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FIBROTIC ACTIVITY IN PATIENTS AFTER PERMANENT PACEMAKER IMPLANTATION

THESIS SUMMARY

of a PhD Thesis

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Scientific Speciality Cardiology

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The thesis contains 135 pages and is illustrated with 5 tables and 34 figures. The bibliography includes … references in Latin. The research was carried out at the Department of Cardiology at Virgin Mary University Hospital – Burgas. The thesis was discussed and referred for defence at a departmental council of the Department of Internal Diseases at the Medical University ‘Prof. Dr. P. Stoyanov’ – Varna.

Note. The numbering of the figures in the thesis summary is kept as the original numbering of figures in the thesis.

The thesis defence will be on 18/10/2024 at 2 PM in .... at an open session of the Scientific Jury. The defence materials are available in the library of the Medical University ‘Prof. Dr. P. Stoyanov’ – Varna.

CONTENTS

FREQUENTLY USED ABBREVIATIONS

ABP	-	Arterial blood pressure
SCD	-	Sudden cardiac death
RV	-	Right ventricle
DCMP	-	Dilated cardiomyopathy
ECM	-	Extracellular matrix
LBBB	-	Left bundle branch block
LV	-	Left ventricle
PPM	-	Permanent pacemaker
AFib	-	Atrial fibrillation
RAAS	-	Renin-angiotensin-aldosterone system
HF	-	Heart failure
MRI	-	Magnetic Resonance Imaging
AV-block	-	Atrioventricular block
BMI	-	Body mass index
BNP	-	B-type natriuretic peptide
CFBs	-	Cardiac fibroblasts
CITP	-	Carboxy-terminal telopeptide of collagen type I
CRT	-	Cardiac resynchronisation therapy
CTGF	-	Connective tissue growth factor
EF	-	Ejection fraction
HBP	-	His Bundle Pacing
ICD	-	Implantable cardioverter defibrillator
IL-1β	-	Interleukin 1 beta
LAVI	-	Left atrial volume index
LBAP	-	Left bundle branch area pacing
LVEDV	-	Left ventricular end-diastolic volume
LVESV	-	Left ventricular end-systolic volume
MMPs	-	Matrix metalloproteinases
myoFBs	-	Myofibroblasts
PAI-1	-	Plasminogen activator inhibitor type 1
PICM	-	Pacemaker-induced cardiomyopathy
PIIINP	-	Amino-terminal propeptide of procollagen type III
PIIICP	-	Carboxy-terminal propeptide of procollagen type III

PICP	-	Carboxy-terminal propeptide of procollagen type I
PINP	-	Amino-terminal propeptide of procollagen type I
ROS	-	Reactive oxygen species
SSS	-	Sick sinus syndrome
TIMP	-	Tissue inhibitor of metalloproteinases
TNFa	-	Tumour necrosis factor
TGFβ1	-	Transforming growth factor beta 1

I. INTRODUCTION

During the second half of the 20th century, technological advances in cardiology and cardiac pacing caused a surge in the number of patients implanted with pacemakers to overcome existing rhythm-conduction pathology [Mond & Proclemer, 2011]. The reported rate of device placement varies between registries, reaching over 1000 devices per 1 million population in developed European countries [Timmis et al., 2018]. This results in prolonged length and quality of life for the affected patient population [Hofer S et al., 2005].

For more than 50 years, the most common method of cardiac pacing has been the implantation of an electrode in the right ventricular apex. On the other hand, right ventricular apical pacing provokes electrical and mechanical dyssynchrony, leading to an increased risk of developing heart failure (HF) and atrial fibrillation (AF) [Nielsen et al., 2003; Sweeney et al., 2003].

Results from analyses have shown that after permanent pacemaker (PPM) implantation, more than 10% of the patients develop manifestations of HF as early as year 2 [Tayal et al., 2019]. Conversely, studies on experimental models suggest that asynchronous ventricular contraction leads to a difference in the workload of cardiomyocytes in the different myocardial areas [Duchenne et al., 2018]. This is followed by changes in cellular metabolism and left ventricular remodelling, which is associated with dynamics in the extracellular matrix (ECM) and collagen deposition in the myocardium [Mollmann et al., 2009]. These results raise the question of the molecular and cellular mechanisms involved in the development of HF and fibrosis in the cardiac muscle in patients after PPM implantation and of an effective non-invasive method for diagnosing this condition.

Dynamic monitoring of changes in the fibrosis status in patients with permanent pacemakers has been the subject of single studies, with inconsistent and insufficient results. This was the reason for conducting the current study, establishing the definition of its aim.

II. AIM AND TASKS OF THE STUDY

1. AIM: To study the fibrotic response as well as mechanical and electrical contraction of the heart in patients after implantation of a biventricular pacemaker and to follow the changes over a period of 6 months after implantation.

2. TASKS. To achieve the aim, the following tasks were identified:

A. To investigate the fibrosis status before implantation of a biventricular PPM, at week 12 and at week 24 after implantation by determining the plasma levels of the signalling molecules TGF-β1and CTGF.

B. To monitor the markers of collagen synthesis levels before implantation of biventricular PPM, at week 12 and week 24 after implantation by examining the plasma levels of PIPC, and PIIINP.

C. To examine echocardiographically left atrial volume by monitoring the change in this parameter baseline on the day after PPM implantation, at the 6th, 12th and 24th weeks onwards.

D. To monitor the width of the QRS complex from the surface ECG to assess myocardial electrical asynchrony induced by apical right ventricular pacing at baseline at the 6th, 12th, and 24th weeks after implantation.

III. MATERIALS AND METHODS

1. Study design

The study was carried out at the Department of Cardiology at Virgin Mary University Hospital – Burgas, Bulgaria, for the period March 2019 – August 2021. The procedure for the inclusion of patients and controls was initiated after approval of the Ethics Research Committee of the Medical University – Varna (82/28.03.2019) and the Ethics Research Committee of Virgin Mary University Hospital – Burgas (502/21.03.2019), following the requirements of the Declaration of Helsinki (The World Medical Association Declaration of Helsinki, 2008). The participants involved in the study were over 18 years of age and were included after receiving prior information and signing informed consent for participation.

Two groups were set up – a patient group and a control group. The participants were selected based on clearly formulated "Study Inclusion and Exclusion Criteria" (see below). The patient group was formed by selecting patients admitted to the ward with an indication for biventricular pacemaker implantation. Controls were volunteers referred by a general practitioner for screening to participate in the study after a yearly preventive medical check-up.

A key point in the study design was to align the demographic and clinical characteristics of the two groups (patient and control) to ensure maximum objective comparison and minimise the possibility of selection bias [Kang et al., 2008; Suresh, 2011]. This contributed to the reliability of the conclusions afterwards and the causal correlation found. The control group was established as similar to the patient group in terms of sex, age and comorbidities.

Clinical evaluation of each participant was performed based on available medical records, detailed medical history, physical examination, electrocardiogram and transthoracic echocardiography.

For the purposes of the study, four indices of fibrotic activity in peripheral venous blood drawn from a cubital vein were examined in each participant.

In the patient group, fibrotic activity indices were determined three times, immediately before PPM implantation (baseline or visit 1 – V1), on the 12^{th} week (visit 2 – V2) and 24^{th} (visit 3 – V3) week after implantation. Patients also underwent an additional visit at week 6 after pacemaker implantation, which was marked as visit V1.1. At this visit, clinical examination, surface electrocardiogram, echocardiography and telemetry of the implanted device were performed.

The control group had the same parameters examined three times, at baseline (visit 1 – V1), at week 12 (visit 2 – V2) and at week 24 (visit 3 – V3) after selection for the study. The

blood was centrifuged as required by the assays used. The resulting plasma was frozen and stored again according to the applied tests. In the control group, a clinical examination with ECG and echocardiography was performed when the volunteering patients were selected for inclusion in the study. At the follow-up visits, blood was drawn to examine fibrosis indices, and the participants were questioned about new-onset complaints and diseases.

Only patients with preserved sinus activity and complete atrioventricular block as an indication for implantation of a biventricular pacemaker were screened for the study. Patients were consecutively screened at the time of hospitalisation in accordance with the set of research tasks.

The selected patients, after signing informed consent, were implanted with a biventricular DDDR pacemaker – according to the requirements described in the ESC/EHRA guidelines [Boriani et al., 2019; Brignole et al. 2018].

2. Participants in the study

Out of 144 patients screened, due to exclusion criteria (see exclusion criteria), 99 patients were dropped from the study. To balance the male-female ratio, 45 patients (25 men, 20 women), mean age of 72.18 ± 1.35 yr, were selected. The inclusion criteria presented below, derived from the study objectives, were used to select the participants.

Inclusion criteria for the patient group

1. Presence of complete atrioventricular block as an indication for implantation of a biventricular pacemaker.
2. 2. Eligible comorbidity – up to grade I arterial hypertension that was well medically controlled.
3. 3. Lack of exclusion criteria.

Inclusion criteria for the control group

1. No history or ECG evidence of rhythm-conduction pathology.
2. Eligible comorbidity – up to grade I arterial hypertension that was well medically controlled.
3. Absence of exclusion criteria.

For the purposes of the study, it was of utmost importance to minimise the influence of fibrotic response from medications and comorbidities in the selected patients and controls. Because of this, both patients and controls were treated only with pharmaceuticals for which there was no evidence of direct influence on the RAAS system (renin-angiotensin-aldosterone system). After inclusion in the study, the participants were prescribed therapy with a

dihydropyridine calcium antagonist, amlodipine, a thiazide diuretic, hydrochlorothiazide, and, if necessary, a centrally acting medication – methyldopa, was added in doses necessary to achieve blood pressure control.

Patients enrolled in the study were followed up at four visits – at baseline, 6, 12 and 24 weeks after PPM implantation, respectively. Blood samples were taken immediately after selection for inclusion at the first venous catheter insertion. For the purposes of the study, echocardiographic examination and ECG recording were performed on the day after pacemaker implantation. Data from baseline blood samples and echographic parameters were marked as visit 1 (V1). To assess and monitor the early effect of restored atrial ventricular synchrony as a result of the pacemaker implantation, a follow-up visit was introduced at week 6, when echocardiographic examination, ECG, and pacemaker telemetry were performed. This visit was marked as visit 1.1 (V1.1) and was not associated with blood sampling for fibrotic activity testing. At the follow-up visits at week 12, visit 2 (V2), and at week 24, visit 3 (V3), a clinical examination, echocardiography, ECG, and telemetry were performed, and blood samples were collected for fibrosis testing. The echocardiographic study was performed according to the current guidelines for evaluation of the cardiac chambers, as well as those for clinical echocardiographic studies proposed and described by the European Association of Cardiovascular Imaging [Lang et al., 2015]. All participants were questioned for symptoms of new-onset cardiac vascular disease, and none was found on the clinical and echocardiographic examinations performed afterwards.

Identical to the patient group exclusion criteria were applied to form the control group (see below). The control group was established as identical to the patient group, with participants recruited at the same time as the patient selection and after signing informed consent. Thus, after a total of 102 were screened, 46 of them (24 men, 22 women) were selected as controls with a mean age of 71.96 ± 1.29 years. There was no medical history and ECG evidence of present rhythm-conduction pathology in the controls. The presence of well-controlled medications for arterial hypertension up to grade I was allowed as a comorbidity. Antihypertensive medications, which have no direct effect on fibrotic response, were used in both patient and control groups. The health status of the study participants (patients and controls) was determined based on medical history, physical examination, laboratory tests, and recorded ECG and transthoracic echocardiography.

The selection of study participants (patients and controls) aimed to exclude all factors that might influence fibrotic activity, namely comorbidities and medication therapy.

Exclusion criteria from the study:

- Presence of cardiovascular disease: coronary artery disease (including acute coronary syndrome, previous myocardial infarction regardless of time past, coronary revascularisation PCI/CABG, stable angina pectoris), heart failure with reduced pump function, uncontrolled hypertension, inflammatory heart disease (myocarditis, pericarditis, infective endocarditis), congenital heart defects, clinically significant acquired heart valve disease, cardiomyopathies, thromboembolic events.
- Presence of other diseases: renal or hepatic insufficiency, diseases of the central nervous system, inflammatory and/or infectious diseases in the last three months, neoplastic or autoimmune diseases, chronic lung diseases, diseases of the endocrine system, surgical interventions in the previous three months.
- Presence of pregnancy, systemic intake of NSAIDs (non-steroidal anti-inflammatory medications), antithrombotic medications and corticosteroids.
- Participants who for any reason were not followed up by the end of the specified period were excluded from the study.

3. Operative technique, intraoperative verification of electrode parameters, programming of the implanted device

Implantation of pacemakers was performed in the angiography unit of the hospital, and strict rules of asepsis and antisepsis were followed [Ramsdale & Rao, 2012]. After a meticulous disinfection of the operative field with povidone-iodine solution, the patient was covered with a sterile set, after which local anaesthesia was administered in the subclavian vein with lidocaine (1% lidocaine hydrochloride solution).

For electrode placement, the subclavian vein was punctured to the left or right to establish venous access. Before the puncture, the position of the vein was verified by injecting 20 ml of Omnipaque contrast medium. Cannulation of the vein was performed according to Seldinger's method using the 7F Peel-Away Introducer Kit [Seldinger, 1953]. After successful insertion of the two guides into the superior vena cava, the two electrodes, one for the ventricle and one for the atrium, are introduced sequentially.

The electrode for the ventricle is positioned in the right ventricular apex with active fixation. Its position is confirmed radiographically in the right anterior oblique projection and the left lateral projection. To confirm electrode position stability, stimulation at 1 V wave amplitude is used after instructing the patient to cough and take several deep breaths in and out. Once the stimulation threshold, resistance, and sensing of the electrode are determined using

Medtronic PSA (pacing system analyser), it is fixed to the fascia of the pectoralis muscle with a non-resorbable ligature.

In order to reduce the risk of asystole during implantation, once a stable position of the right ventricular electrode is ensured, it is left connected to the PSA analyser with a protective stimulation of 30 beats per minute.

The atrial electrode was inserted into the ear of the right atrium via a preset J-shaped stylet. To verify the correct position, the presence of the specific lateral movement of the electrode tip was monitored during radioscopy. After confirming the correct position, it was fixed by releasing the helix in the atrial myocardium. The parameters of sensing, stimulation and resistance were measured with the PSA analyzer, and the electrode was fixed with a non-resorbable ligature to the pectoralis fascia.

The following values were assumed as optimal parameters:

- Atrial sensing above 1 mV.
- Stimulation threshold below 1 V at a pulse width of 0.5 ms.
- Resistance between 400 and 1000 Ohms at a pulse width of 0.5 ms.

For the chamber electrode, the optimal parameters were as follows:

- Chamber sensing above 2.5 mV.
- Stimulation threshold below 1 V at 0.5 ms pulse width.
- Resistance between 400 and 1000 Ohms at 0.5 ms pulse width.

The electrodes were connected to the pulse generator which was placed in a subclavicularly pre-shaped pocket in the prepectoral space. The surgical wound was closed layer by layer with absorbable suture and intradermal stitch, after which a dry sterile dressing was applied. The implanted devices were Medtronic's Sphera DR MRI SureScan and the electrodes were CapSure Fix Novus MRI SureScan, with lengths of 52 mm and 58 mm for the atrium and ventricle, respectively.

After each implantation and before leaving the hospital, patients underwent telemetry to confirm optimal electrode parameters and programming of the devices in DDDR mode. At each follow-up visit, conducted at the 6th, 12th, and 24th weeks after implantation, pacemaker telemetry was carried out to check for any indications of device malfunction. No such malfunction was recorded in any of the participants. The rate of sustained right ventricular pacing was also checked and was found to be over 80% in all patients included in the study.

4. Obtaining and storage of samples

Venous blood was used for the study of the indicators, which was taken from a peripheral vein (left or right cubital vein). The volume of blood collected for each blood sample was 8 ml and was distributed in two serum vacutainers of 4 ml each. Patients had their blood samples collected three times, before PPM implantation (baseline), at 12 and 24 weeks after implantation. Blood samples from the control group were also taken three times – at baseline, after the 12^{th} and 24^{th} weeks after inclusion in the study.

After the blood samples were collected, they were left on an easel for 3 hours at a temperature of 22°C and then centrifuged at 3500 rpm for 15 minutes. The released serum was transferred into plastic tubes. Two tubes were designated for each indicator – one for testing and one as a reserve and then frozen at –20°C. Collected and frozen samples were transported in dry ice within 1 month of collection and then stored at –70°C, where they remained for up to six months.

Thawing and refreezing of samples were not allowed during the study tests.

All samples were coded immediately after receipt. This process prevented their identification during laboratory testing (control or patient sample) as well as determining the day of sample collection.

5. Indicators and laboratory methods

For each study participant (patient or control), a total of four indicators reflecting fibrotic activity were examined in serum: two signalling molecules and two markers of collagen synthesis.

Serum levels of the signaling molecules TGF-β1 and CTGF as well as the collagen synthesis markers PICP and PIIINP were determined. The analysis of the samples was carried out in the Lipogard Medical Diagnostic Laboratory in Sofia.

In the patient group, fibrotic activity markers were determined three times – immediately before PPM implantation (initial value or visit 1 – V1), at week 12 (visit 2 – V2) and at week 24 (visit 3 – V3) after PPM implantation.

In the control group, the same parameters were examined three times, at baseline (visit 1 – V1), week 12 (visit 2 – V2) and week 24 (visit 3 – V3) after selection for the study.

Each indicator was determined three times, and the average of the three measurements was taken into account when calculating the results.

For the purposes of the study, echocardiographic estimates of left ventricular t-diastolic and t-systolic volumes were performed, thus ejection fraction was calculated using Simpson's method. This method is commonly used for assessing the left ventricular pump function according to the current guidelines [Lang et al., 2015]. Left atrial end-systolic volume was also measured from the apical quadruple position, also by Simpson's method. Left atrial volume is used as an indirect marker to assess left ventricular end-diastolic pressure and the effect of right ventricular apical pacing on haemodynamics [Nagueh et al., 2016]. The values obtained were indexed to the body surface area of the patients and then subjected to statistical processing. All methods of echocardiographic measurements adhered to the current guidelines for cardiac cavity assessment described in the European Association of Cardiovascular Imaging [Lang et al., 2015]. To assess the early effect of restored atrioventricular synchrony after overcoming the conduction disturbance with a biventricular pacemaker, an additional echocardiographic study was performed on the patient group 6 weeks after implantation. It was coded as visit 1.1.

A surface ECG was recorded at each patient visit to monitor the width of the QRS complex. This was needed to assess the electrical activation of the myocardium in the biventricular pacemaker mode and to analyse the induced asynchrony.

5.1.Determination of TGF-β1

The TGF-β1 levels were determined by ELISA (Enzyme-linked immunosorbent assay) method using MyBioSource kits for Human TGF-β1 Sandwich – ELISA with the following characteristics: sensitivity 10 pg/ml and detection range 62.5 pg/ml – 4000 pg/ml.

The method functions according to the following principle:

ELISA (Enzyme-linked immunosorbent assay) is a method based on enzyme-linked antibodies. This method allows the qualitative and quantitative determination of specific proteins in complex biological samples. The method was originally described by Engvall and Perlmann in 1971 [Engvall & Perlman, 1971].

ELISA has different formats, the most common being the "sandwich" format [Crowther, 2009] (Figure 13). In this format, the antibodies capture the antigen present in the sample. A second antibody labelled with an enzyme is then used to recognize and detect the complex formed.

The procedure usually involves the following steps:

1. Preparation of the samples and isolation of the antigen (in this case TGF-β1).
2. Antibodies that specifically recognise TGF-β1 are added to the sample. These can be anti-TGF-β1 antibodies that adsorb to the surface of the laboratory dish.

3. If TGF-β1 is present in the sample, then this antigen will be captured by the antibodies and will form a complex.
4. A second antibody, labelled with an enzyme that recognizes the antigen-antibody complex, is added.
5. After adding substrate, the enzyme changes its colour which is measured with a spectrophotometer.
6. The colour change measurement is used to estimate the concentration of TGF-β1 in the sample.

This method is preferred due to its high sensitivity and specificity and is commonly used in molecular and cell biology, as well as in medical research to determine the concentration of various biomarkers in biological samples.

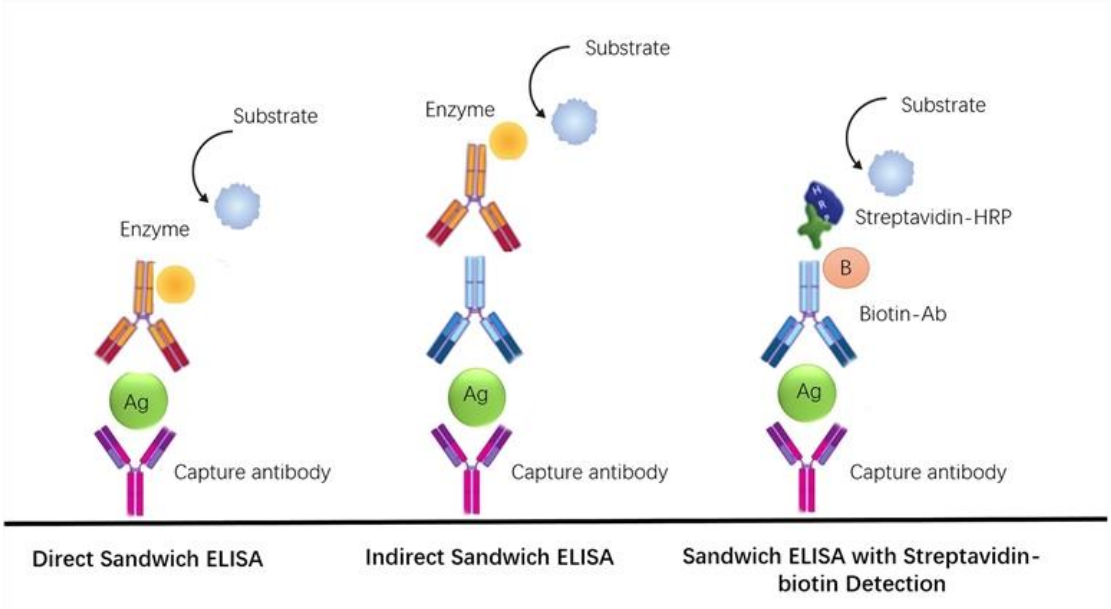

Fig. 13. *Scheme of ELISA methods (Thermo Scientific, 2010)*

A 96-well plate pre-coated with an antibody specific for Human TGF-β1 was used for the study. The serum samples for the assay were added to the cells where the available TGF-β1 bound to the immobilizing antibodies. The plate was then washed with buffer solution, after which biotin-labelled antibodies for Human TGF-β1 were added. After further rinsing of unbound antibodies with buffer solution, streptavidin bound to HRP (horseradish peroxidase) was added to the plate. After sequential washing of the analytical plate, TMB (3,3',5,5'-tetramethylbenzidine; 3,3',5,5'-tetramethylbenzidine) solution was added. This solution changes colour to blue upon oxidation, which occurs as a result of the formation of oxygen radicals generated by the hydrolysis of the hydrogen peroxide by HRP.

The TMB solution colour changes to yellow with the addition of sulphuric or phosphoric acid (stop-solution). Spectrophotometric analysis was performed at 450 nm within 30 minutes after adding the stop-solution. For the purposes of this research, optical densities were measured in duplicate and concentrations were calculated using the ELISA reader software using standard curves derived from standard dilution.

5.2. Determination of CTGF

CTGF levels were determined by ELISA [Engvall & Perlmann, 1971] using OmniKine kits from MyBioSource for Human CTGF Sandwich – ELISA with a detection range of 63 pg/ml to 4000 pg/ml. Prior to the start of analysis, serum samples were diluted according to the manufacturer's instructions. The assay procedure is described above.

5.3. Determination of PICP

PICP levels were determined by ELISA [Engvall & Perlmann, 1971] using kits from MyBioSource for the Human PICP Sandwich, an ELISA with a sensitivity of 2.26 ng/ml and a detection range of 5 ng/ml to 1500 ng/ml. Prior to the start of analysis, serum samples were diluted according to the manufacturer's instructions. The assay procedure is described above.

5.4.Determination of PIIINP

PIIINP levels were determined by the ELISA method [Engvall & Perlmann, 1971] using kits from MyBioSource for the Human PIIINP Sandwich, an ELISA with a sensitivity of 1.0 ng/ml. Prior to the start of analysis, serum samples were diluted according to the manufacturer's instructions. The assay procedure is described above.

6. Electrocardiographic and echocardiographic methods

A surface ECG was recorded at each visit of the patients selected for the study using the SCHILLER CARDIOVIT AT-1 G2 device (manufactured in January 2013 by Schiller Americas, Inc.) with a recording speed of 25 mm/s and a calibration amplitude of 10 mm/1 mV. The width of the QRS complex was measured from the recording on 5 consecutive contractions, and the average value was taken and included in the statistical analysis.

Patients underwent echocardiographic examination at each visit using the GE Healthcare Vivid S6 device (manufactured in September 2013 by GE Medical Systems, Israel Ltd). In the

control group, ultrasound examination was performed only during the inclusion visit. All measurements were performed according to the current guidelines for assessing cardiac structures and functions, and, complying with the guidelines for clinical echocardiographic examination proposed and described by the European Association of Cardiovascular Imaging [Lang et al., 2015].

The two-dimensional disc summation method was used to determine left ventricular volumes according to the rules of the Simpson method. For this purpose, the endocardial border of the left ventricular cavity was delineated from the apical four-chamber and two-chamber views of the transducer, during end-diastole and end-systole, respectively. Images used for analysis had to be of good quality, including papillary muscles and the trabecularization to the left ventricular volume. Thus determined end-diastolic volume (LVEDV – left ventricle end-diastolic volume) and end-systolic volume (LVESV – left ventricle end-systolic volume) were indexed to the body surface, after which the ejection fraction (EF – ejection fraction) was calculated by the following formula: EF = (EDV – ESV) / EDV.

Left atrial volume index (LAVI) was measured from the apical quadruple position using Simpson's method and was also indexed to the body surface area of the individual. For defining a reduced ejection fraction, a value below 50% was taken as recommended by Lang et al. (2015). For defining an increased left atrial volume, a value above 34 ml/m2 was used as recommended in the same reference.

7. Statistical analysis – methods used

Statistical analysis of the data was performed using the specialized software STATISTICA 13.3.0 developed by StatSoft Inc., USA (StatSoft, Inc., STATISTICA Manual (Data analysis software system), Version 11.0, 2018). When statistical hypotheses were performed, the significance level was set at $p = 0.05$. This represents the probability of making a first-order error, meaning that the null hypothesis can be rejected, even when it is true, with a probability of 5%.

The results were presented as mean value ± standard deviation (± SD) or n (%), depending on the nature of the data.

For the purpose of the thesis research, the following statistical methods were applied:

7.1. Descriptive statistics

It was used to calculate mean values of n observations in the sample $\overline{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$, standard deviations $SD = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \overline{x})^2}$, standard errors of the mean $SE = \frac{SD}{\sqrt{n}}$, relative proportions and 95% confidence intervals [Jobson,1991]. Descriptive statistics provides a quantitative description of the basic properties and characteristics of the population under study. The frequency distribution of the indicators considered, broken down by study group, mean values and standard errors was presented in tabular form.

7.2. Student's t-test (T-criterion) for two independent samples

A two-tailed t-test for independent (unpaired) samples was used in our study. The t-tests are effective for testing the hypothesis of equality of means of two dependent or independent samples [Guo & Yuan, 2017]. The t-statistics were introduced by the Irish mathematician Willian S. Gosset (1876–1937) in 1908 when he published his works in the journal Biometrica under the pseudonym Student. The testing of hypotheses of equality of means and relative proportion indices was done by the so-called Student's t-criterion.

When $p < 0.05$ values were obtained, the hypothesis of equality of means was rejected and the hypothesis that the difference between means was statistically significant was confirmed.

7.3. Student's t-test (T-criterion) for two dependent samples

Dependent samples are those in which the units in one sample predetermine those in the second sample. A typical case is the research comparing variables before and after some impact.

The null hypothesis and its alternative statistical hypothesis are tested:

H_0 : the difference d between the means of the two samples is zero

$H_0 : \mu_1 = \mu_2$

i.e. there is no significant difference between the before and after results.

An alternative hypothesis is put forward:

H_A: the difference d between the means of the two samples is different from zero

H_A : $\mu_1 \neq \mu_2$

i.e. there is a statistically significant difference between the before and after results.

The test is performed using the Student's t-criterion with $(N-1)$ degrees of freedom (df), where N is the number of data in the samples at the significance level α (p-level) that is most often chosen $\alpha = 0,05.$ We calculate the differences between the respective measurements in the samples $d_i\,(i = 1,2,...,N)$, $\bar{d}$ of these differences

$$\bar{d} = \frac{\sum_{i=1}^{N} d_i}{N}$$

and the standard deviation of these differences:

$$S_d = \sqrt{\frac{\sum_{i=1}^{N} (d_i - \bar{d})^2}{N-1}}$$

The statistic being tested is

$$t = \frac{\bar{d}}{S_d \sqrt{N}}, \nu = N-1$$

If its value is

$$t < -t_{\frac{\alpha}{2}}(\nu) \quad \text{or} \quad t > t_{\frac{\alpha}{2}}(\nu)$$

then the null hypothesis is rejected with significance level.

7.4. Power analysis of a sample volume test

The sample size is an essential characteristic of a sample. A change in volume results in a proportional increase or decrease in the frequencies of occurrence of individual values. The question of sample size is of utmost importance because it establishes the reliability of the conclusions from the statistical analysis. Since there are no established rules, a Power analysis of the applied test on the preliminary data is necessary as a guidance for the required sample size [Suresh & Chandrashekara, 2012].

In our thesis research, we performed a power analysis of the t-criterion for the variance of fibrosis indices in the study groups.

7.5. Regression analysis

Regression analysis studies and evaluates possible functional correlations between two or more random variables whose values are real numbers [Schober&Vetter, 2021]. It is a statistical method for investigating the presence of a correlation between one variable (the response) and one or more independent variables (predictors, arguments, factor variables). The resulting function is known as a model representing the response as a function of the independent variables. When the statistical significance (adequacy) of the model is established, it is used to predict the value of the dependent variable for values of the factor variable that are not observed in the sample.

Regression analysis is used for modelling the dependence of a dependent (outcome) variable on one or more factor attributes (independent variables), without taking into account that variation in the variables under consideration may be due to external, non-model factors. When the independent variable is one, we refer to univariate regression analysis, and if there are more independent variables, to multivariate analysis. When the outcome variable depends on two or more factors (independent variables), multivariate regression analysis is applied. In regression analysis, the dependent variable is always one.

In univariate analysis, the dependence between two variables is, in the most general case, expressed by the equation:

$y = f(x)$,

where *y* is the dependent variable (a function of *x*) and *x* is the independent variable (an argument).

The change of the function depending on the change of the argument is called regression. The correlation between *x* and *y* can be represented by:

- Regression equation with regression coefficients;
- Graphically – with a regression line.

7.5.1. Linear regression

To investigate a (possible) linear correlation between one dependent variable (*y*) and one or more independent (*x1, x2,..., xn*) variables, the linear regression method is used. Simple linear regression is applied in the presence of one predictor (one independent variable), while bivariate and multivariate regression analyses are performed in the presence of two or more predictors, respectively. The equation of the linear regression model in this case is:

$y(x)=b_0 + b_1 x$,

where b_0 is a constant (a free term in the regression equation) and b_1 is a regression coefficient indicating by how many units the dependent variable changes when the factor variable changes by one of its units. Based on the sample data, estimates of these coefficients are computed using the least squares method. For the statistical significance of the estimated coefficients $\hat{b}_0$ and $\hat{b}_1$ in the regression equation, the null hypotheses $\hat{b}_0 = 0$ and $\hat{b}_1 = 0$ are also tested using Fisher's criterion with degrees of freedom in the numerator and denominator (1, n-2) respectively, where n is the number of data in the sample. When the p-value of the criterion is less than 0.05, the null hypotheses are rejected and the coefficients are assumed to be statistically significant.

Adequacy check of the linear regression model

The adequacy of the constructed linear regression model is performed by the values of correlation coefficient r.

- Correlation coefficient r

The r values vary in the interval [–1;1] and determine the strength of the linear correlation:

$|r|<0.3$ – weak correlation dependence;

$0.3\leq|r|\leq0.7$ – good correlation dependence;

$|r|>0.7$ – strong correlation dependence.

The sign r indicates the type of dependence. For $r > 0$ there is a positive correlation, a growing linear dependence, i.e. as the x values increase, the y values increase as well. For $r < 0$ there is a negative correlation or decreasing linear dependence, i.e. as the x values increase, the y values decrease. At $r = 0$, the quantities are correlation independent.

IV. RESULTS AND DISCUSSION

1. Demographic and clinical characteristics of the study participants

Table 1 shows the demographic characteristics of the patient and control groups.

Table 1. *Demographic characteristics of patient and control groups*

	Patient group	**Control group**	**P values**
N participants in a group	45	46	> 0.05
Mean age (years)	72.18 ± 1.35	71.96 ± 1.29	>0.05
Male/Female	25/20	24/22	>0.05
BMI (kg/m^2)	27.45 ± 0.64	26.51 ± 0.49	>0.05

As evident from the p > 0.05 values, there were no statistically significant differences between them in terms of number of participants included, sex, age and BMI (p > 0.05) (Table 1).

Table 2. *Clinical characteristics of patient and control group. Medication therapy administered after selection for inclusion in the study*

	N of patients (%)	**N of controls (%)**	**P value**
Comorbidities			
Hypertensive disease	39 (86.66 %)	37 (80.43%)	>0.05
Antihypertensive therapy			
Methyl-Dopamine	23 (51.11%)	24 (52.17%)	>0.05
Amlodipine	35 (77.78%)	33 (71.74%)	>0.05
Hydrochlorothiazide	35 (77.78%)	35 (76.09%)	>0.05

Regarding comorbidities, both the patient and control groups were assumed to have only up to grade I well-controlled arterial hypertension. Regarding medication treatment, all participants were treated with medications for which there was no evidence of influence on the fibrotic response and collagen synthesis (Table 2).

Alignment of the groups in terms of demographic and clinical characteristics presented above was incorporated into the study design (see "Materials and Methods"). This ensured a balance between the groups and an objective comparison of the results.

2. Variance in fibrosis parameters

2.1. Deviations in the signalling molecules: TGF-β1 and CTGF

Figure 14 presents the absence of a statistically significant difference between baseline TGF-β1 values in the patient (V1 patients) and control groups (V1 controls) (407.69 ± 27.36 pg/ml vs 369.97 ± 24.20 pg/ml, $p > 0.05$). At week 12 (patients V2) after PPM implantation, significantly increased values were found compared to baseline in the control group (Figure 14) (774.18 ± 47.41 vs 369.97 ±2 4.20 pg/ml, $p < 0.001$). At week 24, the significant increase in the study parameter in patients (V3 patients) persisted compared to baseline in controls (V1) (748.28 ± 40.76 vs 369.97 ± 24.20 pg/ml, $p < 0.001$) (Figure 14).

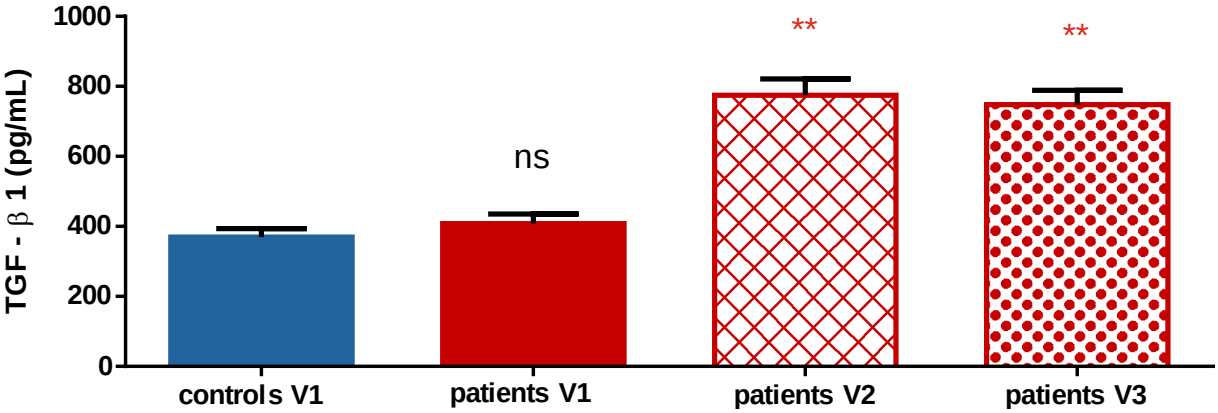

Fig. 14. *Comparison of TGF-β1 values in patients versus baseline values in the control group (Controls V1) (patients V1 – baseline values; patients V2 – values at week 12 after PPM implantation; patients V3 – values at week 24 after PPM implantation) (*–p<0.05; **– p < 0.001; ns – statistically insignificant difference)*

A follow-up of the values in the patient group shows that at week 12 (patients V2) after PPM implantation, the elevated TGF beta 1 levels had reached statistical significance compared to baseline (patients V1) (774.18 ± 47.41 vs 407.69 ± 27.36 pg/ml, $p < 0.001$) (Figure 15). At week 24, the significant increase in values (V3 patients) was sustained compared to baseline (V1 patients) (Figure 15) (748.28 ± 40.76 vs 407.69 ± 27.36 pg/ml, $p < 0.001$).

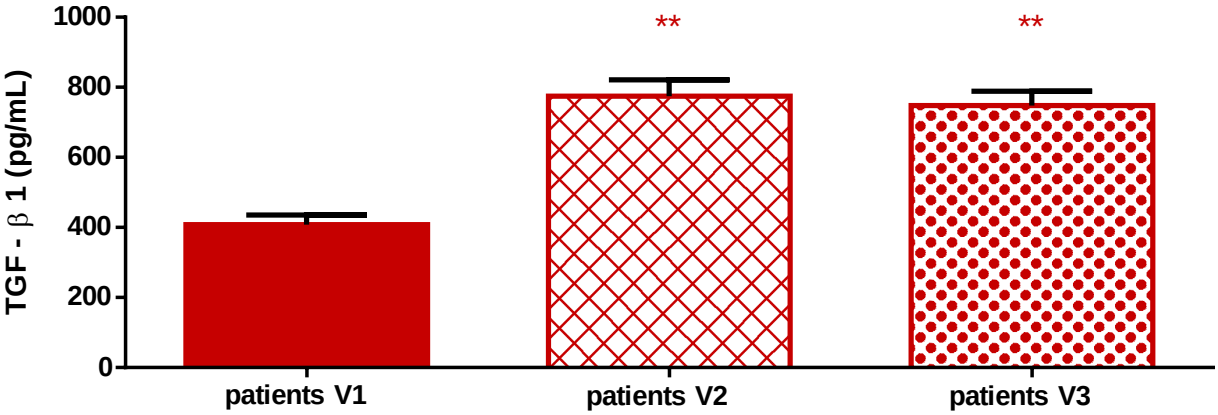

Fig. 15. *Dynamics of TGF-β1 levels in the patient group (patients V1– baseline values of the indicator; patients V2 – values at the week 12 after PPM implantation; patients V3 – values at week 24 after PPM implantation; *–p<0.05; **–p<0.001; ns – statistically insignificant difference*

The TGF-β1 assay performed in the control group did not reveal any significant changes in the plasma levels of the indicator during the follow-up period. There was no significant difference between the values of the indicator at the second and third visits compared to baseline (324.83 ± 23.94 vs 369.97 ± 24.20 pg/ml; 359.55 ± 24.80 vs 369.97 ± 24.20 pg/ml, p > 0.05) (Figure 16). Although not presented in Figure 16, the indicator values were also compared between the third and second visits, and no significant difference was found (359.55 ± 24.80 vs 324.83 ± 23.94 pg/ml, p > 0.05).

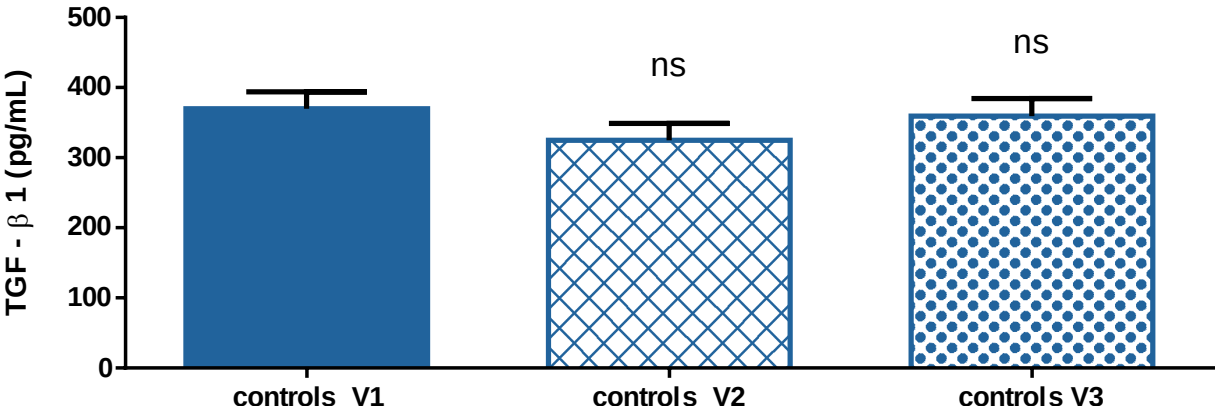

Fig. 16. *Dynamics of TGF-β1 levels in the control group:(controls V1 – baseline values of the indicator; controls V2 – values at week 12 after inclusion in the study; controls V3 – values at week 24 after inclusion in the study. ns – statistically insignificant difference)*

Figure 17 shows that there was no significant difference between the baseline CTGF values of patients (patients V1) and controls (controls V1) (312.66 ± 15.25 vs 313.05 ± 12.98, p > 0.05). At week 12 after PPM implantation, the patient group (patients V2) was found to have significantly increased values compared to baseline values in the control group (Figure 17) (627.74 ± 41.81 vs 313.05 ± 12.98, pg/ml p < 0.001). At week 24 post-implantation, the elevation of the monitored indicator in patients (V3 patients) had remained at statistically

significantly higher levels compared to baseline in controls (V1 controls) (400.83 ± 13.29 vs 313.05 ± 12.98 pg/ml, p < 0.001) (Figure 17).

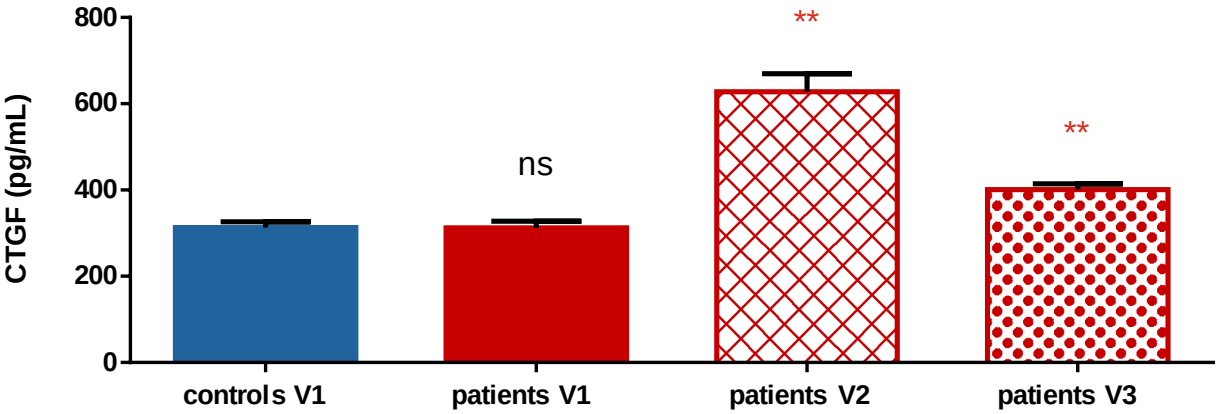

Fig. 17. *Comparison of CTGF values in patients versus baseline values in the control group (Controls V1) (patients V1- baseline values; patients V2- values at week 12 after PPM implantation; patients - V3- values at week 24 after PPM implantation; *- p<0.05; **- p<0.001; ns- statistically insignificant difference)*

When CTGF values were monitored in the patient group at week 12 (patients V2) after PPM implantation, an increase in values was found compared to baseline (patients V1) (Figure 18) (627.74 ± 41.81 vs 312.66 ± 15.25 pg/ml, p < 0.001). At week 24 post-implantation, the increase in CTGF levels in patients (V3) had remained significantly higher compared to baseline (patients V1) (Figure 18) (400.83 ± 13.29 vs 312.66 ± 15.25 pg/ml, p < 0.001).

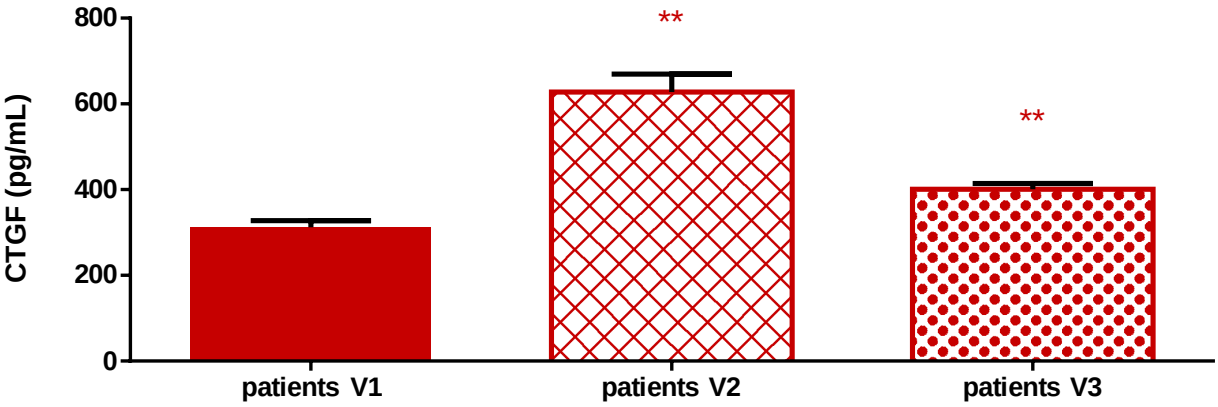

Fig. 18. *Dynamics of CTGF levels in the patient group: (patients V1 – baseline values; patients V2 – values at week 12 after PPM implantation; patients V3 – values at week 24 after PPM implantation; *– p<0.05; ** –p<0.001; ns – statistically insignificant difference)*

In the control group, no significant changes in the plasma levels of the indicator occurred during the follow-up period. There was no significant difference between the values of the indicator at the second and third visits compared to baseline (311.02 ± 11.47 vs 313.05 ± 12.98 pg/ml; 324.74 ± 14.20 vs 313.05 ± 12.98 pg/ml p > 0.05) (Figure 19). Although not presented

in Figure 19, there was no significant difference in the levels of the third and second visit follow-up (324.74 ± 14.20 vs 311.02 ± 11.47 pg/ml, p > 0.05).

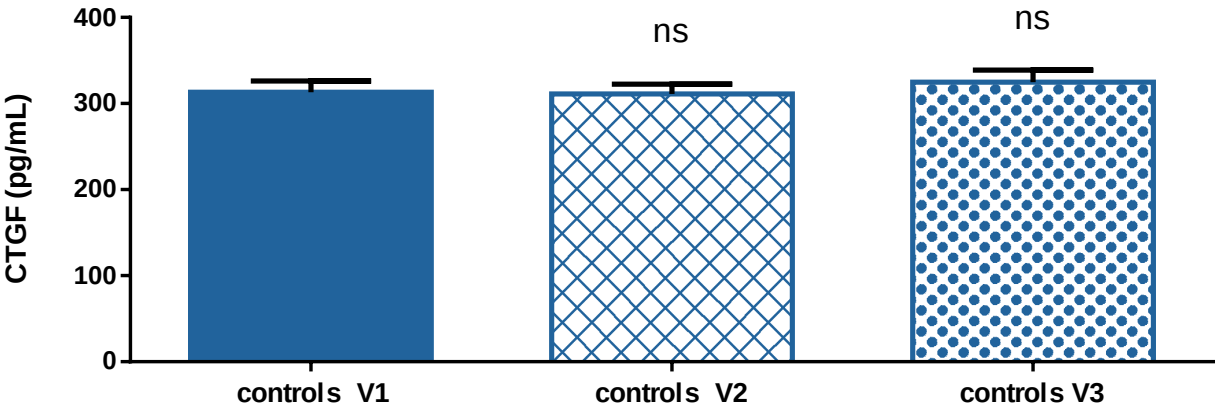

Fig. 19. *Dynamics of CTGF levels in the control group: (controls V1 – baseline values of the indicator; controls V2 – values at week 12 after inclusion in the study; controls V3 – values at week 24 after inclusion in the study; ns – a statistically insignificant difference)*

Abnormal cardiac activation resulting from apical right ventricular stimulation is associated with changes in the myocardial metabolism and perfusion [Tse & Lau, 1997]. This has been demonstrated in both clinical and preclinical studies where regional differences in coronary blood flow, glucose utilization and oxygen consumption by cardiomyocytes during right ventricular stimulation have been found [Lee et al., 1994; Prinzen et al., 1990]. These changes lead to disorganization of myofibrils, deposition of dystrophic calcifications, and heterogeneity in mitochondrial function. Pacemaker-induced left ventricular dysfunction leads to myocardial hypertrophy in later activated areas as a result of local mechanical stress and redistribution of sympathetic nerve endings [Simantirakis et al., 2001; Prinzen et al., 1995]. Through the process of mechanotransduction, cardiomyocytes sense these regional changes in load by responding with structural and biochemical changes (Lee & Libby, 2000). This process results in the transduction of intracellular stimuli from the cell membrane to the cell nucleus by signal-transducing protein phosphorylation [Tamada et al., 2009; Wang et al., 2009]. Molecules such as integrins, cadherins, and titins act as mechanosensors for extracellular, intercellular, and intracellular stimuli, thereby eliciting an adaptive response to the specific stimulus (McCain & Parker, 2011; Curtis & Russell, 2011). As a result of the change in cardiac contraction mechanics due to apical right ventricular stimulation, activation of signalling pathways that influence the fibrotic response and remodelling of the ECM is provoked [Gopalan et al., 2003]. During the development of the profibrotic state, abnormal production of cytokines and growth factors occurs, leading to chronic activation of fibroblasts. Multiple signalling molecules are involved in this process, and TGF-β1 is considered to have a leading role in triggering this cascade [Verrecchia & Mauviel, 2002]. The TGF-β superfamily interacts with TGF-type 1 and

TGF-type 2 receptors which activate effector proteins known as SMAD proteins. These in turn play a central role in the transmission of signals from receptors located on the cell membrane to specific genes in the cell nucleus [Kretzschmar et al., 1997]. It has also been established that the major genes encoding collagen synthesis in ECM are direct targets of SMAD proteins [Verrecchia et al., 2001].

CTGF is another important factor in the fibrotic response, and its expression in fibroblasts is controlled by TGF-β1 with the involvement of the SMAD effector pathway [Holmes et al., 2001]. Activation of CTGF leads to fibroblast proliferation and increased collagen deposition in ECM and has a synergistic effect with TGF-β1. As shown, TGF-β1 and CTGF are actively involved in the activation of the fibrosis response in the presence of a pathological condition in the myocardium [Zou et al., 2021].

The results of our study showed an extremely early increase in TGF-β1 levels in the patient group as early as 12 weeks after pacemaker implantation (Figure 14 and Figure 15). This increase was statistically significant both relative to baseline values in patients and relative to baseline values in the control group. CTGF follow-up also showed a significant increase in the patients at week 12 post-procedure (Figure 17 and Figure 18). These unidirectional changes in the levels of the signalling molecules are likely due to the induced profibrotic state in the myocardium as a result of apical right ventricular stimulation. To minimize the possibility that additional factors might influence the fibrotic system in the cardiac muscle, our follow-up group included only patients without serious concomitant cardiovascular pathology. *This brings us to suggest that the elevated levels of TGF-β1 and CTGF result from apical pacing-induced asynchronous cardiac contraction.*

Research findings indicate that when myocardial dysfunction develops, cardiac fibroblasts produce TGF-β1, leading to the development of interstitial fibrosis [Bujak & Frangogiannis, 2007]. Under *in vitro* conditions, TGF-β1 induces a significant increase in collagen type I and type III synthesis [Eghbali et al., 1991]. Studies of myocardial histological samples have shown that the collagen fraction in the interstitium decreases after CRT implantation and overcoming the asynchrony from the left femoral block [D'Ascia et al., 2006]. Also, plasma TGF-β1 levels decrease at the first year after surgery in patients with aortic valve stenosis and correlate with left ventricular remodelling [Almendral et al., 2010; Villar et al., 2009]. A definite association between TGF-β1 levels and asynchronous ventricular contraction in the LBBB was found by Osmancik et al. (2013). When TGF-β1 levels were followed up in LBBB patients with indications for CRT implantation, a decrease in its levels was found in the respondent group at 6 months after implantation. They also found an increase in this marker in the non-respondents group, with higher levels before CRT implantation associated with a worse

prognosis in the respective patients. Similar results have been shown in animal models, where asynchrony in LBBB is associated with asymmetrical hypertrophy of the left ventricular wall and locally increased CTGF expression [van Oosterhout et al., 1998; van Middendorp et al., 2017]. Sustained apical right ventricular stimulation leads to similar changes. An increase in the fibrotic activity in the left ventricular lateral wall of the myocardium has been found in experimental animal models [Lin et al., 2010].

With age, natural changes occur in the body, and ageing fibroblasts are associated with increased levels of TGF-β1 [Tominaga & Suzuki, 2019]. Non-coding microRNAs (miRNAs) are potent regulators of gene expression and occupy an important place in the pathophysiology of many cardiac diseases [Gebert & MacRae, 2019]. The miR-29 family is one of the first miRNA families closely associated with the development of cardiac fibrosis. They modulate collagen metabolism in the peri-infarct zone in experimental models. A study of miRNA profiles in adult patients who died of sudden cardiac death (SCD) due to primary myocardial fibrosis showed increased expression of miR-1468-3p [Lin et al., 2020]. Also, an *in vitro* study of human cardiac fibroblasts showed that an increase in miR-1468-3p levels induced collagen deposition and cell metabolic activity, resulting in increased expression of type I collagen, CTGF and periostin. Anti-miR-1468-3p antagonized TGF-β1-induced collagen deposition and metabolic activity. This suggests that affecting miR-1468-3p levels in adult subjects may be a way to therapeutically influence fibrotic deposition in the myocardial interstitium.

There is strong evidence that plasma CTGF activity increases with time progression [Ungvari et al., 2017]. This was the reason why we tracked the levels of the signalling molecules TGF-β1 and CTGF simultaneously and dynamically in both the patient and control groups. Our results showed no significant change in these indicators when monitored in the control group (Figure 16, Figure 19). This points us towards the conclusion that age-related changes in the values of these signalling molecules require a longer period, and the indicators' dynamics in the patient group are not due to the time factor.

The results of the 24-week follow-up in the patient group, conducted in our study, showed that the levels of TGF-β1 (Figure 14, Figure 15) and CTGF (Figure 17, Figure 18) remained significantly elevated relative to both baseline values in the control group and baseline values in the patient group. *Considered in the context of the literature data outlined above, these results indicate the presence of a persistent profibrotic state that persists up to six months after PPM implantation.*

2.2. Deviations in the collagen synthesis markers PICP and PIIINP

Baseline serum levels in patients were not different from controls as shown in Figure 20 (85.13 ± 4.68 vs 79.34 ± 3.49 ng/ml, $p > 0.05$). At week 12 (V2 patients), PICP levels were significantly elevated compared to controls (90.51 ± 4.28 vs 79.34 ± 3.49 ng/ml, $p = 0.0445$), and at week 24 (V3 patients) the increasing trend persisted, and the levels were significantly higher compared to baseline in the control group (161.35 ± 14.05 vs 79.34 ± 3.49 ng/ml, $p < 0.001$) (Figure 20).

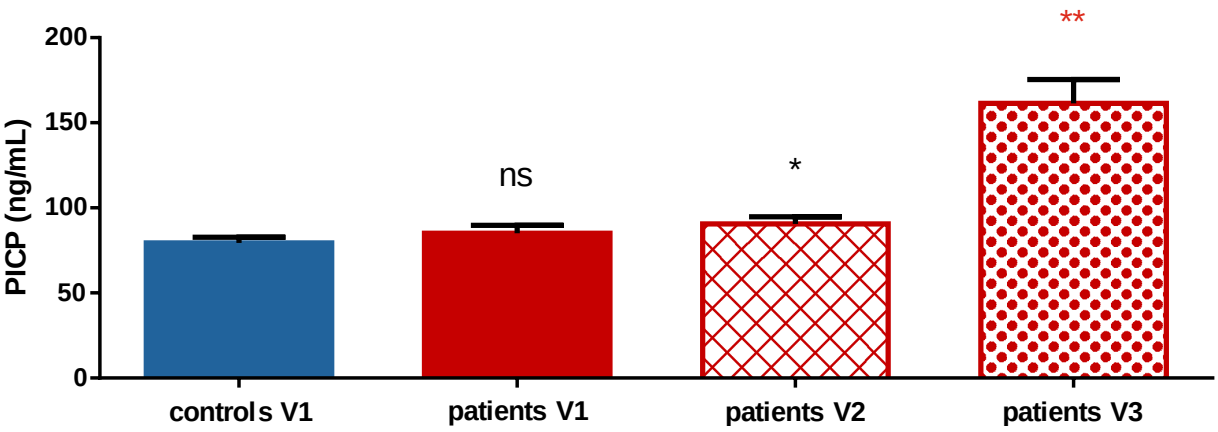

Fig. 20. *Comparison of PICP values in patients versus baseline values in the control group (Controls V1) (patients V1 – baseline values; patients V2 – values at week 12 after PPM implantation; patients V3 – values at week 24 after PPM implantation; *–p<0.05; **–p<0.001; ns – statistically insignificant difference)*

Comparison of the values in the patient group (Figure 21) shows that at week 12 (patients V2) PICP levels had increased compared to baseline values (patients V1) (90.51 ± 4.28 vs 85.13 ± 4.68 ng/ml, $p < 0.05$), but this increase had not reached considerable significance. At week 24 (patients V3), absolute values continued to increase and were now significantly higher compared to baseline levels (patients V1) (161.35 ± 14.05 vs 85.13 ± 4.68 ng/ml, $p < 0.001$).

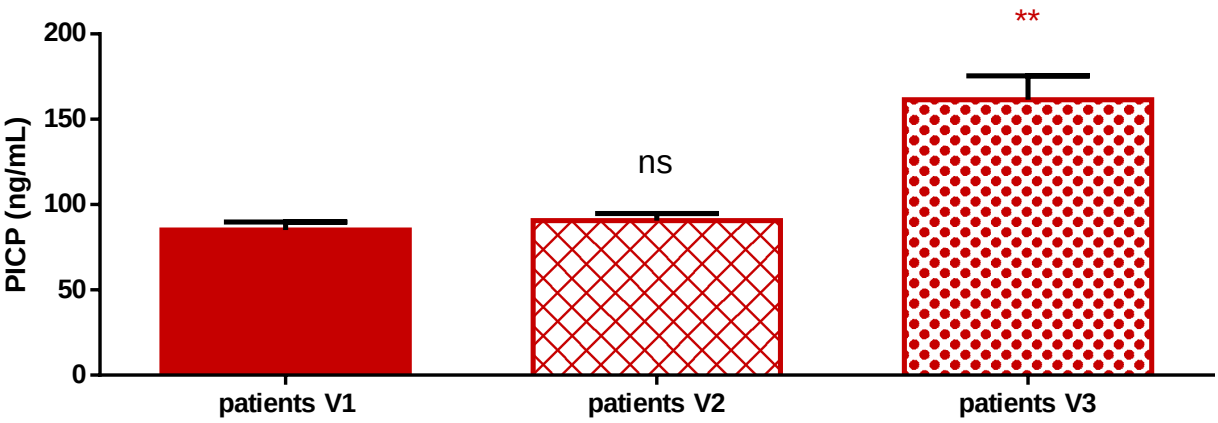

Fig. 21. *Dynamics of PICP levels in the patient group: (patients V1 – baseline values; patients V2 – values at week 12 after PPM implantation; patients V3 – values at week 24 after PPM implantation; *–p<0.05; **–p<0.001; ns – statistically insignificant difference)*

In the control group, no significant changes in plasma levels of this indicator occurred throughout the follow-up period. There was no significant difference between the values of the indicator at the second and third visits compared to baseline (80.91 ± 4.14 vs. 79.34 ± 3.49 ng/ml, p > 0.05; 85.26 ± 4.75 vs. 79.34 ± 3.49 ng/ml, p > 0.05) (Figure 22). Also, there was no significant difference in the indicator levels between the third and second visits although not reflected in Figure 22 (85.26 ± 4.75 vs. 80.91 ± 4.14 ng/ml, p > 0.05).

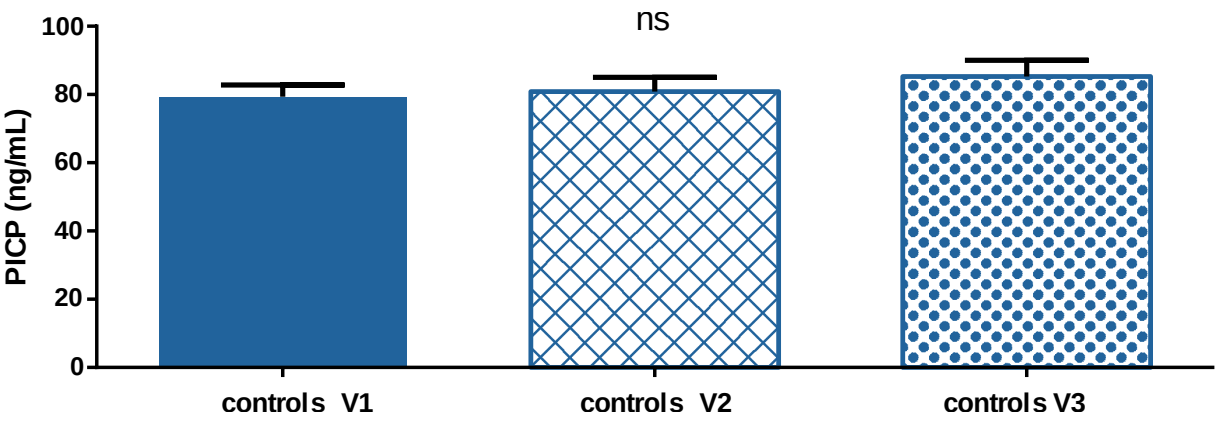

Fig. 22*. Dynamics in PICP levels in the control group: (controls V1 – baseline values of the indicator; controls V2 – values at week 12 after inclusion in the study; controls V3 – values at week 24 after inclusion in the study; ns – a statistically insignificant difference*

The changes in PIIINP levels are presented in the figures below.

Baseline PIIINP values did not differ from baseline values in controls, which is presented in Figure 23 (4.11 ± 0.20 vs 3.94 ± 0.24 ng/ml, p > 0.05) (Figure 23). At week 12, patient levels (V2 patients) were significantly higher compared to baseline controls (6.95 ± 0.56 vs 3.94 ± 0.24 ng/ml, p < 0.001) (Figure 23). At week 24, the patient values (V3 patients) had reached levels higher than controls, but this difference was not significantly significant (4.56 ± 0.20 vs 3.94 ± 0.24 ng/ml, p > 0.05) (Figure 23).

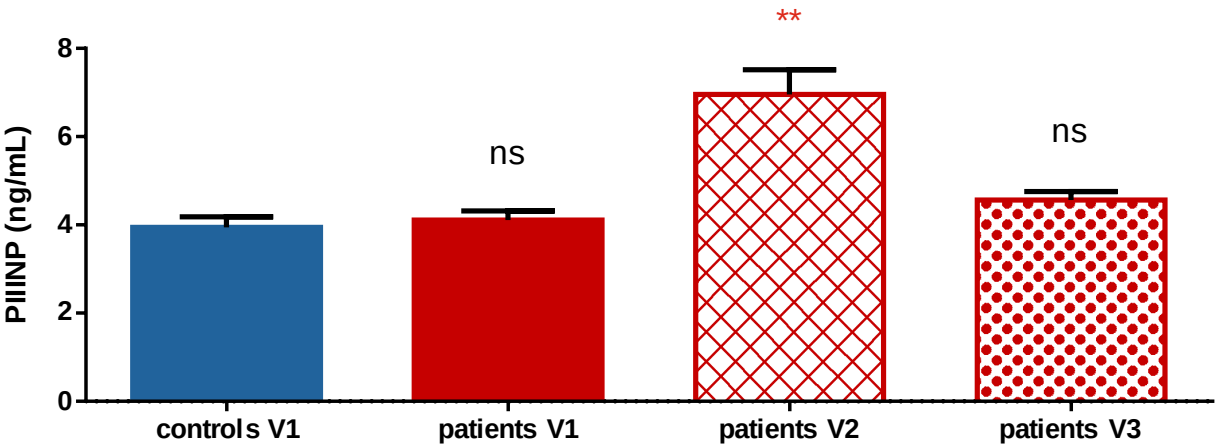

Fig. 23. *Comparison of PIIINP values in patients versus baseline values in the control group (Controls V1) (patients V1 – baseline values; patients V2 – values at week 12 after PPM implantation; patients V3 – values at week 24 after PPM implantation; *–p<0.05; **– p<0.001; ns – statistically insignificant difference)*

Monitoring the PIIINP levels in the patient group (Figure 24) showed that at week 12 (patients V2) PIIINP values had increased significantly compared to baseline (patients V1) (6.95 ± 0.56 vs 4.11±0.20 ng/ml, $p < 0.001$). At week 24 (V3 patients), levels had declined and were still higher than baseline, but this difference was not statistically significant (4.56 ± 0.20 vs 4.11 ± 0.20 ng/ml $p > 0.05$).

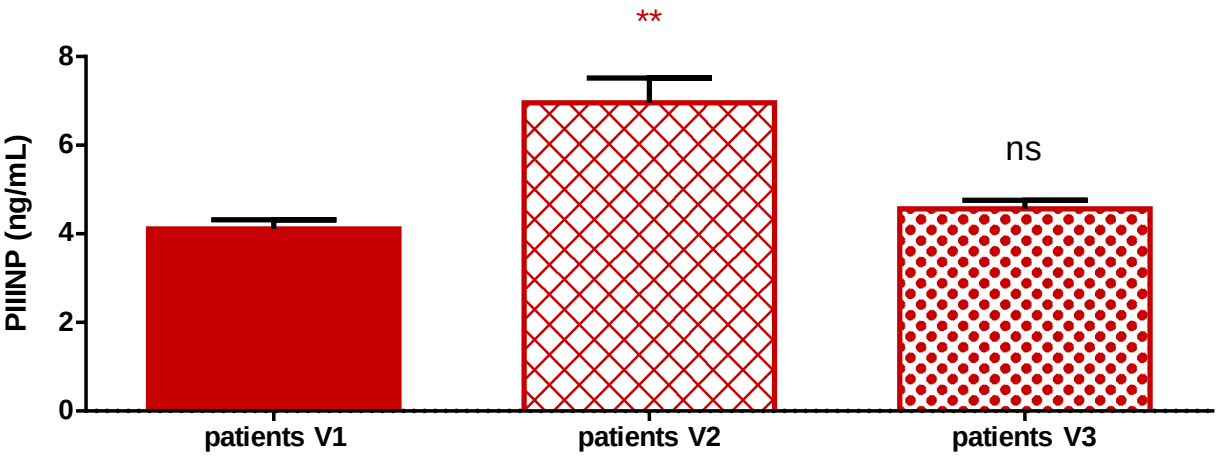

Fig. 24. *Dynamics of PIIINP levels in the patient group: (patients V1 – baseline values of the indicator; patients V2 – values at the week 12 after PPM implantation; patients V3 – values at the week 24 after PPM implantation; *–p<0.05; **–p<0.001; ns – statistically insignificant difference)*

In the control group, there were no significant changes in the plasma levels of the indicator during the follow-up period: no significant difference was found between the values of the indicator at the second and third visits compared to baseline (4.60 ± 0.32 vs 3.94 ± 0.24 ng/ml; 4.06 ± 0.29 vs 3.94 ± 0.24 ng/ml, $p > 0.05$) (Figure 25).

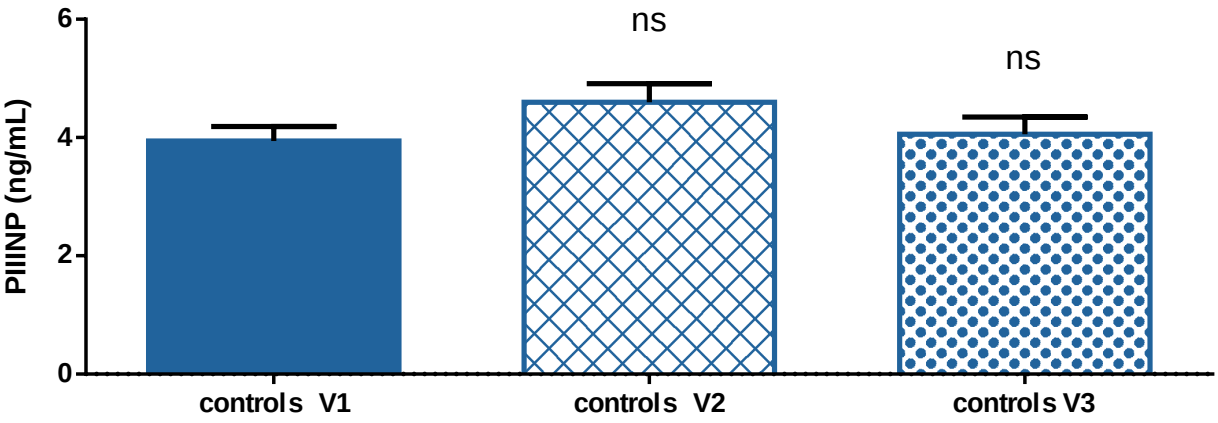

Fig. 25. *PIIINP dynamics in the control group: (controls V1 – baseline values of the indicator; controls V2 – values at week 12 after inclusion in the study; controls V3 – values at week 24 after inclusion in the study; ns – a statistically insignificant difference*

As early as 1994, it was found in experimental models that sustained right ventricular apical pacing for 14 weeks resulted in regional changes in myocardial perfusion, increased catecholamine activity and the development of diastolic dysfunction [Lee et al., 1994]. There is now strong evidence of the negative consequences on cardiac function resulting from apical right ventricular stimulation-induced asynchronous LV contraction [Ravassa et al., 2019]. ECM remodelling underlies the development of systolic and diastolic dysfunction over time in patients with cardiovascular pathology [Ducharme et al., 2000; Spinale, 2002]. Increased collagen deposition in the extracellular matrix of the myocardium leads to the development of fibrosis and structural remodelling [McMurray et al., 2012]. As a consequence, there is reduced cardiac muscle compliance and increased risk of developing myocardial dysfunction, arrhythmias, ischemia and heart failure [Velagaleti&Vasan, 2007].

It is currently believed that the cardiac interstitium is a dynamic network structure with important metabolic activity [Jarvelainen et al., 2009]. Under the influence of a pathological stimulus, fibroblast activation occurs, leading to increased collagen synthesis [Jong et al., 2011; Espeland et al., 2018]. Patients with advanced heart failure are found to have increased collagen content in the interstitium, resulting in a further worsening of the pathophysiological consequences and disease progression [Segura et al., 2014; Kong & Christia, 2014].

In recent years, levels of PICP (carboxy-terminal pro-peptide of pro-collagen type I) and PIIINP (amino-terminal pro-peptide of pro-collagen type III) have established themselves as reliable molecules for assessing collagen metabolism [Prockop and Kivirikko, 1995; Lopez et al., 2015]. Evidence from previous studies suggests that both the amount of interstitial collagen and the ratio of type I to type III collagen are important for developing cardiac dysfunction [Uchinaka et al., 2018]. When this ratio increases, decreased left ventricular wall elasticity and

ventricular dilatation are found. The correlation between disorders of collagen metabolism and the development of various cardiac pathologies has been studied for the past decades.

The results of our study showed that PICP levels increased as early as week 12 post-implantation compared to controls, albeit with a cut-off value of $p = 0.0445$ (Figure 21). At week 24, there was a significant increase in PICP, both relative to patients' baseline values ($p < 0.001$) (Figure 22) and relative to controls' baseline values ($p < 0.001$) (Figure 21). *Considering the dynamics of the changes, these findings provide grounds to believe that apical right ventricular stimulation results in extremely early activation of collagen metabolism.*

Since the half-life of the collagen molecule in the cardiac interstitium is over 6 months, it has been suggested that the myocardial remodelling processes in the presence of a pathological stimulus require time, with the balance between synthesis and degradation shifting towards increased synthesis [Weber et al., 1989]. Type I collagen molecules build thick fibrils with low levels of turnover, whereas type III collagen molecules build thinner and more delicate filaments subject to accelerated turnover [Holm Nielsen et al., 2020]. Type I collagen takes longer to synthesize due to the more complex secondary and tertiary structure of the molecule. The rate of synthesis of collagen molecules, and therefore the extent of their turnover, should be considered when interpreting the results of our study.

The myocardial extracellular matrix has a specific composition and contains mainly type I collagen (85%) and type III collagen (11%) [Kong et al., 2014]. Evidence suggests that the composition of fibrotic tissue in reactive myocardial fibrosis is characterised by an increase in type I collagen, which is composed of thick fibrillar polymers with multiple cross-links between them, and a smaller amount of type III collagen, which has thinner and less cross-linked molecules [Lopez et al., 2015]. Due to the different biophysical characteristics of the two types of collagen fibrils, even a slight increase in the ratio between type I collagen and type III collagen results in a significant increase in myocardial stiffness. This corresponds with our findings showing a substantial increase in the PICP levels in the patient group towards the end of the 24-week follow-up period (Figure 21). When comparing the PIIINP values, it can be seen that at week 24 its levels are elevated relative to both baseline levels in patients and controls, although this increase does not reach significant significance (Figure 23; Figure 24). *As a result, we can conclude that both markers respond with an increase within 6 months to the asynchronous ventricular contraction induced by apical right ventricular stimulation.*

According to the design of our study, the patients had no severe concomitant cardiovascular pathology except for conduction disturbances and up to grade I well-controlled arterial hypertension, which necessitated the implantation of a permanent pacemaker. This brings us to assume that collagen synthesis responds with activation due to the electrical and

mechanical asynchrony induced by stimulation in the right ventricular apex. Similar data were obtained in a study conducted by Lin et al. (2010). They found that after 12 weeks of constant right ventricular stimulation, heterogeneous changes in ECM, increased MMP-2 and MMP-9 activity, and an increase in collagen type I mRNA levels were observed.

In the patient group of our study, there was an increase in PIIINP levels at week 12 post-implantation, both relative to baseline in patients ($p < 0.001$) (Figure 24) and relative to baseline in the control group ($p < 0.001$) (Figure 23). However, at week 24 post-implantation, there was a decline in values to levels close to but higher than baseline.

Regarding the significant increase 12 weeks after implantation, it can be assumed that it may be the result of the surgical intervention. It is a well-established that type III collagen is involved in the construction of skin and subcutaneous tissue and is actively involved in the healing of surgical wounds [Riekki et al., 2004]. Studies of samples taken from surgical wounds found a significant increase in PIIINP levels in the days following intervention [Haukipuro et al., 1987]. Serum levels of this marker have also been found to increase 1000-fold over those of non-operated patients, peaking on postoperative day 5. However, the dynamics of marker levels after this period has not been investigated. Data from histological examinations of tissues from surgical wounds in the process of healing show the most pronounced collagen deposition in samples taken on days 14 and 28 after intervention [White et al., 2002]. It is important to note that immunofluorescence histological analyses on days 14 and 28 showed the presence of type I and type III collagen in equal amounts in the samples. Although these data do not show the dynamics of collagen deposition after this period, it is clear that the healing process of the surgical wound engages both type I and type III collagen equally in the first 4 weeks. The healing of any wound normally goes through the phases of inflammation, proliferation and remodelling [Young & McNaught, 2011]. In minor surgical interventions, such as permanent pacemaker implantation, surgical cicatrixes usually go through these phases quickly, throughout 7 to 10 days. However, pacemaker implantation also requires the formation of a pocket to house the pacemaker [Burri et al., 2021]. Intraoperative pocket formation disrupts the integrity of the subcutaneous connective tissue. The healing processes of soft tissue injuries can lead to a sustained increase in PIIINP synthesis [Haukipuro et al., 1990].

Results from studies in experimental animals have shown an increase in PIIINP levels as granulation tissue forms during soft tissue healing, and this increase can last up to several months [Jensen et al., 1993]. Data from a study conducted by Ulrich et al. (2003) in patients with skin burns undergoing surgical treatment showed a peak in PIIINP levels on day 14 after the intervention, and they remained significantly elevated for up to 6 months onwards. Importantly, PIIINP values at 6 months after surgical reconstruction of extensively damaged

skin dropped by almost half but were significantly higher than those in the control group. This fact highlights the active involvement of collagen synthesis in the process of cicatrix formation and remodelling and proves that even in patients with extensive cicatricial areas, the marker values significantly decrease 6 months after reconstruction. Also, the formation of a mature cicatrix in patients with skin burns can persist for a period exceeding 12 months [Tuan & Nichter, 1998].

Based on the information presented thus far, it can be assumed that the increased PIIINP values at week 12 after PPM implantation may be due to the healing processes and the formation of the capsule around the pulse generator. However, the elevated values after 6 months are likely to result from the activation of collagen synthesis in the myocardium induced by right ventricular apical stimulation. To establish the further course of PIIINP levels, follow-up over a longer period is necessary.

Collagen deposition in reactive myocardial fibrosis increases cardiac muscle stiffness, leading to the development of systolic and diastolic dysfunction [Zile et al., 2011; Michalski et al., 2017; Duprez et al., 2018]. In patients with congestive heart failure, a positive correlation has been found between PICP levels and left ventricular dimensions, BNP levels and the presence of intraventricular asynchrony [Löfsjögård et al., 2014]. Similar results have been found in ICD patients [Flevari et al., 2012]. The data showed that a lower left ventricular ejection fraction and a higher PICP/PIIINP ratio were found in registered tachycardia requiring device therapy. In both studies, serum markers of collagen synthesis were examined simultaneously without analysing their change in dynamics.

As discussed earlier, changes in the myocardial interstitium can occur in various pathological conditions [Velagaleti&Vasan, 2007; McMurray et al., 2012]. Therefore, the patients included in our study were free of serious comorbidities that may affect fibrotic activity during follow-up. Conversely, with the natural processes occurring with the biological ageing of the body, activation of fibrotic processes is observed in various tissues and organs [Jong et al, 2011]. Fibrosis is mainly characterised by increased collagen deposition in the interstitium of the affected organ [Hwang et al., 2007]. Collagen fibrils synthesised in the interstitium of an ageing organism are characterised by increased resistance to metalloproteinases (MMPs) due to the presence of more cross-links, twists and overlaps between molecules [Kwak et al., 2011]. This process leads to an increase in myocardial stiffness, electrical inhomogeneity and the development of left ventricular systolic and diastolic dysfunction [Zile et al., 2011]. For this reason, we monitored the markers of collagen synthesis in parallel and dynamics in both patient and control groups. Our results showed no significant changes in the indicators when tracked in the control group (Figure 22, Figure 25).

It is essential to note that patients' medication therapy at inclusion in the study, as well as during the study itself, did not affect fibrotic metabolism or there is no definite evidence of this. This was addressed in the study design in order to reliably report the "net" effect of PPM on the molecules that were assessed. There is compelling evidence from studies that the medications affecting RAAS influence the extent of collagen deposition in the myocardium and thus improve patient prognosis [Heymans et al., 2015]. Data from a study in humans with hypertension found that after 9 months of treatment with mineralocorticoid receptor antagonist spironolactone, there was a significant reduction in plasma levels of the collagen synthesis markers PIIINP and PICP, as well as an increase in the levels of the collagen degradation marker CITP (C-terminal telopeptide of collagen type I) [Ferreira et al., 2019]. As a result, reverse extracellular matrix (ECM) remodelling and reduction of myocardial fibrosis were observed. Importantly, this effect was independent of the degree of blood pressure lowering, highlighting the direct impact on the myocardial interstitium and leading to an improvement in the long-term prognosis of patients and a reduction in the risk of developing systolic and diastolic cardiac dysfunction. Our enrolled patients were treated with methyldopa, amlodipine, and hydrochlorothiazide (Table 2). There is no literature evidence for these medications to affect collagen metabolism. According to the study design, we attempted to minimise external factors that could influence collagen synthesis and the activation of the fibrotic cascade. On the one hand, the enrolled patients had no severe comorbidities, and on the other hand, antihypertensive therapy was modulated to avoid medication affecting RAAS.

The process leading to myocardial remodelling is extremely dynamic [Hwang et al., 2007]. As it has been shown, it is influenced by a variety of pathological factors as well as by the administered medication therapy [Ferreira et al., 2019]. Also, the body's natural ageing processes that increase collagen deposition in different parenchymal organs should not be overlooked. To assess the extent of myocardial collagen deposition in patients with PPM, a study was conducted by Saunderson et al (2021). They performed contrast-enhanced MRI before PPM implantation and 6 months after in patients with high-grade AV block. It is important to note that in the study the electrode was placed in the right ventricular apex. The results showed that patients with the most pronounced myocardial fibrosis on pre-PPM MRI exhibited LV dysfunction, necessitating an upgrade to a resynchronization system. In those patients who had evidence of increased myocardial fibrosis at 6 months post-implantation, a more significant reduction in EF and increase in LVESV was found. Because contrast-enhanced MRI is the gold standard for assessing the extent of myocardial fibrosis, the data from this study once again confirm the association between right ventricular apical pacing with PPM and cardiac remodelling. A similar study with MRI and follow-up of collagen synthesis markers in

patient and control groups is needed to confirm the results, and the data obtained will be even more representative.

To sum up, our results were presented in a joined graphic, with values expressed as decimal logarithms (Figure 26). Figure 26 shows the unidirectional dynamics of the increase of the studied signalling molecules and the marker of collagen synthesis PICP in the patient group, compared with the plateau course of the values of the indicators in the control group.

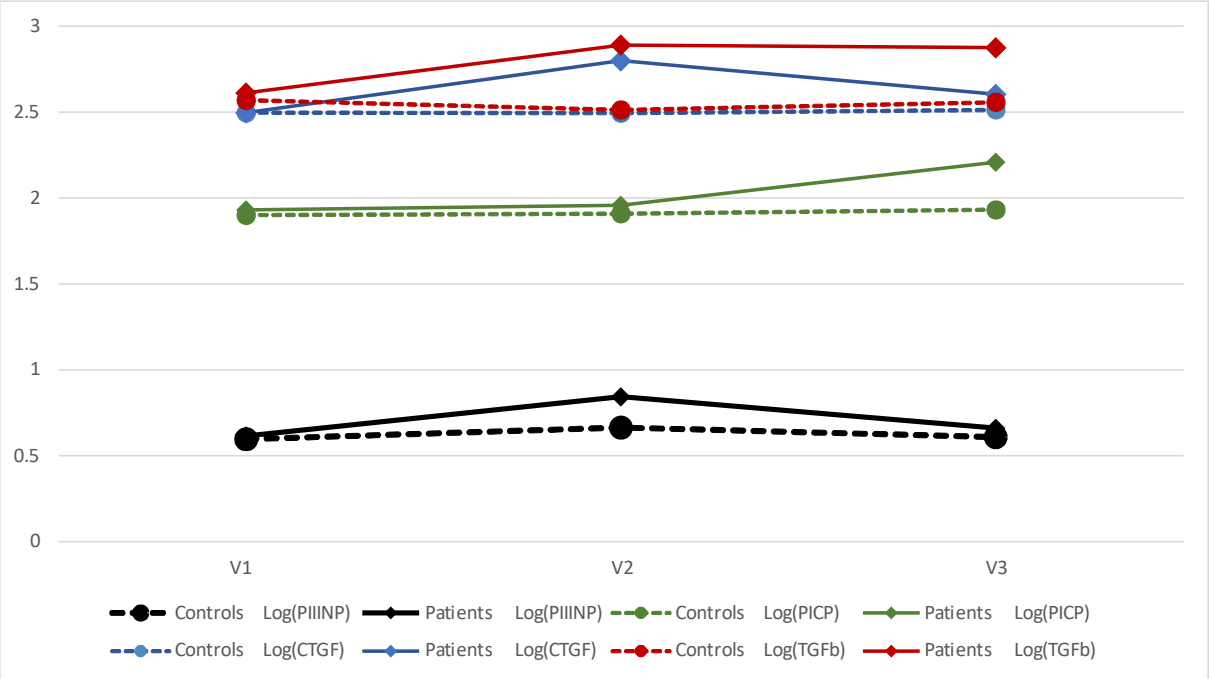

Fig. 26. *Dynamics of values in patients and controls expressed as decimal logarithm*

Analysing simultaneously the changes in the studied indicators, as well as the fact of the absence of severe concomitant pathology, respectively the set inclusion and exclusion criteria of the study, we believe that the external influence on the fibrotic process is minimised. *Taking into account the points made earlier and in the context of the data presented above, we can assume that the increase in PICP and PIIINP levels at week 24 might be credited to pacemaker-induced asynchronous ventricular contraction.*

3. Adequacy of the volume of the studied groups – power analysis of t-criterion for changes in the fibrotic markers

Of particular importance for any study is the sample size, which is determinant of the credibility of the drawn conclusions [Bland, 2000; Munro, 2005]. A study with an incorrect sample size may adversely affect the results of the study. This requires an assessment of the

power of the statistical criterion used (for the thesis presented, this is a two-tailed independent samples t-test used to assess significant differences in levels of the signalling molecules for fibrosis activity and the collagen synthesis marker PICP) at a given level of first order error (level of agreement) depending on sample size, mean and standard deviation (SD). Power below 0.75 is considered insufficient in clinical studies. This means that the statistical criterion used has less than a 75% chance of capturing the specified differences. Therefore, it is commonly accepted that power must be >0.75 to be considered satisfactory [Fitzner & Heckinger, 2010].

Table 3 below shows the power of the t-criterion for testing the hypothesis of equality of the mean values (patients and controls) of the fibrosis indicators studied after 24 weeks of follow-up, given a first-order error level (level of agreement) of 0.05. The results show that there is very good statistical power of the t-criterion (>0.75) in the sample size we used (patient and control group size), except in the case of plasma PISP levels, where the power is at the cut-off (0.76).

Table 3. *Power of the t-criterion for two independent samples at the presented mean and SD of the fibrosis indicators studied at week 24 (V3/V3c)*

Fibrosis indicator	**Mean value ±SD in patients on V3**	**Mean value ±SD in controls on V3**	**Power of the criterion**
CTGF	400.83 ± 78.60	324.74 ± 92.00	0.99
TGF beta1	748.28 ± 257.77	359.55 ± 166.38	0.95
PCIP	161.35 ± 83.12	85.26 ± 31.13	0.76

To visualise the power analysis methodology, we present the calculations (Table 4) and the graph (Figure 27) on the CTGF indicator made by the used statistical package STATISTICA 13.3.0, StatSoft Inc., USA

Table 4. *Calculations with STATISTICA 13.3.0 statistical package, StatSoft Inc., USA*

Power Calculation (FIBR.sta) Two Means, t-Test, Ind. Samples H0: Mu1 = Mu2

	Value
Population Mean Mu1	400.83
Population Mean Mu2	324.74
Population S.D. (Sigma)	78.60
Standardized Effect (Es)	1,0181
Sample Size N1	40.0000
Sample Size N2	46.0000
Type I Error Rate (Alpha)	0.0500
Critical Value of t	1.9886
Power	0.9965

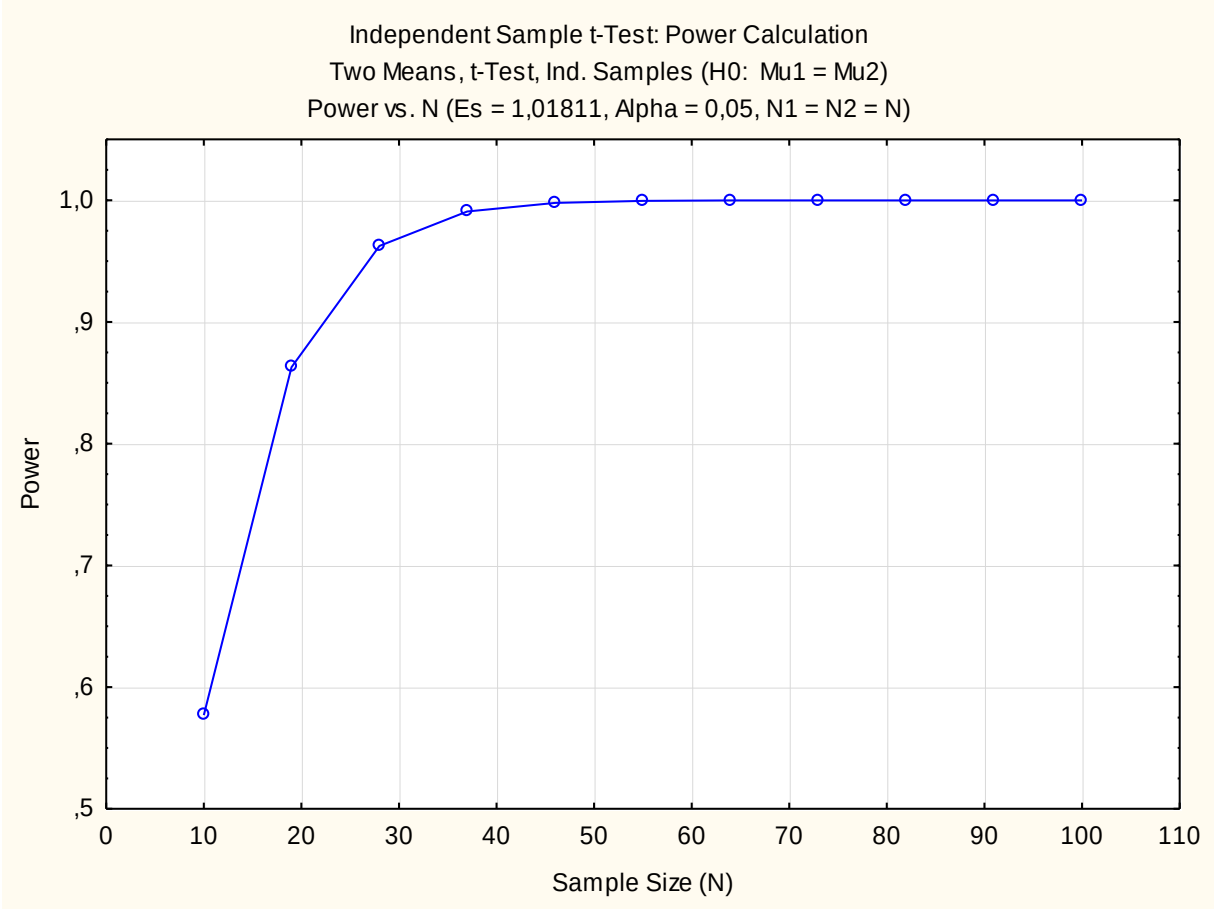

Fig. 27. *The graph presents the correlation between sample volume and t-test power magnitude for the two independent samples of CTGF plasma level values*

In summary, the power analysis performed shows that the number of participants selected for each group is sufficient and adequate for the obtained mean and SD values of the fibrosis indicators studied. The presented results and conclusions made are reliable and correctly describe changes in the collagen synthesis marker and the signalling molecules of fibrotic activity in the populations studied. They are not random findings or the result of experimental error.

4. Deviations in the studied echocardiographic parameters

For the purposes of the study, the following parameters were monitored: LAVI, LVEDV, LVESV and ejection fraction (EF%) was calculated. Table 5 shows no difference between the baseline LVEDV values of the patients and those of the control group ($p > 0.05$). Also, there was no significant difference in values in patients versus controls at follow-up at week 6 (patients V1.1) ($p > 0.05$), week 12 (patients V2) ($p > 0.05$) and week 24 (patients V3) ($p > 0.05$).

Table 5. *Results of transthoracic echocardiography*

ECG ***indicator***	**LAVI** ***(mL/m²)***	**LVEDV** ***(mL/m²)***	**LVESV** ***(mL/m²)***	***EF (%)***
Controls	16.69 *± 0.23*	52.17 *± 1.65*	24.52 *± 0.76*	55.98 *± 0.33*
Patients V1	20.03 *± 0.71*	51.98 *± 1.97*	24.50 *± 0.77*	57.36 *± 0.66*
P-value	*< 0.001*	*> 0.05*	*> 0.05*	*> 0.05*
Patients V1.1	25.48 *± 1.33*	51.95 *± 1.95*	23.45 *± 0.90*	56.16 *± 0.79*
P-value	*< 0.001*	*> 0.05*	*> 0.05*	*> 0.05*
Patients V2	27.42 *± 1,34*	51.94 *± 1.63*	24.23 *± 0.88*	56.23 *± 0.65*
P-value	*< 0.001*	*> 0.05*	*> 0.05*	*> 0.05*
Patients V3	27.05 *± 1.51*	51.62 *± 1.81*	26.05 *± 0.72*	56.13 *± 0.69*
P-value	*< 0.001*	*> 0.05*	*> 0.05*	*> 0.05*

Figure 28 shows that there was no difference between baseline LVESV values in patients (V1) and controls (24.50 ± 0.77 vs 24.5 2± 0.76 ml/m2, $p > 0.05$). At week 6 and week 12 (patients V1.1 and patients V2). There was also no significant difference between the data for patients versus controls (23.45 ± 0.90 vs 24.52 ± 0.76 ml/m2, $p > 0.05$; 24.23 ± 0.88 vs 24.52 ± 0.76 ml/m2, $p > 0.05$). At week 24 after PPM implantation, there was a tendency for increased values in patients compared to controls, but this difference did not reach statistical significance (26.05 ± 0.72 vs 24.52 ± 0.76 ml/m2, $p > 0.05$) ($p = 0.15$)

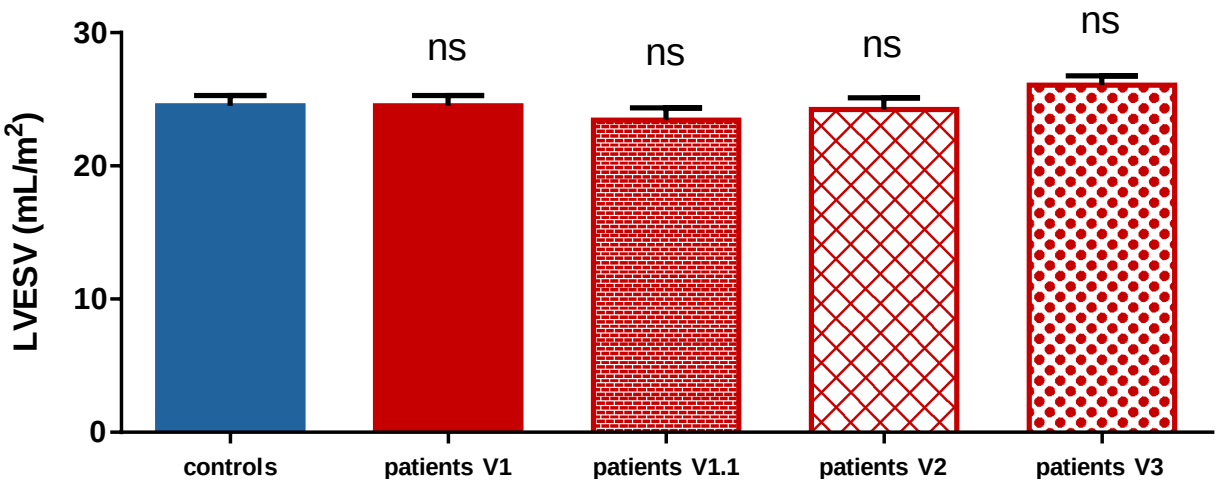

Fig. 28*. Comparison of LVESV values in patients versus baseline values in the control group (Controls V1) (patients V1 – baseline values; patients V1.1 – values at week 6 after PPM implantation; patients V2 – values at week 12 after PPM implantation; patients V3 – values at week 24 after PPM implantation; *–p<0.05; **–p<0.001; ns – statistically insignificant difference)*

Figure 29 shows that there was no significant difference between baseline EF% in patients (V1) and controls (57.36 ± 0.66 vs 55.98 ± 0.33%, p > 0.05). At the follow-up visits at week 6 (patients V1.1) and week 12 (patients V2), there was also no significant difference from baseline in the control group (56.16 ± 0.79 vs 55.98 ± 0.33%, p > 0.05) and (56.23 ± 0.65 vs 55.98 ± 0.33%, p > 0.05). At week 24 post-implantation, there was no significant difference in patients' EF% from baseline in the control group (56.13 ± 0.69 vs 55.98 ± 0.33%, p > 0.05).

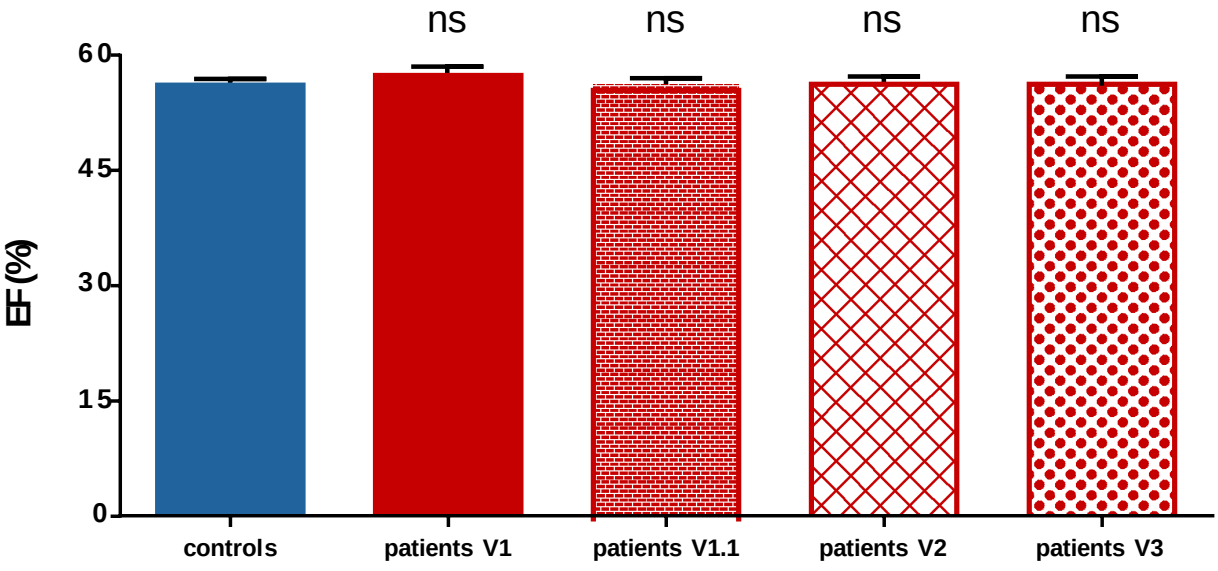

Fig. 29*. Comparison of EF% values in patients versus baseline values in the control group (Controls V1) (patients V1 – baseline values; patients V1.1 – values at week 6 after PPM implantation; patients V2 – values at week 12 after PPM implantation; patients V3 – values at the week 24 after PPM implantation; *–p<0.05; ** –p<0.001; ns – statistically insignificant difference)*

Follow-up of the EF in the patient group (Figure 30) shows that at the follow-up visits at week 6 (patients V1.1) and week 12 (patients V2), there was also no significant difference from

baseline (56.16 ± 0.79 vs 57.36 ± 0.66%, p > 0.05; 56.2 3± 0.65 vs 57.36 ± 0.66%, p > 0.05). At week 24 post-implantation, there was a trend towards lower EF in patients compared to baseline values, with the difference not reaching statistical significance (56.13 ± 0.69 vs 57.36 ± 0.66%, p > 0.05) (Figure 30).

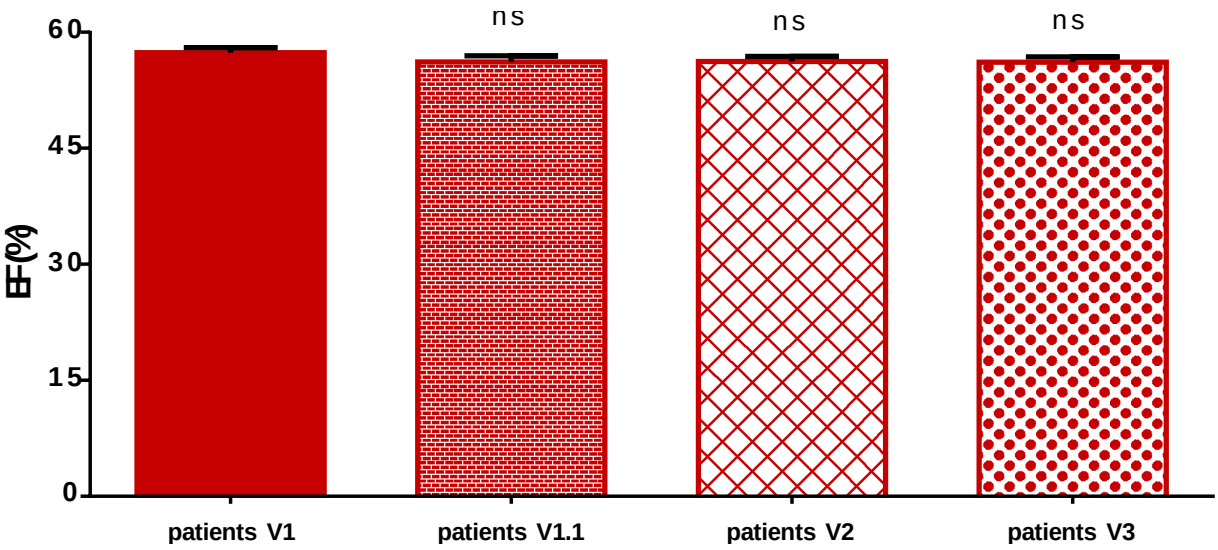

Fig. 30. *Dynamics of EF% values in the patient group: (patients V1 – baseline values of the indicator; patients V1.1 – values at week 6 after PPM implantation; patients V2 – values at week 12 after PPM implantation; patients V3 – values at week 24 after PPM implantation; *–p<0.05; ** 0150p<0.001; ns – statistically insignificant difference)*

Revealing the aetiology of underlying cardiac dysfunction is the basis for the diagnosis of heart failure, with treatment based on a specific pathological cause. Most commonly, heart failure is due to myocardial dysfunction, which may be systolic, diastolic, as well as combined. Left ventricular ejection fraction is the most used marker to classify heart failure. EF values are used as the basis for clinical trial conclusions, to assess the effect of medication treatment and to determine the extent of reimbursement by the health insurance system in most countries [Lam & Solomon, 2014]. Values of the normal ejection fraction remain controversial, with a lower limit of 49 to 57% depending on age, sex, and ethnicity, according to the EchoNoRMAL study [Poppe et al., 2015].

According to the American Society of Echocardiography and the European Association of Cardiovascular Imaging, ranges of 62% (52–72%) in men and 64% (54–74%) in women are assumed as normal [Lang et al. 2015]). According to the EF values, heart failure patients are classified by the European Society of Cardiology (ESC) into three groups: those with preserved EF >50%, those with mildly reduced EF from 40–49% and those with reduced EF below 40% [Ponikowski et al, 2016].

The negative effect of apical right ventricular pacing on the cardiac pump function has now been firmly established [Zhang, 2008]. There is a consensus that apical pacing over 40% of the time is strongly associated with an increased risk of developing manifestations of heart

failure [Khurshid et al, 2014]. A linear correlation between the frequency of right ventricular pacing and the risk of left ventricular dysfunction has also been demonstrated [Kiehl et al, 2016].

According to our study design, patients with complete atrioventricular block with more than 80% apical right ventricular pacing during the entire follow-up period were included at screening. The results showed that the left ventricular EF in the patient group did not undergo significant changes compared with the control group by the end of the 24-week follow-up period. Figure 29 shows that at visit 3, there was a trend of decreasing EF values in the patients compared to those in the control group (Figure 29). There was also an increase in left ventricular ejection fraction (LVESV) values in patients versus controls at visit 3, although this difference also did not reach statistical significance but had a cut-off value of $p = 0.15$ (Figure 28). This is consistent with the established trend of a reduction in the left ventricular ejection fraction.

Although our results do not show a significant decline in left ventricular pump function, it should be noted that the presence of an initial trend toward its reduction should not be underestimated. Data from a number of studies performed to date strongly suggest an increased risk of developing systolic dysfunction after PPM implantation and apical right ventricular pacing. The study by Zhang et al. (2008) in a large group of patients showed a relatively late onset of left ventricular systolic dysfunction in patients with baseline normal pumping parameters. The results showed that 26% of the patients showed a decline in left ventricular EF, with the majority of these occurring after the third year after implantation. It should be noted that some of the included patients had comorbidities such as IHD and DM, which may further contribute to the development of heart failure. This may explain the relatively high incidence of affected patients, as data from other similar studies most commonly show the development of systolic dysfunction with apical right ventricular pacing in about 20% of patients [Dreger et al., 2012].

Conversely, the BLOCK HF Trial, for example, found an extremely early decline in cardiac pumping performance as early as 6 months after implantation [Curtis et al., 2013]. It is important to note, however, that the patients included in this study had borderline or moderately reduced EF and coexisting pathology such as ischemic disease, chronic kidney disease, diabetes mellitus, etc. Randomized patients were followed for approximately 2 years. The effect of biventricular pacing with CRT and right ventricular apical pacing was compared, with results favouring resynchronisation devices in terms of mortality and manifestation of heart failure. An interesting result of this study was that an increase in left ventricular end-systolic volume (LVESV) was associated with a significantly increased relative risk of death and hospitalisation for heart failure.

Other studies have shown the development of systolic dysfunction in 12.3% of paced patients after a period of 4.3 years, with a mean decline in EF of 24% [Kiehl et al., 2016]. However, due to the lack of follow-up visit data, it is unclear at what point in time the decline in the left ventricular pumping performance begins to be recorded.

Similar results were also derived by the team of Bansal et al. (2019), where patients were followed for a period of 14.5 months and new-onset systolic dysfunction was found in 13.8% of patients with permanent apical right ventricular pacing. From what has been said so far, we can conclude that in our study, there was a trend toward a decrease in left ventricular pumping parameters in the patient group compared with the control group. These changes did not reach significance and we can assume this is due to the relatively short follow-up period of 6 months. On the other hand, the fact that our selected patients were free of concomitant cardiovascular pathology, except up to grade I well-controlled arterial hypertension, should not be overlooked. With this design, we aimed to minimize the influence of additional factors and conditions that could affect left ventricular remodelling.

Figure 31 shows that baseline LAVI values were significantly higher compared to controls (20.03 ± 0.71 vs. 16.69 ± 2.23 ml/m2, $p < 0.001$). At the follow-up visits, at the 6th, 12th and 24th weeks, respectively, there was a significant increase in values compared to those in the control group (Figure 31) (25.48 ± 1.33 vs. 16.69 ± 2.23 ml/m2, $p < 0.001$; 27.42 ± 1.34 vs. 16.69 ± 2.23 ml/m2, $p < 0.001$; 27.05 ± 1.33 vs. 16.69 ± 2.23 ml/m2, $p < 0.001$).

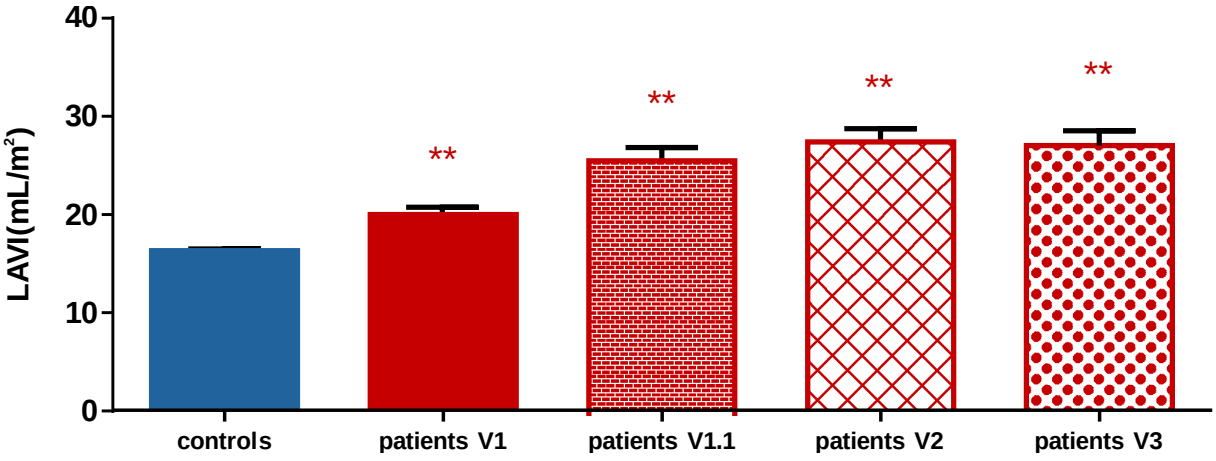

Fig. 31. *Comparison of LAVI values in patients versus baseline values in the control group (Controls V1) (patients V1 – baseline values; patients V1.1 – values at week 6 after PPM implantation; patients V2 – values at week 12 after PPM implantation; patients V3 – values at week 24 after PPM implantation; *–p<0.05; **–p<0.001; ns – statistically insignificant difference)*

LAVI follow-up in the patient group (Figure 32) showed that at the follow-up visits on the 6th, 12th and 24th, respectively, there was a significant increase in the values compared to

baseline (25.48 ± 1.33 vs 20.03 ± 0.71 ml/m2, p < 0.001; 27.42 ± 1.34 vs 20.03 ± 0.71 ml/m2, p < 0.001; 27.05 ± 1.33 vs 20.03 ± 0.71 ml/m2, p < 0.001).

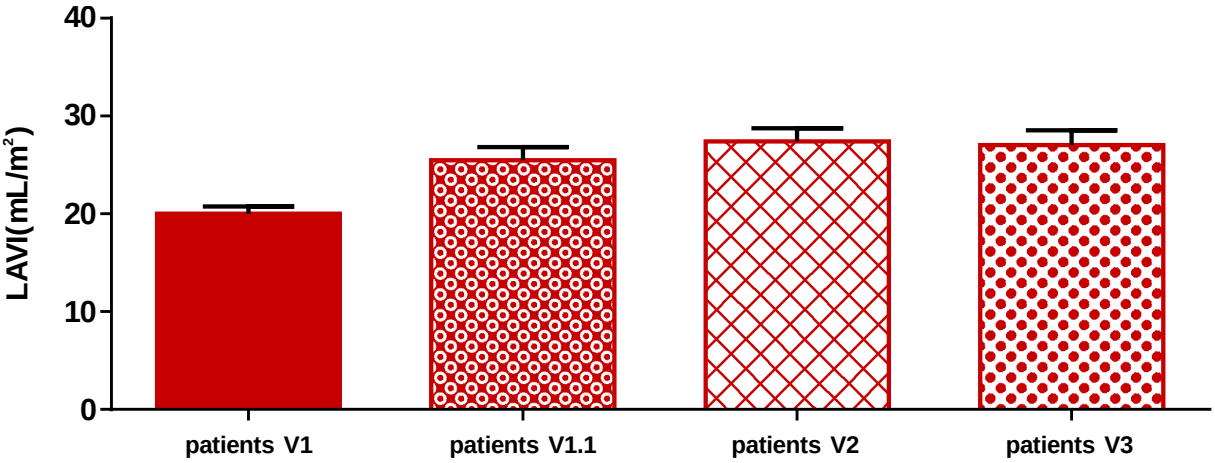

Fig. 32. *Dynamics of LAVI in the patient group – (patients V1 – baseline values of the indicator; patients V1.1 – values at week 6 after PPM implantation; patients V2 – values at week 12 after PPM implantation; patients V3 – values at week 24 after PPM implantation; *–p<0.05; **–p<0.001; ns – statistically insignificant difference)*

The left atrium is important for the efficiency of the cardiac cycle, ensuring adequate left ventricular filling [Obokata et al., 2013]. During the reservoir phase, the LA receives blood from the pulmonary veins and, by increasing its volume due to its compliance, ensures normal LV filling pressure. The LA reservoir phase is also influenced by left atrial relaxation, left atrial contractility, and the apical displacement of the left ventricular base during systole [Goette et al., 2017]. In the early diastole phase after mitral valve opening, blood from the left atrium passively enters the left ventricle as a result of the pressure gradient created between the two cavities. After diastasis or the slow ventricular filling phase, when the atrium acts as a connection between the pulmonary veins and the LV, contraction of the left atrium follows, thereby filling the ventricle [Rossi et al., 2014].

The left atrium is extremely sensitive to volume and preload due to increased left ventricular filling pressures [Dernellis et al., 1998]. Also, disruption of left atrium function results in remodelling of the pulmonary vessels as well as a decrease in their compliance, leading to right ventricular pressure loading and pulmonary hypertension. Therefore, early detection of left atrium dysfunction is important in the evaluation of a number of cardiac diseases [Sugimoto et al., 2017].

In the case of a complete atrioventricular block, the sequence of atrioventricular coupling is disrupted, compromising adequate ventricular filling [Appleton et al., 1991]. In the early period of complete atrioventricular block, there is atrial volume and pressure overload as a result of the two processes. First, there is complete asynchrony between atrial and ventricular

contraction, which at some point results in atrial shortening against the closed atrioventricular valves. As a result, there is a sharp increase in their pressure and blood retrograde flow is directed to the pulmonary and systemic veins, respectively. Conversely, in complete atrioventricular block, a diastolic mitral and tricuspid regurgitation occurs because of delayed or absent ventricular systole to follow atrial contraction. Atrial systole is followed by a reduction in atrial pressure, resulting in a small inverse gradient and partial mitral valve closure. For the mitral and tricuspid valves to close completely, ventricular systole must occur, and if this does not occur, as in the presence of high-grade AV block, diastolic mitral and tricuspid regurgitation occurs [Rokey et al., 1986].

The results of our study showed that the baseline LAVI values of the patients were significantly higher than those of the control group (Figure 32). According to the study design, the enrolled patients had normal echocardiographic parameters according to accepted criteria [Lang et al., 2015] and had no significant valvular pathologies. Considering this, it is safe to say that the baseline increased left atrial volume in the patients compared to the control group is the result of the haemodynamic burden resulting from the atrioventricular block.

The increase in left atrial volume is one of the most commonly used indices to assess its function and serves as an indirect indicator to assess left ventricular pressure [Lang et al., 2015]. Left atrial remodelling results from multiple factors, including tachyarrhythmias in atrial fibrillation, volume and pressure overload due to valvular pathology, left ventricular wall stiffness changes in ischemic heart disease, arterial hypertension, and others [Gottdiener et al., 2006; Owan et al., 2006].

Right ventricular apical stimulation leads to electrical and mechanical asynchrony in the left ventricle, resulting in the development of left ventricular dysfunction and increased filling pressures [Delgado et al., 2009; Puggioni et al., 2004]. Non-physiological activation of the myocardium causes asynchrony in the ventricular systole, impairs ventricular relaxation, and reduces the degree of longitudinal shortening and circular twists [Tops et al., 2007; Tops et al., 2009]. These changes affect the function and structure of the left atrium, which can lead to the development of arrhythmias and other complications [Matsuoka et al., 2009]. Additionally, right ventricular apical pacing induces left ventricular diastolic dysfunction and increases left atrial afterload [Xie et al., 2012a]. This process increases atrial pressure and contributes to left atrial enlargement in the early postoperative period, as shown by the results of Xie et al. study (2012 a).

In our study, a significant increase in LAVI was seen in patients compared to baseline values as early as 6 weeks post-implantation (Figure 32). This confirms the extremely early onset of pressure changes in the left atrium due to the induced asynchronous cardiac

contraction, which also leads to an increase in its volume. Similar results have been reported comparing a group of patients with apical right ventricular pacing and those with HBP (His Bundle Pacing), where cardiac contraction occurs as a result of electrical activation of the native conduction system. At the 6th month after implantation, a significant increase in LAVI was observed in the apical right ventricular pacing group and a decrease in the HBP group [Michalik et al., 2021]. Another point worth mentioning is that the left atrium volume continued to increase in the absence of other echocardiographic abnormalities. Therefore, we believe that it is related to altered LV synchrony and the left atrial remodelling is not a single event (Figure 32). Data from the MOST (MOde Selection Trial) reported 10 years ago strongly associate right ventricular pacing with new-onset AFib [Sweeney et al., 2003]. The study demonstrated that for every 1% of right ventricular pacing, the risk of AFib increased by 1%, and this correlation remained linear over time until 85% pacing was reached. Similar results are available from other studies, where left atrial burden during apical right ventricular stimulation leads to an increase in the risk of AFib new-onset [Sanalaga et al., 2011; Xie et al., 2012b].

This highlights the significance of monitoring left atrial volumes in patients with an implanted pacemaker and risk stratification of atrial arrhythmia.

5. Dependence of changes in the echocardiographic parameters on fibrotic activity markers

In our thesis, we used simple linear regression for modelling the correlation between CTGF and EF, CTGF and LAVI, TGF and EF, TGF and LAVI, PICP and EF, and between EF and QRS. Using the simple linear regression equation with a single predictor, we created models to predict changes in the studied parameters according to the recorded echocardiographic parameters. Assessment of the adequacy of these models was performed using the resulting *r* (correlation coefficient) and *r2* (coefficient of determination).

After analysis, a good linear correlation ($r = –0.57$; $r2 = 0.33$) was found between CTGF and EF values (Figure 33). The adequacy of the model is confirmed by the p-values for the coefficients: $p(b0) < 0.05$ and $p(b1) < 0.05$.

The correlation coefficient is $r = –0.57$, meaning the dependency is inversely proportional (decreasing). From the coefficient of determination $r2 = 0.33$, it follows that 33% of the EF changes can be explained by the corresponding CTGF changes.

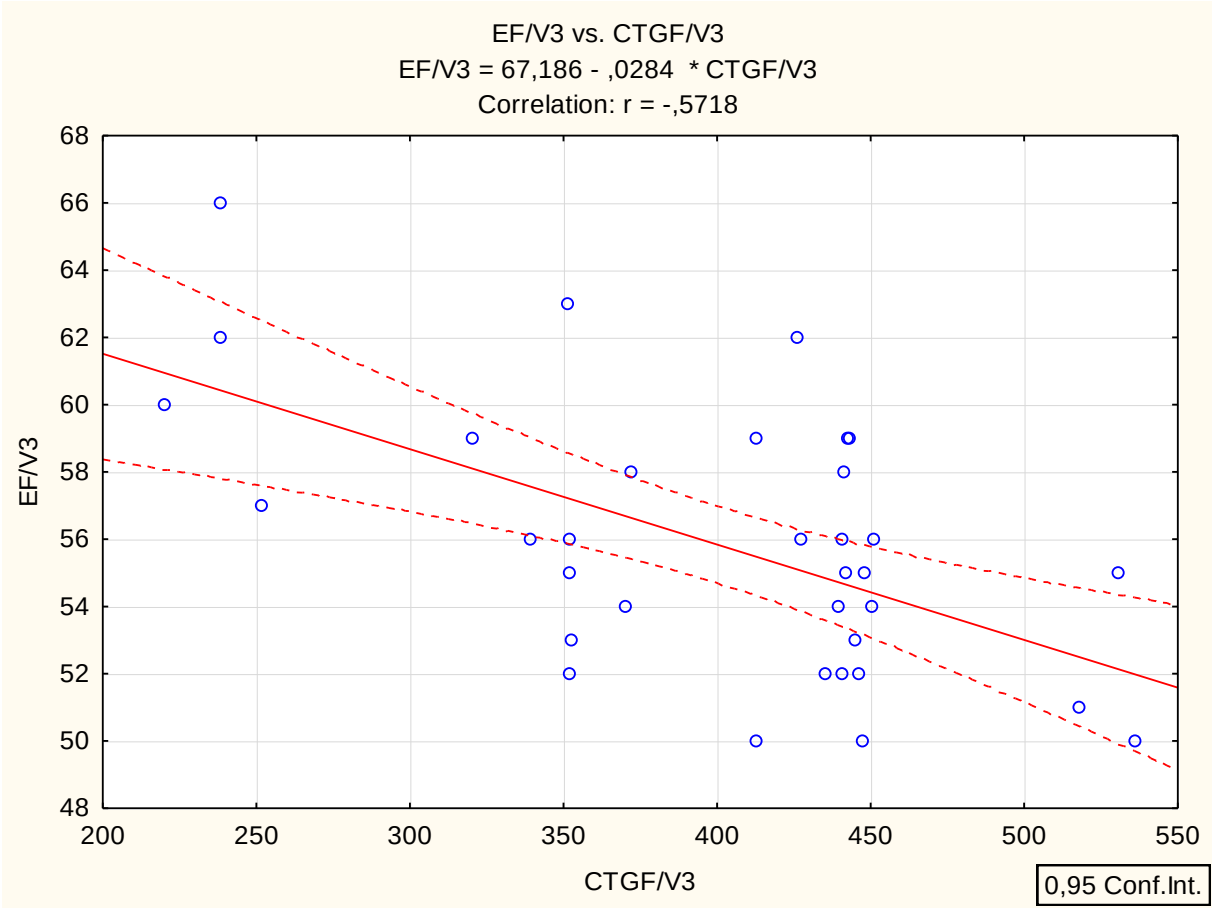

Fig. 33. *Graphical representation of the model correlation obtained by simple linear regression for the CTGF and EF values at the 3rd visit*

Although the subtle mechanisms of left ventricular diastolic dysfunction have not been completely clarified, increases in cardiac fibrosis and cytokine activation are proven factors in the development and progression of its manifestation [Schelbert et al, 2014].

Among the investigated cytokines and fibrosis markers, CTGF and TGF-β1 stand out as some of the most representative and have been targeted in recent years for therapeutic intervention of cardiac fibrosis [Dai et al., 2011].

Elevated CTGF levels are an accepted biomarker of systolic dysfunction in patients with chronic heart failure [Koitabashi et al., 2008]. Studies have demonstrated a significant correlation between CTGF and BNP levels with those of the echocardiographic index for left ventricular filling pressure in the early diastolic phase (E/E') in patients with manifestations of cardiac insufficiency.

Our study confirms these results We found a significant inverse correlation between CTGF levels and left ventricular EF changes (Figure 33). Despite the lack of significant dynamics in the EF at follow-up values in the patient group, it is important to note the need for an extended study to confirm these initial changes.

Data from other studies have also demonstrated a positive correlation between CTGF levels and extracellular volume, determined using nuclear magnetic resonance imaging, in patients with moderately reduced and preserved ejection fraction [Behnes et al., 2014].

These results, together with the data from our study, confirm that CTGF is a reliable biomarker for assessing cardiac fibrosis and its impact on the cardiac function in the patients studied.

6. Changes in the width of the paced QRS complex

Figure 34 shows no significant difference in the width of the paced QRS complex between the values at week 6 (V1.1) and baseline (V1) (154.80 ± 3.37 vs 149.00 ± 3.80 msec, $p > 0.05$). At the follow-up visit, at week 12 (V2), there was an increase in values compared to baseline, but the difference did not reach statistical significance (158.89 ± 3.52 vs 149.00 ± 3.80 msec, $p > 0.05$). At week 24 follow-up, the values increased and the difference reached statistical significance compared to baseline values (159.92 ± 3.83 vs 149.00 ± 3.80 msec, $p < 0.05$).

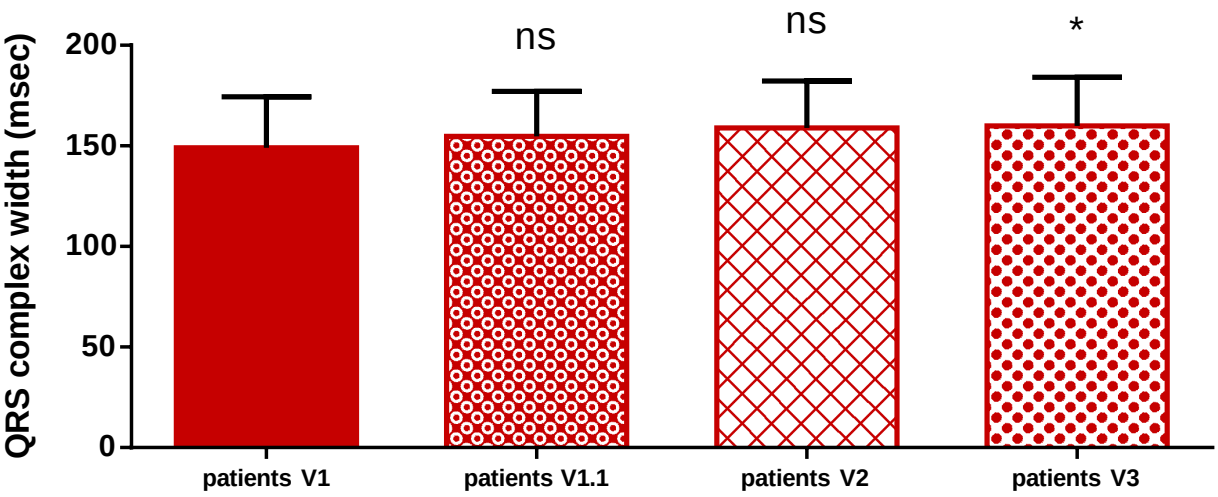

Fig. 34. *Comparison of QRS values in the patient group: (patients V1– baseline values; patients V1.1 – values at week 6 after PPM implantation; patients V2 –values at week 12 after PPM implantation; patients V3 – values at week 24 after PPM implantation; *–p<0.05; **– p<0.001; ns – statistically insignificant difference)*

The results of our study showed that after PPM implantation with an electrode in the RV apex, there was a trend of widening of the paced QRS complex recorded by surface ECG during follow-up visits. At week 12 post-implantation, the QRS complex had increased duration compared to baseline values, but without statistical significance ($p = 0.06$). At 24 weeks, the increasing trend was maintained, and the difference with the baseline values was with a borderline p-value ($p = 0.048$).

Right ventricular apex pacing leads to depolarization of the cardiac muscle, with the impulse being transmitted along the cardiomyocytes rather than along the conduction system of the heart, resulting in delayed and asynchronous left ventricular contraction. Conduction along the native conduction system occurs at an average of about 80 msec, with values of 92.7 ± 9.3 msec for males and 87.1±8.7 msec for females – values which are regarded as the norm [Macfarlane & Lawrie, 1989]. Myocardial contraction resulting from electrical activation of the right ventricular apex is sufficient to maintain the stroke volume and stabilise haemodynamics in patients with complete atrioventricular block but is not mechanically physiological. This leaves patients at risk of developing left ventricular dysfunction, dyssynchrony, and pacemaker-induced cardiomyopathy (PICM) [Lee et al. 2019]. Follow-up data from patients with permanent apical right ventricular pacing suggest that expansion of the paced QRS complex beyond 150 msec is associated with an increased need for hospitalisation due to decompensated HF and a decline in the left ventricular ejection fraction [Khurshid et al., 2016]. This group of patients was followed up for a period of 3.4 years, with a reduction in the left ventricular ejection fraction recorded on average after the second year. It is important to note that in our study an extremely early trend of expansion of the duration of the paced QRS complex was recorded. This potentially explains the reduction of pumping parameters over time. The development of left ventricular dysfunction after PPM implantation is an indication for re-implantation with an upgrade to a resynchronization therapy (CRT system) [Glikson et al. 2021]. Follow-up data from patients with PICM have shown that CRT implantation results in reversible recovery of left ventricular dysfunction in over 70% of patients within 1 year [Khurshid et al., 2018]. *This confirms the concept that pacemaker-induced asynchronous ventricular contraction plays a major role in the development of left ventricular systolic dysfunction.*

7. Final discussion

The pooled analysis of the results will enable evaluation of the change in fibrosis status and how this affects cardiac function in patients after implantation of a biventricular pacemaker. In interpreting the results, the following facts are emphasised:

- Fibrosis activity was monitored over a period of 6 months, which enabled us to evaluate the changing dynamics in the parameters in patients after biventricular pacemaker implantation.

- Both signalling molecules responsible for unlocking the profibrotic state and markers of collagen synthesis were investigated. All four investigated indicators are representative as per the publications data from the literature to date.
- Fibrosis activity was followed dynamically in both patient and control groups. This made it possible to compare the two groups taking into account the effect of the natural aging processes of the organism on cytokine activity and collagen synthesis. Also, all patients selected for the study were free of severe concomitant pathology, thus minimising external factors that may activate the fibrotic system.
- Echocardiographic indices that have been validated to be informative for the assessment of cardiac systolic and diastolic function were monitored. The dynamics in the studied echocardiographic parameters can be used to indirectly assess the effect of increased myocardial collagen deposition on the cardiac function in patients after PPM implantation.

The fact that at baseline there was no difference in the values of the investigated parameters between patients and controls is essential in our research. When the serum levels of the studied molecules were monitored, an extremely early increase compared to baseline was recorded for CTGF ($p < 0.001$), TGF-β1 ($p < 0.001$), PICP ($p < 0.05$) and PIIINP ($p < 0.001$) – as early as week 12 after PPM implantation. Results at week 24 showed retention of the elevated levels at significantly higher levels for CTGF ($p < 0.001$), TGF-β1 ($p < 0.001$) and PICP ($p < 0.001$). Those of PIIINP were also higher than baseline, but the difference did not reach statistical significance ($p > 0.05$). We can speculate that implantation of a biventricular pacemaker provokes fibrotic activation, which occurs early at week 12 after the procedure and persists up to 6 months afterwards.

For the correct interpretation of the results, it is important to note that the patients included in the study had no severe concomitant pathology. This minimises the risk of external influences on the fibrotic system and possible interference by extraneous factors.

In the modern population, arterial hypertension is an extremely common disease, increasing in prevalence with age to over 50% worldwide [Mills& Stefanescu, 2020]. Therefore, patients included in the study were assumed to have up to grade I well-controlled arterial hypertension according to the current guidelines [Williams et al., 2018]. After their selection for the study, all participants had their antihypertensive therapy modulated. Hydrochlorothiazide, amlodipine and methyldopa were administered in the required doses for blood pressure control. This minimised the effect of antihypertensive medications on RAAS and the fibrotic system in the body respectively. This allows us to assume that changes in the values of the studied indicators are most likely provoked by the PPM implantation.

To assess the effect of apical right ventricular pacing on cardiac function, several echocardiographic parameters were monitored – LVESV, LVEDV, EF and LAVI. The dynamics in the width of the paced QRS complex were recorded from the surface ECG.

Analysis of the results showed that there was a trend of increasing LVESV values ($p = 0.15$) (Table 5) and decreasing EF ($p > 0.05$) (Table 5), but these changes did not reach significant significance. The reason could be attributed to the relatively short follow-up period to induce significant left ventricular structural remodelling.

However, the results of LAVI follow-up showed an extremely early increase compared to baseline values as early as the sixth week after PPM implantation ($p < 0.001$), and this increase persisted until the sixth month. Left atrial volume is a proven and reliable indicator reflecting increased LV filling pressures in the absence of significant valvular pathology [Lang et al., 2015]. Right ventricular apical pacing provokes asynchronous ventricular contraction, resulting in incomplete LV emptying, functional mitral regurgitation, manifestations of diastolic dysfunction and an increase in left atrial afterload [Xie et al., 2012a]. It can be assumed that this underlies the extremely early significant increase in the left atrial volume we found as early as the sixth week in patients after PPM implantation.

Analysis of the width of the QRS complex in patients after PPM implantation in our study showed a definite trend of increase, reaching statistical significance at week 24 compared to baseline values ($p < 0.05$).

The data of dilated QRS complex in patients with existing cardiac pathology is a reliable marker for prediction of complications, it is easily applicable and with low variability in the outcome [Shamim et al., 2002]. Also, according to other studies, there is a definite association between an enlarged QRS complex and the presence of diffuse myocardial fibrosis [Nazarian et al., 2010]. *This allows us to assume a correlation between asynchronous ventricular contraction provoked by right ventricular apical pacing, left atrial strain and activation of the fibrotic cascade in the studied patients.*

At the same time, as far as our research revealed activation of collagen synthesis and initial signs of the development of cardiac dysfunction, the question of therapeutic options to improve the prognosis of this group of patients is raised.

In recent years, alternatives to right ventricular apical pacing have been actively sought because of the strong evidence of its negative impact on the structure and function of the heart. However, electrode placement in the RV apex is currently the most widely applicable. This method is still preferred worldwide because the technique is easy to perform and the stability of the electrode parameters in the long term have been proven over time. Therefore, the question of medicating fibrosis and preventing cardiac dysfunction in PPM patients is brought forward.

Currently, there is no specific antifibrotic medication that is routinely used in the clinical practice. Some known molecules for HF treatment have shown promising results in this regard. Such are the representatives of angiotensin-converting enzyme inhibitors [Brilla et al., 2000], the loop diuretic torasemide [Lopez et al., 2007], mineralocorticoid receptor antagonists [Gueret et al., 2016], the neprilysin inhibitor sacubitril [Pfau et al., 2019), etc. Also, some of the available medications not specifically designed for cardiac diseases have a potential role as antifibrotic agents that affect myocardial fibrosis [Neuber et al., 2021].

Conversely, data from a meta-analysis of more than 40 studies conducted in patients with HF and preserved EF (HFpEF) showed conflicting results regarding the effect of treatment with beta-blockers and RAAS inhibitors on mortality and hospitalizations (Martin et al., 2018). Evidence suggests that treatment with MRAs and ARNIs results in a modest reduction in the risk of hospitalization for HF events, and treatment with beta-blockers reduces the risk of cardiovascular mortality. However, similar results have not been demonstrated with treatment with ARBs and ACE inhibitors. Similar results are available from other studies where treatment with MRAs resulted in reducing the risk of HFpEF manifestations and a decrease in the plasma levels of the collagen synthesis markers PIIINP and PICP [Xiang et al., 2019].

These conflicting results may be due to the fact that both the development of interstitial fibrosis and HFpEF have complex aetiologies. For example, a recent study found that one out of seven patients with HFpEF manifestations had proven wild-type transthyretin amyloidosis [Gonzalez-Lopez 2015]. This requires a precise analysis of the phenotypic manifestation and underlying pathology in order to optimize medical treatment.

In conclusion, it can be stated that activation of the fibrotic cascade and enhanced collagen synthesis are observed after PPM implantation. Indirect evidence of the development of diastolic dysfunction and left atrial strain was recorded, most likely provoked by asynchronous ventricular contraction resulting from apical right ventricular pacing. Currently, there is no specific therapeutic strategy in this group of patients and they are treated according to the guidelines for heart failure management. Considering the risk of developing cardiac dysfunction, a rigorous echocardiographic screening of patients with permanent pacemakers and telemetry data of RV pacing above 20% may be warranted to design therapeutic management on time.

V. CONCLUSIONS

1. The present study was conducted on a well-balanced patient and control group. For this reason, we can assume that the specific dynamics found in the studied parameters are very likely closely related to the PPM implantation.
2. Major regulatory molecules responsible for collagen synthesis, TGF-β1 and CTGF, underwent unidirectional significant changes during the follow-up period, with a strong increase in values at week 12 post-intervention, also present at week 24 post-intervention.
3. Serum PICP levels showed a steady increase 24 weeks after PPM implantation and evidence of increasing cardiac interstitium-specific type I collagen synthesis during this period.
4. PIIINP is characterised by specific dynamics in values – a significant rise early after intervention and recovery to baseline levels 6 months after intervention. It is a prerequisite to assume perioperative tissue trauma as a possible source for the increased type III collagen synthesis.
5. The size of the patient sample was adequate, as evidenced by the high statistical power of the t-criterion in testing the hypothesis of equality of the mean values of the studied parameters in serum.
6. Left atrial volume increased gradually during the follow-up period, as evidenced by the fourfold increase in the measured values of the indicator.
7. The paced QRS complex is prolonged, presenting a deepening electrical asynchrony and slowing conduction velocity in the myocardium.

VI. SCIENTIFIC CONTRIBUTIONS

The study results are original.

1. A first-of-its-kind clinical study on the fibrotic process after PPM implantation has been performed by investigating simultaneously, on dynamics, signalling pro-fibrotic molecules and markers of collagen synthesis.
2. For the first time, straightforward evidence of enhanced collagen synthesis after PPM implantation with significant activation of key regulatory mechanisms responsible for it is presented.
3. Specific dynamics in the levels of TGF-β1, CTGF, PICP and PIIINP were found, presenting evidence for the myocardial origin of enhanced collagen synthesis after PPM implantation.
4. It has been shown that early (up to the 6th month) after PPM implantation the development of structural and electrophysiological changes in the myocardium occurs.
5. Regulatory molecules of enhanced collagen synthesis after PPM implantation have been refined, providing an opportunity in the search for new therapeutic options to influence myocardial remodelling.