyses of semen in men with CKD usually show oligozoospermia, asthenozoospermia and a decrease in the ejaculate volume. Additionally, Xu et al. described vastly decreased sperm cell viability, motility and percentage of spermatozoa with normal morphology.[67] The fertility index (defined according to the Harvey formula: sperm concentration × sperm motility × percentage of spermatozoa with normal morphology) was strikingly, almost 60 times, lower than in the control group. Further ultrastructural examinations showed the abnormalities in the heads and tails of spermatozoa as well as the lack of acrosomes.[67]

In histological examinations, the atrophy of Sertoli cells was described.[9] Further studies involving testicular biopsy analyses showed decreased germ cell proliferation and an increase in fibrosis in men with uremia.[68] Moreover, men requiring maintenance dialysis develop a decrease in the testicular volume, which tends to progress along with the consecutive dialysis years.[69] This is also reflected in a markedly decreased serum AMH concentration in men with CKD stage 5. Anti-Müllerian hormone in men is secreted by Sertoli cells to the bloodstream and the seminal fluid, and can be interpreted as a marker of the function of these cells.[64]

It is important to stress that several congenital conditions can be linked to the development of subfertility or infertility in CKD men. The presence of posterior urethral valves may be responsible for the pathogenesis of erectile and/or ejaculatory dysfunction even in young men.[70] Also, autosomal dominant polycystic kidney disease (ADPKD) may be accompanied by, for example, seminal vessel cysts and/or asthenozoospermia.[71]

Impaired fertility in men after kidney transplantation

Kidney transplantation in adult male patients seems to alleviate the derangements in the hypothalamic–pituitary–gonadal axis caused by uremia.[72,73] On the other hand, studies concerning testicular hormone concentration and the semen quality parameters yielded somewhat conflicting results.[74,75]

In a recent study by Eckersten et al., a rapid normalization of testosterone, LH and FSH as well as PRL was observed.[76] However, no significant changes in serum AMH concentration and a decrease in serum inhibin B concentration were noted,[76] suggesting that the Sertoli cell function does not improve as fast or to the same extent as the Leydig cell function.

Kidney transplantation does not fully nullify the morphological abnormalities of the testes and does not normalize the semen quality in comparison with healthy peers. Although an increase in the spermatogonium, spermatozoon and spermatocyte count was observed

in the testicular biopsies of men before and after KTx, no increase in the Sertoli cell number was found.[77] This is in line with a previously mentioned study by Eckersten et al.[76] Moreover, in other studies, only slight improvement in the morphological image of the testicular biopsies was found in men after KTx treated with azathioprine, which raises the important question of the choice of the immunosuppression protocol in young kidney allograft recipients with reproductive plans.

Tainio et al.[77] observed a smaller testes volume in men after successful KTx in childhood or adolescence in comparison with healthy controls.[77] Also, the semen quality was worse, with only 22% of patients with normospermia. This was especially prominent in the subpopulation of men treated with cyclophosphamide in childhood. Interestingly, serum androgen concentrations in kidney allograft recipients and the general population were comparable.[77]

Successful KTx also tends to ameliorate ED,[73] but the degree of improvement varies from more than 50% of patients in some studies,[78] to no improvement or even worsening in others.[79]

Effect of immunosuppression

Calcineurin inhibitors (CNI) – cyclosporine A (CsA) and tacrolimus – seem not to have a significantly negative influence on male fertility, at least within therapeutic blood concentrations,[65,79] even though animal studies showed a tendency to oligozoospermia, reduced sperm cell motility and altered testicular morphology under tacrolimus regimen.[80,81] Reduced fertility in men after KTx can be attributed to mammalian target of rapamycin (mTOR) inhibitors – sirolimus and everolimus. Those compounds may interfere in the hypothalamus–pituitary–gonadal axis and may have a negative impact on the sperm cell count and motility.[82,83] The effect of mycophenolate mofetil (MMF) and mycophenolic acid (MPA) on male fertility is not clear. Both MMF and MPA are teratogenic during pregnancy, but it seems that they have no profound negative impact on spermatogenesis and the paternity rate.[84,85]

Conclusions

Fertility disorders in women with CKD arise from a complex permeation of disturbances. Sexual function disorders, endocrine alterations with the prominent role of the hypothalamus–pituitary–ovarian axis disorders, reduced renal clearance of different hormones as well as the impairment of the ovarian function per se (e.g., a reduced ovarian reserve), all influence each other. These deteriorations together with the accumulation of uremic toxins form a composite network of disturbances, ultimately leading to the development of fertility impairment in women with CKD. In consequence, pregnancy is over 40 times rarer in women on prevalent hemodialysis than in the general population. Successful KTx in women with CKD reduces the accumulation of uremic toxins, restores the function of the endocrine system and improves fertility, but cannot reverse the damage already done to the ovaries by the uremic milieu, which is reflected by a reduced ovarian reserve. Thus, KTx vastly improves, but does not normalize fecundity in women with CKD.

Fertility disorders in men with CKD stem from a permeating network of 3 main disturbances. Erectile dysfunction, endocrine alterations with the prominent role of the hypothalamus–pituitary–testicular axis disorders and hypotestosteronemia, reduced renal clearance of different hormones as well as the direct impact of uremia on Sertoli and Leydig cells, leading to impaired spermatogenesis, all influence each other. Those deteriorations together with the accumulation of uremic toxins ultimately lead to the impairment of fertility in men with CKD. Successful KTx in men with CKD restores the function of the endocrine system and improves fertility up to a point, but cannot fully reverse the morphological damage already done to the gonads by uremia itself. Thus, also in CKD men, KTx improves, but does not normalize fecundity.

ORCID iDs

Piotr Kuczera https://orcid.org/0000-0002-7526-978X
Andrzej Więcek https://orcid.org/0000-0002-8625-4188
Marcin Adamczak https://orcid.org/0000-0002-0804-1718

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Ocular complaints from students during COVID-19 pandemic

Ada Sterczewska[1,A,B,D], Alicja Wojtyniak[1,C,D], Ewa Mrukwa-Kominek[2,E,F]

[1] Students' Scientific Society, Department of Ophthalmology, Faculty of Medical Sciences in Katowice, Medical University of Silesia in Katowice, Poland
[2] Department of Ophthalmology, Faculty of Medical Sciences in Katowice, Medical University of Silesia in Katowice, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Ada Sterczewska
E-mail: adasterczewska@gmail.com

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Abstract

Background. During coronavirus disease 2019 (COVID-19) pandemic, students were obliged to switch to online learning. Nevertheless, a long time spent in front of the screens is one of the risk factors of dry eye disease (DED).

Objectives. To evaluate ocular symptoms typical for DED presented by Polish students during online learning and entertainment before and during the pandemic, as well as to assess the prevalence of these symptoms.

Materials and methods. The original questionnaire was distributed online via social media (Facebook) to Polish students in November 2020. Three hundred sixty-eight anonymous questionnaires were collected and statistically analyzed.

Results. During the pandemic, online learning and screen entertainment time extended on average by 4 h and 40 min, respectively. Only 8% of students admitted to having no ocular symptoms and 77% reported the exacerbation in previous ocular complaints. Reported symptoms included pain/discomfort of the eyes, itchiness, dryness, red eyes, feeling gritty particles under eyelids, and blurred vision. Actions such as using eye drops, having breaks from studying to have distant vision, consultation with an ophthalmologist, using warm and cold compresses, or none of these were undertaken by 45%, 42%, 8%, 7%, and 19% of students, respectively. Nonmedical students reported worsening of previous symptoms more often than medical students ($p < 0.05$). A correlation was observed between the number of new/intensified symptoms and the change in screen learning time ($r = 0.17$, $p < 0.05$).

Conclusions. Eye complaints are prevalent in the population of students. During the pandemic these symptoms intensified, which may have been caused by the extension of the screen time. There is a need for better education on ocular hygiene to improve visual clarity and awareness of risk factors of DED.

Key words: students, online learning, dry eye disease, COVID-19, eye complaints

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Background

The coronavirus disease 2019 (COVID-19) pandemic began in December 2019 in China and changed everyone's life, including students. During the pandemic, Polish students were obliged to switch to online learning from March 2020.[1] Nevertheless, a long time spent in front of the screens is one of the well-known risk factors of the dry eye disease (DED).[2] Dry eye disease is a complex, multifactorial condition of the ocular surface where the loss of homeostasis and hyperosmolarity, as well as the instability of the tear film are observed.[3] Worldwide, this is the most common ocular disease and it is becoming more frequent along with the aging society, increasing prevalence of using digital devices and the development of a more stressful social environment.[4–7] What is more, DED has an influence on the quality of life and vision, and decreases productivity at work.[8–9] The influence of COVID-19 pandemic is much broader; its impact on psychological well-being was observed as the increase in anxiety prevalence in the general population, as well as in the specific communities such as healthcare professionals and medical students.[10–13] During a break in traditional medical training, the latter group experienced the augmented risk of mental or psychological symptoms, such as depression and suicidal ideations, as compared to general population. It can be assessed as a negative effect of COVID-19 on education, especially severe in the group of respondents in our study.[14]

Objectives

Our aim was to evaluate ocular symptoms typical for DED presented by Polish students during online learning and entertainment before and during the pandemic, as well as to assess the prevalence of these symptoms.

Materials and methods

At the beginning of the study, the interviews regarding e-learning and changes in ocular complaints during the pandemic were conducted with 20 students in October 2020. The original online questionnaire based on those students' suggestions, DED symptoms and our own ideas was prepared and distributed to Polish students in November 2020.[3] The survey was created using Google Forms and consisted of 24 questions (17 single-choice, 3 multiple-choice and 4 open questions). The questions concerned mainly basic demographic data, the field of study, DED symptoms before (while attending classes at a university) and during COVID-19 pandemic (from March to November 2020), the change in screen time spent on learning and leisure activities, and actions undertaken because of the symptoms. The survey was posted online on students' Facebook groups with a short invitation to complete it. The data collection was carried out in November 2020. A total of 377 questionnaires were obtained and 9 of them were excluded due to being incomplete or incorrect. The data was collected and analyzed anonymously.

Statistical analyses

Statistical analyses included descriptive methods as well as the association and significance of different methods. The χ^2 tests were performed for the categorical data. When the class size was less than 5, the Fisher's exact test was used. For quantitative data, the Shapiro–Wilk test was used to assess data distribution normality. We applied Mann–Whitney U test in order to compare the differences among the groups as the number of new/intensified symptoms, age (years), the change in screen learning time (h) and the change in screen entertainment time (h) were found to be non-normally distributed (more details available in Table 5). Spearman's rank correlation was calculated to compare the number of new/intensified symptoms with the change in screen learning time. A p-value <0.05 was considered statistically significant.

Results

Data were collected from 368 students, out of which 82% were female. Mean age was 23 years (95% confidence interval (95% CI): [22.7; 23.27]). The median duration of studying was 4 years. Fifty-six percent of the participants were medicine students, others chose nonmedical courses of study. The largest group of respondents were students of the Medical University of Silesia, Katowice, Poland (40%).

An autoimmune disease was reported by 7% of respondents. Seven percent of students were active smokers. As many as 41% wore glasses every day, 22% wore glasses occasionally and 37% did not wear glasses at all. About 8% of students declared everyday use and 14% declared occasional use of contact lenses. There were 34% of students under the ophthalmologist's care before the time of pandemic, out of which 82% were under the ophthalmologist's care due to the refractive error. One interviewee's cause was DED. Other diagnoses included strabismus, lymphangioma, demodicosis, nystagmus, and suspicion of glaucoma.

The online learning time during the pandemic, in comparison to the period before the pandemic, extended according to 94%, remained the same according to 5% and shortened according to 1% of respondents. On average, online learning time extended by 4 h (95% CI: [3.86; 4.15]). A correlation was observed between the number of new/intensified symptoms and the change in screen learning time ($r = 0.1750$, $p < 0.05$, degrees of freedom (df) = 285, Spearman's rank correlation). A prolonged screen entertainment time was reported by 47% of students, no change by 37% and 16% of students decided

Table 1. The ocular symptoms that appeared for the first time during coronavirus disease 2019 (COVID-19) pandemic

New symptoms during COVID-19	Number of students	% of all students (n = 368)	% of students with new symptoms (n = 132)
Itchiness	61	17	46
Pain/discomfort of the eyes	71	19	54
Dryness of the eyes	55	15	42
Feeling gritty particles under eyelids	35	10	27
Red eyes	43	12	33
Discharge	5	1	4
Other	6	2	5

Table 2. The ocular symptoms that intensified during coronavirus disease 2019 (COVID-19) pandemic

More severe symptoms during COVID-19 pandemic	Number of students	% of all students (n = 368)	% of students with more severe symptoms (n = 282)
Itchiness	169	46	60
Pain/discomfort of the eyes	179	49	63
Dryness of the eyes	187	51	66
Feeling gritty particles under eyelids	54	15	19
Red eyes	116	32	41
Discharge	20	5	7
Other	7	2	2

to cut down the entertainment time. It was extended by about 40 min/0.6 h on average (95% CI: [0.44; 0.76]).

Reported symptoms included pain/discomfort of the eyes, itchiness, dryness of the eyes, red eyes, feeling gritty particles under eyelids, and blurred vision. A detailed distribution of new symptoms is demonstrated in Table 1 and the distribution of the symptoms that intensified during COVID-19 is shown in Table 2. The mean number of the new/intensified symptoms was 3.

A deterioration of visual acuity during COVID-19 pandemic was declared by 41% of participants, 44% of whom noticed the improvement after blinking a few times. About 21% of interviewees were uncertain about their vision and 39% denied any problems with vision acuity. Actions such as using eye drops, having breaks from studying to have distant vision, consultation with an ophthalmologist, using warm and cold compresses on eyes, or none of these were undertaken by 45%, 42%, 8%, 7%, and 19% of students, respectively.

Only 3% of students complained about ocular symptoms for the first time during COVID-19 pandemic. Twelve percent of participants stated that their symptoms started before the pandemic and did not change. More symptoms or increased severity of symptoms were observed in 77% of cases. Only 8% of respondents claimed to have no ocular problems. Therefore, we decided to compare students with no change (NC) in symptoms (asymptomatic/with the same intensity in symptoms) with those who experienced more symptoms or their symptoms became more severe during the pandemic – change group (C). Online learning time increased in the change group to a greater extent ($p = 0.0003$, $df = 2$, $\chi^2 = 16.25$), although the group was more eager to decrease the time spent on online entertainment compared to those unaffected ($p = 0.0016$, $df = 2$, $\chi^2 = 12.84$). Moreover, they experienced decreased visual acuity more often than the other group (C: 45%, NC: 20%; $p < 0.0001$, $\chi^2 = 18.53$). Students from the change group used eye drops more frequently (C: 48%, NC: 16%; $p < 0.0001$, $df = 1$, $\chi^2 = 24.88$), and more of them took breaks from studying to look into the distance (C: 47%, NC: 17%; $p < 0.0001$, $df = 1$, $\chi^2 = 21.87$). A more detailed analysis of the results is presented in Table 3.

Nonmedical students (N) reported worsening of the previous symptoms more often than medical students (M) (N: 89%, M: 72%; $p < 0.0001$, $df = 1$, $\chi^2 = 17.01$). The use of eye drops in symptomatic participants was similar in both groups (N: 39%, M: 43%; $p = 0.3369$, $df = 1$, $\chi^2 = 0.92$), as well as consulting the ophthalmologist (N: 6%, M: 9%; $p = 0.1977$, $df = 1$, $\chi^2 = 1.66$). Twenty-two percent of participants from nonmedical fields and 18% of those from the medical field denied taking any actions because of their symptoms ($p = 0.2844$, $df = 1$, $\chi^2 = 1.15$). Taking regular breaks from studying was declared by 48% of nonmedical students compared to 36% of medical students ($p = 0.0195$, $df = 1$, $\chi^2 = 5.46$). The comparison of medical and nonmedical students is presented in Table 4.

Discussion

To our knowledge, this is the first research on the symptoms of DED in Polish students during COVID-19 pandemic. Nevertheless, the recent studies on the influence

Table 3. The comparison of students who experienced no change in their ocular symptoms and those who reported more symptoms or more severe symptoms than before the coronavirus disease 2019 (COVID-19) pandemic

Category		Students with no change in symptoms (no change group (n = 75))	%	Students with more severe/more symptoms (change group (n = 293))	%	Test value	df	p-value
Female		47	63	255	87	24.08[a]	1	**<0.0001**
Male		28	37	38	13			
Age		23 ±3 years	–	23 ±1.5 years	–	8947.00[b]	N/A	**0.0012**
Medical students		58	77	149	51	17.01[a]	1	**<0.0001**
Nonmedical students		17	23	144	49			
Spectacles	everyday	26	35	124	42	1.57[a]	2	0.4570
	sometimes	17	23	63	22			
	no	32	43	106	36			
Contact lenses	everyday	5	7	24	8	0.27[a]	2	0.8749
	sometimes	10	13	42	14			
	no	60	80	227	77			
Currently smoking		7	9	20	7	0.55[a]	1	0.4574
Autoimmunologic diseases		4	5	22	7.5	–[c]	1	0.6211
Medications which can cause DED		27	36	101	34	0.39[a]	2	0.8210
Time spent on online learning	increased	64	85	281	96	16.25[a]	2	**0.0003**
	no changes	11	15	9	3			
	decreased	0	0	3	1			
Time spent on online entertainment	increased	41	55	131	45	12.84[a]	2	**0.0016**
	no changes	32	43	104	35			
	decreased	2	3	58	20			
Under ophthalmologist's care		20	27	107	37	2.56[a]	1	0.1093
Decreased vision acuity		15	20	133	45	18.53[a]	2	**<0.0001**
Breaks		13	17	138	47	21.87[a]	1	**<0.0001**
Eye drops		12	16	140	48	24.88[a]	1	**<0.0001**
No actions		19	25	54	18	1.79[a]	1	0.1810

Tests used: [a] χ^2 test; [b] Mann–Whitney U test; [c] Fisher's exact test; DED – dry eye disease; df – degrees of freedom. Bold indicates statistically significant results ($p < 0.05$).

of increased time in front of a visual display terminal (VDT) on the ocular complaints from students during pandemic – Giannaccare et al.[15] and Cartes et al.[16] – showed many similarities to our observations. The high prevalence of DED symptoms in students, as well as a considerably extended time in front of a VDT during pandemic correlated with the deterioration of symptoms. Moreover, previous studies involving VDT users before the pandemic, pointed the same risk factors of DED, that being female sex and increasing age, similarly to our research.[17,18] Furthermore, the prevalence of ocular symptoms related to DED was higher in our study (92%) in comparison to the paper of Stapleton et al. (5–50%).[19] In our opinion, the small number of students who decided to consult their symptoms with an ophthalmologist (8%) could be justified by a low awareness, a more difficult access to healthcare during COVID-19 pandemic, the self-medication, a bearable intensification of symptoms, or all of the above. We understand that it is the first comparison of ocular symptoms in students of medical and nonmedical courses. Medical students should supposedly be better educated on visual hygiene as well as symptoms and treatment of DED. Nonetheless, knowledge alone is sometimes not enough. It is thought-provoking that nonmedical students decided to reduce their time of online entertainment more often compared to their counterparts studying medicine. We have observed that conducting more and more activities and events such as courses, conferences, meetings, or sport classes online, could potentially intensify ocular symptoms globally. Therefore, there is a need for better education on ocular hygiene, breaks at work for improving visual clarity and awareness campaigns about risk factors of DED.[17] The ocular complaints reported by students involved in e-learning should have prompted them to monitor their time spent in front of the electronic devices, and take proper actions in case of presenting any symptoms.

Considering the rapid growth of use of electronic devices all around the world, there is a need for further research

Table 4. Comparison of medical and nonmedical students

Category		Medical students (n = 207)	% of medical students	Nonmedical students (n = 161)	% of nonmedical students	p-value	Test value	df
Female		152	73	150	93	**<0.0001**	23.97[a]	1
Male		55	27	11	7			
Age		23 ±1.5 years	–	22 ±3.75 years	–	**<0.0001**	9165.00[b]	N/A
Change in ocular symptoms		149	72	144	89	**<0.0001**	17.01[a]	1
No change in symptoms		58	28	17	11			
Spectacles	everyday	85	41	65	40	0.9328	0.1391[a]	2
	sometimes	46	22	34	21			
	no	76	37	62	39			
Contact lenses	everyday	20	10	9	6	**0.0301**	7.01[a]	2
	sometimes	36	17	16	10			
	no	151	73	136	84			
Currently smoking		11	5	16	10	0.0915	2.85[a]	1
Autoimmune diseases		15	7	11	7	0.8778	0.024[a]	1
Medications which can cause DED		78	38	50	31	0.3219	2.2670[a]	2
Time spent on screen learning	increased	190	92	155	96	0.0666	5.42[a]	2
	no changes	16	8	4	2			
	decreased	1	1	2	1			
Time spent on screen entertainment	increased	108	52	64	40	**0.0021**	12.35[a]	2
	no changes	77	37	59	37			
	decreased	22	11	38	24			
Decreased vision acuity		84	41	64	40	0.4916	1.42[a]	2
Breaks to look into the distance		74	36	77	48	**0.0195**	**5.46**[a]	**1**
Eye drops		90	43	62	39	0.3369	0.92[a]	1
No actions		37	18	36	22	0.2844	1.15[a]	1
Ophthalmologist consult		19	9	9	6	0.1977	1.66[a]	1

Tests used: [a] χ^2 test; [b] Mann–Whitney U test; DED – dry eye disease; df – degrees of freedom. Bold indicates statistically significant results ($p < 0.05$).

Table 5. Normality test – the results of Shapiro–Wilk test

Tested variable	W	p-value
The number of new/intensified symptoms	0.9269	**<0.0001**
Age [years]	0.6653	**<0.0001**
Change in screen learning time [h]	0.9441	**<0.0001**
Change in screen entertainment time [h]	0.9243	**<0.0001**

Bold indicates statistically significant results ($p < 0.05$).

aimed at investigating new ways to protect the eyes, as well as for the treatment of symptoms caused by the exposure to prolonged screen time.

In our study, we did not examine anxiety or coping styles of students during COVID-19 pandemic in the context of DED. However, after analyzing current literature, we considered that aspect of the pandemic as significant, especially among Polish students. A high level of anxiety and stress was experienced by that group during the pandemic. Additionally, a negative impact of COVID-19 was observed more often in female rather than male students. Therefore, it was recommended to support students by providing them with psychological help during the time of the pandemic.[20] Furthermore, older Polish population represented by participants of the University of the Third Age showed various levels of anxiety because of the pandemic, but none of the collected results in applied scales were elevated enough to identify high COVID-19-related anxiety.[21]

Limitations

The first limitation is related to the use of the original, not validated, self-report questionnaire. Secondly, the use of contact lenses has not been studied thoroughly. However, a small number of students declared themselves as lens users. Various stages of the pandemic and the regulations considering online and on-site learning may have had varying impacts on student populations. Therefore, the findings of the study should be interpreted in the specific context of November 2020, the time of lockdown in Poland, when online learning was a basis of education on the university level.

Conclusions

Bearing our results in mind, we claim that eye complaints are extremely prevalent in the population of students. What is more, during the pandemic these symptoms intensified, which seemed to be caused by the extension of the screen time. The ocular complaints reported by students involved in e-learning should have prompted them to monitor their time spent in front of the electronic devices and take proper actions regarding the presenting symptoms. Finally, there is a need for better education on ocular hygiene to improve visual clarity and awareness of risk factors of DED.

ORCID iDs

Ada Sterczewska https://orcid.org/0000-0002-0819-8512
Alicja Wojtyniak https://orcid.org/0000-0001-8750-3011
Ewa Mrukwa-Kominek https://orcid.org/0000-0002-1541-4666

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The assessment of pregnant women's risk awareness and dangers resulting from gestational diabetes: A preliminary report

Anna Karpiewska[D], Sonia Jarczak[B,C], Małgorzata Małodobra-Mazur[A,E,F]

Division of Molecular Techniques, Department of Forensic Medicine, Faculty of Medicine, Wroclaw Medical University, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Anna Karpiewska
E-mail: anna.karpiewska@umw.edu.pl

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Abstract

Background. During pregnancy, 2 main types of carbohydrate tolerance disorders may occur: pregestational diabetes mellitus and gestational diabetes mellitus (GDM). Gestational diabetes mellitus constitutes 90% of the cases diagnosed during pregnancy; 10% of the cases are previously undetected type 1 diabetes. In the subsequent pregnancy, in as many as 30% of the women, GDM will occur again.

Objectives. To determine the level of awareness of the women diagnosed with GDM concerning the diagnosis and self-control of diabetes, as well as the risk of poorly controlled or treated gestational diabetes. In particular, the attention was paid to the women's awareness of self-control and dietary behavior, depending on their age, education, number of pregnancies, and quality of medical care.

Materials and methods. One hundred women with gestational diabetes were accepted as the study group. To achieve the research goal, the study used a questionnaire consisting of 46 questions.

Results. As a result of the analysis, a relatively high level of awareness was found among 31.3% of the women aged 19–24, which decreased with age. It was noticed that the level of women's awareness of metabolic complications in pregnancy did not increase along with the potential experience and practically acquired knowledge related to earlier pregnancy. However, with age, the awareness of the need to change the lifestyle with focus on physical activity increased, although it did not matter whether it was the first or the subsequent pregnancy.

Conclusions. The results emerging from this study provide a perfect basis for conducting further research in a given direction, as they highlight many dependencies that can potentially influence the awareness of various aspects related to gestational diabetes.

Key words: pregnancy, diabetes, women, awareness, gestational

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Background

Gestational diabetes mellitus (GDM) constitutes 90% of the cases of hyperglycemia diagnosed during pregnancy. Insulin resistance is considered to be the leading cause of GDM. In most healthy women, blood insulin levels are approx. 50% higher at the end of pregnancy, which covers the increased demand resulting from developing insulin resistance. About 5% of pregnant women have a relative insulin deficiency, which is the cause of the development of GDM, due to the greater demand of the body for insulin.[1–7] Many additional risk factors increase insulin resistance and insulin deficiency: becoming pregnant after 35 years of age, giving birth to a child with the body weight >4000 g, having a child with a developmental defect, intrauterine fetal death, hypertension, obesity or overweight, having a family history of type 2 diabetes, GDM in previous pregnancies, giving birth to many children, or polycystic ovary syndrome.[2,3,8] The American Diabetes Association (ADA) annually publishes recommendations for good quality of medical care in diabetes.[9] High level of medical care characterized in this study should provide the women with the basic knowledge of self-control, dietary or pharmacological treatment, and lifestyle changes.

Current Polish epidemiological data indicate the frequency of GDM at the level of 3–12% in the population of pregnant women (21.64% – in women over 35 years of age). In the USA, the level of about 9% is noted.[10,11] The epidemiological data from the registers of DeSisto et al.[12] and the estimates of the prevalence of GDM in the Pregnancy Risk Assessment Monitoring System (PRAMS) show the frequency of GDM at the level of 1–25% in the population of pregnant women. The percentage discrepancy depends on the pregnant women's ethnicity and the diagnostic criteria used in a given region.

Uncontrolled and untreated GDM can lead to many complications in the mother, fetus and newborn child. Pregnant women's level of knowledge and awareness is crucial. This applies to diagnostics, glycemic control skills, appropriate lifestyle regulation, as well as the risk assessment of poorly controlled or treated GDM. The pharmacological treatment of hyperglycemia in pregnant women with GDM is based mainly on insulin therapy. In most women with GDM, the course of the disease is mild, which allows for the avoidance of insulin therapy. Nevertheless, in approx. 20–30% of women, insulin is necessary to achieve normoglycemia.[8] The most common guidelines for the treatment of hyperglycemia are a proper diet and physical activity, improving the sensitivity of tissues to insulin and having a beneficial effect on psychophysical fitness.[13]

Objectives

The study aimed to determine the level of awareness of the women diagnosed with GDM, concerning the diagnosis and self-control of diabetes, as well as the risk of poorly controlled or treated GDM. In particular, attention was paid to the women's awareness of self-control and dietary behavior, depending on their age, education, number of pregnancies, and quality of medical care.

Materials and methods

One hundred women diagnosed with gestational diabetes were enrolled in the study. The GDM was diagnosed following the recommendations in force in Poland[1–3,14] and oral glucose tolerance test (75 g) (OGTT). The diagnostic criteria for plasma glucose concentration were consistent with those recommended by the Polish Diabetes Association [mg/dL], that is: the inclusion criteria to the study were the results of the OGTT (75 g) 92–125 (fasting), ≥180 (after 60 min), 153–199 (after 120 min). The criteria for the diagnosis of gestational diabetes based on the OGTT test are described in Fig. 1.

Questionnaire

The study was based on a questionnaire distributed among the surveyed women between January and April

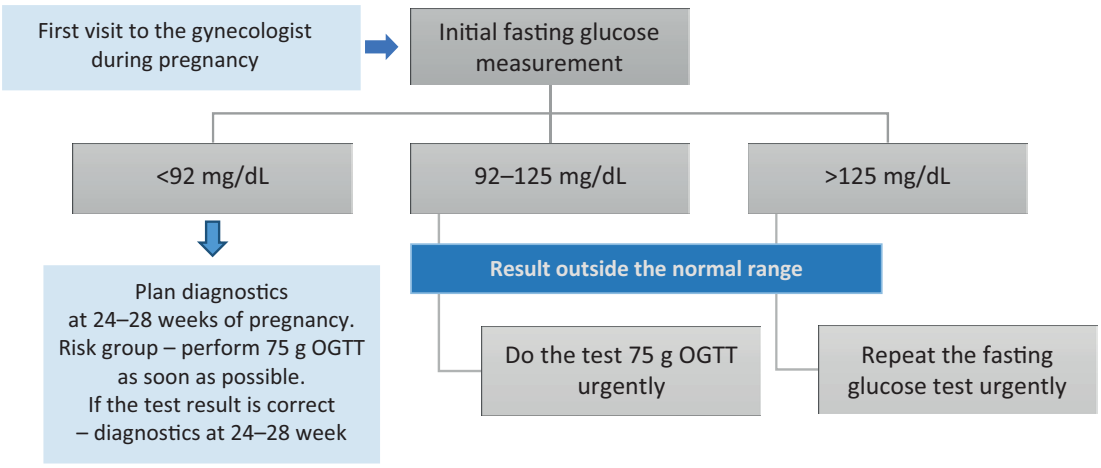

Fig. 1. The criteria for the diagnosis of gestational diabetes based on the oral glucose tolerance test (OGTT)[2,3] (self-modification)

2020. The questions in the survey were divided into 7 parts, depending on the subject of the questions: I – general data concerning age, education and possible previous pregnancies; II – the awareness of the correct values of carbohydrate metabolism; III – the risk of developing diabetes; IV – the risk of GDM for a woman/fetus; V – medical care in pregnancy; VI – self-monitoring and dietary and pharmacological treatment; and VII – change in lifestyle and physical activity.

Statistical analyses

At the beginning of the research procedure, the level of risk awareness and dangers in pregnant women resulting from GDM were assessed. The points considered to be the indicators of the awareness level, awarded for providing the correct answers to the self-questionnaire questions, were summed up. In the case of the multiple-choice questions, points were awarded for not giving incorrect answers. The possible number of earned points was from 0 to 29 points. After the standardization of the results to the value of 100, the percentage of the correct responses was obtained. These results were divided into the following ranges: low level (<66% of the correct answers), average level (66%–80% of the correct answers) and high level (>81% of the correct answers) (Table 1). The percentage of correct answers was used to assess the general awareness of GDM among surveyed women.

To assess the quality of medical care, a new variable was created with 2 categories of responses: better and worse. The respondents who answered "yes" to at least 4 out of 6 questions concerning this area, were considered to be women provided with better medical care.

The level of statistical significance was set at $p < 0.05$. The variables expressed at the ordinal or nominal level were analyzed using χ^2 test. Parametric test (Student's t-test (t)) or its nonparametric equivalents, which compare the medians of the dependent variable in individual groups (Mann–Whitney U (MW) test or Kruskal–Wallis test (KWT)) were used to analyze the quantitative variables by a group. The selection of tests was based on the distribution of the variables, which was verified with the Shapiro–Wilk test (Table 2). The calculations were made in the R v. 3.6.0 statistical environment (R Foundation for Statistical Computing, Vienna, Austria), with the PSPP program (Alberta Pensions Services, Edmonton, Canada; free software supported by the Free Software Foundation, GNU General Public License (GNU GPL) https://www.gnu.org/software/pspp/) and MS Excel 2019 (Microsoft, Redmond, USA).

Results

The study participants were divided into groups depending on their age: 19–24 (16%), 25–34 (63%) and >34 (21%). The vast majority of the women had higher education

Table 1. Descriptive statistics of individual parts of the survey

Parameter	n	M	SD	Min	Max	Q25	Me	Q75
General awareness [points]	100	20.57	4.03	9.00	29.00	18.00	21.00	23.25
General awareness [%]	100	70.92	13.89	31.00	100.00	62.00	72.00	80.00
Correct values of carbohydrate metabolism [points]	100	8.29	1.97	3.00	11.00	7.00	8.00	10.00
Correct values of carbohydrate metabolism [%]	100	75.52	17.91	27.00	100.00	64.00	73.00	91.00
Risk of developing gestational diabetes [points]	100	2.36	1.06	0.00	4.00	2.00	2.00	3.00
Risk of developing gestational diabetes [%]	100	59.00	26.48	0.00	100.00	50.00	50.00	75.00
Risk of GD for a woman/fetus [points]	100	4.67	1.14	1.00	6.00	4.00	5.00	5.00
Risk of GD for a woman/fetus [%]	100	77.78	18.88	17.00	100.00	67.00	83.00	83.00
Medical care in pregnancy [points]	100	3.68	1.68	0.00	6.00	3.00	4.00	5.00
Medical care in pregnancy [%]	100	61.38	27.88	0.00	100.00	50.00	67.00	83.00
Self-monitoring and dietary and pharmacological treatment [points]	100	1.57	0.64	0.00	2.00	1.00	2.00	2.00
Self-monitoring and dietary and pharmacological treatment [%]	100	78.50	1.98	0.00	100.00	50.00	100.00	100.00

n – number; M – mean; SD – standard deviation; Min – minimum; Max – maximum; Q25 – 1st quartile; Me – median; Q75 – 3rd quartile; GD – gestational diabetes.

Table 2. The results of the normal distribution of the studied variables obtained with the Shapiro–Wilk test

Hypothesis	Statistic	p-value
The relationship between age and risk of developing gestational diabetes	0.960	0.004
The relationship between education and normal values of carbohydrate metabolism	0.948	<0.001
The relationship between education and self-control and treatment	0.750	<0.001
The relationship between first or subsequent pregnancy and risk of developing gestational diabetes	0.962	0.005
The relationship between gestational diabetes in a previous pregnancy and normal values of carbohydrate metabolism	0.975	0.534

Table 3. Characteristics of the study group

Parameter	Frequency	Percent
Age		
19–24	16	16.00
25–34	63	63.00
>34	21	21.00
Education		
Primary	1	1.00
Vocational	2	2.00
Secondary	24	24.00
Higher	73	73.00
First pregnancy		
Yes	62	62.00
No	38	38.00
GDM in a previous pregnancy		
Yes	14	14.00
No	24	24.00
Not applicable	62	62.00

GDM – gestational diabetes mellitus.

(73%), 24% secondary, 2% vocational, and 1% primary. Among the surveyed women, 62% were in their first pregnancy, while 14% of the remaining 38% had GDM in the past (Table 3).

First, the relationships between the general awareness of GDM and age, education, number of pregnancies, and the experience of diabetes in the past were examined. However, the individual areas of the respondents' awareness to which the subsequent hypotheses referred to were so detailed that the number of points that could be obtained within each of them indicated relatively small awareness, concerning: correct values of carbohydrate metabolism (11 points), risk of GDM development (4 points), health consequences for woman or fetus (6 points), medical care during pregnancy (6 points), as well as self-control and dietary and pharmacological treatment (2 points). Hence, these areas could not create comparable levels of awareness. Therefore, the analysis was based on the quantified data, i.e., the percentage of the correct answers.

There was no difference in the awareness of the GDM regarding the age of the women, their education (after the removal of minor categories: primary and vocational), the number of pregnancies, and previous GDM in the past (Fig. 2).

However, interesting results were obtained when a detailed analysis of the relationship among the awareness of the correct values of carbohydrate metabolism, the health risks of GDM for the woman and the fetus, medical care during pregnancy, self-control, and dietary and pharmacological treatment, as well as the respondents' age, education and the number of pregnancies

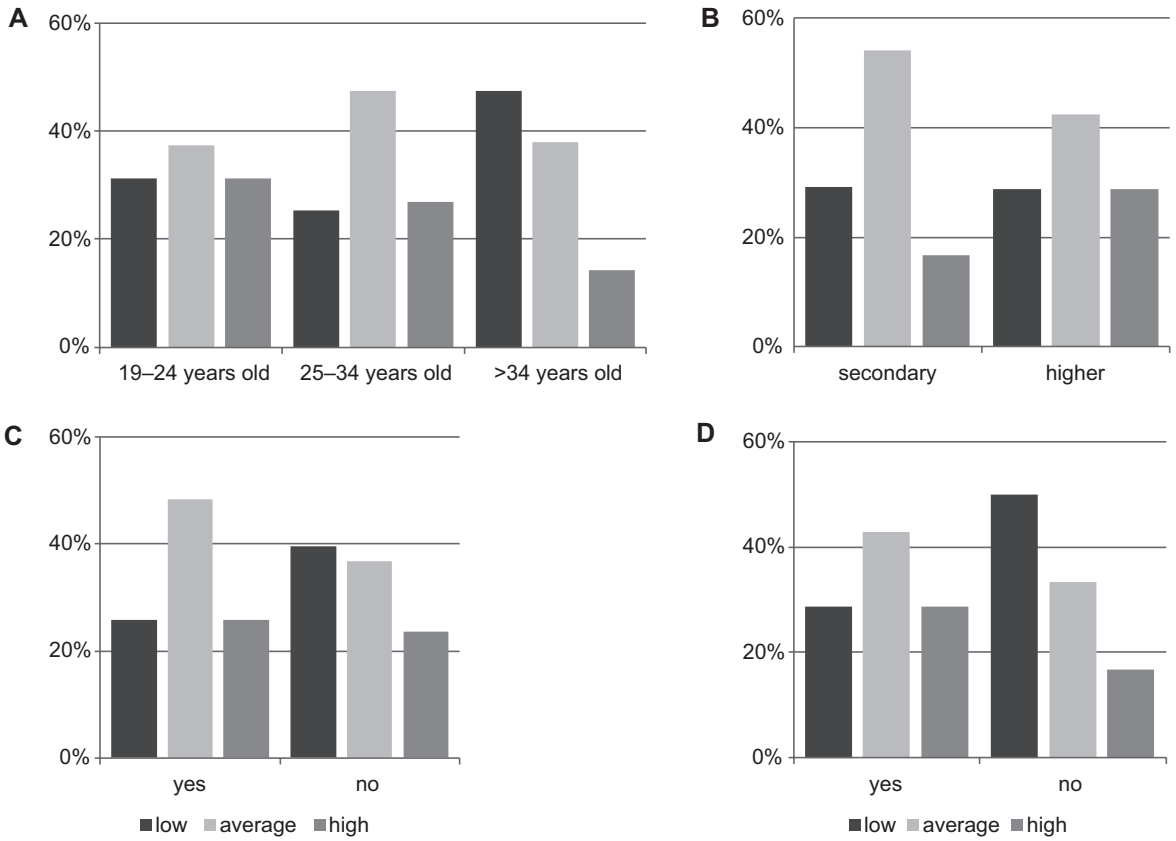

Fig. 2. Relationship between the level of the respondents' general awareness of gestational diabetes mellitus (GDM) and age (A), education (B), the first or subsequent pregnancy (C), and the experience of diabetes in the previous pregnancy (D)

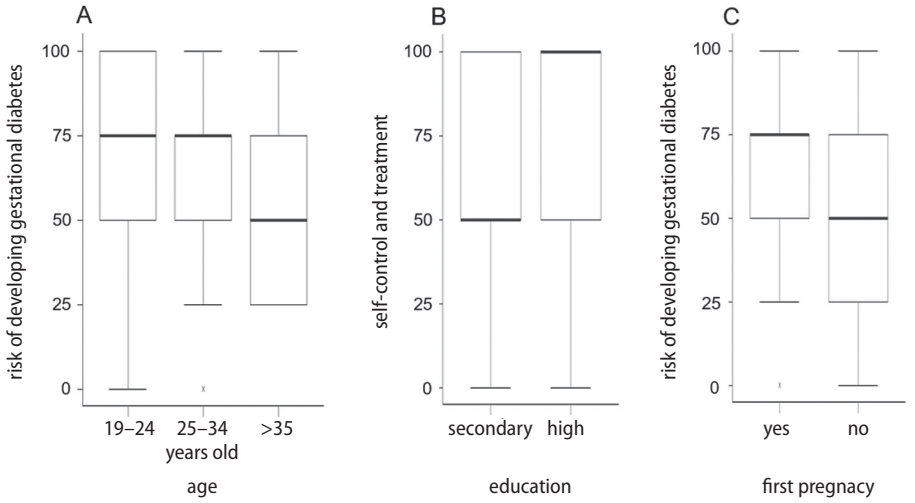

Fig. 3. Relationship between the level of awareness in terms of: A. the risk of developing gestational diabetes and the age of the respondents (Kruskal–Wallis test); B. self-control, treatment and the education of the respondents (Mann–Whitney U test); C. the risk of developing gestational diabetes and the first or subsequent pregnancies of the examined women (Mann–Whitney U test). Box plots show the summary of the data: lower whisker – minimum; central 'box' (lower quartile (Q1), median (Q2) and upper quartile (Q3)); upper whisker – maximum; and outlier (x) – extreme data point

Table 4. Relationship between the awareness of the risk of developing gestational diabetes mellitus (GDM) and the age of the examined women. Results of non-parametric Kruskal–Wallis test

Parameter	Descriptive statistics						
	age [years]	χ^2	df	p-value	Min	Max	Me
Risk of developing gestational diabetes	19–24	6.58	2	**0.037**	0.00	100.00	75.00
	25–34				0.00	100.00	75.00
	>34				25.00	100.00	50.00

df – degrees of freedom; Min – minimum result; Max – maximum result; Me – median. Values in bold are statistically significant.

Table 5. Relationships between the level of the awareness of the risk of developing gestational diabetes and the age of the respondents. Results of non-parametric Kruskal–Wallis test

Parameter	Age groups [years]		p-value
Risk of developing gestational diabetes	19–24	25–34	0.421
	19–24	>34	**0.042**
	25–34	>34	0.312

Values in bold are statistically significant.

(including pregnancies with accompanying GDM) were performed. A statistically significant ($p < 0.05$, KWT) higher level of awareness of the risk of developing GDM was observed among the examined women aged 19–24 compared to those aged >34 (Table 4,5; Fig. 3A). The surveyed women also differed in the level of the awareness of carbohydrate metabolism in a statistically significant way ($p < 0.05$, MW), depending on their education level (Table 6; Fig. 4B). The women with higher education achieved significantly higher results than the respondents with secondary education. A statistically significant ($p < 0.05$, MW) higher level of the awareness of glycemic self-control and adherence to the recommended (dietary or pharmacological) treatment was also observed among the women with higher education compared to the women with secondary education (Table 6; Fig. 3B). The women

Table 6. The relationship between the awareness of the correct values of carbohydrate metabolism and glycemic self-control, adherence to the recommended (dietary or pharmacological) treatment and respondents' education, and between the awareness of gestational diabetes and the respondents' first or subsequent pregnancy. Results of non-parametric Mann–Whitney U test

Dependencies among themselves		U	p-value	Min	Max	Me
Education	Normal values of carbohydrate metabolism	636.50	**0.043**		N/A	
	secondary	N/A		36.00	100.00	73.00
	high	N/A		27.00	100.00	82.00
Education	Self-control and treatment	660.50	**0.031**		N/A	
	secondary	N/A		0.00	100.00	50.00
	high	N/A		0.00	100.00	100.00
First pregnancy	Risk of developing gestational diabetes	838.50	**0.012**		N/A	
	yes	N/A		0.00	100.00	75.00
	no	N/A		0.00	100.00	50.00

Me – median; Min – minimum result; Max – maximum result; N/A – not applicable. Values in bold are statistically significant.

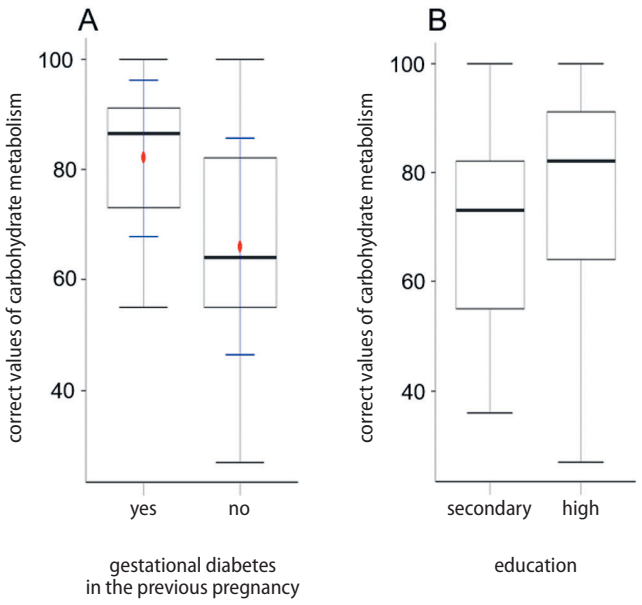

Fig. 4. Relationship between awareness: A. concerning the normal values of carbohydrate metabolism and the experience of diabetes in a previous pregnancy (Student's t-test); Box plots show the summary of the data: lower whisker – minimum, blue transverse line on the lower and upper whisker – standard deviation (SD), central box (mean values are indicated by the red dot symbol (M)); upper whisker – maximum; B. concerning the correct values of carbohydrate metabolism and the education of the respondents (Mann–Whitney U test); Box plots show the summary of the data: lower whisker – minimum, central 'box' (lower quartile (Q1), median (Q2) and upper quartile (Q3)); upper whisker – maximum; and outlier (x) – extreme data point

who experienced GDM in previous pregnancies had a much higher level of knowledge of the correct values of carbohydrate metabolism than the women who had never dealt with diabetes before (p = 0.012, t; Table 7; Fig. 4A). At the same time, in the analysis of the awareness of the pregnant women related to the risk of developing GDM, a statistically higher level of awareness was found among the women giving birth for the first time than among the multiparous women (p = 0.012, MW; Table 6; Fig. 3C). However, the examined women did not differ in the level of awareness in the remaining relationships related to age, education and the number of pregnancies, in a statistically significant way (p > 0.05, MW).

It can also be noticed that 43.8% of the women aged 19–24 years significantly more often did not pay attention to the glycemic index (GI) of products (p = 0.033, χ^2; Table 8; Fig. 5), compared to the women aged 25–34, among whom only 14.3% did not pay attention to the GI, and to the women over 34 years old, among whom only 23% did not pay attention to GI. The impact of the quality of medical care on the type of food consumed was observed. Most women (89.2%) who received better medical care and 60.0% of those who received worse medical care paid attention to the GI of products found in their daily diets. The pregnant women with better medical care significantly more often (p = 0.002, χ^2) noticed the GI of the consumed products (Table 8; Fig. 5). At the same time, the women with varying degrees of education equally often paid attention to the GI of products in their diets. They did not differ in physical activity in different age and education groups. Their number of training units per week was similar, and their level of lifestyle changes due to GDM did not differ statistically (p > 0.05, χ^2).

Discussion

The aim of the study was to assess the level of the women's awareness concerning various health aspects resulting from the diagnosis of GDM. It may be noted that the percentage of women with a high level of awareness

Table 7. Relationship between gestational diabetes mellitus (GDM) awareness and previous pregnancy diabetes experience. Results of parametric Student's t-test

Parameter		t	df	p-value	M	SD
Normal values of carbohydrate metabolism		2.6	36	0.012	N/A	
Gestational diabetes in a previous pregnancy	yes	N/A			82.00	14.12
	no	N/A			66.04	19.68

df – degrees of freedom; M – mean; SD – standard deviation; N/A – not applicable. Values in bold are statistically significant.

Table 8. Relationship among lifestyle changes and physical activity in pregnancy and the age of the respondents and the quality of medical care in pregnancy. Results of the χ^2 test

Parameter			Age [years]			Medical care	
			19–24	25–34	>34	better	worse
Paying attention to the glycemic index of products found in the daily diet	yes	n	9	54	16	58	21
		%	56.3	85.7	76.2	89.2	60
	no	n	7	9	5	7	14
		%	43.8	14.3	23.8	10.8	40
Results of the test			χ^2 = 6.803, df = 2, **p = 0.033;** χ^2 = 10.021, df = 1, **p = 0.002**				

df – degrees of freedom; p – p-value. Values in bold are statistically significant.

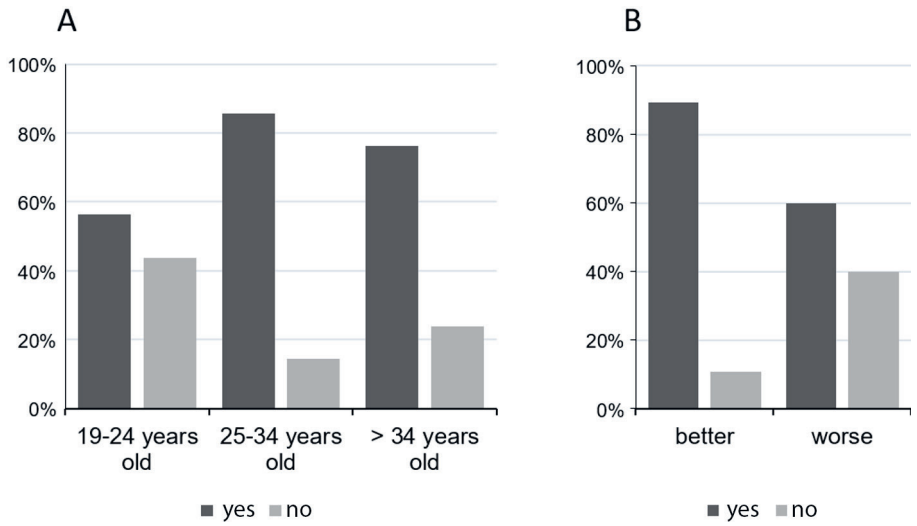

Fig. 5. Relationship between paying attention to the glycemic index and A. the age of the pregnant women, B. the level of medical care

decreases with age. With increasing age, the society spends less and less time on education, whereas the age of 19–24 is one of the most intensive stages of learning, which may be the reason for the increased awareness in this group of the surveyed women. The study participants of >34 years of age may use outdated knowledge, which may also be the reason for the smaller number of the women with a high level of awareness in this group. Higher education determined the greater percentage of the women with a high level of awareness compared to the pregnant women with secondary education. Nevertheless, no statistically significant relationship was found between the respondents' level of awareness and their education. Although the result is not statistically significant, the difference is noticeable. The results showed no correlation between the number of experienced pregnancies and the level of general awareness of GDM. We have also noticed a difference in the low level of awareness between the 1st and 2nd or subsequent pregnancies. There were significantly fewer women giving birth for the first time with a low level of awareness than the women giving birth in the past. This suggests that the level of the women's awareness of metabolic complications in pregnancy does not increase with the potential experience and the practically acquired knowledge related to the experience of earlier pregnancy. The experience of gestational diabetes in a previous pregnancy resulted in a slightly higher level of women's awareness, compared to the respondents who did not experience GDM. Worth noting is the result related to the high number of pregnant women having a low level of awareness; they were not burdened with GDM in a previous pregnancy.

Similar research using the questionnaire had already been carried out in the world before in such countries as Australia, India and Samoa.[15–17] The authors of these studies pointed to the need for education in order to improve the awareness of GDM. In 2019, the questionnaire was used in India as a research method to examine 523 pregnant women.[18] Thomas et al. drew attention to the need for raising women's awareness and knowledge of GDM, as it may have an impact on identifying the disease at an earlier stage and thus reducing its effects on the mother and child.[18] A similar study was carried out in 2019 in Norway (in the Oslo region) among 238 pregnant women in a multiethnic population, out of which 108 (45.4%) were native speakers of Norwegian, while 130 (54.6%) were from Asia, Africa and Eastern Europe. Based on a survey conducted in 3 languages (Norwegian, Urdu and Somali), the authors focused on the issue of the language barrier, as native Norwegian women had a much greater understanding of GDM compared to the women from other ethnic groups. However, they noticed a more significant correlation between poor knowledge of GDM and low education, regardless of the ethnic group.[19] The results of the surveys on various aspects of the women's awareness of GDM carried out in different countries confirm the outcomes observed in our study. However, in some aspects, the obtained results were surprising.

Based on the detailed results of our study, we have observed that the women aged 19–24 years had a much higher level of the awareness of the risk associated with the development of carbohydrate disorders during pregnancy than the women >34 years old. In recent years, more and more preventive measures have been used. A great emphasis is placed on the risk factors influencing metabolic disorders, including GDM, such as obesity, overweight or polycystic ovary syndrome. Articles and programs related to pregnancy, in which GDM is discussed, appear increasingly in social media and press. The women aged 19–24 have a potentially greater interest in the topic of pregnancy, and therefore they have a greater understanding of the risk of GDM. Contradicting studies were obtained by Thomas et al., who showed a positive relationship between the knowledge and awareness of DGM and age.[18]

The women with higher education were characterized by a higher awareness of the correct values of carbohydrate metabolism as well as self-control, dietary and

pharmacological treatment, because of their generally greater common knowledge. Some of them may have been professionally related to medical faculties, which determines more extensive medical knowledge and increased awareness of metabolic disorders.

Similarly, women who were pregnant for the first time were much more aware of the risk of developing GDM. Perhaps, it is related to the new situation and the desire to broaden their knowledge on the physiology of pregnancy. Hence, the women in the 2nd or subsequent pregnancy might have not updated or expanded their previously acquired knowledge. For this reason, their level of awareness was significantly lower. Again, the obtained results went against those obtained by Thomas et al.,[18] as in their study the number of pregnancies and the advancement of those pregnancies correlated positively with the level of awareness of GDM.

However, women who have experienced GDM in a previous pregnancy know more about the normal carbohydrate metabolism values. Experienced with GDM in their first pregnancy, they had to become familiar with the carbohydrate metabolism values in order to control GDM properly. Practicing GDM control in their first pregnancy, they became well-acquainted with the norms and proper values of blood glucose. The knowledge of the abovementioned issue was assimilated practically, so the level of its awareness was higher. The obtained results were similar to those obtained by the other researchers, which showed higher knowledge of GDM in women who experienced GDM in a previous pregnancy. This was confirmed by the fact that the pregnant women participating in the study who had a history of diabetes in their families, had broader knowledge of various aspects of GDM than those who did not. Electronic and written media are the primary tools to raise the level of knowledge of GDM.[18]

We have searched for a relationship between the surveyed women's changes in lifestyle and physical activity, as well as between the women's age and education. The age of women was statistically significant in terms of the control of products consumed during pregnancy with GDM, with less attention paid to this issue among the youngest group. The results may be linked to a very intensive lifestyle, a lack of knowledge of GI of products and no involvement in preparing meals, as well as low awareness of the proper diet in GDM. There was no significant correlation between education and lifestyle changes as well as physical activity.

The study also investigated the relationship between the change in lifestyle, including physical activity and the first or subsequent pregnency. We also looked at the relationship between lifestyle changes and GDM in a previous pregnancy. The majority of the women from all groups declared a change in lifestyle after becoming pregnant. Changing lifestyle and physical activity is one of the most crucial aspects of pregnancy. Most of the women tried to adapt their lifestyle to the situation, but – unfortunately – their physical activity was limited. Meanwhile, they should often decide to introduce their daily physical activity, which significantly impacts the improvement of metabolic disorders such as GDM.

It was observed that the women with high awareness changed their lifestyle more often; this concerns the quality of meals, resulting from the analysis of the GI and physical activity introduced into the daily or weekly schedule. The obtained results prove the importance of prophylaxis, broadening the knowledge of the women through various types of campaigns and improving the access to the current knowledge of the areas related to GDM.

The last topic discussed in this study was the relationship between the quality of medical care in pregnancy and the awareness of proper self-control, dietary or pharmacological treatment, and lifestyle change. Generally, women provided with better medical care significantly more often paid attention to the quality of products consumed in their daily diets and declared daily physical activity much more frequently than in the case of the worse level of healthcare. The level of medical care seemed to be very important in terms of activity related to lifestyle changes. Thus, numerous social programs aimed at increasing medical care in pregnancy should be carried out, as this might result in a higher awareness of pregnant women with GDM, and at the same time limit the health risk to a pregnant woman and the fetus.

Limitations

The main limitations are the restriction to the Polish population and the relatively small study group. Our results indicate the need for further research that would allow for more accurate identification of the causes of low awareness concerning GDM of women.

Conclusions

The results emerging from this study provide a perfect basis for conducting further research in a given direction, as they highlight many dependencies that can potentially influence the awareness of various aspects related to GDM, especially the results obtained for the quality of medical care.

ORCID iDs

Anna Karpiewska https://orcid.org/0000-0002-4448-5594
Sonia Jarczak https://orcid.org/0000-0003-3073-9511
Małgorzata Małodobra-Mazur https://orcid.org/0000-0002-9864-5928

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