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The Editorial Board kindly informs that since 2014 *Nowiny Lekarskie* has been renamed to *Journal of Medical Science*.

The renaming was caused by using English as the language of publications and by a wide range of other organisational changes. They were necessary to follow dynamic transformations on the publishing market. The Editors also wanted to improve the factual and publishing standard of the journal. We wish to assure our readers that we will continue the good tradition of *Nowiny Lekarskie*.

You are welcome to publish your basic, medical and pharmaceutical science articles in *Journal of Medical Science*.

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Proteomic analysis of subarachnoid hemorrhage – liquid-phase isoelectric focusing in complex protein sample

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ABSTRACT

Aim. The aim of this study was to present the proteomic approach based on liquid phase isoelectric focusing fractionation coupled to nLC-MALDI-TOF/TOF-MS/MS analysis to characterize cerebrospinal fluid from control patients and those suffering from subarachnoid hemorrhage. The new perspective in characterization of this brain neuropathology is in constant demand to point a valuable panel of indicators which could improve the treatment outcome.

Material and Methods. The cerebrospinal fluid samples were applied to a commercial liquid phase isoelectric focusing apparatus and separated into 10 fractions by pI. Further, the untargeted mass spectrometry investigations were performed with data dependent acquisition mode for full-scan MS analysis with subsequent MS/MS fragmentation using nLC-MALDI-TOF/TOF-MS/MS.

Results. In total, the detection of 1664 and 2187 unique tryptic peptides provided biological evidence for 134 and 271 proteins in control and subarachnoid hemorrhage sample, respectively. The interpretation of liquid phase separation was performed by intersection analysis of two items between groups of ten fractions. The cumulative intersection exploration revealed the highest concentration of the detected components in the middle fractions of the focusing chamber, whereas the gradual dilution appeared on its extreme.

Conclusions. The employed strategy ensured overall screening of investigated material presenting the proteins abundance in the current state of analysis. Few proteins such as proenkephalin A, peroxiredoxin-6, cathepsin B, thrombospondin-1, glial fibrillary acidic protein and α-spectrin were recognized as potential indicators, according to literature, pointing the possibility for monitoring in further studies as panel of valuable biomarkers.

Keywords: MALDI mass spectrometry, proteomic strategies, protein separation, cerebrospinal fluid, subarachnoid hemorrhage.

Introduction

Cerebrospinal fluid (CSF) surrounds the central nervous system including the ventricular system of the brain, spinal canal and subarachnoid space cranial cavity. It is produced by choroid plexus of the brain cells, lining cells and microglia [1]. The main functions of the CSF are the protection of the brain and spinal cord from the mechanical injuries, compensation of the intracranial pressure and transfer of the humoral information. Analysis of this fluid may access the state of the environment in human central nervous system (CNS) [2].

Aneurysmal subarachnoid hemorrhage (SAH) belongs to cerebrovascular diseases and contributes to 6–8% of all cerebral stroke events. It is caused by the extravasation of blood into the sub-arachnoid space [3]. The pathophysiological process of cellular and molecu-

lar changes following SAH are still not fully characterized [4]. Therefore, the proteomic examination of CSF may contribute to detection of potential cerebral indicators, which will lead to better prediction of patients deterioration and treatment outcome [3, 5–6]. Currently performed proteomic analyses are mostly based on mass spectrometry (MS) with application of soft ionization methods: MALDI (matrix-assisted laser desorption/ionization) and ESI (electrospray ionization). Due to significant specificity and sensitivity, these techniques are commonly used in numerous clinical studies e.g. monitoring of selected compounds [7]. However, direct analysis of complex biological samples is associated with problems of analyte suppression and high dynamic range in protein content [8, 9]. Thus, the preparation steps including separation and concentration of analyzed sample are often required before MS analysis.

Cerebrospinal fluid suffers protein dynamic range problems with the small number of proteins constituting the sample. Preparation techniques are important in order to identify the largest number of constituents and thus appropriate biomarker within sample. One of the widely used method is two dimensional gel electrophoresis (2-DE), which combines two independent separation processes: due to isoelectric point and molecular weight of proteins [10]. Its compatibility with mass spectrometry techniques is defined as bottom-up strategy in clinical proteomic research [11].

An alternative approach to gel electrophoresis in modern proteomics is fractionation based on gel free liquid phase isoelectric focusing (LP-IEF) [12], where the protein charge is changing until its pI is reached. By adding carrier ampholytes [13], smooth and relatively stable pH gradient with greater buffering capacity could be achieved. Moreover, the main advantage of gel free technology is associated with the possibility of protein recovery in the liquid phase [14]. Therefore, this methodology becomes an attractive tool as a separation technique prior to the nano-LC/MS analysis, widely applied into various biological samples like a human erythroleukemia cell line [15], ovarian carcinoma cells [16–17] or brain tissue [18]. Regarding to our study, LP-IEF in combination with gel electrophoresis was reported to be an important tool for identifying low abundant proteins in human cerebrospinal fluid and membrane proteins in frontal cortex [19].

Aim

The aim of this study was to present method for proteomic analysis of CSF for screening analysis of SAH. We used LP-IEF combined with nLC-MALDI-TOF/TOF mass spectrometry to characterize the protein content of analyzed cases with and without SAH. The usefulness of gel free strategy was estimated by intersection analysis.

Material and Methods

Study cases

The research project has been approved by the Regional Ethics Committee of the Poznan University of Medical Sciences (decision No. 503/15). The SAH case was admitted due to the subarachnoid hemorrhage. A computed tomography (CT) scan demonstrated intracerebral hemorrhage of the left frontal lobe and intraventricular bleeding causing mass effect (**Figure 1**). Digital subtraction angiography demonstrated ruptured anterior communicating artery aneurysm which was coiled. The patient required insertion of external ventricular drainage during which 10 mL of cerebrospinal fluid was collected for the analysis. The material was characterized: blood stained with opalescent transparency; white cell count (WCC) – 118/μL; red blood cell count (RBC) – 20000/μL; protein concentration – 1944 mg/dL. The blood in the ventricles was gradually absorbed and patient`s condition improved. After 14th day the patient was woken up and the ventricular drainage was removed. Control case was admitted with six months history of gait disturbance, urinary incontinence and mental decline. The patient was diagnosed with the normal pressure hydrocephalus (**Figure 2**) and the programmable ventriculo-peritoneal shunt (Sophy Mini SM8 valve, Sophysa) was inserted where 10 mL of cerebrospinal fluid was collected. The material was characterized: colorless with clear transparency; white cell count (WCC) – 2/μL; red blood cell count (RBC) – 320/μL; protein concentration – 166 mg/dL. The neurological condition of the case gradually improved after the operation. Immediately after collection of cerebrospinal fluid, the material was centrifuged at 3000 rpm for 10 min and stored at -80°C until analyzed.

Isoelectric focusing fractionation

Prior the fractionation, the filter membrane Amicon Ultra 100K (Millipore, Bedford, MA) devices were used for ultrafiltration procedure to cut off the high molecular weight proteins, according to the manufacturer's instructions [20]. Firstly, the membrane was rinsed with deionized water, then 2000 μL of cerebrospinal fluid was centrifuged at 5400 × g for 30 min. The collected filtrate was mixed with n-octylglucoside to a concentra-

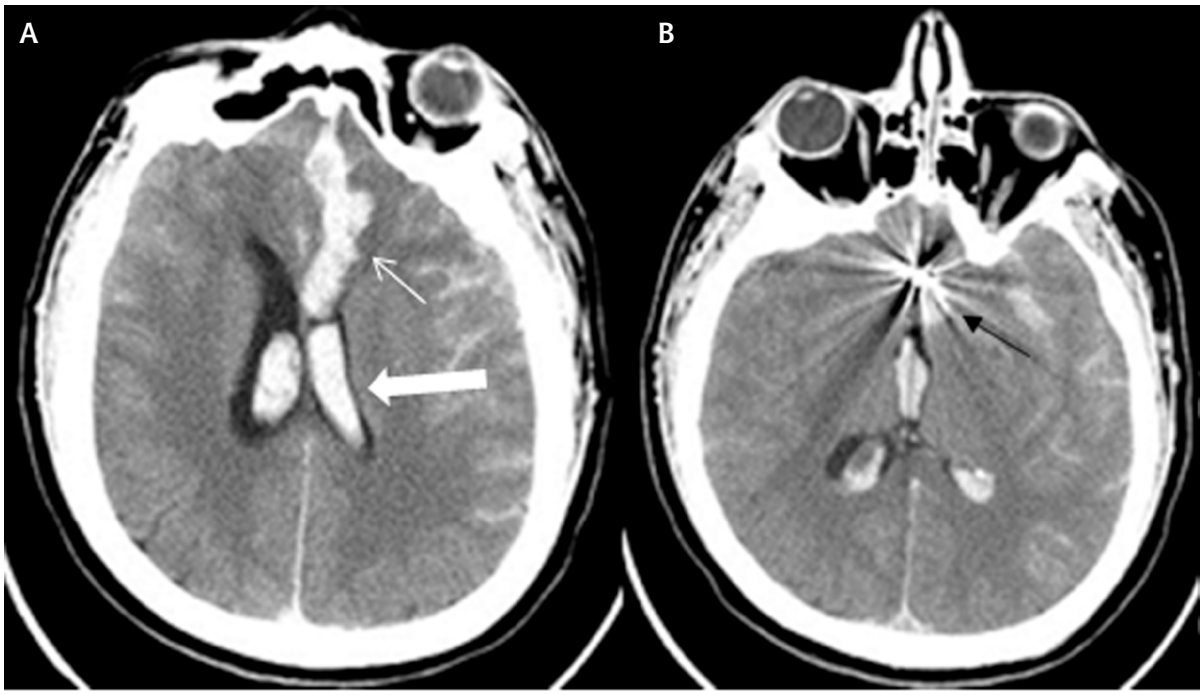

Figure 1. A computed tomography (CT) scan of the patient with subarachnoid hemorrhage on admission demonstrating: (A) intracerebral hemorrhage (white thin arrow) with intraventricular extension (white thick arrow); (B) coiled aneurysm of anterior communicating artery and the blood in fourth ventricle (black arrow)

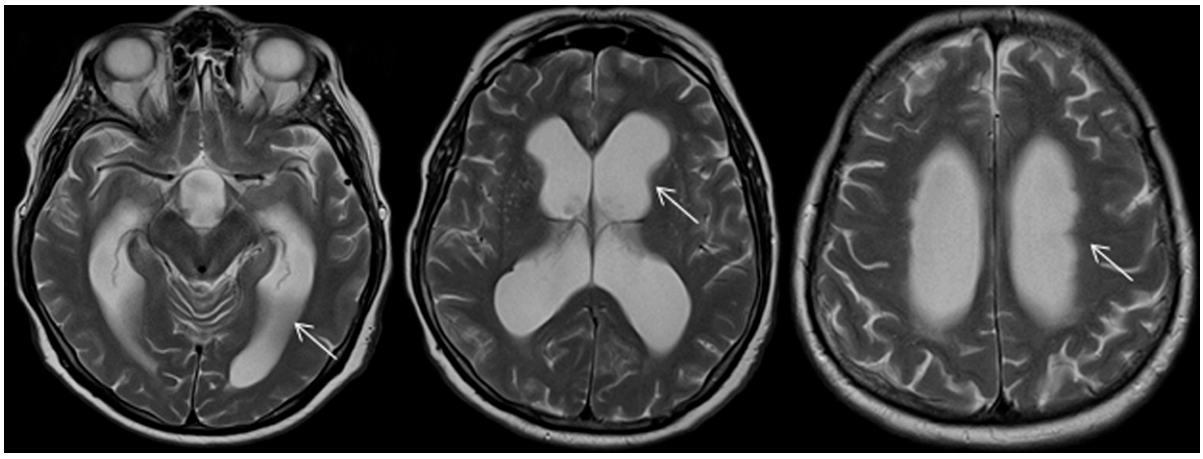

Figure 2. A magnetic resonance imaging (MRI) of the control patient with normal pressure hydrocephalus demonstrating dilated ventricles (white thin arrow)

tion of 0.1% and with ampholyte (40%, pH range 3–10 isodalt, Bio-Lyte, Biorad) to a concentration of 2.5%. The 500 µg of CSF protein was loaded into the Micro-Rotofor chamber (Bio-Rad Laboratories, Hercules, CA, USA) for focusing without further treatment. Constant power (1 W) was applied. Initial voltage was approximately 80 V and a plateau of 600 V was reached after 2 h. Ten separate fractions for each investigated case were rapidly harvested and purified using ReadyPrep 2-D (Bio-Rad Laboratories, Hercules, CA, USA) cleanup kit in accordance to manufacturer's instruction [21]. The precipitated proteins were resolved in 30% ACN/0.1% TFA solution and subjected to MALDI-TOF/TOF-MS/MS analysis acquiring gel free and SDS-PAGE strategies.

Gel electrophoresis

Additionally, the IEF fractions from SAH case were separated using SDS-PAGE. The 20 µL of sample mixture was loaded on a 14% Tris-glycine–SDS-PAGE gel and the process was carried out at 200 V for 40 min. Gel separation was performed on a Mini-Protean Tetra Cell (Bio-Rad Laboratories, Hercules, CA, USA) and was continued until the blue bromophenol front reached the bottom of the gels. The gel was stained with Coomas-

sie Brilliant Blue G250 and the background was washed out by use of 9% acetic acid.

In-gel and In-solution digestion

All in-solution CSF fractions obtained from MicroRotofor were subjected to digestion protocol according to the procedure modified from Pierce In-Solution Tryptic Digestion Kit. While the most intense proteins bands in the mass range from 20 to 37 kDa of Coomassie stained gel were processed with in-gel digestion according to adopted Shevchenko et al. protocol [22], the digested peptides were extracted from the gel by incubation in 50 µL of 50% ACN/ 0.1% TFA solution.

MALDI-TOF/TOF-MS/MS protein identification

The digested peptides were subjected to nano-LC analysis. The system consisted of EASY nano-LC II (Bruker Daltonics) and fraction collector Proteineer-fc II (Bruker Daltonics). Firstly, loaded peptides were concentrated on a trap column NS–MP-10 BioSphere C18 5 µm particle size, 120-Å pore size, 100 µm inner diameter, 20 mm length (NanoSeparations, Nieuwkoop, Netherlands), then separated on a Acclaim PepMap 100 column C18, 3 µm, 100 Å, 75 µm × 150 mm (Thermo Scientific, Sunnyvale, CA, USA) by linear gradient of water (mobile phase A) and 90% ACN (mobile phase B), both containing 0.05% TFA. The gradient elution method was: 2–50% B in 96 min. The flow rate was maintained at 300 nL min-1 and the injection volume was 6 µL. In total, 384 fractions of each separated fraction were automatically mixed with matrix solution and spotted onto a AnchorChipTM target (Bruker Daltonics). Per fraction 80 nl of eluent was mixed with 420 nl matrix solution. Matrix solution was generated by mixing: 748 µL of 95:5 (v/v) acetonitrile: 0.1% TFA, 36 µL of saturated solution of HCCA in 90:10 (v/v) acetonitrile:0.1% TFA, 8µL of 10% TFA and 8µL of 100 mM ammonium phosphate monobasic. The system was controlled using HyStar 3.2 software (Bruker Daltonics). Afterwards, the tandem mass spectrometry analysis was performed using the MALDI-ToF/ToF UltrafleXtreme instrument equipped with a SmartBeam II laser (Bruker Daltonics). Typical instrument setting for MS mode was as follows: ion source 1, 25.09 kV; ion source 2, 22.59 kV; lens voltage, 7.89 kV; pulsed ion extraction time, 120 ns; matrix suppression mass cut off, m/z 700. All spectra were acquired by accumulating 4000 shots from 40 non-overlapping positions with a repetition rate of the pulsed laser of 2 kHz. By routine, a standard peptide calibration mixture in the mass range of 700–3500 Da (Bruker Daltonics) was analyzed for external calibration of the mass spectrometer. The MS/MS mode for protein identification was applied with the following setting: ion source 1, 7.50 kV; ion source 2, 6.75 kV; lens, 3.50 kV; reflectron 1, 29.50 kV; reflectron 2, 14.00; lift 1, 19.00 kV; lift 2, 3.00 kV, pulsed ion extraction time, 80 ns; fragments only. Precursors with a signal-to-noise ratio above 10 were automatically subjected to MS/MS analysis. The maximum number of MS/MS per fraction was set to 20. The control of the instrument, data acquisition, processing and evaluation was performed using the following software platforms: flexControl 3.4, FlexAnalysis 3.4 and WARP-LC 1.3 and ProteinScape 3.1 (Bruker Daltonics). The MS/MS spectra were processed with ProteinScape 3.0 platform by searching the SwissProt database with Mascot 2.4.0 search engine (Matrix Science, London, UK). The LC-MALDI results were filtered to a false discovery rate at a peptide-spectrum match level of less than 1% based on decoy counts and only proteins with at least one unique peptide were included. The LC-MALDI data were used with the significant threshold of $p > 0.05$ set by the search engine. The general protein search parameters were included: trypsin and semi trypsin digestion, 1 and 2 missed cleavages, peptide precursor mass tolerance: 35 ppm; fragment mass tolerance: 0.7 Da; peptide charge: +1; monoisotopic mass; carbamidomethylation of cysteine as fixed modification; oxidation and dioxidation of methionine as variable modification. The results were compiled into one protein list.

Statistical analysis

The interpretation of isoelectric focusing separation was performed by intersection analysis of two items between groups of ten fractions, which contained measured masses of peptides obtained by nLC-MALDI-TOF/TOF-MS/MS. The 45 selections of interaction (as a result of combination of two items choose ten fractions) were showed as an upper triangular image plot. For clarity, the symmetrical lower triangular part was presented as a blackened area. The cumulative intersection analysis was made in both left and right directions starting from the fifth fraction, as a point of injection of the sample. We define $m \in \{1,2,...10\}-\{5\}$, as a number of fraction and $n = 5$ (fifth fraction). Therefore, Lm a result for m fractions can be presented using following notations:

$$\bigcap_{n=5}^{n-m} = L_m$$

for the left direction and

$$\bigcap_{n=5}^{m-n} = L_m$$

for right one. Furthermore, the membership of set was created by using of all peptides included by search engine to identify two proteins: peroxiredoxin-6 and cathepsin B, which were described as potential indicators for this brain pathology and had enough number of peptide to analyze. For all steps, MATLAB v.8.1.0.604 software with the Bioinformatics Toolbox was used.

Results

Gel-free LC-MS/MS approach

Using gel free approach consisting of the LP-IEF set and nLC-MALDI-TOF/TOF-MS/MS, the CSF digested peptides derived from patient with and without SAH were analyzed. The data dependent acquisition (DDA) mode was acquired, where full-scan MS analysis was performed and subsequently MS/MS fragmentation on a defined number of the most intense ions. The approach is valuable in the untargeted investigation of samples with no hypothesis about which parent ions should be fragmented as a priority. The identified proteins and peptides from the different fractions were collected into compilation list. In total, 1664 and 2187 unique tryptic peptides provided biological evidence for 134 and 271 proteins in control and SAH sample, respectively (**Supplementary material 1 and 2**). Among these the characteristic of high abundant components of CSF were acknowledged. Due to high dynamic range between analyzed samples, in CSF with SAH up to 177 unique proteins were recognized. The data obtained from particular IFE fractions of SAH specimen were compared. The highest identification number was found in fraction 5, where from 167 detected proteins as many as 67 were unique. Further, the fraction 4, 8 and 7 also gave valuable contribution to proteomic characterization of the analyzed case, whereas fraction 1, 2 and 10 presented poor description in protein content. Analyzing the specificity of separation process, it was observed that many common species were overlapping with the neighboring fractions. The pI of detected proteins were varying from 3.9 to 16.4. Thus, in overall distribution of biomolecules in artificial pH gradient (**Figure 3**), we were able to observe higher repletion of proteins with pI 5–6 in IEF fraction 3, 4 and 5, whereas the proteins with pI 8–9 were more abundant in the last fractions. The interaction analysis of masses identified by MALDI-TOF/TOF-MS/MS between two selected fractions was shown as the upper triangular image plot

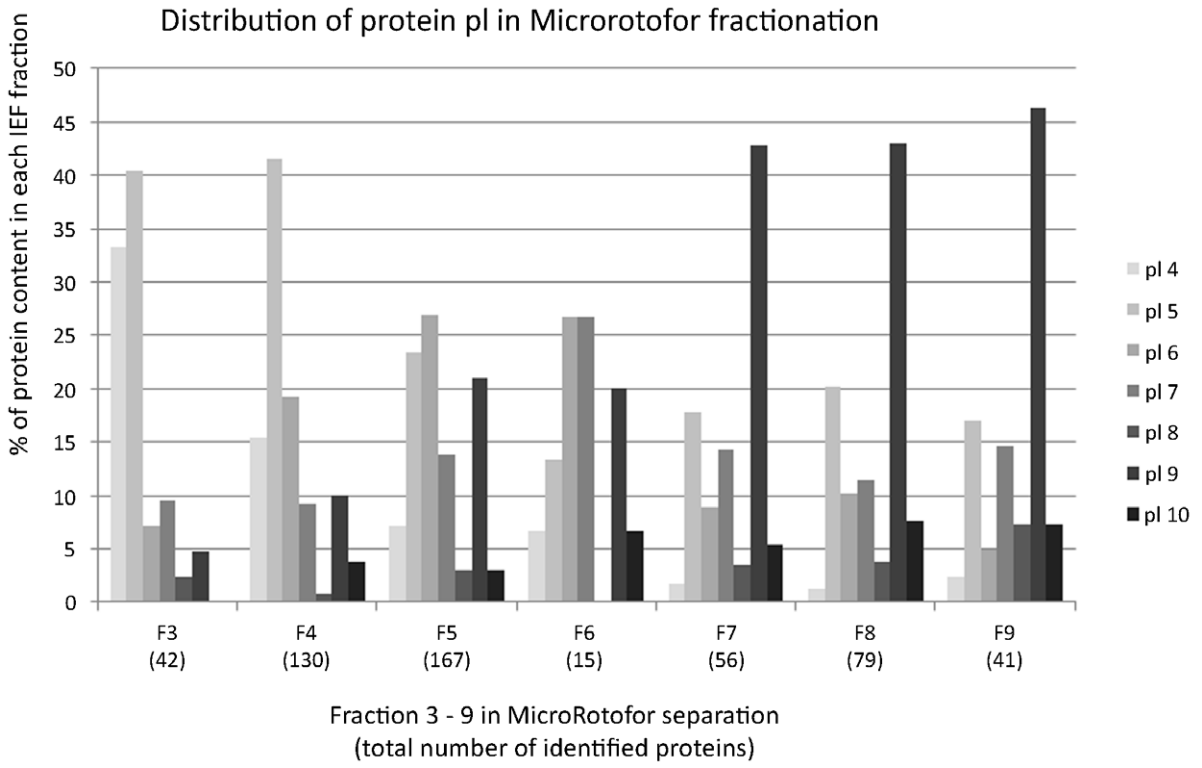

Figure 3. A bar graph represents the distribution of protein isoelectric point (pI) in Microrotofor separation between fractions 3 to 9. On the horizontal axis the investigated fractions and identified per each proteins with the percentage content according to its pI

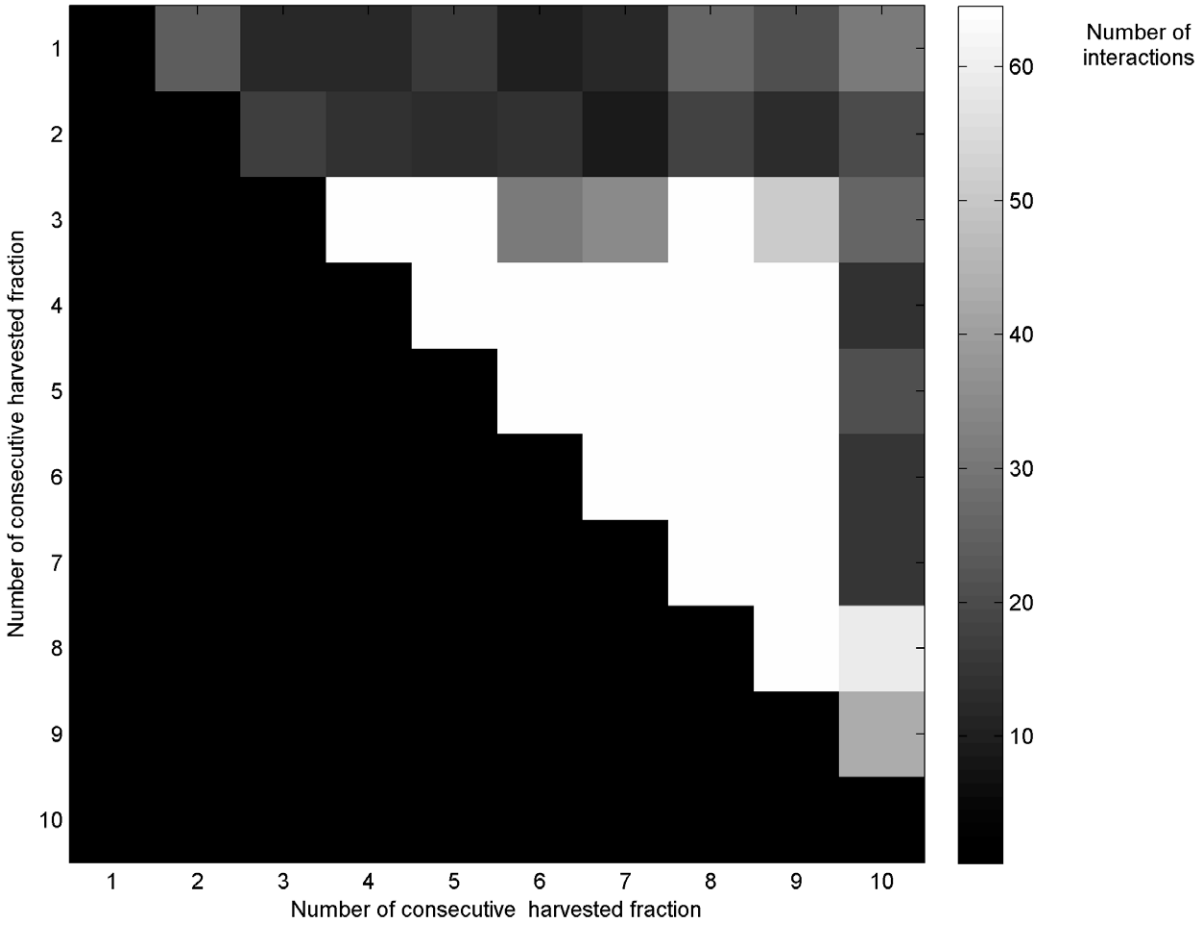

Figure 4. Upper triangular image plot of cumulative intersection analysis of two items in group of ten fractions. For clarity symmetrical lower triangular part was presented as a blackened area

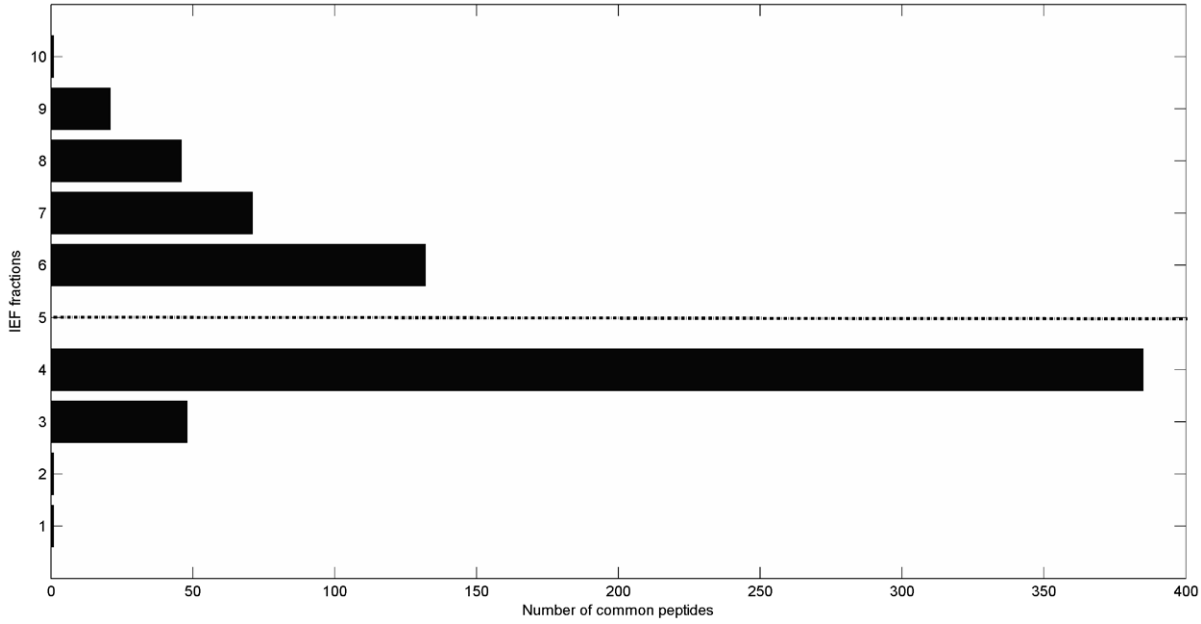

Figure 5. Cumulative intersection analysis for all measured compounds

(**Figure 4**). The intersection analysis of two items in group of ten fractions resulted in 45 compared areas. The performed analysis indicated a large number of common compounds in the adjacent fractions, especially true for the fractions in the center. Moreover, it was noticed that larger distance between the fractions ensured the identification of more unique elements. The best separation performance was obtained between pairs of 1–6 and 2–7. The highest concentration of the detected components was observed in the middle fractions, whereas the gradual dilution appeared on the extremes of the MicroRotofor chamber. The dilution process occurred better in the left direction from the center. This effect is clearly seen in **Figure 5**, which shows the cumulative intersection of the fractions starting from the fraction number 5 in the decreasing direction on the left and increasing on the right. Regarding to the quantity, the fractions are not arranged symmetrically with respect to a fraction number 5. The total number of all detected compounds (included peptides) were the highest in fraction 4 (2995) and 5 (3667). Although, some of their measured masses were presented also in other fractions (**Figure 6**), which is confirmation for dilution phenomenon. The **Figure 6** shows the membership of the peptides masses used for identification of chosen proteins: cathepsin B and peroxiredoxin-6. The measured m/z of enzyme protease cathepsin B were additionally observed in fraction 2, 3, 7 and 8. With regard to protein peroxiredoxin-6 a similar conclusion was proposed. The characteristic masses assigned to this protein have been detected in the whole range of analyzed fractions.

Gel-based LC-MS/MS approach

The most intense protein bands in the mass range from 20 to 37 kD from SDS-PAGE analysis were excised from the gel and subjected into in-gel digestion procedure (**Supplementary material 3**). The common identified proteins in both strategies (SDS-PAGE vs gel free) were compared with respect to percentage of sequence coverage and number of identified tryptic peptides (**Table 1**). We observed that approximately 80% of the analyzed proteins was better characterized using gel free strategy, thus we concluded that this methodology is valuable in screening analysis of various biological samples.

Discussion

In our study we implemented the LP-IEF based on distribution of current via the electrolytes system gradually increasing pH from anode to cathode. The principle underlies in placement of proteins and others components between both electrodes where the pH is equal with the isoelectric point of analyzed molecules. However, it is difficult to predict or achieve the optimal length of focusing run. Moreover, the separation of all components in pH gradients should be perceived more as a quasi-equilibrium process [23]. The Rotofor

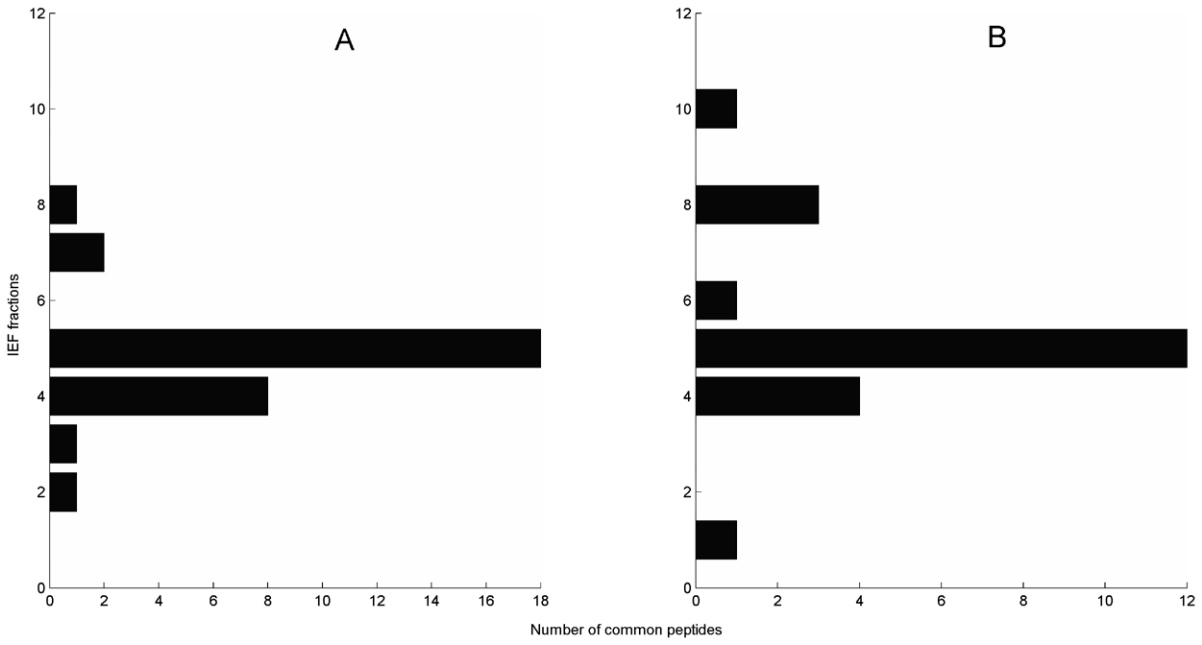

Figure 6. The membership of the detected peptides used for identification of protein cathepsin B (A) and peroxiredoxin-6 (B) in particular IEF fractions

Table 1. The common proteins and peptides identified in cerebrospinal fluid with subarachnoid hemorrhage using MALDI gel free and SDS-PAGE approach. The comparison based on number of identified tryptic peptides and obtained sequence coverage (SC)

No.	Accession	Protein name	MW [kDa]	Peptides SDS-PAGE	Peptides gel free	SC [%] SDS-PAGE	SC [%] gel free
1	APOA1_HUMAN	Apolipoprotein A-I	30.8	6	19	33.7	61.8
2	B2MG_HUMAN	Beta-2-microglobulin	13.7	4	19	46.2	63.9
3	PMGE_HUMAN	Bisphosphoglycerate mutase	30.0	2	6	16.2	32.4
4	CAH1_HUMAN	Carbonic anhydrase 1	28.9	66	54	80.1	86.2
5	CAH2_HUMAN	Carbonic anhydrase 2	29.2	28	30	68.8	68.5
6	CAH3_HUMAN	Carbonic anhydrase 3	29.5	5	7	42.7	41.9
7	COTL1_HUMAN	Coactosin-like protein	15.9	2	3	27.5	35.2
8	COF1_HUMAN	Cofilin-1	18.5	2	10	28.9	66.3
9	DOPD_HUMAN	D-dopachrome decarboxylase	12.7	3	3	28.0	30.5
10	HEM2_HUMAN	Delta-aminolevulinic acid dehydratase	36.3	2	3	12.1	16.1
11	NPC2_HUMAN	Epididymal secretory protein E1	16.6	2	2	17.9	25.8
12	BLVRB_HUMAN	Flavin reductase (NADPH)	22.1	19	15	60.2	63.6
13	GSTO1_HUMAN	Glutathione S-transferase omega-1	27.5	7	10	34.9	33.2
14	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	36.0	10	9	40.6	34.3
15	HEMO_HUMAN	Hemopexin	51.6	2	17	8.7	46.1
16	IGHA2_HUMAN	Ig alpha-2 chain C region	36.5	2	11	7.9	42.1
17	IGHG1_HUMAN	Ig gamma-1 chain C region	36.1	4	26	24.2	61.8
18	IGKC_HUMAN	Ig kappa chain C region	11.6	6	7	80.2	80.2
19	LAC3_HUMAN	Ig lambda-3 chain C regions	11.2	4	3	55.7	46.2
20	IBP7_HUMAN	Insulin-like growth factor-binding protein 7	29.1	3	12	21.6	55.3
21	KLK6_HUMAN	Kallikrein-6	26.8	3	5	23.4	33.2
22	LXN_HUMAN	Latexin	25.7	2	2	9.5	12.2
23	PPAC_HUMAN	Low molecular weight phosphotyrosine protein phosphatase	18.0	3	4	35.4	43.7
24	TIMP1_HUMAN	Metalloproteinase inhibitor 1	23.2	3	9	15.5	67.1
25	PPIA_HUMAN	Peptidyl-prolyl cis-trans isomerase A	18.0	3	19	24.8	70.9
26	PRDX2_HUMAN	Peroxiredoxin-2	21.9	15	14	58.1	76.8
27	PRDX6_HUMAN	Peroxiredoxin-6	25.0	9	14	47.8	67.4
28	PEBP1_HUMAN	Phosphatidylethanolamine-binding protein 1	21.0	3	14	34.8	79.7
29	PTGDS_HUMAN	Prostaglandin-H2 D-isomerase	21.0	7	22	46.8	74.7
30	PARK7_HUMAN	Protein DJ-1	19.9	4	17	30.7	65.1
31	PNPH_HUMAN	Purine nucleoside phosphorylase	32.1	12	12	40.1	50.9
32	TRFE_HUMAN	Serotransferrin	77.0	2	67	3.9	69.8
33	ALBU_HUMAN	Serum albumin	69.3	11	126	18.1	85.7
34	SODC_HUMAN	Superoxide dismutase [Cu-Zn]	15.9	4	9	38.3	64.9
35	TTHY_HUMAN	Transthyretin	15.9	38	18	86.4	69.4
36	TPIS_HUMAN	Triosephosphate isomerase	30.8	13	15	65.7	71.0

separation has no barriers and it is common that the same proteins can be found in several focused fractions, causing the fractionation less sufficient [24]. In our study the effectiveness of the IEF fractionation was assessed with combination of direct MALDI analysis. For MS analysis the computer-controlled data dependent acquisition mode related to ion abundance levels in analyzed sample was used. The ions selection for MS/MS analysis is associated with the width of the chromatographic peaks or with the concentration of peptides comprised in complex mixtures. In biological fluids the number of peptides co-eluting can considerably exceed the number of ions subjected for MS/MS acquisition. Therefore, the data acquisition can be biased against the low abundance signals that corresponded to proteins at low concentration. Also if the ionization technique favors certain peptide features, then ions section is determined towards those ions. Thus, more concentrated proteins will be selected, reflecting the abundance level in sample as the peptide hits and spectral count correlated to protein abundance [25]. Regarding to our results, the identified proteins were presented mostly in higher concentration as neither immunodepletion methods nor combination with additional chro-

Table 2. The identified proteins with potential value as biomarkers in cerebrospinal fluid after subarachnoid hemorrhage using gel free nLC-MALDI-TOF/TOF-MS/MS mass spectrometry approach

Accession	Protein name	Score	SC%	m/z meas.	Peptide Sequence
PRDX6_HUMAN	Peroxiredoxin-6	813.62	67.40	2098.1237	M.PGGLLLGDVAPNFEANTTVGR.I R.FHDFLGDSWGILFSHPR.D
				2030.9957	R.DFTPVCTTELGR.A K.LIALSIDSVEDHLAWSK.D
				1395.6609	K.DINAYNCEEPTEK.L
				1897.0140	K.LPFPIIDDR.N
				1582.6731	R.ELAILLGMLDPAEKDEK.G + Oxidation
				1085.6053	R.ELAILLGMLDPAEKDEK.G
				1900.9822	R.VVFVFGPDKK.L
				1884.9907	K.LSILYPATTGR.N
				1135.6600	R.NFDEILR.V
				1191.6760	K.DGDSVMVLPTIPEEEAK.K
				906.4737	R.VVFVFGPDK.K
				1829.8861	R.VATPVDWK.D
				1007.5473	
				915.4934	
PENK_HUMAN	Proenkephalin A	294.4	14.2	2222.0680	R.LVRPADINFLACVMECEGK.L
				1853.8109	R.PADINFLACVMECEGK.L
				2125.1529	K.ELLQLSKPELPQDGTSTLR.E
				1528.8011	L.SKPELPQDGTSTLR.E
TSP1_HUMAN	Thrombospondin-1	74.11	2.80	2195.0619	R.IPESGGDNSVFDIFELTGAAR.K
				1394.7346	R.FVFGTTPEDILR.N
GFAP_HUMAN	Glial fibrillary acidic protein	139.3	10.9	1098.6294	K.ALAAELNQLR.A R.DNLAQDLATVR.Q
				1215.6248	R.LEAENNLAAYR.Q
				1263.6353	
SPTA1_HUMAN	Spectrin alpha chain, erythrocyte	129.9	2.2	1468.8693	Y.GRDLQGVQNLLKK.H
				1667.7788	K.HEALENDFAVHETR.V
CATB_HUMAN	Cathepsin B	1413.4	54.9	2102.0655	R.SRPSFHPLSDELVNYVNK.R
				1858.9369	R.PSFHPLSDELVNYVNK.R
				1527.8217	R.LCGTFLGGPKPPQR.V
				982.4931	R.VMFTEDLK.L
				1855.9301	R.VMFTEDLKLPASFDAR.E
				1839.9336	R.VMFTEDLKLPASFDAR.E
				1286.6291	R.EQWPQCPTIK.E
				2172.9245	R.DQGSCGSCWAFGAVEAISDR.I K.GLVSGGLYESHVGCR.P
				1590.7879	K.GLVSGGLYESHVGCRPY.S
				1850.8756	K.ICEPGYSPTYK.Q
				1314.6047	K.HYGYNSYSVSNSEK.D
				1634.7256	K.NGPVEGAFSVYSDFLLYK.S
				2005.9942	K.SGVYQHVTGEMMGGH.A K.SGVYQHVTGEMMGGHAIR.I K.
				1589.6918	SGVYQHVTGEMMGGHAIR.I N.SWNTDWGDNGFFK.I R.
				1945.9297	GQDHCGIESEVVAGIPR.T
				1929.9390	K.LPASFDAR.E
				1573.6741	
				1823.8596	
				876.4665	

matographic columns (i.e. weak cation exchange chromatography) were applied to overcome the problem of protein dynamic range concentration. Moreover, keeping in mind that blood contamination directly disturbs the detailed proteome investigation; our goal was to show the current state of the protein composition during the SAH. The extensive data obtained from MALDI analysis contribute much to overall screening of the analyzed material, pointing the protein abundance of in this brain pathology. However, not all of the collected MS/MS spectra were correctly assigned to sequence database. The reason could be seeing that in complex mixtures the majority of confidently identified peptides are based on tryptic ends [26]. Thus, limits of this approach can be related to digestion process which increased sample complexity [27].

In the recent studies few reports were published describing SAH as a neuropathology complication that significantly increased mortalities of the cases. Despite rapid hospitalization, the treatment outcome is difficult to predict. Therefore, the biomarkers of SAH are needed to improve knowledge about this condition and to

monitor all changes occurring in the central nervous system. From our protein identification list we were able to recognize proteins which were already recognized as potential indicators by other groups (**Table 2**). Proenkephalin A, a stable precursor fragment of the encephalin, was detected in our study based on four peptides. It was reported that high level of plasma proenkephalin A was associated with poor clinical outcome of aneurysmal SAH and might carry predictive value for 6-month mortality [4, 28]. Further, the glutathione S-transferase P (GSTP1) and peroxiredoxin-6 (PRDX6) significant increases were observed in extracellular microdialysate of stroke patients [29]. By using MALDI proteomic approach we were able to identify the peroxiredoxin-6 with 67.4% sequence coverage. Additionally, the protein cathepsin B was also recognized with high sequence coverage on the level of 54.9%. Interestingly, it was highlighted by Yu et al. that cathepsin B/D was up-regulated in the neurons of rat cortexes after SAH [30]. The time course investigation reveals the expression of cathepsin B/D peaked at 48 h suggesting that these proteases may be released into neurone cytoplasm after SAH, where the lysosomal iron overload may lead to the activation of the apoptotic signaling. Moreover, three other potential indicators of early brain injury such as thrombospondin-1, glial fibrillary acidic protein and α-spectrin were identified. So far they were not analyzed in the CSF of patients with subarachnoid hemorrhage [31]. The thrombospondin-1 is a glycoprotein known to take part in hemostasis and angiogenesis. The increased expression was observed in experimental intracerebral hemorrhage. Consequently, the higher plasma level was also found to be related with clinical severity and long-term prognosis [32]. Further, the glial fibrillary acidic protein (GFAP) a brain specific biomarker is seeking to have diagnostic potential and prognostic value as two experimental studies pointed to higher GFAP levels in serum of patients with greater SAH severity and poorer patient outcomes [33–34]. Lastly, the a-II spectrin breakdown products (SBDPs) released from degenerating neurons, were also proposed and identified in higher level in the CSF with SAH [31].

Perspectives

The proteomic approach based on liquid phase isoelectric focusing fractionation combined with nLC-MALDI-TOF/TOF-MS/MS analysis was proposed to characterize CSF with SAH. The cumulative intersection analysis of in-solution sample separation revealed the highest concentration of the detected components in the middle fractions of the focusing chamber with gradual dilution on its extreme. Thus, rather pointing to the MicroRotofor utility as a tool for concentration of complex protein sample than selectively fractionation technique. The employed strategy ensured overall screening of investigated material presenting the proteins abundance in the current state of analysis. Few proteins such as proenkephalin A, peroxiredoxin-6, cathepsin B, thrombospondin-1, glial fibrillary acidic protein and α-spectrin were recognized as potential indicators, according to literature, pointing to the possibility for their monitoring in further studies as panel of valuable biomarkers. Nevertheless, the main limitation is connected with difficulties in availability of the cerebrospinal fluid in routine collection. Further examination should be conducted in term of quantitative analysis of the proposed proteins with inclusion of different time points during the SAH event.

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Conflict of interest statement
The authors declare no conflict of interest.

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Delivering bad news by physicians – Polish reality check

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ABSTRACT

Introduction. Disclosing unfavorable information is a very important moment in both diagnostic and therapeutic processes. It is also a highly stress-inducing factor, both among patients and physicians. During our research we tried to establish how exactly bad news is communicated to patients and the amount of stress that Polish physicians are under in such situations.

Material and Methods. Quantitative research was conducted in a university clinical hospital. With the use of an anonymous questionnaire, physicians (n = 100) from oncology, internal diseases, cardiac surgery, gynecology, obstetrics, and urology clinics were asked about the sources and the intensity of stress involved in BBN (Breaking Bad News). Similarly, patients (n = 378) of said clinics were asked to evaluate the relationships they had with their doctors.

Results. Most (66.7%) clinicians declared they always conveyed unfavorable information to their patients fully and in detail. Exactly 50.0% admitted they were experiencing high or very high level of stress while doing so. They were mostly (56.1%) anxious about depriving their patients of hope and (38.5%) feared they were letting their patients down. 37.3% of clinicians were afraid of emotional response. Significantly fewer physicians (43%) than patients (84.6%) were of the opinion that all of the medical orders must be followed to the letter.

Conclusions. Results suggest that BBN was a stressful experience for physicians. It was mostly related to the fear of disrupting the patient's well-being. Low level of effective communication was caused by the insufficiency of BBN skills. Social and cultural aspects also played a role.

Keywords: Clinical-Patient Communication, Doctor-Patient Relationship, Breaking Bad News.

Introduction

Breaking bad news is one of the hardest duties physicians must face during their professional practice [1]. As numerous studies have shown, the level of soft skills demonstrated by clinicians while disclosing unfavorable information is directly proportional to therapeutic results [2]. Proper communication has a substantial impact on the quality of medical influence, increasing the level of trust in doctor-patient relationship, among other things [3]. On one hand, not only is this communication essential in achieving the patient's full cooperation during medical treatment and their involvement in the therapeutic process, it also improves the patient's psychological endurance. It results in faster recovery and/or less severe symptoms of the disease. On the other hand, the level of distress, including the intensity of pain and discomfort the patient is experiencing, increases with anxiety and stress-inducing circumstances, which are correlated to the feeling of being ill-informed or confused. The use of soft skills while breaking bad news serves another vital purpose, which is to protect physicians from excessive stress. The feeling of

duty well performed reinforces one's self-confidence as a professional and helps to prevent the risk of occupational burnout [4].

With our study we tried to establish how exactly bad news is delivered to patients and what amount of stress Polish physicians are under in such situations. What we wanted to find out was how doctors actually cope, seeing that their responsibility is not only the duty of care, but also to teach and socialize students and young physicians professionally. We were interested in the comparison of the physician and patient preferences in the context of mutual influence in the doctor-patient relationship. We concluded it was an important socio-cultural variable, which determines the method of disclosing medical information.

Material and Methods

The survey was conducted between February and June of 2015 in the University Clinical Centre in Gdansk. It is a clinical hospital functioning at the Medical University of Gdansk. It has been classified under the highest, third level of specialization. According to the Polish Ministry of Health standards, it is a model health, research and training facility.

The first group of respondents consisted of physicians (n = 100) from thirteen different clinics: oncology, internal diseases, cardiac surgery, gynecology, obstetrics, and urology. Adult patients of said clinics (n = 378), fully responsive and being prepared to leave the hospital when the survey was taking place, constituted the second group. The selection of both clinicians and patients meeting the above criteria was random (**Table 1**).

The data was collected with the help of a survey questionnaire created by an interdisciplinary team of experts specializing in clinical psychology, medical sociology and medical law. The research instrument contained questions about the sources and level of stress connected with the necessity of disclosing unfavorable prospects to patients as well as the evaluation and nature of the doctor-patient relationship. Statistica v.12 software was used for statistical analysis. The respondents' opinions and evaluations were compared with the demographic, health and medical variables. The analysis of the relationship between the discrete variables and the statistical heterogeneity of the respondent groups was made with the use of the Pearson's chi-square test. The differences were considered statistically significant if the $p < 0.05$.

Table 1. Characteristics of the Respondents

Doctors	n	% of *n*	Patients	n	% of *n*
Sex			Sex		
Women	52	53.6	Women	246	65.8
Men	42	46.4	Men	128	34.2
Age			Age		
⩽ 30	23	23.0	18–30	46	12.3
31–40	42	42.0	31–40	55	14.7
41–50	25	25.0	41–50	65	17.4
51–60	10	10.0	51–60	63	16.8
			⩾ 60	145	38.8
Academic degree			Marital status		
M.D.	55	60.4	Single	57	15.2
Ph.D.	29	31.9	Married	267	71.2
Sc.D.	7	7.7	Divorced	24	6.4
Specialty			Widow / Widower	27	7.2
None	44	45.4	Education		
One	23	23.7	Junior high school	3	0.8
Two	28	28.9	Vocational	91	24.6
Three and more	2	2.1	Secondary	119	32.2
Position			Higher	135	36.5
Junior doctor	3	3.5	Hospitalization time in a year		
Resident doctor	31	36.5	⩽ 7 days	191	51.3
Junior assistant	11	12.9	8–14 days	93	25.0
Senior assistant	38	44.7	15–21 days	26	7.0
Senior registrar	2	2.4	⩾ 22 days	62	16.7

Results

Ways of delivering bad news

We began our study with an attempt to determine the physicians' approach to breaking bad news (**Table 2**). Most of them declared they always inform their patients personally and in full detail about unfavorable medical diagnosis and/or prognosis (66.7%). More than every tenth clinician admitted they convey only carefully pre-selected information, which means their patients are not being fully informed about their clinical state. Exactly 58% of the doctors claimed they would be interested in a communication procedure, which could provide some effective methods of disclosing unfavorable news, were it available in Poland.

Levels of stress

A significant majority of the physicians recognized the moment of delivering bad news as extremely stressful, regardless of the way it was done (**Table 3**). More than half of the respondents declared intense stressor overload. Specialists described their experiences as highly stressful significantly more often than residents (p = 0.03929).

Sources of stress

According to the surveyed clinicians, the fear of depriving their patients of hope was the main (56.1%) cause of stress (**Table 4**). A substantial percentage (38%) of respondents admitted they felt uncomfortable knowing patients were expecting to

Table 2. Physicians' approach to breaking bad news

Categories of response	% of n = 100
I always inform my patients personally and in full detail	66.7
I do not inform my patients in hope they will figure it out themselves	0.0
I prefer to disclose unfavorable news only to the patient's family	1.0
My patients get information from the medical documentation they are given when leaving the hospital	1.0
I issue a referral for my patient to see a specialist, hoping the information will be given there	0.0
I convey only pre-selected information	12.1
I have other methods than mentioned above	19.2

Table 3. Declarative level of perceived stress

Level of stress*	n = 92	% of n
No stress	4	4.3
Very low and low level of stress	15	16.3
Moderate level of stress	25	27.2
High and very high level of stress	46	50.0
Maximum stress intensity	2	2.2

* The respondents were asked to indicate their answer on an 11-point scale, where 0 meant "no stress" and 10 meant "maximum stress intensity". The answers were categorized as follows: 0 = "no stress", 1–3 = "very low and low level of stress", 4–6 = "moderate level of stress", 7–9 = "high and very high level of stress", and 10 = "maximum stress intensity".

Table 4. Causes of stress involved in breaking bad news

Categories of response*	% of n = 83
Depriving the patient of hope	56.1
Patient's emotional response	37.3
Lack of sufficient training	9.6
Time limit	14.4
Prognostic uncertainty	15.7
Family members insisting on nondisclosure of unfavorable information	4.8
The feeling of inadequacy or hopelessness	12.0
A long-term relationship with the patient	4.8
Patient's expectations as to the positive outcome of treatment	38.5

* The respondents could pick only two of the answers.

hear good news about their treatment, and communicating unfavorable information would mean letting them down. Exactly 37.3% of the doctors disclosed they were afraid of emotional response. Almost every seventh respondent (15.7%) picked out prognostic uncertainty and the discomfort caused by insufficient amount of time they were able to offer their patients when delivering bad news (14.4%). Every tenth physician experienced the stress-inducing feeling of inadequacy and hopelessness while delivering unfavorable news. Only 9,6% of the clinicians recognized lack of training and the resulting skill deficiency in terms of communicating bad news as considerably stress-inducing.

Key aspects of the doctor-patient relationship

Clinical communication is substantially determined by cultural and social references. Thus, we asked doctors and patients for evaluation of the main components constituting the physician-patient relationship (**Table 5**). Majority of respondents from both groups declared they preferred partnership in the doctor-patient interaction. In their opinion, the patient should also have the right to participate in conscious decision-making concerning therapeutic choices. Most of the interviewees recognized the physician's obligation to inform the patient fully about their health. There was a considerable asymmetry as to the issue of following medical orders. A significant majority of patients (84.6%) believed they must follow all medical orders to the letter. Only 43% of the clinicians held the same view, while every fourth physician decided that their patients are not obliged to adhere to treatment recommendations.

Discussion

Numerous statistics show that delivering unfavorable information is a highly stressful task for physicians [1, 5–7]. As can be seen from the results of our survey, Polish doctors face the same problem. More than 52% of the clinicians admitted they felt intensely stressed while disclosing bad news (see **Table 3**). In our opinion, however, these results need to be interpreted with the socio-cultural aspect in mind. Even though it affects the doctor-patient relationship noticeably [8], it is rarely taken into consideration during research. We believe that the principle of autonomy, fundamental in the Anglo-Saxon countries, translates into how physicians understand their duty of delivering unfavorable information. Suitable preparation for this task, offered to future clinicians as part of medical education [9], notification protocols [10–12] and psychological support, are further elements of importance owing to their stress-reducing function.

Our study has shown that, in case of Polish doctors, basic stress-inducing categories connected to breaking bad news involve anxieties concerning the patient's well-being (fear of depriving the patient of hope or being unable to meet the patient's therapeutic expectations; see **Table 4**). Although the majority of both patients and clinicians have declared they prefer partnership in doctor-patient interactions, the percentage of neutral opinions has also been sizeable. As many as 40% of the surveyed patients expressed their negative view about the idea of physician-patient therapeutic partnership (see **Table 5**). The paternalistic model of practice seems to remain deeply rooted not only as a physicians' attitude but also as some patients' expectation.

Table 5. Comparison of respondents' preferences as to key aspects of the doctor-patient relationship

Evaluative statement		Disagree*	Neutral*	Agree*
		n (%)		
Patient and physician are partners in the therapeutic process	Doctors	6 (6.0)	39 (39.0)	55 (55.0)
	Patients	40 (11.6)	74 (21.4)	231 (67.0)
Patients must follow all medical orders to the letter	Doctors	25 (25.0)	32 (32.0)	43 (43.0)
	Patients	15 (4.3)	39 (11.1)	297 (84.6)
Patients have the right to participate in conscious decision-making about their own health	Doctors	2 (2.0)	10 (10.0)	88 (88.0)
	Patients	8 (2.3)	23 (6.6)	318 (91.1)
Physicians are obliged to disclose all information concerning the patient's health to the patient	Doctors	2 (2.0)	18 (18.0)	80 (80.0)
	Patients	8 (2.3)	15 (4.3)	329 (93.4)

* The respondents were asked to indicate their answer on an 6-point scale, where 1 "I strongly disagree" and 2 "I disagree" were put into the "I disagree" category, whereas 6 "I strongly agree" and 5 "I agree" were categorized as "I agree". Answers 3 and 4 were neutral and as such they were placed in the "Neutral" category.

The basic rule of the Polish medical model of ethics is the commitment to the priority of the patient's well-being. Any news which may disrupt it may not be delivered should the physician, in their subjective certainty, find its predictable consequences iatrogenic. Article 17 of the Polish Medical Code of Ethics includes the following guideline: "Information about diagnosis and unfavorable prognosis may not be disclosed to the patient only if the physician strongly believes that such disclosure will cause the patient great harm or affect the patient's health negatively in any other way; should the patient, however, explicitly demand otherwise, full information ought to be given". Put into practice, it means that clinicians with especially low level of soft skills and experiencing chronic stress tend to use the exception described in Article 17 to justify nondisclosure of information or to communicate only its pre-selected, shortened version.

The way bad news are delivered is another issue. High stress intensity is directly related to faulty clinical decision-making and results in premature closure [6]. Notification protocols may offer a satisfactory solution here. Unlike Anglo-Saxon countries, where these are considered standard, Polish educational system is not widely familiar with communication procedures of this kind [13]. As of today, basic communication skills shaping courses still have not found their place among regular academic modules offered to medical students in Poland. If they exist in any form, it is rudimentary and rather theoretical. Practical training of soft skills is hence available solely with the help of commercial courses organized outside of universities, i.e., hospices, private institutions, foundations, and associations. Over the course of the years merely two protocols have been created which may be considered useful in academic training and competence development. "The 5 Steps Method" is a procedure for communicating news about the death or a serious illness of a child to the parents [14]. „EMPATHY" is a protocol for disclosing unfavorable information to the parents of oncological patients [15]. No procedure has been established with adult patients in mind, even though almost six in ten clinicians would be interested in using it, as the results of our study have shown. It is true that clinical psychologists are being hired more and more often to assist doctors in breaking bad news. It is still not common practice, however. Furthermore, psychological services are provided exclusively to the patients. Formally, Polish physicians do not receive any support when coping with difficult clinical situations.

Those key factors seem to contribute to the low quality of clinical communication in general. In consequence, Polish doctors have ranked the lowest among all of the 18 countries participating in OECD research [16] in all of the categories, (1) "Spending enough time with patient" maximum: Belgium 97.5, OECD18 84.9, USA 80.9, minimum: Poland 69.6,; (2) "Easy-to-understand explanations" maximum: Belgium 97.8, OECD18 87.9, USA 86.3, Poland 69.6; (3) "Giving opportunity to ask questions or raise concerns" maximum: Belgium 97.7, OECD18 85.0, USA 86.7, minimum: Poland 33.6; (4) "Involving patient in decisions about care and treatment" maximum: Luxembourg 95.4, OECD18 81.3, USA 83.9, minimum: Poland 47.9. It should be emphasized that, according to the OECD report, Poland holds third place in regard to the number of consultations provided by doctors per person per year. The organization of the health care system also seems to be an important variable, as it limits clinicians with excessive bureaucracy, hence reducing their time for direct contact with patients.

Through this study a number of issues emerged surrounding contraceptive method decision-making that could inform development of messaging and policy changes. First, communication campaigns could work to de-mystify the process that health 15 professionals use to support contraceptive decision-making. Communication campaigns should also help clients understand their important role in method choice by increasing their internal locus of control about contraceptive method decision-making. These campaigns would work best if done in tandem with training among contraceptive providers on client-centered counseling, including the important role of clients in the selection of the contraceptive method. Second, given how important switching contraceptive methods is in response to unmanageable side effects, contraceptive providers should be trained to discuss the strategy of switching to all clients – potential future clients, new clients, and continuing clients. Third, all persons who provide contraceptive methods, including those in the private sector, would benefit from training on client-centered counseling, especially related to counseling all clients – new and returning – on potential side effects. Widely disseminating accurate information about the importance of individual preference in contraceptive method choice, and the ability to switch methods, could increase contraceptive use in Nigeria through increased use among non-users, satisfaction with use among current users, and the power that comes from feeling in control.

As our study has shown, BBN was an intensely stressful experience for Polish physicians. This can be largely attributed to the fear of BBN disrupting the patient's well-being. Low level of soft competencies is, in our opinion, only one of the reasons for such an attitude. It has also a lot to do with paternalism, still present in some form and visible in the patients' expectations. Thus, we suggest that physician-targeted educational content should include notification protocols for BBN as part of soft skills shaping training programs. Even if the aforementioned tools (e.g. SPIKES protocol), which are created in Anglo-Saxon countries, happen to be less applicable for the other European patients [17], we do believe the above suggestion is valid, considering the low quality indicators of clinical communication in Poland. System solutions, such as communication skills training courses and psychological assistance for physicians experiencing extreme stress, also require due support.

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Informed consent and ethical approval
Informed consent was obtained from all individual participants included in the study. The research was positively evaluated and approved by the Independent Bioethics Commission for Research at the Medical University of Gdansk.

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Cognitive function in patients with childhood-onset combined pituitary hormone deficiency not treated with growth hormone

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ABSTRACT

Introduction. Growth hormone deficiency is a known factor leading to impairment of psychological performance but there are very few studies on cognitive function in adult patients with childhood-onset combined pituitary hormone deficiency (ChO-CPHD). Therefore, the aim of our study was to assess cognitive skills in adult patients with this disorder.

Material and Methods. The study was performed in a unique group of 28 adult ChO-CPHD patients, never treated with growth hormone (mean age 42.5 +/- 16.3 years; 12 women and 16 men). The assessment of cognitive performance (WAIS-R) comprised scores of verbal IQ, non-verbal IQ and particular qualitative analysis of subtests.

Results. The mean score of full scale (IQ = 81,4) was below the normal when compared to the population norm (IQ = 100, SD +/- 15), although their verbal score (IQ = 85,7) was a bit higher than their nonverbal score (IQ = 81,7).

Conclusions. The obtained result of total intelligence quotient (IQ) had shown equable subnormal results and a cognitive level below average in the study group. Decreased subscales of WAIS-R are related to fluid intelligence (this may reflect abnormal brain development or could be linked to the influence of hormonal disorders in early life) as well as, emotional traits of personality.

Keywords: cognitive function, intelligence quotient, growth hormone (GH), combined pituitary hormone deficiency (CPHD), Wechsler Intelligence Scale for Adult (WAIS-R).

Introduction

Hypopituitarism is a clinical syndrome of deficiency in pituitary hormone production. This may result from disorders involving the pituitary, hypothalamus, or surrounding structures [1]. Outcome of this disease depends on the type of hormonal deficiency [2]. Effects of hypopituitarism may be gradual or sudden and calamitous, because pituitary dysfunction affects other endocrine organs. Impaired wellbeing was reported as one of the symptoms in adults with growth hormone (GH) deficiency [3, 4] and improvement of life quality has been noted after implementation of recombinant human GH (rhGH) treatment [5, 6]. There is evidence from neuropsychological studies that growth hormone has a severe impact on cognitive function. Patients with growth hormone deficiency often complain of attention deficits, as well as poor memory. It seems well established that GH deficiency is associated with emotional and cognitive problems and the presence of a scholastic underachievement [7, 8]. Reasonably, short stature 'itself' might predispose these patients to psychosocial difficulties. The higher incidence of academic failure,

in the presence of normal intellectual performance, has been attributed to environmental and psychosocial factors, including over-protective parents and low self-esteem associated with the short stature [9, 10]. How does everyday existence look to these patients in adulthood? How does combined childhood-onset pituitary hormone deficiency, (not only restricted to GH deficiency), influence their cognitive processes? Which cognitive functions in adult patients with such a deficiency, had been most affected? To answer these questions we undertook a study aiming to assess cognitive skills in the group of adult ChO-CPHD never treated with rhGH.

Material and Methods

Patients

The study was carried out on 28 patients (16 males and 12 females) referred to the Department of Endocrinology due to childhood-onset CPHD. Age range at the moment of psychological studies was 17–65 (average 42.5 +/- 16.3) years. The mean age when CPHD diagnosis was made was 11.21 +/- 6.11 years. In all patients, GH, thyrotropin (TSH), and gonadotropins deficiency was diagnosed, and hypoplasia of the anterior pituitary lobe was found on MRI. More than half of the patients were receiving hydrocortisone because of the early or late occurring adrenocorticotropin (ACTH) deficiency. In addition, 12 patients also exhibited prolactin (PRL) deficiency. All individuals were receiving hormonal replacement therapy including levothyroxine and sex hormones, but no one was treated before with recombinant human GH, which determines the importance and unique character of the studied group. This criteria resulted in narrowing down of the overall number of examined subjects. Patients' characteristics including age at diagnosis, age at start of hormonal replacement therapy and their level of education is shown in **Table 1**.

Methods

Psychiatric diseases were excluded in the preliminary psychological consultation. The psychologist used the Polish adaptation of the Wechsler Adult Intelligence Scale (WAIS-R) to assess cognitive functions. The WAIS-R (PL) examination consists of 11 subtests, measuring a different facet of intelligence. The patient's attainments on various measures are summarized into three composite scores of IQ: the verbal, nonverbal (performance), and full scale, which provide estimates of the individual's intellectual ability. In addition, the WAIS-R (PL) provides four extra index scores (perceptual organization, freedom from distractibility, processing speed and verbal comprehension) allowing for a more detailed examination of the strengths and weaknesses of an individual's performance. The verbal tests were: Information, Digit Span, Vocabulary, Arithmetic, Comprehension and Similarities. The non-verbal (performance) tests were: Picture Completion, Picture Arrangement, Block Design, Object Assembly, and Digit Symbol. Based on the UK normative data and in line with the IQ scores the Index Scores have a mean of 100 and standard deviation of 15. There are 4 levels of deviations: Mental Retardation (MR) less than or equal to 69, Borderline MR 70–79, Dull Normal 80 to 89, Normal 90 to 109, Bright Normal 110 to 119, Superior 120 to 129, Very Superior 130 and above.

Psychometric tests employed for this study are standardized, and therefore additional research conducted in the parallel control group is not required.

Table 1. Characteristics of patients with CPHD

Variable	CPHD patients (n = 28)
Sex – n (%):	
– Males	16 (57.1)
– Females	12 (42.9)
Age (years) – mean ± SD (range) at the time of psychological study	41.7 ± 11.1 (18–59)
Age, when the testosterone or estradiol/progesterone therapy was initiated – mean ± SD (range)	19.8 ± 4.6 (9–30)
Age, when the thyroid hormone therapy was initiated – mean ± SD (range)	15.6 ± 6.8 (6–29)
Education – n (%):	
– Elementary level	15 (53.6)
– High school	10 (35.7)
– University level	3 (10.7)

The local Bioethical Committee approved the study and patients gave informed consent.

Statistical analysis

Continuous variables were expressed as the mean, standard deviation, minimum, median and maximum score. The D'Agostino-Pearson test was used to check the normality of the data distribution. Statistical analysis was performed using paired t-test, Pearson correlation analysis and Spearman rank test. If the p value was below 0.05, the results were considered as statistically significant.

Results

Quantitative analysis

Majority of subscale scores were closely centered and decreased. The obtained score of global IQ had shown subnormal and below intelligence quotient (mean 81.4), likewise verbal IQ (mean 85.7) and nonverbal IQ (mean 81.7). No significant variance ($p > 0.05$) between verbal IQ and nonverbal IQ was found in this study, indicating a level of balanced development of different cognitive functions (**Table 2**).

The principle finding of our study is that CPHD patients have an intellectual performance below average, compared to the population norm. As a reminder, median results of WAIS-R in the normal population is a score of IQ 100 (SD +/- 15). The patients' full-scale IQ scores were all below average (**Figure 1**). There was no significant difference between verbal IQ and non-verbal IQ, and no statistical discrepancy between women and men was observed.

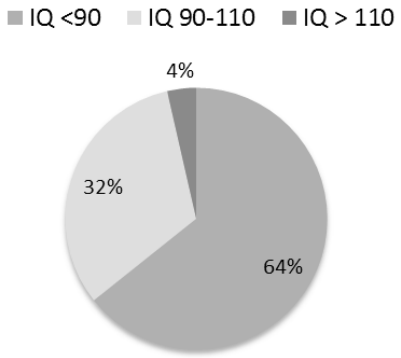

Figure 1. Distribution of WAIS-R test scores in patients with ChO-CPHD

Analysis of total, verbal and non-verbal IQ scores and age when thyroid hormone or appropriate sex hormone treatment was implemented and revealed a weak correlation between the age when testosterone or estradiol/progesterone therapy was started and total IQ, and verbal IQ scores ($r = -0.4115$; $p = 0.03$ and $r = -0.3982$; $p = 0.04$ respectively). There was no such correlation between IQ scores and the time of initiation of thyroid hormone therapy. Furthermore, no statistical significant difference between males and females was found.

Qualitative analysis

However, all subtests were below the average score (< 10). The highest scores (best-developed cognitive operations) in the studied group were reported within:

1. Verbal subtests (*Information, Comprehension, Vocabulary*) which estimate**:** word knowledge and verbal fluency**,** degree of general information acquired from culture, and long-term memory.

Table 2. Results of WAIS-R test in patients with ChO-CPHD

Test/Subtest	Mean	Standard Deviation (Park. #36)	Minimum	Median	Maximum
Total score	81.43	17.39	45	79	116
Verbal score*	85.75	18.14	45	84	120
Information	8.79	2.88	2	9	14
Digit Span	7.29	2.92	4	6	13
Vocabulary	8.21	2.92	1	8	13
Arithmetic	6.42	3.82	1	6	18
Comprehension	8.43	3.07	1	8	13
Similarities	8.00	3.62	1	9	14
Nonverbal score*	81.71	15.62	52	83	109
Picture Completion	7.04	3.21	1	7	14
Picture Arrangements	7.57	2.83	1	7	13
Block Design	8.25	2.77	1	9	12
Object Assembly	8.34	2.13	5	8	12
Digit Symbol	6.93	3.66	1	6	13

* Difference between verbal and nonverbal score was not statistically significant ($p > 0.05$)

2. Non-verbal subtests (*Object assembly, Block design*) which reflect: visual performance, synthesis, and construction, visual-spatial skills and ability to see how parts make up a whole, they also reveal level of creativeness.

The lowest scores (least developed, the most decreased cognitive operations) were noticed within:

1. Verbal tests (*Arithmetic, Digit Span*) that indicate mathematical word problems which are performed mentally: immediate auditory restoration and working memory, decreased numerical reasoning, distractibility (sensitive on emotional disturbance and distraction), attention/concentration while manipulating mental mathematical problems logical and abstract reasoning. All these scores could also be influenced by emotional tension and anxiety.
2. Performance subtests (*Picture Completion, Digit Symbol-Coding*) that describe**:** long-term visual memory, visual perception – ability to quickly perceive visual details, perceptual organization, attention to fine detail, and the ability to differentiate essential from non-essential details, visual-motor coordination, motor, perceptual and mental speed, including learning abilities.

Discussion

Hormones are the chemical messengers that serve as signal carriers for various cells. Several hormones have the potential for influencing different types of behavioral and psychological symptoms. They can change personal behavior by modulating emotions and mood [11]. Variable expressivity of sex hormones, and their different levels might be associated with cognitive impairment [12]. People with combined pituitary hormone deficiency (CPHD) suffer from quantitative and qualitative abnormalities of pituitary hormone production [13].

Early behavioral descriptions of hormonal effects on emotional disorders have long been disclosed [14]. According to several studies conducted in GHD patients, and only a few reports regarding CPHD, patients show emotional instability, a lack of energy, difficulties in social and sexual functioning, and often suffer from sleeping problems. They exhibit lower marriage frequency, higher unemployment rate and more often, inability to obtain a driving license [15, 16]. However, it was not known until the 1960s that hormones of the pituitary gland influence learning and memory [17]. During the past years several studies have evidenced that growth hormone (GH) may exert distinctive effects on the central nervous system and induce beneficial effects on psychological capabilities [18]. There is also expanding evidence in the neuropsychological literature that growth hormone (GH) deficiency is associated with cognitive impairment and this impairment may be ameliorated with GH replacement therapy [16, 19, 20]. Recent reports are also concentrating on the cognitive function of those patients including their intellectual functions; the processes of perceiving, imagining, remembering, reasoning, and judging. Most of the studies indicated that GHD can lead to small, but clinically relevant changes in memory, processing speed and attention focus in patients [21]. Children with isolated GHD are reported to have specific educational deficits, in particular learning disability and attention-deficit disorders, which have been tentatively attributed to a compromised intellectual potential [22]. The general picture of cognitive functioning in adult CPHD patients is that their mental status (including IQ) is subnormal. Many of these patients show memory impairment and subnormal intelligence quotient (IQ) scores. Their cognitive functioning appeared subnormal with lapses of attention, difficulty in concentrating and forgetfulness [23]. Also in the present study, the cognitive functions of adult patients with ChO-CPHD never hormonally substituted with rhGH and not systematically treated with other hormones, appeared subnormal. Obtained results show that these patients indeed have apparent cognitive dysfunction, although not severe. The principle finding of this report is that people with CPHD have an intellectual ability that is within the lower than average range when compared to the population norm and with reduced ability to perform tasks involving antidistractory skills. This might suggest educational problems in the past, with necessary special individual attention and lower expectations in school programs, as was confirmed in psychological interviews. Remarkable information provided qualitative analysis of individual psychometric charts with scale score analysis. Decreased scores on arithmetics indicate impaired immediate auditory memory, and at the very least, inadequate attention and concentration.

Although abnormalities of visual-motor integration among children with GHD and hypopituitarism have been described previously [9, 15], the present study also revealed that the lowest results were obtained in tasks comprising the perceptual organization index. This involves the interpretation and organization of visually presented information and is due to process efficiency. In turn, the most decreased subtest Digit Span and Digit Symbol-Coding revealed decreased

ability to receive, store, process, and use information through a series of cognitive ordering procedures called sequential processing. These building blocks are not only essential for listening, learning, reading and communication, but also every mental process that is dependent on these processing abilities. Factor comparisons showed that impaired verbal comprehension and perceptual organization were not significantly varying in the studied group, and both of them are essentially equivalent to working memory, which is consistent with the level of general intellectual abilities. According to D. Wechsler, intelligence is an individual's ability to adapt and constructively solve problems in the environment [24] and is also influenced by personal traits and other non-intellective components, such as anxiety, persistence and goal awareness. Therefore the assessment of intellectual performance depends on emotional dependence. These personal traits could significantly decrease scores of Arithmetic and Digit span. The WAIS-R subtests were classified into two categories: fluid intelligence and crystallized intelligence. Fluid intelligence is related to biology. This is the ability to find meaning in confusion and solve new problems, the ability to draw inferences and understand the relationships of various concepts, independently of acquired knowledge [25]. This is also the ability to reason in an abstract way, defined as our "on-the-spot reasoning ability, a skill not basically dependent on our experience." Belsky [1990] indicated this type of intelligence, being active when the central nervous system (CNS) is at its physiological peak [26]. Fluid intelligence includes such abilities as problem-solving, learning, and pattern recognition, that generally correlates with measures of abstract reasoning. Fluid intelligence includes those types of basic intelligence that make learning quick and thorough. Underlying abilities such as short-term memory, abstract thought, and speed of thinking are usually considered as a component of fluid intelligence. Crystallized intelligence in contrast, is the ability to utilize previously acquired knowledge and experience. Crystallized intelligence correlates with abilities that depend on knowledge and experience, appearing as a function of brain regions responsible for the storage and usage of long-term memories (such as the hippocampus) [27]. It is the store of information that a given society has accumulated over time. Crystallized intelligence includes those types of intellectual abilities that reflect accumulated learning [28]. The WAIS measures fluid intelligence on the performance scale and crystallized intelligence on the verbal scale. Results of our study group suggest that patients with CPHD have impaired fluid intelligence more significantly than crystallized intelligence, because the higher scores were noted in the verbal part (comprehension, vocabulary, information). The study group could better deal with understanding social conventions, common sense, and social rules and expressions (Verbal comprehension), rather than perceptual organizational learning, and pattern recognition.

As mentioned before, abnormalities of visual-motor integration among patients with hypopituitarism have been described previously [15]. A range of assessments, including the WAIS, was used in this study. Authors concluded that overall performance of patients was in the low-average range. A link between academic performance and socioeconomic status and short stature is indicated and might predispose CPHD patients to some of their psychosocial difficulties, such as educational and professional achievements. The higher incidence of academic failure in presence of normal intellectual skills has been attributed to environmental and psychosocial factors, including over-protective parents and low self-esteem resulting from the impact of short stature. But, it is possible that their problems are related to congenital predispositions; for example impaired fluid intelligence. If so, then an overprotective approach by family members should not be recommended and children with hypopituitarism should be encouraged to intensive and systematic compensatory stimulation of the CNS.

Congenital disturbances in pituitary development, as well as postnatal and later life insults such as hypoglycemia, hypothyroidism, abnormal delayed growth and sexual development in patients with CPHD could all impact normal developmental processes. In summary, our results suggest that patients with ChO-CPHD have lower than average cognitive function with specific difficulties in attention, working speed, memory and perceptual organization. Diminished cognitive functions such as deterioration in memory, reduced ability to process information quickly, and reduced verbal fluency has been observed. An important question is whether the cognitive dysfunction seen in CPHD patients can be related to other common symptoms of CPHD. For example, these patients often are also depressed, and cognitive dysfunction occurs in people who are depressed. Conceivably, the cognitive dysfunction in CPHD could be simply present due to depression. Depressed patients are often slow and the score of speed performance tasks were significant decline in our group of patients. Due to those reasons, we excluded depressive symptoms in our study.

The lowest scores were in tasks sensitive to emotions, tension and anxiety, comprising: concentration, attention, numeric reasoning, short term memory, visual perception, perceptual organization, visual memory, visual-motor coordination, motor and mental speed. Educational attainments of parents were not recorded, but genetic factors as well as social class might provide some explanation for the lower IQ scores in patients.

Conclusions

In addition to decreased overall cognitive performance in the study group, moderate deficits in terms of verbal and performance scales were also observed. Furthermore, the study showed decreased IQ and associated reduced scores of subtests, primarily concerning conscious perceptual processing (Perceptual Organization and Working Memory). Patients also appeared to have more impaired fluid intelligence than crystallized intelligence; but this may be related to purely biological disturbances, such as impaired brain development or other dysfunctions (hypoglycemia, low thyroxin concentrations) particularly appearing in early life. One noteworthy problem is also the relationship between fluid intelligence and the emotional component of personality.

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Polyphenol-rich extract of Aronia melanocarpa inhibits TNF-α induced apoptosis in H9c2 cells

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ABSTRACT

Introduction. In certain pathological states within cardiovascular system, cardiomyocytes may exhibit overexpression of TNF-α that results in induction of myocardial oxidative stress and cardiac cells apoptosis. Thus, they may participate in development and progression of post-infarct congestive heart failure. The aim of this study was to evaluate the effect of polyphenol-rich *Aronia melanocarpa* extract (AME) on TNF-α induced apoptosis in cardiomyoblast H9c2 cells.
Material and Methods. Apoptosis was measured in H9c2 cells preincubated with increasing concentrations of commercial extract of aronia – Aronox (10–50 μg/mL) for 24h and then treated with TNF-α: 100 ng/mL for 24h as well. The MTT assay was used to determine cardiomyoblasts viability. Alteration of the mitochondrial membrane potential, specific for apoptotic cells, was evaluated with caspase-3 activity assay kit.
Results. Our results showed that AME significantly inhibited TNF-α induced apoptosis (IC_{50} = 55.84 μg/mL) and cytotoxicity in H9c2 cells. Significant inhibition of apoptosis was observed in all tested concentrations of AME. The highest anti-apoptotic and cytoprotective effect was observed at the highest concentration (50 μg/mL), while in lower the concentrations cytoprotective effect was statistically insignificant.
Conclusions. Polyphenol-rich AME exhibits anti-apoptotic and cytoprotective effect in H9c2 cardiomyoblasts treated with TNF-α. Further studies are required in context of its possible application in prevention and/or therapy of cardiovascular diseases.

Keywords: *Aronia melanocarpa*, plant extract, H9c2 cardiomyoblasts, oxidative stress, apoptosis, cardiovascular disease.

Introduction

The cardiovascular diseases (CVD) are a major cause of death worldwide (World Health Organization, 2014). The prevailing view is that oxidative stress may play a crucial role in CVD development and progression [1]. It is postulated that increased expression of proinflammatory cytokine TNF-α in cardiomyocytes can have an important impact on induction of oxidative stress in myocardium. Hence, it may lead to increased apoptosis of cardiomyocytes and endothelial cells, further ventricular remodeling, down-regulation of myocardial contractility, that results in chronic heart failure, wherein the intensity of those alterations depends on TNF-α expression level [2, 3]. Therefore, researchers are looking for a new compounds that can be used in prevention or/and treatment of cardiovascular diseases associated with oxidative stress.

Polyphenols are naturally occurring antioxidants highly effective in scavenging of free radicals (FR) and reactive oxygen species (ROS) [4, 5]. They are commonly found in food products like: fruits, vegetables, legumes or red wine, green and black tea [5]. One of the richest source of plant-derived polyphenols are berries of *Aronia melanocarpa* (Michx.) Elliott (Black Chokeberry).

Researchers reported numerous beneficial health properties of aronia products including anti-inflam-

matory and cardio-protective activity [4, 6 ,7]. Several studies have also indicated cytoprotective and anti-apoptotic activity of aronia extract, but the exact mechanism is still unclear [8–10]. The reliable experimental in vitro model of human myocardial cells widely used for evaluation of antioxidant and anti-apoptotic effect of a number of plant extracts and plant-derived antioxidants in conditions of induced apoptosis is the cardiomyoblasts cell line – H9c2 [10–14]. Therefore, in our study, we treated H9c2 cells with TNF-α to induce oxidative stress-mediated apoptosis in vitro. The impact of polyphenol-rich AME – Aronox on TNF-α induced apoptosis was investigated.

Material and Methods

We used a natural, polyphenol-rich plant extract derived from berries of *Aronia melanocarpa* – Aronox (Adamed Ltd, Czosnów, Poland) containing polyphenols 60% w/w, including at least 20% w/w of anthocyanins, according to manufacturer's data. High-performance liquid chromatography (HPLC) analysis showed that total concentration of phenolics in the Aronox extract was 309.6 mg/g wherein concentration of phenolic acids (isomers of chlorogenic acid) was 149.2 mg/g, anthocyanins (anthocyanin glycosides: cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, cyanidin 3-xyloside) was 110.7 mg/g and flavonoids (quercetin glycosides) – 49.7 mg/g of extract [15].

The adherent cardiomyoblast cell line H9c2 primarily derived from embrionic rat heart (Sigma Aldrich, St. Louis, MO, USA) was cultured in Dulbecco Modified Eagle Medium (DMEM, Sigma Aldrich, St. Louis, MO, USA), containing 4500 mg/L glucose and L-glutamine, supplemented with 10% v/v fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany) and antibiotic-antimycotic solution (AAS, Sigma Aldrich, St. Louis, MO, USA). The cell culture was maintained in monolayer on standard Petri dishes (Becton Dickinson, East Rutherford, NJ, USA) at 37°C, 5% CO_2 in a fully humidified atmosphere. The culture medium was replaced by fresh medium every 2 days. In our experiment we used cells from passages 4 ± 2. Cardiomyoblasts were preincubated with 10, 20, 40, 50 µg/mL of Aronox dissolved in Phosphate Buffered Saline (PBS, Biomed-Lublin, Lublin, Poland) for 24h, and subsequently treated with 100 ng/mL of TNF-α for 24h. Three control groups have been performed (including negative control group): after 24h of incubation without additives cells were treated with 50 µg/mL of Aronox,100 ng/mL of TNF-α for 24h, and a negative control group was incubated without any additives for 48h.

Cells viability was evaluated with the MTT Cell Proliferation Assay Kit (Biotium, Hayward, CA, USA) based on their metabolic activity status, while alterations of the mitochondrial membrane potential related with cells apoptosis were evaluated using the Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, Mountain View, CA, USA).

Statistical analysis

All experiments were repeated at least three times. Data were analyzed using R Project software version 2.15.1 (www.r-project.org). The continuous variables were expressed as means ± SD. Data comparison was executed by the one sample Student's T-test, one-way ANOVA and post-hoc Tukey HSD test for one-way ANOVA. The statistical significance was adopted at p-value < 0.05.

RESULTS

Inhibition of H9c2 cells cytotoxicity induced by TNF-α

Data collected from the MTT cell viability assay are shown as means ± SD, lower absorbance values are related with reduced metabolitic activity of H9c2 cells (**Figure 1**). Viability of H9c2 cells preincubated with 10–50 µg/mL AME and treated with TNF-α were evaluated in comparison to 50 µg/mL AME control group.

Pretreatment with AME significantly inhibited cytotoxicity induced by TNF-α ($p = 0.04652$, T-test; $p = 0.00454$, ANOVA). The strongest cytoprotective effect of AME was observed in its highest concentration of 50 µg/mL ($p = 0.00630$). There was no significant difference in viability of cells preincubated with 10, 20 µg/mL of AME and and AME control samples. In concentration 40 µg/mL, cytoprotective effect of AME was low and also statistically insignificant. Surprisingly, we have found that H9c2 cells viability was decreased in control samples treated with 50µg/mL of AME only, that may be caused by the lower initial H9c2 cell count in some evaluated samples.

The obtained values of relative cell viability, presented in **Figure 2** as a percentage relative to the untreated control cells, showed protective effect of AME in H9c2 cells in conditions of induced apoptosis. Obviously, cytoprotective effect was observed in samples preincubated with AME in concentration higher than 20 µg/mL.

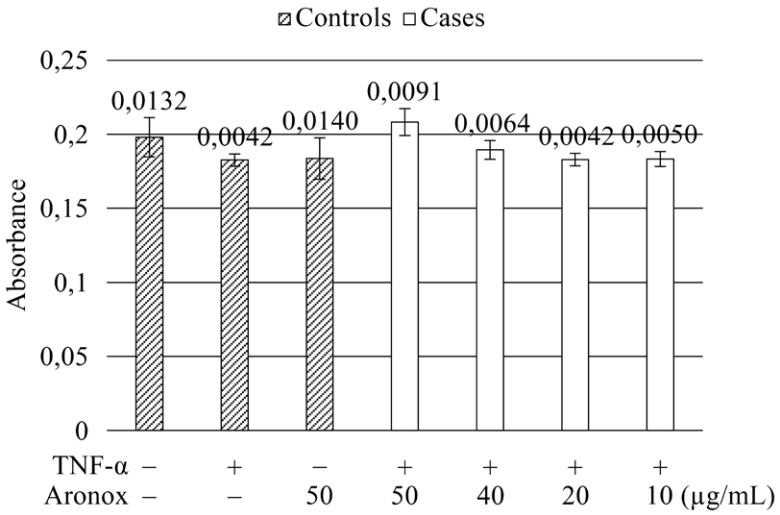

Figure 1. Cytoprotective effect of Aronia melanocarpa extract (Aronox: 10, 20, 40, 50 µg/mL) in H9c2 cells treated with 100 ng/mL TNF-α

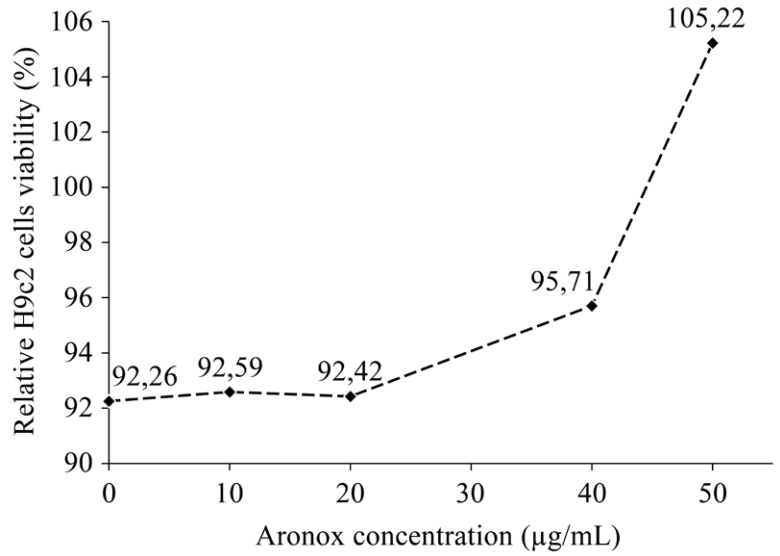

Figure 2. Concentration-dependent cytoprotective effect of Aronia melanocarpa extract (Aronox) in H9c2 cells treated with 100 ng/mL TNF-α

Anti-apoptotic activity of AME extract in H9c2 cells induced by TNF-α

Analysis of the caspase-3 activity in H9c2 cells preincubated with AME and treated with TNF-α. showed anti-apoptotic activity of AME, regarding to control samples Results shown in **Figure 3** are presented as mean absorbance value ± SD, where lower absorbance values were related with decreased apoptotic cells ratio. Apoptotic cells count was significantly lower in case samples (10–50 µg/mL AME; 100 ng/mL TNF-α) compared to AME treated controls ($p = 4.146x10^{-14}$, T-test; p = 0.0012, ANOVA). Statistical analysis revealed that AME in all tested concentrations significantly inhibited TNF-α induced H9c2 cells apoptosis, wherein the strongest anti-apoptotic effect was observed in the highest applied concentration of AME 50 µg/mL (p = 0.00049, Tukey HSD). The caspase-3 activity was comparable between control negative samples and controls incubated with 50 µg/mL of extract, thus AME in its maximal concentration did not induce H9c2 cells apoptosis.

The inhibition of apoptosis (in %) was presented on the basis of caspase-3 activity measurements. We found that AME inhibited apoptosis induced by TNF-α starting with its lowest tested concentration (10 µg/mL) (**Figure 4**).

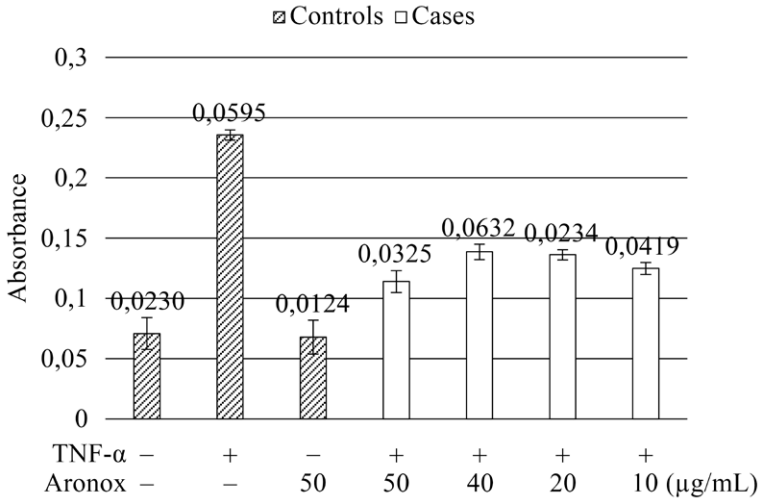

Figure 3. Anti-apoptotic effect of Aronia melanocarpa extract (10, 20, 40, 50 µg/mL) in H9c2 cells treated with 100 ng/mL TNF-α

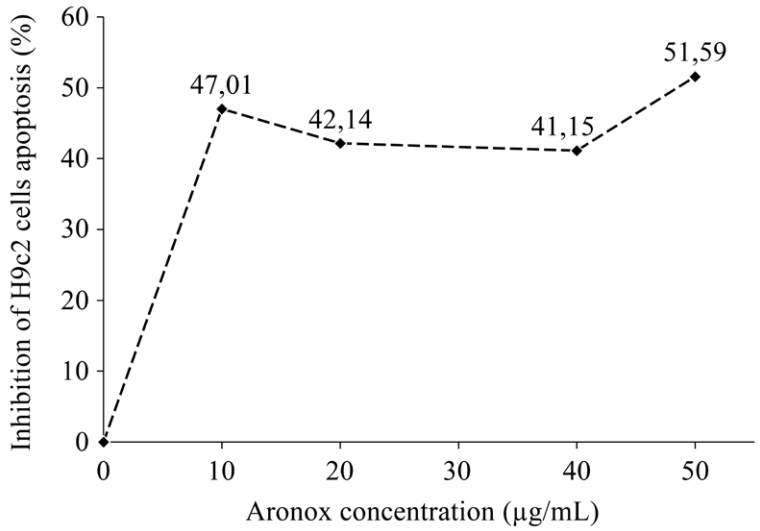

Figure 4. Inhibition of TNF-α (100 ng/mL) induced apoptosis in H9c2 cells preincubated with Aronia melanocarpa extract (10, 20, 40, 50 µg/mL)

The estimated value of half maximal inhibitory concentration (IC50) for AME in conditions of TNF-α induced H9c2 cell apoptosis was 55.84 µg/mL.

Discussion

According to the epidemiological data in group of non-communicable diseases, the cardiovascular diseases (CVD) are the major cause of death in developed countries [16, 17]. It is estimated that annual CVD mortality will rise rapidly from 17.5 mln in 2008 to 22.2 mln in 2030 [16]. These facts contributed to intense research for a new compounds that could be used in prevention and treatment of CVD. Ones of the most promising group of nutrients identified as possibly cardioprotective are plant derived antioxidants – polyphenols, commonly found in *Aronia melanocarpa* berries [4, 7].

Study performed by Olivetti *et al.* on postmortem samples of human hearts revealed the presence of apoptotic cells in „border zone" of myocardium during acute myocardial infarction. Therefore, apoptosis appears to be a significant factor involved in cardiomyocytes loss in the post-infarcted heart [18]. Additionally, it is suggested that apoptosis may lead to post-infarct ventricular remodeling and development of heart failure [19]. Thereby, a crucial issue in myocardial infarction treatment is prevention of myocardial cell loss as it is an important determinant of patients morbidity and mortality. In most cases of chronic heart failure the cardiomyocytes apoptosis incidence is low, however its

long-term deleterious effects on myocardium and participation in the loss of cardiac cells may be a clinically significant factor for these patients [20].

As was already mentioned, important role in both: induction of cardiomyocytes and endothelial cells apoptosis, and development of myocardium structural-functional alterations plays an increased expression of TNF-α in cardiac myocytes in certain pathological conditions [2, 3]. The key role in programmed cell death induced by TNF-α plays the increased mitochondrial ROS generation. This specific mechanism is still not fully understood, however, Kim *et al.* suggested that mitochondrial ROS modulator 1 (Romo1) may be involved in TNF-α dependent induction of mitochondrial ROS generation [21]. Thus, it can be considered that inhibition of TNF-α induced intracellular oxidative stress and following cardiomyocytes apoptosis by antioxidant compounds (polyphenols) found e.g. in the AME could find a practical application in prevention and treatment of heart failure in a group of patients with CVD.

We studied the effect of polyphenols from AME on TNF-α induced apoptosis in H9c2 cardiomyoblasts. In our study the cytoprotective and anti-apoptotic effect of AME (Aronox) on H9c2 cells under exposure to TNF-α was observed. This result confirmes other reports about AME and plant-derived polyphenolic compound effect on a different cell lines under conditions of induced oxidative stress. In 2012, Zapolska-Downar *et al.* reported dose-dependent cytoprotective effect of Aronox in TNF-a treated human aortic endothelial cells (HAEC), associated with inhibition of intracellular ROS production [8]. It confirms our observations of increased viability of H9c2 cells preincubated with AME compared to untreated controls under conditions of TNF-α induced apoptosis and its dependence on the AME dosage. Moreover, earlier study by Zapolska-Downar *et al.* also confirms our observation of AME anti-apoptotic activity based on reduced caspase-3 activity in case samples regarding to controls [9]. Our findings are consistent with results by Angeloni *et al.* as well. Their study showed that quercetin, the glycosides of which are present in AME, reduces intracellular ROS synthesis, prevents oxidative cell damage and inhibits caspase-3 activation in cardiomyoblasts treated with H_2O_2. On the other hand, O-methylated quercetin metabolites appear to inhibit intracellular generation of ROS but do not affect cells viability nor caspase-3 activation [10]. Recently, the anti-apoptotic activity of polyphenols (epigallocatechin gallate) in H9c2 cells treated with H_2O_2 has also been reported [14]. Other researchers also show the protective effect of plant polyphenols (cyanidin-3-O-β-glucoside) on oxidative stress-induced apoptosis in different cell lines: human umbilical vein endothelial cell (HUVEC), liver hepatocellular carcinoma (HepG2) and mouse insulin-producing pancreatic β-cells (MIN6N) [22–24].

Additionally, existing reports from clinical trials prove the beneficial effects of polyphenols from *Aronia melanocarpa* on the overall condition of the cardiovascular system in vivo. Naruszewicz *et al.* evaluated the clinical utility of statins-flavonoids combined therapy for patients with hypercholesterolemia following acute myocardial infarction. Their study revealed several beneficial effect of flavonoids supplementation e.g. reduced blood pressure, decreased serum levels of C-reactive protein (CRP), cell adhesion molecules – glycoprotein VCAM and immunoglobulin ICAM and oxidized low density lipoprotein (ox-LDL) [6]. In later work, Naruszewicz *et al.* described the decrease in the serum level of interleukin 6 (IL-6) and monocyte chemoattractant protein (MCP-1) and an increase in adiponectin level in a group of patients following myocardial infarction after 6-week supplementation with Aronox regarding to placebo group [7].

However, considering the possible clinical applications of plant polyphenols it should be mentioned that they may also exhibit pro-oxidative and pro-apoptotic activities. It was reported that it may depends e.g. on their applied concentration. According to Watjen *et al.*, some polyphenols (flavonoids) may be cytotoxic or cytoprotective to the hepatoma cells (H4IIE) treated with H_2O_2 depending on their concentration in culture medium [25]. It is reported that compounds contained in *Aronia melanocarpa* berries show selective pro-apoptotic activity in leukemic cells, leaving regular T-cells unaffected [26]. Thus, the cited researches confirm the general view of the selective cytotoxic activity of polyphenolic compounds that do not affect the regular cells viability. Yet, in our study we observed reduction of H9c2 cells viability in control samples incubated with AME at its highest tested concentration.

In fact, this study seems to confirm anti-apoptotic activity of polyphenol-rich AME in H9c2 cardiomyoblast treated with TNF-α. However, it should be considered that in vivo animal/human studies revealed low bioavailability and decline in antioxidant activity of polyphenol compounds after oral ingestion [4, 27–30]. For this reason, it is postulated that in vitro studies metabolites of these compounds should be applied, rather than their native forms present in plant products [29]. It also should be noted that despite the generally low bioavailability of polyphenols, anthocya-

nins are determined in the peripheral blood plasma in the native form (as glycosides) [30]. Regarding to low bioavailability of orally ingested polyphenols, and the fact that in cardiovascular system they are circulating generally in metabolized form additional in vitro studies of polyphenol metabolites are required.

Our results confirm the available literature data and provide evidence on anti-apoptotic activity of *Aronia melanocarpa* derived polyphenols on H9c2 cardiomyoblasts in conditions of TNF-α induced apoptosis**.** However, further studies with higher concentrations of aronia extract are needed to evaluate its possible cytotoxic effect. An in-depth understanding of molecular mechanism underlying the anti-apoptotic activity of polyphenols in cells like i.a. cardiomyocytes as well as further in vivo studies would be useful in context of their possible application to clinical practice for patients with cardiovascular system diseases.

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Conflict of interest statement
The authors declare no conflict of interest.

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Interleukin-7 receptor Thr244Ile gene polymorphism and the risk of systemic lupus erythematosus

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ABSTRACT

Aim. Recently, the *IL-7 receptor* (*IL-7R*) C>T (rs6897932) single nucleotide polymorphism (SNP), which causes a Thr244Ile substitution in the IL-7R α-chain, has been suggested as a risk factor for SLE.
Material and Methods. Using high-resolution melting curve analysis we studied the distribution of the *IL-7R* C>T polymorphism in SLE patients (n = 281) and control subjects (n = 541) in the Polish population.
Results. We did not find significant differences in the distribution of the *IL-7R* C>T genotype and alleles between SLE patients and controls. However, in the dominant model (T/T and C/T vs C/C genotypes), we observed a protective effect of the *IL-7R* C>T polymorphism against the presence of neurological manifestations of SLE [OR = 0.3631 (95% CI = 0.1895–0.6954), p = 0.0017, p_{corr} = 0.0323] and the presence of anti-Scl-70 antibodies (Ab) [OR = 0.3141 (95% CI = 0.1503–0.6561), p = 0.0014, p_{corr} = 0.0266].
Conclusion. Our studies suggest that the *IL-7R* C>T (rs6897932) polymorphism might be involved in the neurological manifestations and the presence of anti-Scl-70 Abs in patients with SLE.

Keywords: Interleukin-7 receptor, SNP, SLE.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder in which the immune system of the host attacks its own tissues [1]. The SLE immune cells are characterized by abnormal signaling in $CD4^+$ T cells as well as abundant autoantibody biosynthesis by B cells [1–3]. This disease can affect the kidneys, joints, skin, lungs, brain, and other organ systems, resulting in defective functioning of organs, as observed in clinical findings of SLE [1]. Familial and genome-wide association studies have suggested many genes that potentially play a role in SLE development, phenotypes and antibody profiles. Exposure to various exogenous factors such as ultraviolet light, drugs, chemicals, pollutants, and bacterial and viral infections all contribute to SLE development. The underlying cause of SLE remains unknown; however, it is accepted that the genetic components of the host and environmental factors make the host vulnerable to this autoimmune disorder [4, 5].

Recently, an increased body of evidence has demonstrated an association of abnormal interleukin-7 (IL-7) signaling with aberrant functions of immune cells and autoimmunity [9]. IL-7 signaling plays an elementary role in B lymphopoiesis, thymocyte maturation, peripheral T cell homeostasis and immune tolerance [10–12]. IL-7 receptor (IL-7R) is a heterodimer comprising IL-7Rα and the common γ-chain, which is also shared by IL-2R, IL-4R, IL-9R, IL-15R, and IL-21R [13, 14]. The *IL-7R* C>T (rs6897932) polymorphism causes a Thr244Ile substitution in the IL-7R α-chain, thereby changing the ratio of membrane-bound to soluble IL-7R, which is implicated in the pathogenesis of autoimmune diseases [15,

16]. It has been demonstrated that the Thr244Ile substitution can be associated with some autoimmune diseases [15, 17–19]. Recently, the *IL-7R* C>T SNP has also been recognized as a risk factor for SLE development [18]. Therefore, we evaluated whether the *IL-R7* C>T SNP is a genetic risk factor for SLE in the Polish population. Because SLE is a heterogeneous disorder, we also examined the association of this polymorphism with different disease phenotypes and antibody profiles.

Material and Methods

Patients and controls

Medical records data for two hundred and eighty-one women fulfilling the American College of Rheumatology Classification criteria for SLE were collected for the study in a random manner at the Institute of Rheumatology in Warsaw, Poland [20, 21]. The control group comprised five hundred and forty-one unrelated healthy female volunteers that were selected during medical examination at the Institute of Mother and Child in Warsaw, Poland. The women with SLE and the controls were of Polish Caucasian origin and of similar age. The mean age was 37 ± 8 years for the SLE patients at diagnosis and 36 ± 7 years for the controls. All participating subjects provided written consent. The study procedures were approved by the Local Ethical Committee of Poznan University of Medical Sciences in Poznan, Poland.

Genotyping

DNA was isolated from peripheral leukocytes using a salting-out procedure. The *IL-7R* C > T (rs6897932) DNA fragment (135bp) was amplified using the primers 5′ TGAGACCCTACCCCCACT 3′ and 5′ GCCAAGATGACCAACAGAG 3′. This polymorphism was then genotyped by high-resolution melting curve analysis (HRM) on a Light Cycler *480* system (Roche Diagnostics, Mannheim, Germany). The *IL-7R* C>T polymorphisms were verified by commercial sequencing analysis.

Statistical analysis

The prevalence of genotypes in patients and controls was examined for deviation from Hardy-Weinberg equilibrium using exact and log likelihood ratio chi-squared (χ^2) tests [http://ihg.gsf.de/cgi-bin/hw/hwa1.pl]. The polymorphism was tested for association with the SLE incidence using the χ^2 test for trend (p_{trend}). The χ^2 test was employed to examine differences in genotypic and allelic distribution between patients and controls, and a p value <0.05 was considered statistically significant. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated. The association of the *IL-7 receptor* (*IL-7R*) C>T SNP polymorphism with clinical manifestations and the presence of autoantibodies was evaluated by χ^2 test. The Bonferroni correction for multiple comparisons was used, and both p values, before (p) and after correction (p_{corr}), were evaluated.

Results

Prevalence of *IL-7R* C>T (rs6897932) genotypes and alleles in SLE patients and controls.

The genotypic prevalence of the *IL-7R* C>T polymorphism did not significantly deviate from Hardy-Weinberg equilibrium between patients with SLE and healthy controls. The number of genotypes and the ORs and 95% CIs for the *IL-7R* C>T SNP are listed in **Table 1**. We did not observe association of *IL-7R* C>T *SNP* with SLE development. The OR for SLE patients with the *IL-7R* TT genotype was 0.5906 (95% CI = 0.2927–1.192, p = 0.1378) the OR for the CT genotype was 1.008 (95% CI = 0.7401–1.373, p = 0.9602), the OR for the TT and C/T genotypes was 0.9397 (95% CI = 0.6988–1.264, p = 0.6803), and the OR for the *C* allele was

Table 1. Prevalence of the *IL-7R* C>T (rs6897932) polymorphism in SLE patients and controls

IL-7R **C>T**	SLE n = 281	Controls n = 541	OR	95%CI	P-value[e]	P_{trend}
Genotype frequency						
C/C	174 (0.62)	327 (0.61)	**Reference**			
C/T	96 (0.34)	179 (0.33)	1.008[a]	(0.7401–1.373)	0.9602[a]	
T/T	11 (0.04)	35 (0.06)	0.5906[b]	(0.2927–1.192)[b]	0.1378[b]	0.3600
C/T + T/T	107 (0.38)	214 (0.39)	0.9397[c]	(0.6988–1.264)[c]	0.6803[c]	
Minor allele frequency						
T	0.21	0.23	0.8891[d]	0.6941–1.139[d]	0.3517[d]	

The Odds Ratio (OR) was calculated for patients [a](C/T vs C/C genotype), [b](T/T vs C/C genotype),[c] (T/T and C/T vs **C/C genotype). We also determined the OR for the patients' minor allele**; [d](T allele vs C allele); [e]χ^2 test.

0.8891 (95% CI = 0.6941–1.139, p = 0.3517). The p value of the χ^2 test for the trend observed for the *IL-7R* C>T polymorphism was also not statistically significant (p_{trend} = 0.3600).

Association of the *IL-7R* C>T SNP with the presence of autoantibodies and clinical manifestations in patients with SLE.

In the dominant model (T/T and C/T vs C/C genotype), we observed a significant protective effect of the *IL-7R* C>T polymorphism against the presence of neurological manifestations of SLE [OR = 0.3631 (95% CI = 0.1895–0.6954), p = 0.0017, p_{corr} = 0.0323] (**Table 2**). We also found a statistically significant protective effect of the *IL-7R* C>T SNP against the presence of anti-Scl-70 Abs [OR = 0.3141 (95% CI = 0.1503–0.6561), p = 0.0014, p_{corr} = 0.0266] (**Table 3**). However, we did not find any significant differences between the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) at diagnosis and the *IL-7R* C>T genotypes.

Discussion

Abnormal concentrations of IL-7R and IL-7Rα on T cells have been demonstrated in blood plasma from patients with SLE [22–24]. The IL-7R levels were significantly higher in SLE patients than in controls and correlated with SLEDAI scores, especially nephritis [22]. In addition to this finding, Kim [23] demonstrated increased levels of IL-7Rα in low effector memory $CD8^+$ T cells, which may affect tissue damage via CD244-mediated cytotoxicity in patients with SLE. Furthermore, Wang [24], using a mouse model of SLE-like serology, found that the function of IL-7R was required for reintroducing RAG proteins into antigen-activated early memory plasma B cells or pre-plasma B cells and contributed to the maintenance of humoral tolerance. Therefore, the genetic variants of *IL-7R*α might influence different SLE phenotypes and antibody profiles.

In conclusion, we did not observe a contribution of the *IL-7R* C>T polymorphism to SLE development in the

Table 2. Distribution of the *IL-7R* C>T (rs6897932) polymorphism among SLE patients with different clinical manifestations

Characteristic	Genotype distribution				MAF[d]	
	C/C SLE[a] / SLE[b]	C/T SLE[a] / SLE[b]	T/T SLE[a] / SLE[b]	**Odds ratio (95% CI), p[c]** T/T + C/T vs C/C	SLE[a]	SLE[b]
Malar rash	91 / 83	49 / 47	6 / 5		0.21	0.21
Discoid rash	52 / 122	28 / 68	4 / 7		0.21	0.20
Phototosensitivity	79 / 95	43 / 53	7 / 4		0.22	0.20
Oral or nasopharyngeal	67 / 107	39 / 57	5 / 6		0.22	0.20
Arthritis	39 / 135	22 / 74	3 / 8		0.22	0.21
Serositis	31 / 143	17 / 79	2 / 9		0.21	0.21
Renal	84 / 90	46 / 50	7 / 4		0.22	0.20
Neurologic	51 / 123	12 / 84	2 / 9	0.3631 (0.1895–0.6954), p = 0.0017	0.12	0.24
Hematologic	56 / 118	30 / 66	5 / 6		0.22	0.21
Immunologic	84 / 90	43 / 53	10 / 1		0.23	0.19
ANA	174 / 174	96 / 96	11 / 11			

Comparison of genotype frequencies between patients (SLE[a]) with and patients (SLE[b]) without a particular manifestation was performed by [c]χ^2 test, minor allele frequency[d].

Table 3. Effect of the *IL-7R* C>T (rs6897932) polymorphism on the presence of various autoantibodies in patients with SLE

Autoantibodies	Genotype distribution				MAF[d]	
	C/C SLE[a] / SLE[b]	T/C SLE[a] / SLE[b]	T/T SLE[a] / SLE[b]	Odds Ratio (95% CI)[a], p[c] T/T and T/C vs C/C	SLE[a]	SLE[b]
anti-dsDNA	58 / 116	31 / 65	7 / 4		0.23	0.20
anti-Smith	15 / 159	8 / 88	2 / 9		0.24	0.21
anti-snRNP	33 / 141	17 / 79	6 / 5		0.26	0.20
anti-Ro	28 / 146	15 / 81	3 / 8		0.23	0.21
anti-La	23 / 151	12 / 84	3 / 8		0.24	0.21
anti-Scl-70	43 / 131	7 / 89	3 / 8	0.3141 (0.1503–0.6561), p = 0.0014	0.12	0.23

Comparison of genotype frequencies between patients (SLE[a]) with and patients (SLE[b]) without an autoantibody was performed by [c]χ^2 test.
minor allele frequency[d].

Polish population. Our results were contradictory to the findings of Wang [18], who demonstrated the IL-*7R* C gene variant as a risk factor for SLE in their studied Chinese population. However, in our study we observed a significant association between the IL-*7R* C>T polymorphism and the presence of neurologic manifestations in patients with SLE and the presence of anti-Scl-70 Abs. In contrast, Wang [18] did not observe an association of this SNP with any clinical features of SLE.

The IL-*7R* C gene variant has been demonstrated as a risk factor for multiple sclerosis (MS), type I diabetes (T1D), chronic inflammatory arthropathies and atopic dermatitis [15, 17, 19, 25, 26]. The other SNP, rs10213865, being in complete linkage with *IL-7R* C>T, has been associated with sarcoidosis [27]. The IL-*7R* C>T polymorphism has also been associated with the risk of hematopoietic cell transplantation relapse in patients with hematological malignancies, and with mortality among untreated HIV-infected Zimbabwean individuals [28, 29]. Moreover, other genetic variations in *IL-7R* are implicated in inhalation allergy, Omenn syndrome (MIM 603554), graft-versus host disease, inflammatory bowel disease and primary biliary cirrhosis [30–34].

The role of the *IL-7R* C>T polymorphism in the development of autoimmunity has been evaluated in some studies [15, 16, 35–37]. Gregory [15] demonstrated that this polymorphism is situated inside of the alternatively spliced exon 6 of *IL-7R* and disrupts an exonic splicing silencer, which alters the ratio of soluble and membrane-bound IL-7R isoforms. McKay [35] demonstrated that two *IL-7R* haplotypes having the *IL-7R C>T* SNP contributed to the levels of mRNA encoding the sIL-7R isoforms. McKay [35] also showed that this MS susceptibility haplotype was accompanied by the over-presentation of sIL-7R isoforms in the peripheral blood of patients with primary progressive MS. These findings were confirmed by Lundström [16], who observed that individuals with MS with the IL7R CC genotype displayed an increased level of circulating sIL-7Rα. They also demonstrated that sIL-7Rα potentiates IL-7 bioactivity, contributing to the increased risk of autoimmunity in subjects with a genotype linked to heightened sIL7Rα [16]. The sIL-7Rα levels also correlated with the IL-*7R* C risk allele in patients with T1D [36]. Recently, Kreft [37] demonstrated that sIL-7Rα levels corresponded to the *IL-7R* C risk allele and abnormal IL-7; therefore, the IL-7Rα concentration may influence the responsiveness of IL-7Rα^{+} T cells.

In conclusion, our study suggests that the *IL-7R* T gene variant may protect against neurological manifestations of SLE and the presence of anti-Scl-70 Abs. However, to confirm the role of the *IL-7R* C>T SNP in SLE, similar studies should be conducted with larger samples of different ethnicities.

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Conflict of interest statement
The authors declare no conflict of interest.

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REVIEW PAPER

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The role of inflammation in cardiac arrhythmias pathophysiology

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ABSTRACT

The pathophysiology of cardiac arrhythmias is highly complex. Inflammation causes electrophysiological changes that contribute to increased vulnerability of arrhythmias, a process known as electrical remodeling. There have been many biomarkers and proteins associated with pathophysiology of cardiac arrhythmias such as C-reactive protein, tumor necrosis factor-α, interleukin-2, interleukin-6, interleukin-8, and monocyte chemoattractant protein-1. At present large attention is paid to the clinical studies on the inflammation in the pathogenesis of the atrial fibrillation (AF), given the limited effectiveness of the current therapeutic approaches. Understanding the main process, molecular mechanism and signaling pathways underlying the pathogenesis of the inflammatory dilated cardiomyopathy (iDCM) will be essential for developing new effective therapeutic strategies.

Keywords: inflammation, atrial fibrillation, biomarkers, remodeling.

Introduction

There have been numerous clinical studies supporting the important role of inflammation in the pathophysiology of many cardiovascular diseases including: coronary artery disease, heart failure, hypertension, cardiac arrhythmias. These systemic diseases are associated with low-grade inflammation and increased levels of proinflammatory cytokines [1].

Inflammation plays an important role in pathophysiology of atherosclerosis. It is involved in atherosclerosis from its formation and progression to its final stage, i.e. systemic thrombosis [2]. Inflammation is also involved in the initiation and maintenance of atrial fibrillation as well as its thromboembolic complications [3]. The inflammatory system, comprised of specialized cells and mediators, is responsible for complex responses to all forms of injuries. It stimulates inflammatory reactions by activating leukocytes, macrophages, mast cells, fibroblasts, and endothelial cells. Acute inflammation leads to the tissue repair, while the chronic inflammation can cause damage and pathological remodeling. Nevertheless, these two dimensions of inflammation may coexist with each other.

The immune system, inflammation and oxidative stress are closely related to common pathological responses. Immune factors (e.g. complement) initiate and propagate the inflammatory reactions. If this reaction is not properly controlled, it can exacerbate the chronic inflammation (e.g. granuloma) [4]. Considering the significance of the inflammatory/immune aspects of cardiac diseases, it needs to be emphasized that inflammation might play an important role in the cardiac cells' function, survival and death. Indeed, inflammatory processes lead to structural and electrical remodeling of the myocardium. Hence, the main pathophysiological mechanisms responsible for the formation of arrhythmias in the cardiac myocardial tissue include ectopic beats, triggered activity and reentry mechanisms. Additionally, inflammation alters electrophysiological properties of the myocardium leading to vari-

ous forms of arrhythmias: from benign supraventricular ectopic beats to malignant forms of ventricular tachycardias and ventricular fibrillation [6]. At present large attention is paid to the clinical studies on the inflammation in the pathogenesis of the AF, given the limited effectiveness of the current therapeutic approaches**.** In this paper we elaborated pathophysiological aspects of inflammation process in cardiac arrhythmias, emphasized the role of proinflammatory biomarkers involved in atrial fibrillation.

Pathophysiology

Klein *et al,* have documented the adverse role of inflammation in the pathogenesis of arrhythmias. They described pathological changes in the membrane potential and heterogeneous impulse conduction due to fibrosis and scarring of the myocardial tissue. They also proposed that the formation of cardiac arrhythmias in the inflamed myocardium occurs due to three factors: structural changes, ventricular dynamics and vascular factors. They further remarked that the parameters of ventricular dynamics such as increased wall tension, increased myocardial oxygen consumption and diminished coronary reserve in case of disturbed systolic or diastolic left ventricular function also contribute to the increased incidence of arrhythmias. It was observed that vascular factors can further increase the arrhythmogenicity of the inflamed myocardium through the disturbance of micro- and macrovascular perfusion and the resulting myocardial ischemia. Hence, myocardial biopsy carried out to detect myocarditis is an important additional examination which can improve the differential diagnosis and treatment of patients with cardiac arrhythmias of obscure etiology [7]. Although the inflammatory process of the myocardium may itself be arrhythmogenic, the change in the electrophysiological properties of the myocardium (i.e. electrical remodeling) may also play a role in cardiac arrhythmias [8].

For example, in experimental autoimmune myocarditis (EAM) in rats, demonstrated the changes in the ventricular electrophysiologic properties and in the level of gene expression of cardiac ion channels. During the acute phase of the myocarditis, the ventricular vulnerability was increased and, as a result, the triggered activity was considered to be one of the mechanisms. Also, the ventricular effective refractory period (ERP) and the duration of monophasic action potential (MAPD) were prolonged, showing the "dome-formation" in phase2 of the MAP trace [9].

It has been noted that the results of atrial biopsies taken from patients with AF have demonstrated the inflammatory infiltrates and oxidative damage within the atrial tissue [5]. In the study of Frustaci *et al*. abnormal atrial histology was found in multiple biopsy from patients with lone AF. The histological findings in biopsies taken from the interatrial septum were coincided with a diagnosis of myocarditis in 66% of patients [10]. Presence of circulating autoantibodies against heavy chains of myosin in a the vast majority of patients with idiopathic paroxysmal AF (PAF), raises the possibility of an inflammatory autoimmune processes [11].

AF and inflammatory biomarkers

AF is the most common sustained cardiac arrhythmia in clinical practice [12]. It is associated with an increased risk in mortality and morbidity, and may exert an adverse effect on the quality of life [13]. The pulmonary veins are the key trigger sites initiating AF. So far the exact stimulus for this focal triggering is unknown. Nevertheless, inflammation may provide one explanation. Indeed, inflammation can also act as an ongoing accelerator in the remodeling process [14]. The pathophysiology of AF is highly complex. It is usually associated with organic heart disease but it may also occur without clinically evident abnormalities. The main pathophysiological mechanisms contributing to the AF's development and progression include both electrical and structural remodeling of the atria. Moreover, AF itself can cause progressive electrophysiological changes during atrial remodeling, which perpetuates the arrhythmia—known as 'AF begets AF' phenomenon [15].

There have been many biomarkers associated with pathophysiology of AF such as C-reactive protein (CRP), tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 [16]. Inflammatory biomarkers related to atrial fibrillation are listed in **Table 1**. The vascular markers that have been studied most frequently include high-sensitivity C-reactive protein (hs-CRP) and interleukin (IL)-6.

High sensitivity CRP is an acute-phase protein, factor known initially for its capacity to bind to the c-polysaccharide of *Streptococcus pneumoniae*. It has developed as the most robust and reproducible marker of vascular inflammation. CRP is synthesized primarily by the liver in response to pro-inflammatory cytokines. CRP is involved in the immune response, phagocytosis/opsonisation of the infectious agent and modulates the function of granulocytes and monocytes. Current-

Table 1. Biomarkers of inflammation involved in the pathophysiology of atrial fibrillation [5, 16]

Inflammatory Biomarkers	Targets and Effects
IL-2	Secreted by T cells stimulates growth and differentiation of T cell response. Il-2 is also a predictor of AF after cardioversion and after surgery.
IL-6	Secreted by macrophages and endothelial cells influences adaptive immunity. IL-6 has both proinflammatory and cytoprotective functions. It has been proven that IL-6 is also a predictor of AF after cardioversion and after ablation. Combined with CRP it independently predicts strokes in patients with AF.
IL-8	Chemokine is produced by macrophages and epithelial cells associated with atrial fibrillation.
hs-CRP	Secreted by hepatocytes is a predictor of the development of AF after cardiac surgery and after ablation. Increased levels of CRP have been found in patients with new-onset AF. Longer duration of AF is associated with higher CRP levels and larger LA diameter.
MCP-1	One of the key chemokines that regulate migration and infiltration of monocytes/macrophages. Increased levels of MCP-1 have been found in lone atrial fibrillation
TNF-α	Produced by macrophages is a cytokine involved in systemic inflammation. Increased levels of TNF-α have been found in patients with AF as opposed to individuals with sinus rhythm. In patients with chronic non-valvular AF it has been found that TNF-α is a predictor of ischemic stroke. TNF induces abnormal Ca^{2+} handling and arrhythmogenicity in pulmonary vein cardiomyocytes.
HSP27	HSP-27 can downregulate TNF expression and upregulate IL-10 levels. HSP-27 is a predictor of AF recurrence after catheter ablation.

ly, there is a consistent and significant association, in all populations, between baseline hs-CRP levels and risk of future cardiovascular events like: (i) stroke, (ii) peripheral vascular disease, (iii) sudden cardiac death, (iv) AF, (v) plaque rupture, (vi) recurrent ischaemia, and (vii) myocardial infarction [17]. In patients with AF, inflammatory cytokines IL-6 and CRP were related to the higher risk of vascular death and thromboembolic events independent of clinical risk factors [18]. In another study the circulating biomarkers of inflammation, CRP, and oxidative stress, oxLDL, were higher in lone AF patients compared to healthy individuals and both biomarkers were associated with an increased risk of hypertension in lone AF [19].

The neutrophil/lymphocyte ratio (NLR) has been evaluated as a new predictor of cardiovascular risk, which is an inexpensive, easy to obtain, and widely available marker of inflammation. Additionally, it can be of use as the risk stratification marker in patients with various cardiovascular diseases. It could be used as an alternative to the traditional markers. For example, in one study, elevated pre- and postoperative N/L ratios were associated with an increased occurrence of AF after coronary artery bypass grafting [20]. Mesut Aydin et al. in retrospective study found significantly higher neutrophil/lymphocyte ratio (NLR) values in patients with documented supraventricular tachycardia (SVT) compared with their control group [21].

AF and hypertension

The relation between inflammation and AF in patients with hypertension has not yet been established [5]. So far, in the animal model of hypertension, leucocyte infiltration into the atria as well as inflammation followed by atrial fibrosis have been investigated [22]. It has been observed that the atrial stretch, due to the elevated left ventricular diastolic pressure in patients with hypertension, might activate regional renin–angiotensin–aldosterone system (RAAS), cardiac apoptosis, and oxidative stress, which can subsequently induce the regional inflammation in the heart [23]. Experimental studies have also revealed that angiotensin II possesses several pro-inflammatory properties. Blocking of the RAAS by either ACE-inhibitors or angiotensin II receptor blockers have shown to improve endothelial function and reduce inducibility of AF [24].

Cardiosurgery and ablation

AF remains one of the most frequent complications after coronary artery bypass graft surgery (CABG). Bruins et al. were first to propose the inflammation-AF hypothesis, following their observations of an increased frequency of AF after CABG [25]. After studying the activation of a complement system during and after CABG, they reported the biphasic complement activation. The first phase occurs during cardiopulmonary bypass itself while the second phase occurs during the first 5 days after surgery, which involves CRP elevation associated with atrial arrhythmias. Interestingly, in the study of Ishida et al. which included patients undergoing off-pump CABG surgery, statistic analysis indicated that the highest quartile of IL-6 level immediately after the surgery varied by patients' age and can independently predict postoperative AF [26]. It was observed that nonsteroidal anti-inflammatory medications given in the imme-

diate postoperative period of CABG surgery are relatively safe and effective in reducing the incidence of AF and favorably affect the length of hospitalization. In addition, Marcus et al. have proved that CRP and IL-6 levels were elevated in patients with atrial flutter (AFl). These levels of biomarkers fall significantly after ablation of AFl, which proved that arrhythmia is the cause of the inflammation [27]. Indeed, Richter et. al showed that markers of oxidative stress and inflammation were up-regulated 2 days after ablation of atrial fibrillation and their up-regulation was associated with an early recurrence of AF, but it didn't predict long-term outcome [28].

In the study of Lim the increased levels of inflammatory biomarkers were observed within the first 3 days after radiofrequency (RF) ablation for AF. The level of inflammatory response was directly associated with early AF recurrence. The elevation in the prothrombotic markers, fibrinogen and D-dimer occurred 7 days after ablation and was correlated with the inflammatory response [29].

In another study which compared cryoablation and open-irrigated RF ablation for AFl, the increase in inflammatory markers was significantly greater in the second method. The higher CRP levels during the RF energy might have occurred due to endothelial damage and surface thrombosis. Patients who received cryoablation, had higher levels of myocardial injury (CK, CKMB, TnT) due to cardiomyocyte swelling and irreversible cell death by the rupture of membranes [30].

In the study of Malmborg *et. al* cryoballon cather ablation was also associated with greater myocardial injury than the one performed by RF energy-based pulmonary vein ablation catheter (PVAC). Nevertheles, there was no difference in the coagulation and inflammation activity between these two techniques [31].

AF and electrical remodeling

The key features of electrical remodeling are shortening of the atrial refractory period, the loss of rate adaptation, an increase in dispersion, slowing down of conduction velocity, and the presence of triggering foci. The shortening of the atrial effective refractory period takes place due to accumulation of calcium within atrial myocytes and, as a result, leads to the reduction of the inward L-type Ca^{2+} current, which in turn contributes to the promotion and maintenance of multiple wavelet-reentry circuits. Decreased Na current (I_{Na}), impaired connexin channel function and enhanced fibrosis deposition, by contrast, slow conduction and promote AF-maintaining reentry [32]. Calcium homeostasis in cardiomyocytes is regulated by TNF, PDGF, and IL-2, which are associated with increased triggering and shortening of the action potential duration. Abnormal intracellular Ca^{2+} handling is emerging as a key contributor to AF that promotes both ectopic activity and reentry. Delayed afterdepolarization is initiated by diastolic Ca^{2+} leak from the sarcoplasmic reticulum [5, 32] According to Lee *et. al,* TNF induces abnormal Ca^{2+} handling and arrhythmogenicity in pulmonary vein cardiomyocytes [33]. TNF can also decrease the expression of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a) by enhancing methylation in the promoter region [34]. PDGF-A, which promotes cell proliferation and collagen expression in cardiac fibroblasts, increases atrial fibrosis and susceptibility to AF [35]. In clinical studies, high CRP and IL-6 levels, are associated with increased atrial size, which further contributes to inflammation in atrial remodeling [36]. Indeed, the levels of myeloperoxidase in atrial tissue are higher in patients with AF than in individuals with sinus rhythm. Also, the increased blood myeloperoxidase level has been associated with early AF recurrence after catheter ablation [37].

Ventricular arrhythmias, inflammation and the prevention of sudden cardiac death

Approximately 50% of cardiac arrests occur in case of individuals without a known heart disease but most of them suffer from a concealed ischaemic heart disease. Nevertheless, it is known that inflammatory heart diseases lead to malignant ventricular arrhythmias and sudden cardiac death [38]. In nested, case-control study of apparently healthy male physicians, baseline plasma CRP levels were positively associated with risk of sudden cardiac death (SCD) over the ensuing 17 years of follow-up. The meantime to SCD was 9.2 years (range 0.7 to 16.8 years). One clinical implication of these data is that markers of inflammation may be useful in identifying men at higher risk of SCD who have no obvious signs of coronary heart disease (CHD) many years before the fatal event [39]. In another study of six hundred seventy eight healthy middle-aged and elderly subjects with no apparent heart disease and frequent ventricular premature complexes (VPC), a CRP values $\geqslant$ 2.5 µg/mL were associated with a significantly higher risk of death and acute myocardial infarction [40].

Myocarditis

Myocarditis is the pathological myocardial infection and autoimmunity that causes active inflammatory destruction of myocytes. Aetiologically, a wide spectrum of infectious agents, including viruses, bacteria as well as toxic and hypersensitivity reactions might be involved. Enteroviruses, especially Coxsackie B, adenoviruses and parvovirus B19 are among the most common causal agents. The typical microscopic image involves the presence of inflammatory cells together with necrotic myocytes [41].

Ventricular premature beats are typical forms of cardiac arrhythmias in myocarditis. Nevertheless, they may also include malignant ventricular tachycardia or ventricular fibrillation. Myocarditis can also be a factor responsible for sudden cardiac death in young people [42]. The electrocardiogram (ECG) is a sensitive tool used for diagnosis of myocarditis (in case of abnormal ST – T waves and conduction block are frequently observed in myocarditis). ECG manifestations are diverse, and include atrioventricular block (I to III degree), intraventricular conduction delay (widened QRS complex), frequent premature beats, supraventricular tachycardia, atrial fibrillation, sinus arrest, ventricular tachycardia, ventricular fibrillation, and asystole [43].

Considering malignant arrhythmias associated with myocarditis, two distinct clinical settings have to be distinguished: acute fulminant myocarditis and inflammatory cardiomyopathy. The first is associated with refractory malignant ventricular tachyarrhythmias in the context of severe acute heart failure (HF), and adverse short-term prognosis with early death due to multisystem failure. On the other hand long-term evolution to inflammatory cardiomyopathy with LV dysfunction results in a high risk of sudden cardiac death (SCD) similar to that for dilated cardiomyopathy (DCM). Patients with fulminant myocarditis have a high mortality rate and a severe risk of life-threatening refractory ventricular tachyarrhythmias. The important association between undiagnosed myocarditis and SCD is emphasized by post-mortem data, which have implicated myocarditis in SCD of young adults at rates of 8.6–44% [38].

In fulminant myocarditis most patients have markedly increased leucocytes (WBC) and CRP. Moreover in the study of Aoyama *et al.*, all readmitted patients had abnormally increased WBC and CRP during their follow-up [44]. In another study creatine kinase (CK) and CRP levels declined sharply during the therapy of fulminant myocarditis by intravenous methylprednisolone, which was associated with hemodynamic improvement measured by the improved left ventricule ejection fraction (LVEF) [45].

In the study of Bironaite *et al.* the concentration of IL-6 and hsCRP in the inflammatory dilated cardiomyopathy (iDCM) was increased. Persistent myocardial stressors increase T-lymphocytes in myocardium of iDCM patients. Moreover, chronic myocardial inflammation affected mitochondria and triggered significant release of Hsp-60, MMP-9/TIMP1 suggesting apoptotic pathways [46].

The pathogenesis of cardiac arrhythmias in the myocarditis is complex. The process was explained in the murine model of myocarditis. One of the triggers is the active process of inflammation related to the presence of the virus in the first stage of the disease. This process leads to the activation of immune system, characterized by the invasion of the natural killer cells and macrophages followed by T-lymphocytes. In this stage various cytokines including IL-1β, TNF-α, IFN-γ, and IL-2 are produced. The next process called subacute myocarditis is defined by activated virus-specific T lymphocytes. These cells plays critical role in eliminating infected myocardial cells and limiting viral replication [47]. Pathological changes in the myocardium especially myocardial necrosis and replacement fibrosis (favoring re-entry mechanism) seem to be the major arrhythmogenic substrate. Proarrhythmic effects of the cytokines and inflammatory mediators may be related to the changes in the ion channel function (especially in the potassium and calcium channels). These modifications leads to the prolongation of the ventricular effective refractory period.

Pericarditis

The etiology of pericarditis may be similar to myocarditis with infective (viruses, bacteria) an non-infective (i.e. autoimmunity) agents. Acute pericarditis in isolation does not seem to be frequently associated with ventricular arrhythmia but is often present as perimyocarditis with a burden of arrhythmia related to the myocardial component [43]. Furthermore, SCD in these patients has mostly a haemodynamic and not an arrhythmic cause.

According to Imazio *et al.* C-reactive protein, WBC levels were higher in patients with pericarditis compared with those with myopericarditis/perimyocarditis [48]. In another study hs-CRP levels were elevated at the initial presentation in 78% of patients and com-

prised the independent risk factors for recurrence of pericarditis. The higher CRP levels also may also support the clinical suspicion of pericarditis, but there is also a small percentage of patients with persistently negative hs-CRP which provides evidence that negative hs-CRP does not role out pericarditis [49]. There is also evidence that anti-inflammatory therapy is associated with the reduction in pericardial and systemic inflammation [50]. The levels of CRP in idiopathic recurrent acute pericarditis (IRAP) may be useful in monitoring the disease activity and choosing the appropriate length of therapy.

Conclusion

Inflammation plays an important role in the formation and maintenance of cardiac arrhythmias. Inflammatory biomarkers may prove useful in predicting the onset of cardiac arrhythmias and their complications. Inflammation and oxidative stress directly affect myocyte apoptosis and cardiac fibrosis, thus lead to progressive electrophysiological changes of the myocardium, which consequently contribute to arrhythmias. A better knowledge of the role of inflammation in the pathophysiology of cardiac arrhythmias is necessary to individualize treatment and develop future therapeutic strategies. New therapies might require the specific targeting of inflammatory biomarkers in addition to the modification of other factors to produce beneficial results [5]. Also, further clinical trials are necessary for the development of anti-inflammatory therapies in the prevention of AF. Identifying unique methods to improve imaging, autonomic modulation, inflammatory biomarkers will help us to better understand the patients with cardiac arrhythmias.

List of abbreviations

AF – atrial fibrillation
PAF – paroxysmal atrial fibrillation
CRP – C-reactive protein
IL – interleukin
TNF – Tumor necrosis factor
MCP – Monocyte chemoattractant protein
EAM – experimental autoimmune myocarditis
NLR – neutrophil/lymphocyte ratio
SVT – supraventricular tachycardia
RAAS – renin–angiotensin–aldosterone system
CABG – coronary artery bypass graft
AFl – atrial flutter
RF – radiofrequency
SCD – sudden cardiac death
CHD – coronary heart disease
VPC – ventricular premature complexes
ECG – electrocardiogram
HF – heart failure
DCM – dilated cardiomyopathy
IRAP – idiopathic recurrent acute pericarditis
MAPD – monophasic action potential duration
ERP – effective refractory period

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REVIEW PAPER

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The role of diet and antioxidants in the prevention of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia among elderly. It is a progressive, neurodegenerative disorder of the brain which leads to the deterioration of cognitive, behavioral and impaired daily functioning and causes the gradual loss of independence. A significant portion of risk for dementia in old age is associated with lifestyle. Three important protective factors are diet, which should be rich in antioxidants, exercise and good cardiovascular health. It is believed that Mediterranean diet has a protective effect from dementia. This diet, rich in fruit and vegetables, legumes, olive oil, whole wheat bread, fish and seafood, with reduced consumption of red meat is also protective from cardiovascular diseases and promotes a healthy long life. There were some studies on the etiology of AD which noted an important role of vitamin B6, B12 and folic acid. All of them are involved in the metabolism of homocysteine, which is regarded as an independent risk factor for the development of AD, atherosclerosis and thrombosis. We also know that supplementation of vitamins C and E in the diet can be protective from AD. On the other hand we know that obesity and undernutrition can increase the risk of development of AD. As we can observe the aging of population we should remember that nutrition constitutes an interesting approach for the prevention of age-related brain disorders.

Keywords: Alzheimer's disease, reactive oxygen species, nutrition.

Introduction

Alzheimer's disease (AD) is progressive, neurodegenerative disorder of the brain which leads to the deterioration of cognitive, behavioral and impaired daily functioning and causes the gradual loss of independence. Multi-infarct dementia (vascular dementia), Parkinson's disease, Lewy body disease, Creutzfeldt-Jakob disease, Pick's disease, Huntington's disease are less common forms of dementia among elderly [1]. AD currently affects about 2% of the population in developed countries and its incidence is forecasted to increase significantly with the aging of the population [2]. US Census Bureau data predict that the number of people over 65 years amounting to about 35 million in 2000, will increase to 70 million in 2050, while the population of people over 85 years old will increase from 4.2 million in 2000 to 21 million in 2050 [3]. AD is the leading form of dementia in North America and Europe and it constitutes the significant part of care cost in these countries. It is considered as a global public health priority [4, 5]. Etiology of AD is multi-factorial and it compounds of genetic and environmental factors including diet, physical activity, smoking and alcohol abuse. Barnes and Yaffe suggested that diabetes mellitus, midlife hypertension, midlife obesity, smoking, depression, and cognitive and physical inactivity are the main modifiable risk factors and represent around 50% of all cases of AD [6]. Diabetes, hypertension, and obesity are the civilization diseases where proper diet and physical activity are important elements of prevention and

treatment. Dietary intake and nutritional state appear to be environmental lifestyle- related factors that might contribute to pathogenesis and slowing down AD [7–9]. Genetic factors are important especially in the generally accepted division of Alzheimer's disease into two types: sporadic AD and family AD [10]. Sporadic AD also known as late-onset AD constitutes more than 90% of all cases and occurs in patients aged 65 years old and older. The risk increases having the APOE ε4 allele of apolipoprotein E (APOE) genotype [11]. Family AD is more sporadic form of early onset AD as its symptoms occur in younger age. Genetic variants of the amyloid precursor protein (APP) gene, presenilin 1 (PSEN 1) gene and presenilin 2 (PSEN 2) gene have been suggested to be associated with family AD [12].

Alzheimer Disease and oxidative damage

Amyloid ß (Aß) and tau are two main proteins playing an important role in pathogenesis of AD [13]. Formation of neuritic plaques composed of insoluble form of Aß in the brain and the accumulation of hyperphosphorylated form of tau in neurofibrillary tangles are the most important steps in the pathogenesis of AD [14–16]. Aß accumulation in the hippocampal region of the brain is thought to induce toxic effects, oxidative stress and immune response that lead to cognitive impairment [17]. The hyperphosphorylation of tau protein worsens the axonal transport and causes neuronal dysfunction in the central nervous system. Abnormal neurofibrillary tangles induce inflammatory response and activate cells (microglia, astrocytes, macrophages and lymphocytes) which causes exaggerated production of cytokines and chemokines [18]. Both, Aß and tau protein, induce inflammation in the vulnerable regions of the brain. This is considered to be an important factor in the onset of AD. The inflammatory process leads to neuronal dystrophy, overproduction of reactive oxygen species (ROS) and increased formation of Aß in the cerebral cortex and subcortical regions [19, 20]. ROS are produced in the oxidative process which is the part of physiological reaction of the brain as a part of cellular signalling, metabolism and keeping the homeostasis [21, 22]. The lack of balance between the formation of free radicals and their removal is the cause of oxidative stress. Oxidative stress results in increased oxidation of lipids, proteins and nucleic acids which leads to cellular dysfunction. Chronic oxidative stress along with oxidative damage of the cerebral microvasculature and brain cells have become a potential pathogenesis of many neurodegenerative diseases such as age-related mild cognitive impairment, Parkinson's disease and Alzheimer's disease [23, 24]. Authors of the prospective study made in 2014 emphasize the increased oxidative stress in AD [25]. Exposing human body cells to chronic oxidative stress may exacerbate production of ROS and cause the death of cells including neurons. Amyloid ß can provoke oxidative stress by itself which is thought to be a crucial factor in the pathogenesis of AD [26, 27]. The collection of Aß in the brain causes the dysfunction of mitochondria and metabolic disturbances as well as increased formation of the ROS [28]. Amyloid beta, as a derivative of transferrin, has strong affinity to transition metal ions such as iron, copper and zinc. It is able to reduce Fe3+ and Cu2+ producing ROS [29]. The human body has a mechanism to inactivate and detoxify ROS. The main antioxidants in the nervous system are superoxide dismutase, glutathione peroxidase and catalase. Some data show decreased levels of antioxidants in the plasma of patients with AD. The most important one is glutathione peroxidase which is a selenium-containing enzyme. Its activity in the blood is well correlated to the level of plasma selenium (Se) [30]. Pillai et al. have shown that Se deficiency is associated with cognitive decline, and that selenoproteins may inhibit the neurodegeneration in AD [31]. On the other hand Loef et al. concluded that there is no evidence for the role of Se in the treatment of AD [32]. However it still allows speculation on a potential preventive relevance. In a prospective study Krishnan and Rani noted that the blood Se may not be involved in regulating oxidative stress in AD. A longitudinal study which correlates plasma and cerebrospinal fluid Se and selenoprotein levels should be done [25]. The similar conclusions were reached by authors of systematic review and meta-analysis analyzing plasma nutrients among patients with AD [33]. The meta-analysis has shown significantly lower plasma levels of folate and vitamins A, B12, C and E in a group of AD patients compared to control population. There is a strong belief that vitamin A, vitamin C and vitamin E which are antioxidants may be beneficial in slowing the progression and preventing AD [34–36].

Alzheimer Disease and diet

Dietary intake and nutritional state are important environmental factors that affect the human body regardless of gender, age or comorbidity [37]. Proper nutrition is an important element of lifestyle, which plays

a significant role in the human biological aging process. The quality of the food is also an important part of prevention and treatment of non-communicable, chronic metabolic diseases, the incidence of which increases with age [38]. Among these diseases is AD. They are included into neurodegenerative disorders.

An increasing number of publications emphasize the role of diet and dietary components in the development and treatment of dementia [39–41]. It is well known that nutritional deficiencies and a poorly balanced diet can lead to disturbances in the functioning of the body. Thus, some of the food components can serve a protective function, reducing the risk of developing diseases. Conducted epidemiological studies emphasize the role of the Mediterranean diet in reducing the risk of developing dementia and AD [42–44]. This diet, rich in fruit and vegetables, legumes, olive oil, whole wheat bread, fish and seafood, taking account of reduced consumption of red meat, is the source of all components of food. The importance of the individual components of the Mediterranean diet as a prevention of cognitive impairment is confirmed by numerous independent studies. Berr et al. conducted four-year follow-up and showed that a diet rich in olive oil reduces the risk of cognitive deficits among the elderly [45]. A prospective study in France over seven years and of 1416 people aged at least 68 years, assessed the effect of consumption of fish and meat on the risk of developing dementia [46]. Increased consumption of fish and seafood (at least 1 time per week) was associated with a significantly lower incidence of dementia. The benefits from consumption of fish was confirmed by the study made on a much larger population – 6158 people aged at least 65 years [47]. Hung et al. demonstrated that fatty fish consumption was associated with a lower risk of dementia and AD in people without the APOE ε4 [48]. During long-term follow-up, conducted among Swedish twins, it has been shown that fruit and vegetable intake was associated with a lower risk of developing dementia and AD in women [49]. It has been demonstrated in a study of 193 healthy volunteers (aged 45 to 102 years) that regardless of gender, age, BMI and lipid parameters, people with a higher daily intake of fruit and vegetables performed better cognitive tests [50]. It has been stated that healthy subjects of any age with a high daily intake of fruit and vegetables have higher antioxidant levels, lower levels of biomarkers of oxidative stress, and better cognitive performance than healthy subjects of any age consuming low amounts of fruit and vegetables. Diet composition similar to the Mediterranean diet seems to be the most appropriate and may exert a long-term beneficial effect on the functioning of the brain [51, 52]. This diet is worth recommendation as it lessens cognitive decline, reduces the progression of mild cognitive impairment to AD and the overall risk of developing AD, and decreases mortality among patients with AD. Thus, high consumption of fish, olive oil, vegetables and fruit with a low glycemic index, seeds, beans, moderate consumption of wine and diary products such as cheese or yogurt, and low consumption of red meat and products without additional sugar is recommended. This diet is also recommended to reduce the risk of cardiovascular diseases, obesity, diabetes and hypertension. Therefore it seems to be a good way not only to prevent dementia but also to stay in a better health [53].

The high content of antioxidants in fruit and vegetables is one of the advantages of the Mediterranean diet. Omega-3 acids (α-linolenic acids) and omega-6 acids (linoleic acids) which belong to polyunsaturated fatty acids (PUFA) are equally important. It is also known that PUFA are precursors of leukotrienes, prostaglandins and cell membranes. Vegetable oils (sunflower, soybean, corn, rapeseed) and the acid of the omega-3 family – fish oil, linseed oil and walnuts are the source of linoleic acids in the human diet.

The importance of fish and seafood consumption in the prevention of AD was punctuated by Barberger-Gateau et al. [46] and by the authors of Rotterdam Study [54]. Kalmijn et al. found that a high intake of total fat, saturated fat and cholesterol was associated with an increased risk of dementia. On the other hand they have assessed that the diet rich in fish reduced the risk of AD.

Increased content of omega-3 in a serum and in a diet was reducing the risk of dementia which was noted in few studies [55–57]. Some authors say that there is no significant correlation between PUFA intake and the risk of dementia, including AD [58–60]. Therefore the importance of fats, especially PUFA, in the etiopathogenesis of AD requires further investigation.

Alzheimer Disease and vitamins

Healthy eating and proper diet will ensure proper nutrition regardless of age and provide the body with all the essential nutrients, including micro- and macronutrients. Please note that in the elderly, we often deal with comorbidities, and above all the involuntary changes in the process of digestion and absorption. The unbalanced diet and increased demand for

protein and vitamins in the elderly is caused by the decreased absorption from the digestive tract which causes significantly reduced health [61]. It is commonly known that the brain is susceptible to oxidative stress and damage as a result of its high metabolic rate and relatively lower regenerative capacity. The brain uses approximately 20% of total body oxygen consumption. A number of publications emphasize the role of both, the antioxidant vitamins (E, C, carotenoids including vitamin A) and vitamins involved in the metabolism of homocysteine (vitamin B6, B12 and folic acid). An excessive amount of ROS, in addition to the development of the inflammatory processes, also contributes to faster aging. The neural tissue manifests large oxygen consumption, increased mitochondria density and a high content of polyunsaturated fatty acids in the cell membranes. This causes elevated sensitiveness to harmful action of the free radicals and peroxides [62]. The significant role in the prevention of this damage plays vitamin E (tocopherol). In the 90s a group of scientists from Australia, showed a relationship between the concentration of vitamin E in the serum and cognitive function among people over 60 years of age. Their studies have been conducted to increase the prevention of stroke [63]. Perkins et al. reported a relationship between low serum level of vitamin E and cognitive impairment [64]. In study, which was carried out among 4809 participants showed that there had been no correlation between the level of vitamin C, carotenoids including vitamin A and cognitive function. Also, there was a study conducted in a group of 442 people which showed the reversed result – ascorbic acid, carotenoids, and their plasma concentration only were associated with a better performance of memory [65]. The protective role of the products containing vitamin E in diet was observed in seven-year prospective study made among 815 healthy people over 65 years of age. They found it as a preventing factor against the development of AD [66]. Ortega et al. emphasized the importance of an adequate supply of vitamin E in the diet. They made an analysis of the diet of elderly people for 5 days [67]. The smaller intake of vitamin E in the diet and its lower concentration in the serum was associated with worse outcomes of the cognitive tests. It is punctuated that not only the alpha-tocopherol, but different forms of vitamin E as well, play an important role in the prevention of AD [68]. Vitamin E which comes from dietary supplements has not been shown to reduce Alzheimer's disease risk. Many common supplements provide alpha-tocopherol only. Most of them do not replicate the range of forms of vitamin E that we find in regular food. It has been shown that high intake of alpha-tocopherol reduces serum concentrations of gamma- and delta-tocopherols [69]. It could be the explanation why the higher intake of vitamin E with food was associated with reduced Alzheimer's disease incidence in the study made by Morris et al. [66]. Similarly, in the Rotterdam Study, high vitamin E intake from natural sources was associated with reduced dementia incidence [70]. There was also a prospective study of 4,740 people over 65 years old, that has shown that supplementation of vitamin E and C was associated with a reduction in the incidence of AD [71]. Equally, another study made among 5395 participants, aged over 55, showed that a high intake of vitamin E and C was associated with a lower risk of AD. This association was noticed regardless of the level of education and the presence of APOE ε4 [72]. However, the reports describing the role of other antioxidative vitamins are divergent. In a large prospective cohort study made by Devore at al., intake of vitamin C, carotenoids and flavonoids had no effect on the risk of dementia including AD [70].

Studies on the role of diet in pathogenesis and course of AD underlined an influence of vitamins B6, B12 and folic acid. These vitamins are involved in the metabolism of homocysteine, which is in the human body as an intermediate product formed from exogenous, derived from protein intake methionine on the way to endogenous cysteine. Homocysteine is regarded as an independent risk factor for atherosclerosis and thrombosis. High blood levels of homocysteine (hyperhomocysteinemia) are also a risk factor for the development of cognitive disorders and dementia including AD [73, 74]. Elevated level of homocysteine and decreased level of vitamin B12 in the plasma of patients with AD was demonstrated by Malaguarnera et al. [75]. In a nearly 10 years study conducted among older people without dementia we had observed that folate intake was associated with a lower risk of developing AD [76]. This dependency was not observed for vitamin B12, vitamin C and carotenoids. There was a publication punctuating that serum levels of vitamin B12 and folic acid may be important in preventing AD [77]. In another study of 370 people without senile dementia over the age of 70 it has been observed that low levels of vitamin B12 and folic acid in their blood correlated with an increased risk of developing AD. The level of folic acid below 10 pmol/L and vitamin B12 below 150 pmol/L doubled the risk of dementia. Walker et al. demonstrated that long-term supplementation with folic acid at a daily dose of 400 mg and vitamin B12 at dose of 100 mg, improved cognitive function. It was

made on the basis of two years of observation [78]. On the other hand we have also found reports of large prospective studies with no association between the consumption of vitamin B6, vitamin B12 and folic acid and the risk of AD [79, 80]. We also know that elevated levels of homocysteine are neurotoxic and linked with cardiovascular dysfunction, cognitive decline, increased risk of dementia and brain atrophy [81].

The role of vitamins in the prevention and course of AD is not clear. There is still a need to make further research where several other dependencies should be considered like comorbidities or the way of supplementation of vitamins.

Conclusion

AD is the most common form of dementia and affects about 2% of the population in developed countries. It will still increase with the aging of the population. Therefore, effective prevention and alleviation of symptoms associated with AD is so important. The proceedings should aim to delay the time of development and reduce the prevalence of cognitive impairment which would allow elderly to live longer and independently. Currently available medications may reduce the symptoms of AD and slow down the progression of the disease however these do not lead to a complete cure. It explains why the knowledge of risk factors of AD should become important part of proceedings. We know that diabetes mellitus, midlife hypertension and obesity, smoking, depression and cognitive and physical inactivity are the main modifiable risk factors which represent about 50% of AD cases. Lifestyle, including proper nutrition and proper weight are important factors in prevention of diseases mentioned before. We also know that diet is a part of non-pharmacological prevention of cardiovascular diseases. The Mediterranean diet improves the health and reduces the risk of many diseases including AD. The supplementation of antioxidative vitamins, such a vitamin C and E, seems to reduce the risk of AD. What we should remember is that malnutrition accompanies AD quite often. There has been some studies saying that accelerated loss of weight may precede the diagnosis of AD [82, 83]. The importance of the single components of the diet, like berries, has not been documented well and requires further analysis [84].

Nevertheless the components of diet play an important role in reducing oxidative stress, modulating the immune response, reducing inflammation process and in providing elements for the body construction. Following that we may understand why the diet is so important for proper brain function and that it may be protective from AD. However, there are only few randomized clinical trials that have been designed to test the role of diet in cognitive decline and in dementia including AD. We still need more studies to prepare the multi-nutrient strategy for people with dementia and to unravel the specific influence of each dietary component on cognitive functions. We should be aware of synergistic interactions between different nutrients and we should keep in mind that there is a positive impact of antioxidants on our brain. We should also remember that connecting medications with non- pharmacological treatment like appropriate diet, physical and mental activity may improve the overall functioning of patients with dementia.

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Single nucleotide polymorphism in coronary artery disease as in-stent restenosis risk factor

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ABSTRACT

In-stent restenosis (ISR) is a challenging complication of coronary stent implantation in both stable coronary artery disease (CAD) and acute coronary syndrome (ACS). Recent scientific aproach enables looking closely into its genetic background. This article briefly highlights the already known to increase susceptibility of ISR genetic polymorphisms and summarizes which were not proven to increase the risk of ISR and which may seem protective among CAD patients.

Keywords: polymorphism, genes, in-stent restenosis, coronary artery disease.

Introduction

In-stent restenosis (ISR) is recently challenging complication of coronary stent implantation in both stable coronary artery disease (CAD) and acute coronary syndrome (ACS). Since coronaroplasty is not only done using balloons, the stent era has pushed invasive cardiology further also to the new era of procedures' complications, including drawback due to ISR [1]. Single nucleotide polymorphism (SNP) is a genetic variation leading to change in one specific location causing significant change in coded protein. It leads to over 90% of genetic variation in human species and may vary among different population groups [2]. Linkage between ISR and SNPs may answer the question why in the era of modern cardiology we still need to struggle with repeating invasive procedures. The next question is whether we could isolate these patients and support them with genetic screening and increased number of control visits.

Many factors contribute to CAD, but only some have impact on ISR. SNPs would be a nice marker to examine before we implant stents in stable CAD subject. SNPs of genes coding angiotensin converting enzyme (ACE – rs1799752), angiotensinogen (rs699), basic fibroblast growth factor (bFGF – rs308395) and renin (rs5705) lead to CAD, but not to ISR [3–5].

In CAD and ISR SNPs proven correlations exist for genes coding transforming growth factor beta 1 (TGF-β1 – rs1800470), platelet-derived growth factor beta (PDGFB – rs2285094), vascular endothelial growth factor A (VEGF-A – rs699947) and connexin 37 (CX-37 – rs1764391) [3, 6, 7]. ISR was found also among patients with CAD and genetic variant of interleukin 18 (IL-18 – rs187238) -137G/G which has not been proven for -137G/C and -137C/C variants obtained from non-CAD controls [8]. Also endothelial nitric oxide synthase (eNOS – rs1799983) was suspected to contribute to susceptibility of ISR in CAD patients its 298G/T variant was investigated together with glutation peroxidase (GPx-1 rs1050450) 599C/T SNP and both of them were found to play role in ISR significantly [9]. eNOS CAD patients studied in other project were proven that carriers of the 298Asp allele of the eNOS (rs1799983) polymorphism showed a higher frequency of restenosis compared to 298Glu homozygotes [10]. Also cyclin-dependent kinase inhibitor p27

Table 1. Correlation between SNP and increased ISR prevalence

Gene	Polymorphism rs number	In-stent restenosis	Reference number
Angiotensin converting enzyme	Rs1799752	no	4
Angiotensinogen	Rs699	no	4
Renin	Rs5705	No	5
Basic fibroblast growth factor	Rs308395	No	3
Transforming growth factor beta 1	Rs1800470	yes	3
Platelet-derived growth factor beta	Rs2285094	yes	3
Vascular endothelial growth factor A	Rs699947	yes	3
Connexin-37	rs1764391	yes	7
Interleukin-10	Rs1800872	no	
Interleukin-18	Rs187238	yes	8
Glutation peroxidase	Rs1050450	yes	9
Endothelial nitric oxide synthase	Rs1799983	yes	9, 10
Cyclin-dependent kinase inhibitor p27	Rs36228499	yes	13
Cyclin-dependent kinase inhibitor p27	Rs2066827	no	13
Cyclin-dependent kinase inhibitor p27	Rs34330	no	13
Cyclin B1	Rs350099	Yes	11
Cyclin B1	Rs350104	yes	11
Cyclin B1	Rs164390	yes	11
Toll-like receptor 4	Rs4986790	no	15
Toll-like receptor 4	Rs4986791	no	15
Receptor for advanced glycation end products	Rs1800625	No, but rotective effect	12
CYP2C19*2	Rs4244285	no	14
Epidermal growth factor	rs4444903	no	3

SNP -838C/A (rs36228499) leads to ISR [11]. Cyclin B1 SNPs themselves demostrate higher ISR risk (rs350099, rs350104, and rs164390) [11]. Interesting may seem protective SNP of receptor for advanced glycation end products (RAGE -374T/A – rs1800625) in -374AA lower susceptibility to ISR was found among 276 subjects [12]. All above data are listed in **Table 1** showing which genetic polymorphism is connected to CAD and ISR.

Although some findings may hopefully show that a kind of genetic marker perfect for screening for ISR would appear, other are unlikely to become one of them. Genes investigated for ISR and correlating neither with increased prevalence of CAD nor with ISR include: cyclin-dependent kinase inhibitor p27 (rs2066827 and rs34330), epidermal growth factor (EGF rs4444903) [3, 13]. Another case might be research done in the field of cytochrome P450 which is responsible for clopidogrel – leading antiplatellet drug – metabolism. 2015 findings indicated that CYP2C19*2 (rs4244285) allele in Arabic origin subjects with a functional CYP2C19*1 allele showed no linkage with ISR [14]. Involvement of immune system in case of Toll-like receptor 4 (TLR4 rs4986790 and rs4986791) [15], IL-10 in Chinese Han population (-592 C/A rs1800872) [16] was not supported.

Taking into consideration hormones, blood vessels controlling molecules, immune system particles and oxidative stress-related molecules that may influence process of ISR and require further investigation, also by means of genetic analysis from large cohorts of patients worldwide. Already provided data support the thesis of genetic background of higher susceptibility to CAD itself, some to atherosclerosis, also located in other than coronary vessels, but ISR is still in phase of analysis. According to the data yet obtained, genetics may play important role in development of ISR among CAD patients by means of either hormonal (ACE, angiotensinogen) or blood vessels controlling agents (eNOS, GPx-1, CX-37, cyclin, cellular growth factors). Thesis of contribution of immune system, supported by logical assumption about inflammatory background of atherosclerosis may not be totally correct since IL-18 genetic polymorphism was solely the only one supported by literature. Neither TLR4, nor IL-10 did not match this hypothesis in large investigated groups compared to patients without ISR. Some referential data exclude one another, but looking closely – investigator take into consideration genetically different populations, i.e. Caucasian and Asian groups should not be considered the same in the fields of genetic analysis. Different

populations although might be (and should be) investigated for finding the same SNPs increasing susceptibility to ISR, need to be also checked for their genetic potential to cause CAD first and, which seems crucial yet omitted in plenty of articles – compared to presence of the same SNP among non-CAD patients, not only investigating CAD subjects group. Another issue worth investigation might be differentiation between ISR, increased by genetic susceptibility and stent type. Important information include: do genes play role in ISR development in different types of stents, does the drug covering drug eluting stents matter among patients with higher presence of particular polymorphisms or does type of stent in comparison with higher susceptibility of ISR genetically proved leads to poorer prognosis. That kind of data would be useful as predictive value for ISR and prognosis among CAD patients, furthermore would lead to individualized therapy among patients subjected to coronaroplasty.

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THOUSAND WORDS ABOUT...

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Thousand words about cervical cancer and epigenetics

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ABSTRACT

Epigenetic modifications include DNA methylation, DNA demethylation along with the major role fulfilled by TET protein. Epigenetic modifications refer to the regulation of gene expression without the alteration of the DNA sequence. Some of the most common epigenetic modifications include DNA methylation and demethylation, as well as the functional role of TET proteins. Epigenetic alterations are heritable traits, therefore one of the key elements to understanding the mechanisms of cancer development is to further our knowledge on the role and function of epigenetic modifications.

This mini-review takes into consideration the overview of the literature on the impact of epigenetic changes in cancer development, especially in the development of CC. Researchers believe that certain compounds are capable of inhibiting the process of DNA methylation and may play an important role in future cancer therapy.

Keywords: cervical cancer, epigenetic, DNA methylation, TET.

Introduction

Cervical cancer (CC) is one of the most common malignancies among women worldwide. An important diagnostic method of CC is the Papanicolau test, also called 'pap test'. The test consists of collecting a sample of cells from the cervix and assessing them for cytopathological changes, to diagnose the stage of the disease [1]. Despite the low cost of research, low risk of complications, high availability and low invasiveness of this diagnostic method, morbidity and diagnosis of CC is still high. Each year, there is about 500,000 new cases of CC among women and 250,000 deaths [2, 3]. This assessment takes into account exposure to risk factors such as age, early onset of coitus, number of pregnancies and deliveries [1]. There are also many studies linking cigarette smoking and the use of oral contraceptives to the development of CC [1, 4].

Progressively more studies have been focusing on epigenetics and its regulatory mechanism in the development of cancer [5], as well as leukemia [6] and CC [7]. The understanding of epigenetic regulation in normal and cancer cells has been rapidly growing since the beginning of the new millennium. This progression of knowledge has been mainly due to new technological developments and has opened the door to new opportunities such as the development of epigenetic therapies [5].

DNA methylation and demethylation

Epigenetic modifications, which include DNA methylation and histone modifications are included in the regulation of gene expression. Gene expression disorders which may occur in any population, might contribute to the emergence and progression of cancer [8]. Although infection with human papillomavirus (HPV) is obligatory, it is not the only factor contributing to the full malignant transformation. Other factors such as epigenetic modifications, might be risk factors for the devel-

opment of CC [2, 8]. It is still unclear how many different factors are linked to the pathogenesis of CC [6].

The pattern of DNA methylation is determined in the early stages of embryonic development and maintained during the whole life of the DNA methyltransferases [9]. The normal genome is not subjected to CpG island methylation, however, researchers have observed excessive methylation of CpG islands and global hypomethylation in tumorigenic transformed cells [10]. During tumor formation, epigenotype cells are significantly altered due to changes in DNA methylation. The possible changes in DNA methylation include hypermethylation of CpG islands, hypomethylation of genes normally methylated, transposition in cancer cells and induction of chromosome instability [11].

Role of hypomethylation in cancer

Hypomethylation is the overexpression of oncogenes due to the demethylation of promoter regions, causing excessive stimulation of cellular proliferation [12]. Hypomethylation may lead to alterations in gene expression, which can cause genomic instability [5]. The genes altered by hypomethylation are usually regulating growth, which is an important factor for the development of the organism or encoding enzymes [13]. Hypomethylation is frequently observed in solid tumors such as colorectal cancer or gastric cancer [12, 13].

Role of hypermethylation in cancer

Research studies have shown that hypermethylation of CpG islands is associated with transcriptional silencing of tumor suppressor genes and DNA repair genes [10]. Inhibition of the expression of these genes causes the cell to be deprived of the normal cell cycle, which leads to cell proliferation and tumor growth [12]. Some of the genes in which methylation is observed include: BRCA1, p16, hMLH1, GSTP1 or APC [12, 13].

Role of TET proteins

The family of TET (ten-eleven translocation, TET1, TET2 and TET3) proteins play an important role in the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylocytosine (5-hmC) [7]. This family of enzymes has significant biological functions in embryonic stem cells and plays an important role in development, aging and disease [14]. It has been observed that a reduction in the concentration of 5-hmC is strongly correlated with the development of cancer. In many studies, researchers have observed the loss of TET1 expression in colorectal cancer, gastric cancer and/or in CC [7, 8, 14–16].

Epigenetic "risk factors" and cervical cancer

In a study conducted by Yin *et. all* (2016), STK31 hypomethylation was observed in the HPV16/18-positive HeLa, SiHa and Caski cell lines. In contrast, HPV-negative cell lines exhibited hypermethylation and silenced expression [17]. Li *et. all* (2015) discovered that RASSF1A promoter hypermethylation increased the risk of CC. These studies provide proof for a possible correlation between HPV infection and RASSF1A promoter methylation in the development of CC [18]. Blanco-Luquin *et. all* (2015) observed significantly longer disease-free survival and overall survival periods in adenocarcinoma (of the uterine cervix) patients with RASSF1A hypermethylation. Researchers suggest that the involvement of DNA hypermethylation in CC varies depending on the histological type and prognosis factors [19], similarly to TET2 in colorectal cancer [15].

A study by Narayan *et. all* (2004) showed the involvement of BRCA1 gene by promoter hypermethylation or down-regulated expression in CC. Researchers also observed important inactivation of genes in the FA-BRCA pathway by epigenetic alterations. This suggests that epigenetic modifications play a major role in this pathway, and therefore in the development of CC [20]. Jha *et. all* (2012) demonstrated significant hypermethylation of p73 and p53 genes by CC patients. They also observed an important correlation between tested genes with some risk factors parameters of CC [21].

Bronowicka-Kłys *et. all* (2016) showed the important reduction of TET1 transcripts in tumoral tissues compared to histopathologically unchanged tissues of CC. Simultaneously, they observed the connection between TET1, TET2 and TET3 transcripts with various clinicopathological data [7]. Similar results were observed in other studies, where a reduction of TET family proteins was demonstrated in colorectal cancer or radically lower levels of TET1 transcripts and proteins were observed in gastric cancer [15, 16].

Conclusion

Advances in genetic and epigenetic research have provided us with appropriate knowledge regarding the development of cancer. Many studies have shown that the development of cancer such as CC, can be linked to genetic and epigenetic factors. It is believed that epigenetic modifications may have an effect on the course of a disease as well as the treatment. There is still a growing interest on this topic, and a number of stud-

ies suggest that understanding the role of epigenetic mechanisms may become a key element in developing cancer therapy and prognosis factors in the future.

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Maturation, pharmacogenomics and metabolomics as factors determining pharmacokinetic and pharmacodynamics profile of alpha-agonist in pediatric intensive care unit patients

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ABSTRACT

Research Project Objectives. Project entitled "Maturation, pharmacogenomics, and metabolomics as factors determining pharmacokinetic and pharmacodynamic profile of alpha-agonist in pediatric intensive care unit patients" was founded by the Polish National Science Center (NCN) under project number: 2015/17/B/NZ7/03032. The duration of the grant is 36 months, and the total grant value is 688800 PLN. The project is run by the Medical University of Gdansk and Poznan University of Medical Sciences. The aim of this grant is to examine the influence of maturation, pharmacogenetics, metabolomics and physiological (or pathophysiological) status of the patients on the pharmacokinetics and pharmacodynamics (PK/PD) of α2-adrenergic drugs (dexmedetomidine and clonidine) in pediatric population. The project was proposed to explain the unusual PK of dexmedetomidine reported in literature and in our preliminary experiments, in which a two-fold increase in dexmedetomidine clearance was observed during prolonged (lasting more than 24 hr) infusions in the intensive care unit patients.
General information. Project entitled "Maturation, pharmacogenomics, and metabolomics as factors determining pharmacokinetic and pharmacodynamics profile of alpha-agonist in pediatric intensive care unit patients" was founded by the Polish National Science Center (NCN) under project number: 2015/17/B/NZ7/03032. The duration of the grant is 36 months, from 2016-04-27 to 2019-04-26 and the total grant value is 688800 PLN. The project is run by the Medical University of Gdansk and Poznan University of Medical Sciences. The research group consists of: principal investigator dr hab. Paweł Wiczling and co-investigators: dr hab. Agnieszka Bienert, dr Alicja Bartkowska-Śniatkowska, dr Joanna Bartkowiak-Wieczorek, mgr Justyna Mocarska, prof. Edmund Grześkowiak, dr Jan Matysiak, mgr Agnieszka Klupczyńska, dr Danuta Siluk, mgr Agnieszka Borsuk and prof. dr hab. Zenon J. Kokot. The Ethical Committee permission number is 261/15.

Keywords: pharmacogenomics, metabolomics, pharmacokinetic, pharmacodynamics, dexmedetomidine, clonidine.

Research plan

The project will be implemented in three steps:

1. The first research task will include collection of the required experimental data from the patients: arterial blood samples, the values of two sedation scores (Ramsay scale and Glasgow Coma Scale modified by Cook and Palma (GSCS) scale), blood pressure, heart rate, body temperature, cardiac

output, blood oxygen saturation, biochemical and demographic parameters. Pediatric risk of mortality (PRISM), as well as Pediatric Multiple Organ Dysfunction Score (P-MODS) will be used to characterize the health status.

2. The second research task will cover: 1) development of sensitive and reliable analytical methods (LC-MS/MS) applicable for quantitative determination of clonidine and dexmedetomidine; 2) untargeted (exploratory) and targeted metabolomics analysis. The exploratory phase will focus on the analysis of the whole metabolic fingerprints in plasma samples without distinction of the particular compounds or group of analytes. It will be followed by the targeted approach which will be focused on dexmedetomidine and clonidine metabolites and other compounds indentified during the exploratory phase; and 3) the pharmacogenomic analysis to determine the frequency of particular gene polymorphisms such as ADRA2B, ADRA2A, UGT, CYP2A6 affecting the pharmacokinetic and pharmacodynamic processes of the studied drugs.
3. The third research task will involve the development of a population (hierarchical) PK/PD model to describe the drug concentrations and effects (Ramsay scale and Glasgow Coma Scale) time-course, to quantitate inter-individual variability and to find factors significantly affecting the PK/PD profiles of the studied drugs through the covariance analysis.

Basic Concept

Individualization of pharmacotherapy plays a crucial role in the treatment, especially among PICU patients. In that population the differences in drug response might be associated with various demographic or physiological parameters, i.e. age, metabolizing enzyme activity and genetic variability of enzymatic pathways [8]. The main objective of our study is to elucidate the mechanism responsible for the time-dependent increase in dexmedetomidine clearance observed in PICU patients during infusion longer than 24 hr. We hypothesized that this effect depends on the genetic variability of metabolizing enzymes and α2-adrenergic drugs receptors. However, another explanation are also possible, i.e. through changes in patients' cardiac output during the long-term infusion. The patients' health status can change during hospitalization and as a result the drug clearance might change for high/moderate hepatic extraction ratio drugs, such as dexmedetomidine. In order to verify these hypotheses we are going to analyze the influence of maturation (age), pharmacogenetics (genetic variability), metabolomics (difference in metabolic profiles), and physiological (or pathophysiological) status of the patients on the pharmacokinetics and pharmacodynamics (PK/PD) of two α2-adrenergic drugs using the state-of-the-art methodology (nonlinear mixed-effect modeling).

Research Methodology

Patients will be included in the study based on the pre-specified inclusion and exclusion criteria. Dexmedetomidine and clonidine will be administered as intravenous infusion through microbore tubing into a central catheter with the initial dosing of 0.8 mcg/kg b.w./hr and 1 mcg/kg b.w./hr, respectively. Drugs will be delivered based on the level of sedation up to a maximum rate of 1.4 mcg/kg b.w./hr for dexmedetomidine and 2 mcg/kg b.w./hr for clonidine.

The sedation monitoring will be provided by the use of the Glasgow Coma Scale modified by Cook and Palma (GSCS) scale, Comfort Scale and the Ramsay score. The GSCS and Comfort Scale have previously been validated as a sedation scale for mechanically ventilated patients [9]. Arterial blood samples (2 ml) will be obtained before administration of the loading dose, during the infusion of dexmedetomidine or clonidine, and after infusion cessation.

Experimental design

The optimization and simulations methods will be used to evaluate the experimental design. Population study (i.e. the total number of subjects to be studied, number of groups and number of individuals per group, dosing and sampling schedules) will be established before performing a prospective analysis with a parametric method (such as NONMEM) in order to ensure the best precision of the population parameter estimates. The population studies design will be based on a free software programs as POPed version 2.13 (http://poped.sourceforge.net/index.php).

Dexmedetomidine and clonidine quantification

Extraction of the studied compounds from plasma samples will be performed with the use of solid-phase extraction technique. Thawed samples at a volume of 200–300 µl will be used. SPE Plexa cartridges (30 mg, 1 ml, Agilent Technologies, Palo Alto, CA, USA) will be employed in order to extract the analytes. The solvent mixtures composition and their volumes, necessary for efficient SPE processing, will be determined

during the final method optimization. Extracted samples will be evaporated to dryness at a miVac Quattro Sample Concentrator (Genevac, Suffolk, UK), reconstituted with 100 µl of the mobile phase, and injected into the chromatographic system. Analyses will be performed using an HPLC system (Agilent Technologies). The mobile phase, pumped at a flow rate of ~0.3 ml/min, will be composed of a mixture of methanol and water with 0.1 formic acid. The optimal temperature for the separation (column compartment temperature) will be tested while the autosampler will be thermostated at 4°C.

Metabolomics

The plasma samples will be analyzed with high performance liquid chromatography coupled with time of flight mass spectrometry (HPLC-TOF-MS, Agilent Technologies), gas chromatography hyphenated to triple quadrupole mass spectrometry (GC-MS/MS, Shimadzu) as well as capillary electrophoresis coupled with the time-of-flight mass spectrometry (CE-TOF-MS, Agilent Technologies). To extract the information on putative biomarkers, the data sets will subjected to for chemometric preprocessing methods, such as peak alignment, filtering and normalization [4, 5]. Subsequently, the normalized data will be analyzed using multivariate statistical analysis (multilevel regression models) in order to indentify the metabolites of studied drugs and endogenous compounds with unusual (in comparison to the mean behavior) time-courses. The preprocessing methods will be performed with the use of Mass Profiler Professional Software (Agilent Technologies). The regression will be performed using MATLAB 9.1 (The MathWorks, Inc., U.S.A.) [5, 6]. For the identification of unknown metabolites, the scientific databases will be used.

The second analytical approach in this part will be a targeted metabolomic analysis. The analytical methods will use a triple quadrupole mass spectrometry detection coupled with high performance liquid chromatography technique (HPLC-MS/MS), capillary electrophoresis (CE-MS/MS) and gas chromatography (GC-MS/MS). In targeted analysis we will use multiple reaction monitoring mode (MRM mode) to determine the known compounds (dexmedetomidine and clonidine metabolites). The obtained data sets will be analyzed using multilevel regression modeling.

Pharmacogenomics

The presence of gene polymorphism in ADRA2B, ADRA2A, UGT, CYP2A6 will be established. Genomic DNA will be isolated from whole blood using "QiAamp DNA Blood Mini Kit" (Qiagen). Genotyping will be performed by real-time PCR method using probes or PCR-RFLP method (polymerase chain reaction and restriction analysis). Reactions of real-time PCR will performed with the use of LightCycler®480.

Pharmacokinetics and pharmacodynamics

The concentrations of dexmedetomidine and clonidine and clinical endpoints will be described by a population pharmacokinetic and pharmacodynamic model [7]. Population nonlinear mixed-effect modeling will be done using NONMEM (Version 7.2.0, Icon Development Solutions, Ellicott City, MD, USA) and the gfortran compiler 9.0. NONMEM runs will be executed using Wings for NONMEM (WFN720, http://wfn.sourceforge.net). The first-order conditional estimation with interaction (FOCE) method will be used. The self-written differential equations will be used as model equations. The NONMEM data processing and plots will be done in Matlab® Software version 9.1 (The MathWorks, Inc., Natick, MA, USA). The minimum value of the NONMEM objective function, typical goodness-of-fit diagnostic plots, and the evaluation of the precision of PK/PD parameter and variability estimates will be used to discriminate between various models during the model-building process. The hypothesis testing (i.e. covariance analysis) will be based on a minimum value of objective function (MOF). The difference in MOF of 10.8 for one degree of freedom and 13.8 for two degrees of freedom between two hierarchical models will be considered statistically significant at $p < 0.001$. The visual predictive checks will be performed to asses each model predictive performance. The uncertainty of all PK/PD parameters will be obtained from the non-parametric bootstrap method.

Measurable Effects

To our knowledge it is the first such a comprehensive study in the field of PK/PD modeling in patients from the pediatric intensive care unit. In our opinion the findings of this project may extend the fundamental knowledge of pharmacokinetics and pharmacodynamics of α2-adrenergic drugs and quantitate the pharmacokinetic and pharmacodynamic differences among patients from special population, i.e. children in intensive care units. This may be further used to improve the PK/PD models and the drug dosing regiments.

Expected Results

The results obtained in this project will allow for a thorough examination of the dynamics of CYP2D6 and CYP2A6 enzymes maturation and its effect on the clonidine and dexmedetomidine metabolism. In this are the number of reported studies is limited and the current literature results do not clearly address the questions concerning the degree of variability of metabolism of both drugs. Therefore, it seems to be important to search for association between polymorphic variation and the level of mRNA expression of selected metabolic enzymes for clonidine and dexmedetomidine and clinical parameters, especially in children and newborns. In our opinion it is also worth to study the metabolomic profile of the patient as the metabolites concentration should also manifest clinically relevant inter-individual and intra-individual differences. The results of the project will provide a rationale for the dose adjustments and might increase the safety of sedation in children at different ages and under different pathophysiological conditions.

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Conflict of interest statement
The authors declare no conflict of interest.

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