Poznan University of Medical Sciences
Poland

JMS Journal of Medical Science

previously Nowiny Lekarskie

Founded in 1889

2019
Vol. 88, No. 4

QUARTERLY

Indexed in:
Polish Medical Bibliography, Index Copernicus,
Ministry of Science and Higher Education, Ebsco, Google Scholar

eISSN 2353-9801
ISSN 2353-9798

www.jms.ump.edu.pl

SECRETARIAT ADDRESS
27/33 Szpitalna Street
60-572 Poznań, Poland
phone: +48 618491432, fax: +48 618472685
email: jms@ump.edu.pl
www.jms.ump.edu.pl

DISTRIBUTION AND SUBSCRIPTIONS
70 Bukowska Street, 60-812 Poznań, Poland
phone/fax: +48 618547414
email: sprzedazwydawnictw@ump.edu.pl

PUBLISHER
Poznan University of Medical Sciences

eISSN 2353-9801
ISSN 2353-9798

Publishing Manager: Grażyna Dromirecka
Technical Editor: Bartłomiej Wąsiel

WYDAWNICTWO NAUKOWE
UNIWERSYTETU MEDYCZNEGO
IM. KAROLA MARCINKOWSKIEGO
W POZNANIU

60-812 Poznań, ul. Bukowska 70
tel./fax: +48 61 854 71 51
www.wydawnictwo.ump.edu.pl

Ark. wyd. 7,2. Ark. druk. 9,3.
Zam. nr 104/20.

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*.

Ethical guidelines

The Journal of Medical Science applies the ethical principles and procedures recommended by COPE (Committee on Conduct Ethics), contained in the Code of Conduct and Best Practice Guidelines for Journal Editors, Peer Reviewers and Authors available on the COPE website: https://publicationethics.org/resources/guidelines

CONTENTS

ORIGINAL PAPER

DOI: https://doi.org/10.20883/medical.388

Association of *ABCB4* and *ABCB11* nucleotide variants with intrahepatic cholestasis of pregnancy

Milena Gruszczyńska-Losy[1, a], Adrianna Mostowska[2, b], Łukasz Adamczak[3, c], Paweł P. Jagodziński[2, d], Ewa Wender-Ożegowska[3, e], Małgorzata Kędzia[3, f, *]

[1] Gynecologic and Obstetrical University Hospital in Poznan, Poland
[2] Department of Biochemistry and Molecular Biology, Poznan University of Medical Sciences, Poland
[3] Division of Reproduction, Department of Obstetrics, Gynecology and Gynecological Oncology, Poznan University of Medical Sciences, Poland

* *Corresponding Autor:* Małgorzata Kędzia, Division of Reproduction, Department of Obstetrics, Gynecology and Gynecological Oncology, Poznan University of Medical Sciences, 33 Polna Street, 60-535 Poznań, Poland, phone: +48601700022, fax: +48618419625, email: mal.gin@poczta.fm

[a] https://orcid.org/0000-0003-2630-2026
[b] https://orcid.org/0000-0003-4181-9402
[c] https://orcid.org/0000-0002-2511-1407
[d] https://orcid.org/0000-0002-9046-6802
[e] https://orcid.org/0000-0002-5492-8651
[f] https://orcid.org/0000-0002-8115-2019

ABSTRACT

Introduction. Intrahepatic cholestasis of pregnancy (ICP) is the most common liver disorder during gestation. The exact pathogenesis of ICP is multifactorial and still unclear. Therefore, our study aimed to check whether the selected *ABCB4* and *ABCB11* nucleotide variants are associated with an increased risk of ICP.
Material and Methods. ICP was diagnosed based on clinical symptoms characteristic of this disease, and confirmed by an increase in serum bile acids and transaminases, spontaneous resolution of clinical symptoms, and normalisation of laboratory tests after delivery. A total of 86 pregnant women meeting the criteria were included in the study. Healthy pregnant women with uncomplicated pregnancy served as a control group (n = 310). Six common nucleotide variants in the *ABCB11* and *ABCB4* genes were genotyped with the use of high-resolution melting curve analysis.
Results. All tested nucleotide variants did not show significant deviation from the Hardy Weinberg equilibrium in both ICP patients and healthy women. None of the *ABCB4* and *ABCB11* variants were significantly correlated with the risk of ICP ($p_{trend} > 0.05$). Similar results were also obtained after the division of patients based on the TBA levels. However, in the group of patients with moderate and severe ICP, a trend toward association between the *ABCB4* rs2109505 variant and cholestasis was observed ($p_{trend} = 0.063$; $OR_{allelic} = 1.87$, 95% CI: 0.92 — 3.80; $OR_{dominant} = 1.90$, 95% CI: 0.83 — 4.36 and $OR_{recessive} = 12.24$, 95% CI: 0.74 — 201.75).
Conclusions. Our study did not show any significant association of the analysed *ABCB4* and *ABCB11* nucleotide variants with an increased risk of intrahepatic cholestasis of pregnancy.

Keywords: Intrahepatic Cholestasis of Pregnancy, *ABCB4, ABCB11*, nucleotide variants.

Introduction

Intrahepatic cholestasis of pregnancy (ICP) is the most common, but short-lived, liver-specific pregnancy disorder. The incidence of ICP in the Caucasian population varies between 0.5–1.5% [1]. This illness usually occurs in the second and third trimester of pregnancy and resolves shortly after partum. Although it may have a very early-onset, as early as nine weeks of gestation, it

can persist for several months after delivery [2]. ICP is very oppressive for the mother because of pruritus, which intensifies at night, but is generally a benign disease. However, from the perspective of foetal complications, there is a correlation between high serum bile acids levels, and an increased risk of an abnormal obstetric outcome connected with an elevated risk for the foetus and newborn [3]. Kawakita et al. [4], based on total bile acid (TBA) levels in maternal serum, distinguished three ranges in the course of cholestasis: mild, with TBA 10–39.9 μmol/L moderate with TBA 40–99.9 μmol/L, and severe with TBA ≥ 100 μmol/L. The authors detected a significant association between severe ICP and adverse outcomes, with increased risk of stillbirth [3, 4].

The exact pathogenesis of ICP is multifactorial and still unclear. Pregnant women with ICP have a deficiency in the excretion of bile salts to bile, which causes an increase in serum bile acids.

Intrahepatic cholestasis of pregnancy is significantly more common in the same families. The relative risk for an affected first-degree relative is 12% [5]. The risk of recurrences in the next pregnancy reaches 45% [6]. In addition, there is an increase in the frequency of ICP in geographical regions and specified ethnic groups [7, 8]. However, the genetic basis of ICP indicates familial clustering and endemic occurrences.

The genetic basis of bile transport disorders across canalicular membranes was based on rarely occurring familial syndromes, including progressive familial intrahepatic cholestasis (PFIC), and benign recurrent intrahepatic cholestasis (BRIC) [9]. These diseases result from the functional deficiency of canalicular ATP-binding cassette (ABC) transporters. In recent years, research on the contribution of genetic factors involved in bile transport disorders were also performed in pregnant women with cholestasis [10, 11].

The most extensively studied candidate gene in intrahepatic cholestasis in pregnancy is *ABCB4* (OMIM *171060). The human *ABCB4* gene is located on the 7q21 chromosome. This gene encodes phosphatidylcholine floppase, an ATPase also known as multidrug resistance protein 3 (MRP3). This protein belongs to the super-family of transporter proteins possessing ATP-binding cassette. A reduction of phosphatidylcholine in the bile causes an escalation of nonmicellar toxic bile acid.

The subsequent gene examined in intrahepatic cholestasis is *ABCB11* (OMIM *603201). This gene is located on chromosome 2q24. The product of *ABCB11* is an ABC transporter named bile salt export pump (BSEP). It actively transports conjugated bile salts into biliary canaliculi against a concentration gradient. Defective function of BSEP results in abnormal bile salt excretion to bile, leading to cholestasis [2 ,11]. Additionally, biliary transporter gene mutations were also detected in severe intrahepatic cholestasis of pregnancy, which is in the main spectrum of interest due to the consequences for the foetus [13].

Therefore, the aim of our study was to check whether the selected *ABCB4* and *ABCB11* nucleotide variants are associated with an increased risk of ICP. In addition, we decided to examine whether their association with the risk of ICP may depend on the severity of this disease.

Material and Methods

Patients and controls

Peripheral blood samples from women with intrahepatic cholestasis in pregnancy, and healthy pregnant control subjects with uncomplicated pregnancy were collected at the Gynaecologic and Obstetrical University Hospital, Division of Reproduction at the Poznan University of Medical Sciences.

ICP was diagnosed based on clinical symptoms: pruritus in the absence of any dermatologic or other systemic medical condition causing pruritus. Confirmation of the diagnosis was made with a rise in serum bile acids (> 10 μmol/L) and transaminases (> 31 U/L), and spontaneous resolution of clinical symptoms and normalisation of laboratory tests after delivery. The exclusion criteria were: viral or autoimmune hepatobiliary disease or extrahepatic biliary obstruction. A total of 86 pregnant women meeting the criteria were included in the study. In this group, there were 67 women with single pregnancy and 19 patients with multiple pregnancies (16 twins and three triplets). The women with ICP were divided into 2 groups (n = 60 and n = 26) according to their TBA level (10–39.9 and ≥ 40.0 μmol/L, respectively). The control subjects were healthy, lean (BMI < 25 kg/m^2) pregnant women with uncomplicated pregnancy (n = 310).

Written informed consent was obtained from all participating individuals. The study procedure was approved by the Local Ethical Committees of Poznan University of Medical Sciences, and was performed in accordance with the code of ethics of the Declaration of Helsinki.

SNP selection and genotyping

Single nucleotide polymorphisms (SNPs) in the *ABCB4* and *ABCB11* genes were identified from the relevant literature and public databases, including the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and the 1000 Genomes Browser (http://browser.1000genomes.org/index.html). SNP selection was based on their functional significance, association with the risk of ICP in previous studies, and minor allele frequencies (MAF, ≥ 5% in the Caucasian population from the 1000 Genomes Project). The characteristics of the SNPs selected for analysis (n = 6) are presented in **Table 1**. Genomic DNA was isolated from peripheral blood lymphocytes with the use of a DNA extraction kit (Blirt-DNA Gdansk, Gdansk, Poland). Genotyping was carried out by high-resolution melting curve analysis (HRM) on a LightCycler 96 system (Roche Diagnostics, Mannheim, Germany) with the use of 5x HOT FIREPol EvaGreen HRM Mix (Solis BioDyne, Tartu, Estonia). Quality control was ensured by including 10% of the samples as duplicates. Samples that failed genotyping were removed from the statistical calculations. The primer sequences and HRM conditions are presented in Supplementary **Table 1**.

Statistical analysis

Each SNP was tested for deviation from the Hardy-Weinberg equilibrium (HWE) in both the patients and controls using the chi-square (χ^2) test. The association of the *ABCB4* and *ABCB11* SNPs with ICP was tested with the Cochran-Armitage trend test. Odds Ratios (ORs) with 95% Confidence Intervals (95% CIs) were used to assess the strength of the association. The allelic, dominant, and recessive models were analysed. The Bonferroni correction was applied to account for multiple testing, and p-values < 0.0083 (0.05 / 6 SNPs) were considered to be statistically significant. The pair-wise linkage disequilibrium (LD) between the tested SNPs (D' and r^2 statistics) was evaluated using the Haploview 4.2 software package (www.broadinstitute.org/haploview/haploview). The same software was used to conduct a haplotype-based association analysis (sliding window approach). Statistical significance was assessed using the 1,000-fold permutation test. All statistical calculations were performed for the whole sample, and after division of the patients based on the TBA levels. In addition, separate association testing was performed after the exclusion of cases with multiple pregnancies.

Table 1. Characteristics of the *ABCB11* and *ABCB4* nucleotide variants

Gene	rs no.	Location (bp)[a]	Consequence type	Alleles[b]	MAF[c]
ABCB11 2q31.1	**rs2287622**	chr2:168973818	missense (p.Val444Ala)	C / T	0.33
	rs3815676	chr2:169013869	intronic	A / G	0.05
	rs7577650	chr2:169034700	upstream	A / G	0.28
ABCB4 7q21.12	**rs4148826**	chr7:87445103	intronic	A / G	0.17
	rs2109505	chr7:87450090	synonymous (p.Ile237Ile)	A / T	0.17
	rs2302386	chr7:87462628	intronic	A / G	0.13

[a] GRCh38 / hg38.
[b] Underline denotes the minor allele.
[c] MAF — minor allele frequency based on 1000 Genomes genotype data (CEU sample).

Results

All tested SNPs did not show significant deviation from HWE in both ICP patients and healthy women (p > 0.05). In the controls, the MAF for the analysed variants was between 2 and 42% (**Table 2**). In the tested sample, the *ABCB4* gene variants are moderated LD (average r^2 = 0.65 and D' = 0.92; **Table 3**), while the *ABCB11* SNPs are in weak LD (average r^2 = 0.05 and D' = 0.34; **Table 4**). None of the *ABCB4* and *ABCB11* SNPs were significantly correlated with the risk of ICP (p_{trend} > 0.05; **Table 3**). Under the assumption of all analysed

Table 2. Association of the *ABCB11* and *ABCB4* nucleotide variants with the risk of ICP

Gene	SNP	Alleles[a]	MAF			OR (95%CI); p-value[b]		
			Cases	Controls	p_{trend}-value	Allelic model[c]	Dominant model[d]	Recessive model[e]
ICP (n = 86)								
ABCB11	**rs2287622**	C / T	0.42	0.42	0.899	0.98 (0.69–1.38); 0.897	1.02 (0.62–1.70); 0.929	0.90 (0.48–1.68); 0.734
	rs3815676	A / G	0.00	0.02	0.096	0.17 (0.01–2.97); 0.131[f]	0.17 (0.01–2.93); 0.128[f]	NA
	rs7577650	A / G	0.34	0.40	0.213	0.79 (0.56–1.13); 0.201	0.86 (0.53–1.41); 0.557	0.54 (0.26–1.15); 0.107
ABCB4	**rs4148826**	A / G	0.16	0.14	0.497	1.18 (0.73–1.88); 0.501	1.10 (0.64–1.88); 0.733	2.77 (0.61–12.63); 0.177[f]
	rs2109505	A / T	0.15	0.13	0.473	1.19 (0.73–1.93); 0.492	1.11 (0.64–1.91); 0.707	7.27 (0.65–81.38); 0.122[f]
	rs2302386	A / G	0.11	0.10	0.695	1.12 (0.64–1.96); 0.693	1.13 (0.61–2.07); 0.699	1.19 (0.12–11.60); 1.000[f]
Mild ICP (n = 60)								
ABCB11	**rs2287622**	C / T	0.43	0.42	0.972	1.01 (0.68–1.50); 0.971	1.14 (0.63–2.07); 0.657	0.84 (0.40–1.75); 0.636
	rs3815676	A / G	0.00	0.02	0.163	0.25 (0.01–4.25); 0.338[f]	0.24 (0.01–4.19); 0.374[f]	NA
	rs7577650	A / G	0.35	0.40	0.350	0.82 (0.54–1.23); 0.337	0.93 (0.53–1.64); 0.812	0.52 (0.21–1.26); 0.141
ABCB4	**rs4148826**	A / G	0.14	0.14	0.934	0.98 (0.55–1.74); 0.935	0.87 (0.45–1.67); 0.670	2.66 (0.48–14.86); 0.249[f]
	rs2109505	A / T	0.12	0.13	0.780	0.92 (0.50–1.69); 0.791	0.84 (0.43–1.64); 0.610	5.19 (0.32–84.14); 0.301[f]
	rs2302386	A / G	0.09	0.10	0.934	0.93 (0.49–1.91); 0.933	0.92 (0.83–1.93); 0.826	1.72 (0.18–16.88); 0.511[f]
Moderate and severe ICP (n = 26)								
ABCB11	**rs2287622**	C / T	0.40	0.42	0.757	0.91 (0.52–1.64); 0.749	0.80 (0.35–1.83); 0.589	1.05 (0.38–2.90); 1.000[f]
	rs3815676	A / G	0.00	0.02	0.360	0.57 (0.03–9.88); 1.000[f]	0.56 (0.03–9.76); 1.000[f]	NA
	rs7577650	A / G	0.33	0.40	0.341	0.74 (0.40–1.35); 0.322	0.73 (0.32–1.62); 0.434	0.61 (0.18–2.09); 0.591[f]
ABCB4	**rs4148826**	A / G	0.21	0.14	0.140	1.67 (0.83–3.38); 0.150	1.74 (0.76–4.00); 0.185	3.03 (0.33–28.16); 0.336[f]
	rs2109505	A / T	0.21	0.13	0.063	1.87 (0.92–3.80); 0.078	1.90 (0.83–4.36); 0.125	12.24 (0.74–201.75); 0.150[f]
	rs2302386	A / G	0.13	0.10	0.365	1.47 (0.63–3.41); 0.367	1.66 (0.67–4.15); 0.272	1.62 (0.08–32.23); 1.000[f]

[a] Underline denotes the minor allele.
[b] Chi-square analysis.
[c] d vs D; d is the risk allele.
[d] dd + Dd vs DD; d is the risk allele.
[e] dd vs Dd + DD; d is the risk allele.
[f] Fisher exact test.
MAF — minor allele frequency; OR — odds ratio; 95%CI — 95% confidence interval; NA — not applicable.

Table 3. Linkage disequilibrium values D' and r^2 for nucleotide variants tested in the *ABCB4* gene

	rs4148826	rs2109505	rs2302386
rs4148826	–	0.977	0.904
rs2109505	0.857	–	0.875
rs2302386	0.539	0.557	–

Numbers denote D' and r^2 values expressed as a percentage of maximal value (1.0). D' values are presented above the diagonal, r^2 values are presented below the diagonal.

Table 4. Linkage disequilibrium values D' and r^2 for nucleotide variants tested in the *ABCB11* gene

	rs2287622	rs3815676	rs7577650
rs2287622	–	0.115	0.422
rs3815676	0.000	–	0.485
rs7577650	0.152	0.004	–

Numbers denote D' and r^2 values expressed as a percentage of maximal value (1.0). D' values are presented above the diagonal, r^2 values are presented below the diagonal.

Table 5. Association of the *ABCB11* and *ABCB4* nucleotide variants with the risk of ICP in the group of patients after exclusion of cases with multiple pregnancies

Gene	SNP	Alleles[a]	MAF			OR (95%CI); p-value[b]		
			Cases	Controls	p_{trend}-value	Allelic model[c]	Dominant model[d]	Recessive model[e]
ICP (n = 67)								
ABCB11	**rs2287622**	C / T	0.40	0.42	0.658	0.91 (0.62–1.34); 0.647	0.82 (0.47–1.41); 0.464	1.03 (0.53 -2.01); 0.938
	rs3815676	A / G	0.00	0.02	0.143	0.22 (0.01–3.85); 0.225[f]	0.22 (0.01–3.80); 0.222[f]	NA
	rs7577650	A / G	0.37	0.40	0.519	0.88 (0.60–1.29); 0.505	0.92 (0.54–1.58); 0.768	0.72 (0.34–1.54); 0.399
ABCB4	**rs4148826**	A / G	0.17	0.14	0.285	1.31 (0.79–2.18); 0.289	1.21 (0.68–2.17); 0.516	3.61 (0.79–16.52); 0.109[f]
	rs2109505	A / T	0.16	0.13	0.310	1.30 (0.77–2.19); 0.331	1.20 (0.67–2.17); 0.539	9.42 (0.84–105.45); 0.084[f]
	rs2302386	A / G	0.12	0.10	0.379	1.30 (0.72–2.35); 0.377	1.33 (0.70–2.53); 0.390	1.54 (0.16–15.04); 0.547[f]
Mild ICP (n = 48)								
ABCB11	**rs2287622**	C / T	0.42	0.42	0.907	0.97 (0.63–1.51); 0.904	0.97 (0.51–1.83); 0.916	0.97 (0.44–2.11); 0.931
	rs3815676	A / G	0.00	0.02	0.214	0.31 (0.02–5.36); 0.375[f]	0.30 (0.02–5.29); 0.372[f]	NA
	rs7577650	A / G	0.39	0.40	0.838	0.95 (0.61–1.48); 0.833	1.14 (0.60–2.14); 0.696	0.66 (0.27–1.64); 0.372
ABCB4	**rs4148826**	A / G	0.16	0.14	0.579	1.18 (0.65–2.15); 0.584	1.07 (0.54–2.12); 0.854	3.37 (0.60–18.92); 0.183[f]
	rs2109505	A / T	0.14	0.13	0.773	1.09 (0.58–2.05); 0.784	1.01 (0.50–2.05); 0.971	6.51 (0.40–105.94); 0.253[f]
	rs2302386	A / G	0.12	0.10	0.523	1.25 (0.63–2.48); 0.519	1.22 (0.57–2.60); 0.608	2.17 (0.22–21.36); 0.440[f]
Moderate and severe ICP (n = 19)								
ABCB11	**rs2287622**	C / T	0.36	0.42	0.479	0.77 (0.38–1.55); 0.463	0.53 (0.20–1.38); 0.186	1.20 (0.38–3.77); 0.761[f]
	rs3815676	A / G	0.00	0.02	0.437	0.79 (0.05–13.73); 1.000[f]	0.77 (0.04–13.59); 1.000[f]	NA
	rs7577650	A / G	0.32	0.40	0.343	0.70 (0.35–1.42); 0.321	0.56 (0.22–1.42); 0.216	0.87 (0.25–3.10); 1.000[f]
ABCB4	**rs4148826**	A / G	0.21	0.14	0.208	1.66 (0.74–3.74); 0.218	1.63 (0.62–4.28); 0.319	4.21 (0.45–39.64); 0.261[f]
	rs2109505	A / T	0.21	0.13	0.112	1.86 (0.82–4.21); 0.131	1.77 (0.67–4.67); 0.241	17.00 (1.02–283.21); 0.113[f]
	rs2302386	A / G	0.13	0.10	0.470	1.43 (0.54–3.81); 0.406[f]	1.61 (0.56–4.66); 0.367[f]	2.20 (0.11–44.18); 1.000[f]

[a] Underline denotes the minor allele.
[b] Chi-square analysis.
[c] d vs D; d is the risk allele.
[d] dd + Dd vs DD; d is the risk allele.
[e] dd vs Dd + DD; d is the risk allele.
[f] Fisher exact test.
MAF — minor allele frequency; OR — odds ratio; 95%CI — 95% confidence interval; NA — not applicable.

Table 6. Haplotype analysis of the *ABCB11* and *ABCB4* nucleotide variants

Gene	Nucleotide variants	Haplotypes	Frequency	Case, Control Frequencies	χ^2	p-value	p_{corr}-value[a]
ABCB11	**rs2287622_rs3815676**	CA	0.576	0.596, 0.570	0.397	0.528	0.598
		TA	0.412	0.403, 0.414	0.065	0.799	0.880
	rs3815676_rs7577650	AG	0.616	0.665, 0.602	2.254	0.133	0.193
		AA	0.371	0.335, 0.381	1.268	0.260	0.342
	rs2287622_rs3815676_rs7577650	CAG	0.454	0.499, 0.442	1.803	0.179	0.453
		TAA	0.248	0.237, 0.251	0.139	0.710	1.000
		TAG	0.163	0.166, 0.162	0.013	0.910	1.000
		CAA	0.122	0.098, 0.129	1.229	0.268	0.607
ABCB4	**rs4148826_rs2109505**	AT	0.855	0.835, 0.861	0.761	0.383	0.810
		GA	0.127	0.153, 0.119	1.435	0.231	0.462
		GT	0.015	0.012, 0.016	0.195	0.659	1.000
	rs2109505_rs2302386	TA	0.860	0.846, 0.864	0.362	0.547	0.942
		AG	0.087	0.113, 0.080	1.925	0.165	0.606
		AA	0.042	0.040, 0.043	0.025	0.874	1.000
		TG	0.011	0.001, 0.014	2.128	0.145	0.510
	rs4148826_rs2109505_rs2302386	ATA	0.847	0.834, 0.851	0.288	0.591	1.000
		GAG	0.087	0.113, 0.080	1.923	0.166	0.541
		GAA	0.040	0.040, 0.040	0.002	0.969	1.000
		GTA	0.013	0.012, 0.013	0.016	0.899	1.000

[a] p value calculated using permutation test and a total of 1,000 permutations

inheritance models, the tested variants showed no evidence of an association with the increased risk of developing intrahepatic cholestasis during pregnancy. Similar results were also obtained after the division of patients based on the TBA levels (**Table 2**). Only in the group of patients with TBA levels > 40 (moderate and strong ICP), there was a trend towards association between the *ABCB4* rs2109505 variant and cholestasis (p_{trend} = 0.063; $OR_{allelic}$ = 1.87, 95% CI: 0.92 — 3.80; $OR_{dominant}$ = 1.90, 95% CI: 0.83–4.36, and $OR_{recessive}$ = 12.24, 95% CI: 0.74–201.75). Separate statistical calculations conducted in the group of patients after exclusion of cases with multiple pregnancies showed comparable results. For all tested nucleotide variants, there was no evidence for either allelic or genotyping association with the risk of ICP (**Table 5**). The result close to being statistically significant was also found for the *ABCB4* rs2109505 variant. Under the assumption of a recessive model, this SNP was associated with 9.42-fold (95% CI: 0.84–105.45, p = 0.084) increase in the risk of ICP (all types). Haplotype analysis of *ABCB4* and *ABCB11* SNPs did not reveal any common haplotypes (frequency > 0.01) associated with ICP (p_{corr} > 0.05; **Table 6**). Negative results were observed for both the whole sample and after the exclusion of cases with multiple pregnancies (results not shown).

Discussion

In recent years, the association between nucleotide variants of *ABCB4* and *ABCB11* and liver cholestatic diseases has become increasingly apparent [14]. Research on the genetic aetiology of the development of the disease was also carried out among pregnant women with cholestasis of pregnancy [15].

In 2004, Pauli-Magnus et al. [16] performed in a group of 21 unrelated pregnant women with cholestasis and a control group of 40 healthy pregnant women, an analysis of genetic variants of the *ABCB4* gene. The results showed that nearly half of the affected pregnant women have a specific *ABCB4* mutation. However, the study of the genetic variants of the BSEP encoding gene (*ABCB11*) failed to confirm its role in the development of cholestasis of pregnancy.

Floreani et al. [17] also proved the presence of three novel non-synonymous mutations in exon 14 of the *MDR3* gene (*ABCB4*) among 3 of 80 patients suffering from cholestasis of pregnancy (4%) and in none of the healthy women.

In pedigree studies, Schneider et al. [18], after examining 55 relatives, showed splicing mutations in the *MDR3* (*ABCB4*) gene, which can cause cholestasis in pregnancy and may be associated with stillbirths.

In the publication by Eloranta et al. [19] a relation was shown between the existence of cholestasis and the presence of a single nucleotide polymorphism SNP (rs473351) of the *ABCB11* gene in the Finnish population (57 affected and 115 healthy individuals).

However, a subsequent study by Painter et al. [20] conducted on a larger group of affected patients (n= 142), also from the Finnish population, failed to confirm these findings, suggesting that ICP is a genetically heterogeneous disease.

In 2009, Dixon et al. [21] published a study of 491 Caucasian pregnant women with ICP and 261 controls, and demonstrated that a single nucleotide polymorphism (c.1331C > T, p.Val444Ala, rs2287622) of the *ABCB11* gene might affect hepatic BSEP expression and be a significant risk factor for ICP.

In our study, we analysed six common nucleotide variants of *ABCB4* and *ABCB11* genes but failed to show any association between them or their haplotypes and the risk of cholestasis development. The allele and genotype frequencies for all tested SNPs were similar in both patients and properly selected controls. In addition, the *ABCB4* and *ABCB11* variants showed no evidence of association with the severity of this disorder. However, it is worth noting that in the group of patients with moderate and severe ICP, the results for the *ABCB4* rs2109505 variant were close to reaching the nominal significance threshold. Under the assumption of an allelic and dominant model, this SNP was associated with a 1.9-fold increase in the risk of ICP. For homozygous carriers of rs2109505, the risk was increased more than 12-fold. A trend towards the association between the *ABCB4* rs2109505 variant and cholestasis was also demonstrated after the exclusion of all cases with multiple pregnancies from the statistical calculations. In this case, the presence of rs2109505 in a homozygous form was associated with a 17-fold greater risk for developing ICP.

Dixon et al. [22] demonstrated a connection of the polymorphic variant rs2109505 in the *ABCB4* gene with the risk of cholestasis, along with two subsequent nucleotide variants in the *ABCB11* gene (rs3815676 and rs7577650). The examination was carried out on a group of 563 pregnant women with cholestasis and 642 healthy pregnant women. This was the largest cohort of pregnant women with ICP examined in relation to genetics. This association was previously reported in a smaller population [23]. The rs2109505 polymorphism is a synonymous variant located at codon 237 (p.Ile237Ile) in exon 8 of the *ABCB4* gene. Its contribution to disease risk via a number of different mechanisms were intensively examined. The effect of this SNP on protein function and response to inducing agents was not ascertained. It cannot be excluded that this association exists because of linkage disequilibrium between rs2109505 and a still unidentified pathogenic *ABCB4* variant.

The sequencing examination of the selected genes that may be connected to cholestasis showed the presence of 12 *ABCB4* mutations, 4 potential mutations of the *ABCB11* gene and a donor splice site mutation (intron19) [24].

Wasmuth et al. [13] analysed the association of selected gene variants of gene encoding hepatobiliary transporters for phospholipids (*ABCB4*) and bile acids (*ABCB11*) in patients with the severe form of intrahepatic cholestasis of pregnancy in a Swedish cohort. The study, conducted among 52 patients with a TBA level > 40 µmol/L, and 52 pregnant women in the control group, revealed that specific *ABCB4* gene haplotypes could represent etiological factors for the development of the severe form of ICP. The authors did not confirm this finding for genetic variants of the *ABCB11* gene. Yeap et al. [2] reported nine pregnancies complicated by severe cholestasis (maximum BA level 74–370 µmol/L) in 5 women. They detected two *ABCB11* mutations with significant loss of BSEP function and one homo- and four heterozygous mutations in *ABCB4*.

The limitation of our study is the relatively small group of patients with intrahepatic cholestasis. Identification of cholestasis based on elevated levels of bile acid applies to around 1% of pregnant women in the Caucasian population. Among those who developed cholestasis, there were patients with multiple pregnancies, for whom the mechanism of developing the ailment is most often the result of a significantly elevated level of steroid hormones (oestrogens and sulphate progesterone metabolites) in 2nd and 3rd trimesters [6], although genetic origins of the ailment may not be ruled out in that group. Hence, it is probable that the real percentage of pregnant women for whom nucleotide variants of the *ABCB4* and *ABCB11* genes may play a role in the ailment's etiopathogenesis is significantly lower.

In conclusion, our study did not show any significant association of the analysed *ABCB4* and *ABCB11* nucleotide variants with an increased risk of intrahepatic cholestasis of pregnancy. The negative result may originate from the relatively low number of the analysed patients and controls, as well as the limited number of examined polymorphic variants. Therefore further studies are necessary to confirm the role of *ABCB4* and *ABCB11* variants in the etiopathology of ICP.

Acknowledgements

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

Funding sources
The study was supported by grant no. 502-01-01110142-05618 from Poznan University of Medical Sciences.

The technical assistance of MSc Justyna Dąbrowska is gratefully acknowledged.

References

1. McIlvride S, Dixon PH, Williamson C. Bile acids and gestation. Mol Aspects Med. 2017 Aug; 56:90–100.
2. Yeap SP, Harley H, Thompson R, Williamson KD, Bate J, Sethna F, et al. Biliary transporter gene mutations in severe intrahepatic cholestasis of pregnancy: Diagnostic and management implications. J Gastroenterol Hepatol. 2019 Feb;34(2):425–435.
3. Geenes V, Chappell LC, Seed PT, Steer PJ, Knight M, Williamson C. Association of severe intrahepatic cholestasis of pregnancy with adverse pregnancy outcomes: a prospective population-based case-control study. Hepatology. 2014 Apr;59(4):1482–1491.
4. Kawakita T, Parikh LI, Ramsey PS, Huang CC, Zeymo A, Fernandez M, et al. Predictors of adverse neonatal outcomes in intrahepatic cholestasis of pregnancy. Am J Obstet Gynecol. 2015 Oct;213(4): 570.e1–8.
5. Eloranta ML, Heinonen S, Mononen T, Saarikoski S. Risk of obstetric cholestasis in sisters of index patients. Clin Genet. 2001 Jul;60(1):42–45.

6. Webb GJ, Elsharkawy AM, Hirschfield GM. The etiology of intrahepatic cholestasis of pregnancy: towards solving a monkey puzzle. Am J Gostroenterol. 2014 Jan;109(1):85–88.
7. Reyes H, Gonzalez MC, Ribalta J, Aburto H, Matus C, Schramm G, et al. Prevalence of intrahepatic cholestasis of pregnancy in Chile. Ann Intern Med. 1978 Apr;88(4):487–493.
8. Lee RH, Goodwin TM, Greenspoon J, Incerpi M. The prevalence of intrahepatic cholestasis of pregnancy in a primarily Latina Los Angeles population. J Perinatol. 2006 Sep;26(9):527–532.
9. van der Woerd WL, van Mil SW, Stapelbroek JM, Klomp LW, van de Graaf SF, Houwen RH. Familial cholestasis: progressive familial intrahepatic cholestasis, benign recurrent intrahepatic cholestasis and intrahepatic cholestasis of pregnancy. Best Pract Res Clin Gastroenterol. 2010 Oct;24(5):541–553.
10. Piątek K, Kurzawińska G, Magiełda J, Drews K, Barlik M, Malewski Z, et al. The role of ABC transporters' gene polymorphism in the etiology of intrahepatic cholestasis of pregnancy. Ginekol Pol. 2018;89(7):393–397.
11. Nicolaou M, Andress EJ, Zolnerciks JK, Dixon PH, Williamson C, Linton KJ. Canalicular ABC transporters and liver disease. J Pathol. 2012 Jan;226(2):300–315.
12. Anzivino C, Odoardi MR, Meschiari E, Baldelli E, Facchinetti F, Neri I, et al. ABCB4 and ABCB11 mutations in intrahepatic cholestasis of pregnancy in an Italian population. Dig Liver Dis. 2013 Mar;45(3):226–232.
13. Wasmuth HE, Glantz A, Keppeler H, Simon E, Bartz C, Rath W, et al. Intrahepatic cholestasis of pregnancy: the severe form is associated with common variants of the hepatobiliary phospholipid transporter ABCB4 gene. Gut. 2007 Feb;56(2):265–270.
14. Aamann L, Ørntoft N, Vogel I, Grønbaek H, Becher N, Vilstrup H, et al. Unexplained cholestasis in adults and adolescents: diagnostic benefit of genetic examination. Scand J Gastroenterol. 2018 Mar;53(3):305–311.
15. Reichert MC, Lammert F. ABCB4 Gene Aberrations in Human Liver Disease: An Evolving Spectrum. Semin Liver Dis. 2018 Nov;38(4):299–307.
16. Pauli-Magnus C, Lang T, Meier Y, Zodan-Marin T, Jung D, Breymann C, et al. Sequence analysis of bile salt export pump (ABCB11) and multidrug resistance p-glycoprotein 3 (ABCB4, MDR3) in patients with intrahepatic cholestasis of pregnancy. Farmacogenetics. 2004 Feb;14(2):91–102.
17. Floreani A, Carderi I, Paternoster D, Soardo G, Azzaroli F, et al. Intrahepatic cholestasis of pregnancy: three novel MDR3 gene mutations. Aliment Farmacol Ther. 2006 Jun;23(11):1649–1653.
18. Schneider G, Paus TC, Kullak-Ublick GA, Meier PJ, Wienker TF, Lang T, et al. Linkage between a new splicing site mutation in the MDR3 alias ABCB4 gene and intrahepatic cholestasis of pregnancy. Hepatology. 2007 Jan;45(1):150–158.
19. Eloranta ML, Häkli T, Hiltunen M, Helisalmi S, Punnonen K, Heinonen S. Association of single nucleotide polymorphisms of the bile salt export pump gene with intrahepatic cholestasis of pregnancy. Scand J Gastroenterol. 2003 Jun;38(6):648–652.
20. Painter JN, Savander M, Sistonen P, Lehesjoki AE, Aittomäki K. A known polymorphism in the bile salt export pump gene is not a risk allele for intrahepatic cholestasis of pregnancy. Scand J Gastroenterol. 2004 Jul;39(7):694–695.
21. Dixon PH, van Mil SW, Chambers J, Strautnieks S, Thompson RJ, Lammert F, et al. Contribution of variant alleles of ABCB11 to susceptibility to intrahepatic cholestasis of pregnancy. Gut. 2009 Apr;58(4):537–544.
22. Dixon PH, Wadsworth CA, Chambers J, Donnelly J, Cooley S, Buckley R, et al. A comprehensive analysis of common genetic variation around six candidate loci for intrahepatic cholestasis of pregnancy. Am J Gastroenterol. 2014 Jan;109(1):76–84.
23. Müllenbach R, Weber SN, Krawczyk M, Zimmer V, Sarrazin C, Lammert F, et al. A frequent variant in the human bile salt export pump gene ABCB11 is associated with hepatitis C virus infection, but not liver stiffness in a German population. BMC Gastroenterol. 2012 Jun; 12:63.
24. Dixon PH, Sambrotta M, Chambers J, Taylor-Harris P, Syngelaki A, Nicolaides K, et al. An expanded role for heterozygous mutations of ABCB4, ABCB11, ATP8B1, ABCC2 and TJP2 in intrahepatic cholestasis of pregnancy. Sci Rep. 2017 Sep;7(1):11823.
25. Gonzalez MC, Reyes H, Arrese M, Figueroa D, Lorca B, Andresen M, et al. Intrahepatic cholestasis of pregnancy in twin pregnancies. J Hepatol. 1989 Jul;9(1):84–90.
26. Eloranta ML, Heiskanen JT, Hiltunen MJ, Mannermaa AJ, Punnonen KR, Heinonen ST. Multidrug resistance 3 gene mutation 1712delT and estrogen receptor alpha gene polymorphisms in Finnish women with obstetric cholestasis. Europ J Obstet Gynecol Reprod Biol. 2002 Nov;105(2):132–135.

Supplementary Table 1. Primers and HRM conditions for genotyping of the *ABCB11* and *ABCB4* nucleotide variants

Gene	rs no.	Chromosome location[a]	Alleles[b]	Primers for PCR amplification (5'–3')	PCR product length (bp)	Annealing temp. (°C)	Melt. temp. range (°C)
ABCB11 2q31.1	**rs2287622**	**chr2:168973818**	C / T	F: AGCTGTCATTTCCCCTGGT R: CACAAAGCATCTGCACCTGT	132	55	76–91
	rs3815676	**chr2:169013869**	A / G	F: GATGCCATTGCCAAGTAGA R: TCTCAGGATGGAGGCATTTC	121	55	74–89
	rs7577650	**chr2:169034700**	A / G	F: GCCAGCATGAGTCAGTTAACAC R: GAAATTGTGTCCTTCCACACAG	143	55	74–89
ABCB4 7q21.12	**rs4148826**	**chr7:87445103**	A / G	F: GTCACATTCTGGCATTCAT R: GCCTTGCAAATGTTGCTCT	120	55	70–85
	rs2109505	**chr7:87450090**	A / T	F: CTTTGTCACTAAATGCCGAGA R: TAAAGGGTTGACCAGAGTGC	97 analysis without and with spiking DNA	55	74–89
	rs2302386	**chr7:87462628**	A / G	F: TTCCTGTGTATTTCCTTCACC R: TTTGGATATCTGGTTGACTCC	139	58	72–87

[a] GRCh38 / hg38.
[b] Underline denotes the minor allele.

Acceptance for editing: 2019-11-09
Acceptance for publication: 2019-12-30

ORIGINAL PAPER

DOI: https://doi.org/10.20883/medical.368

Immunohistochemical evaluation of cellular composition of the immune system of lymph nodes in acute appendicitis

Jakub Żurawski*[1, a], Patrycja Talarska[1, b], Stanisław Łazowski[2], Marcin Grochowalski[3, c], Jacek Karoń[3, d]

[1] Department of Immunobiochemistry, Poznan University of Medical Sciences, Poland
[2] Specialised City Hospital of Jozef Strus Hospital, Laboratory of Pathological Anatomy, Poznań, Poland
[3] Clinic of General Surgery, Poznan University of Medical Sciences, Poland

* *Corresponding Autor:* Jakub Żurawski, 8 Rokietnicka Street, 60-806 Poznań, Poland, email: zurawski@ump.edu.pl

[a] https://orcid.org/0000-0002-5838-0451
[b] https://orcid.org/0000-0003-1305-0990
[c] https://orcid.org/0000-0002-0249-8434
[d] https://orcid.org/0000-0003-4330-7224

ABSTRACT

Introduction. There is not much data about the composition of populations of the immune system in acute appendicitis. The basic histopathological criterion for the diagnosis of acute appendicitis is neutrophil infiltration of the muscle membrane.
Aim. The subject of this publication is a semi-quantitative evaluation of B lymphocytes (CD20+), T lymphocytes (CD3+) and macrophages (CD68+), and the determination of the number of active lymph nodes during the course of inflammation.
Material and Methods. The study material was obtained from 79 patients who had an appendectomy due to acute appendicitis. In this group, the tissue was obtained from: 34 women (aged 20 to 91) and 45 men (aged 20 to 72).
Results. In the course of acute appendicitis, there is involvement of lymph node B lymphocytes, T lymphocytes and macrophages. Independent of the type of inflammation, the cellular make-up of the nodes is similar. The number of lymph nodes decreases with age and is gender dependent.
Conclusions. In the course of acute appendicitis, there is involvement of lymph node B lymphocytes, T lymphocytes and macrophages. The number of lymph nodes decreases with age and is gender dependent. A statistically significant number of the examined cells of the immunological system in the lymph nodes changed due to inflammation ($p < 0.001$). B and T lymphocytes in the lymph nodes and in the mucous membrane of the appendix differed depending on the sex, and the presence of B lymphocytes in the mucous membrane was significantly higher in the group of 20–40 years of age. T lymphocytes were predominant in the centres of the lymph nodes in groups 20–40 and 61–91 years of age, and in the peripheral zones in the group of 41–60 years of age.

Keywords: appendicitis, lymph nodes, histomorphometry.

Introduction

Periumbilical pain is a frequent early symptom of appendicitis, which later migrates to the abdominal right lower quadrant. The pain is characteristically accompanied by nausea, vomiting, and fever. Sharp pain at McBurney's point (two-thirds of the distance from the navel and the anterior superior iliac spine) is pathognomonic of appendicitis. In most cases, an appendectomy is the best treatment choice.

The anatomic location, as well as intestinal stasis and the abundance of lymphoid tissue in the intestinal wall create favourable conditions for the development of inflammation.

The disease may be caused by bacterial, viral and parasitic infections. The inflammation is facilitated by anatomy, such as the length, flexion and close location to the colon. Acute appendicitis occurs as a result of increased pressure in its lumen, and consequently obstruction of venous outflow. In most cases, it is also associated with occlusion by faecal mass, or stercolith, with the proliferation of bacteria, which are a direct cause of inflammation [1].

Inflammatory changes can be seen as hyperplasia of the lymph nodes (appendicitis follicularis). Neutrophil infiltration (appendicitis purulenta) may also occur. When inflammation crosses the appendiceal wall, necrotic foci may appear (appendicitis gangrenosa).

Lymph nodes are round or ovoid structures of stroma and reticular fibres, filled with B lymphocytes, dendritic cells and macrophages.

There are two types of lymph nodes: primary and secondary. Primary lymph nodes have a homogeneous structure and contain small, inactive B lymphocytes (naïve lymphocytes).

Secondary lymph nodes have a more differentiated structure. The centre, the so-called reactive centre, is less dense due to the loose pattern of chromatin in the proliferative nuclei of the centroblasts. B lymphocytes in germinal centres differentiate into plasma cells that secrete antibodies, or into memory lymphocytes. The peripheral zone of the lymph node is a significantly darker zone, called the mantle zone [2].

The presence of the reactive centre and the mantle zone is the result of a response to stimulation by antigens. Hyperplasia of the lymph nodes is caused mainly by the activity of centroblasts in germinal centres.

There is not much data about the composition of populations of the immune system in acute appendicitis, which is the focus of this paper.

Aim

The subject of this publication is a semi-quantitative evaluation of B lymphocytes (CD20+), T lymphocytes (CD3+) and macrophages (CD68+), and the determination of the number of active lymph nodes during the course of inflammation.

Material and Methods

The study material was obtained from 79 patients who had an appendectomy due to acute appendicitis. In this group, the tissue was obtained from: 34 women (aged 20 to 91) and 45 men (aged 20 to 72). The average and median age was higher for the women than than for the men. The difference was statistically significant according to the Mann-Whitney test; $p = 0.0427$

For antigen retrieval, antibodies against: CD3 (Dako A0452), CD20 (Dako M0755) and CD68 (Dako M0814) were used. Preparations were incubated in a water bath at 96°C in a citrate buffer (pH 6.0) for 50 minutes. Endogenous peroxidase was inhibited by 3% H_2O_2. The tissue samples were incubated with antigen for 60 minutes at room temperature. After that, they were rinsed for 10 minutes in TBS followed by incubation in an EnVision system (DakoCytomation, K5007) for 30 minutes.

In all preparations, the DAB-3,3 chromogen was used, which helped to locate the antigen. Following that, preparations were stained with Mayer's hematoxylin and dehydrated in a series of alcohols to xylene.

For immunohistochemical determination, a negative control was performed by omission of the primary antibody.

Photographs of all lymph nodes present in the examined samples were taken with an Olympus BX43 light microscope and XC 30 digital camera, at 100x magnification. Based on photographic documentation, semi-quantitative evaluation of the immunohistochemical reactions was performed, using the cellSens commercial software by Olympus (**Figures 1**, **2**). Statistical calculations were performed with the STATISCTICA 10 statistical package.

Results

Lymph nodes

In all cases, activated lymph nodes were observed (on average 18) (**Figure 3**). Their number depended on the patient's gender. For men, it was 21, and

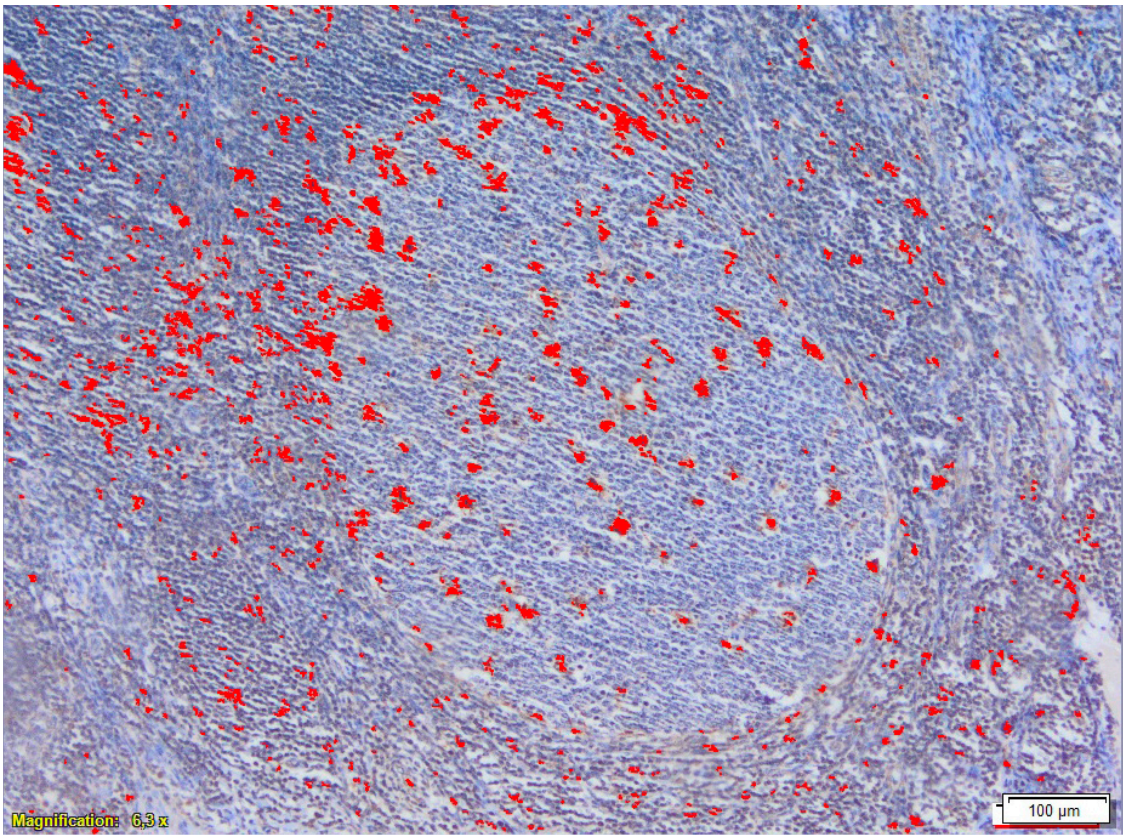

Figure 1. Print screens of cellSens dimension software by Olympus — before image analysis

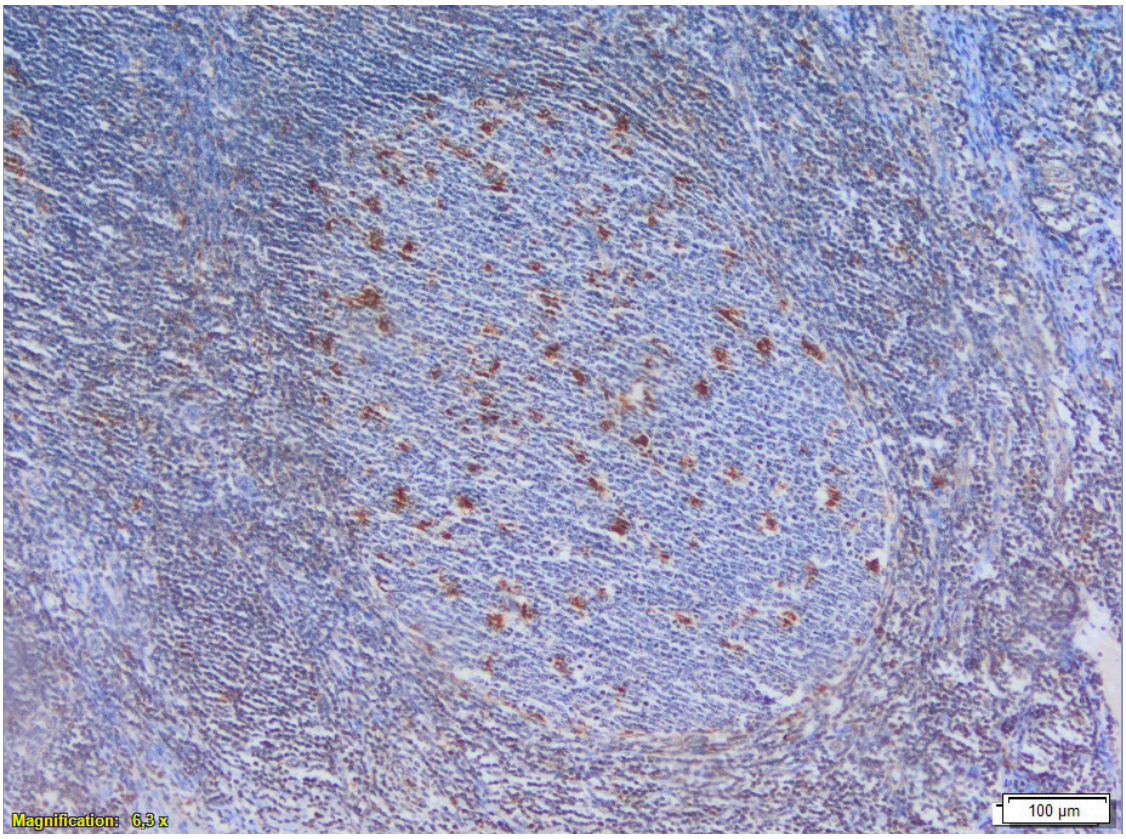

Figure 2. Print screens of cellSens dimension software by Olympus — after image analysis

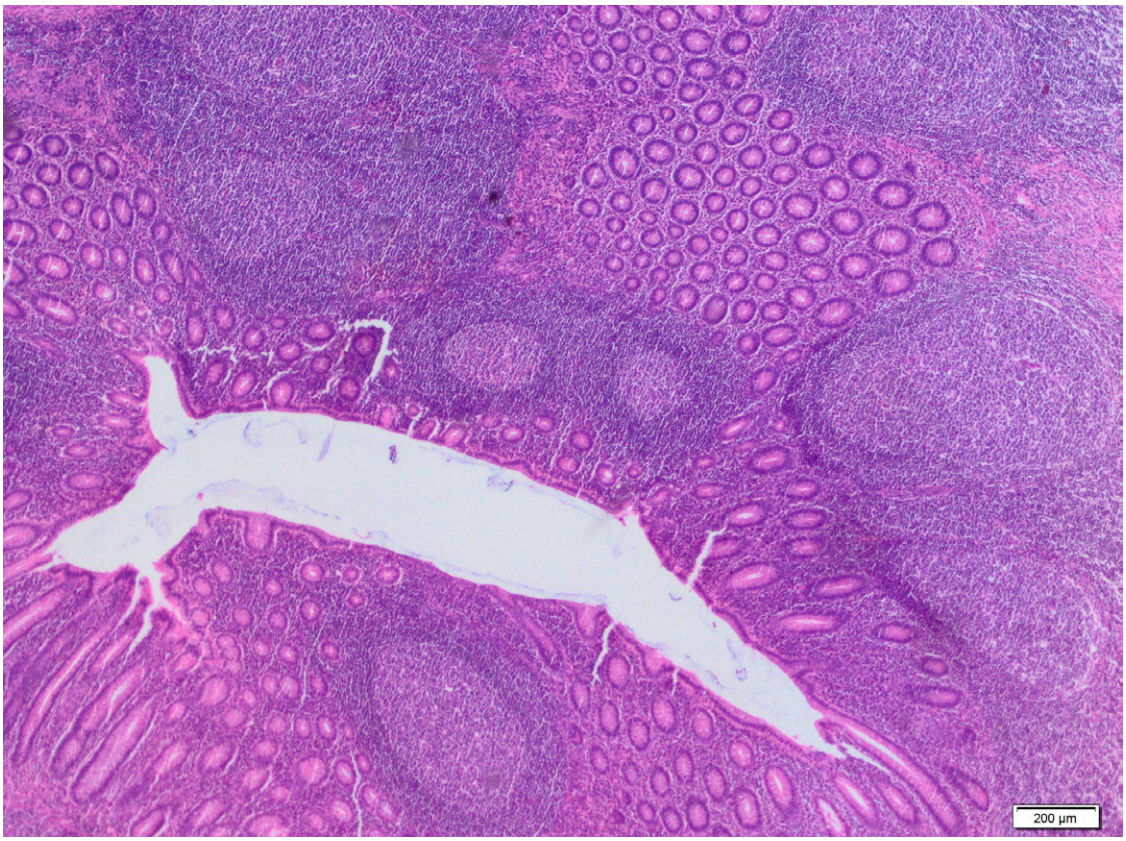

Figure 3. Lymph nodes. HE staining. Magnification 40x

for women, 15, on average. The Mann-Whitney test did not show statistically significant differences (p = 0.2669).

The number of lymph nodes decreased with age. In patients aged 20–40, there were on average 20 nodes, with patients aged 41–60, there were 17, whereas in patients aged 61–91, there were only 10 nodes. The Kruskal-Wallis test did not show a statistically significant difference in the number of lymph nodes in age groups (p > 0.05).

Sex and location of T and B lymphocytes and macrophages

In men, T lymphocytes were more frequently found in the centre (C), and simultaneously in the peripheral zone of the lymph node and in the mucous membrane (PZ/MM) (p = 0.0280).

In women, T lymphocytes were more frequently found in the peripheral zone (PZ), in the mucous membrane (MM), simultaneously in the centre and in the peripheral zone (C/PZ), or simultaneously in the centre of the lymph node and in the mucous membrane (C/MM) (**Table 1**).

In men, there were more B lymphocytes in the centre of the lymph node (C), in the mucous membrane (MM), simultaneously in the centre and in the peripheral zone (C/PZ), or simultaneously in the centre, in the peripheral zone and in the mucous membrane (C/PZ/MM).

In women, on the other hand, B lymphocytes were more frequently observed in the peripheral zone (PZ), and simultaneously in the centre and

Table 1. Location of T lymphocytes in women and men. PZ – peripheral zone of the lymph node, C – centre of the lymph node, MM – mucous membrane, PZ/MM – in the peripheral zone of the lymph node and in the mucous membrane, C/MM – in the centre of the lymph node and in the mucous membrane

Location	Men n = 45		Women n = 34		p
	n	%	n	%	
PZ	12	26.7	13	38.2	0.2765
C	13	28.9	8	23.5	0.9493
MM	2	4.4	2	5.9	0.7630
C/PZ	3	6.7	6	17.6	0.1311
PZ/MM	11	24.4	2	5.9	**0.0280***
C/MM	3	6.7	3	8.8	0.7273

* statistically significant, p < 0.05

in the mucous membrane (C/MM). The test of significance in this case did not show any significant differences in the frequency of B lymphocytes in women and men ($p > 0.05$) (**Table 2**).

Table 2. Location of B lymphocytes in women and men. PZ – the peripheral zone of the lymph node, C – the centre of the lymph node, MM – the mucous membrane, C/PZ – the centre and peripheral zone of the lymph node, C/MM – the centre of the lymph node and the mucous membrane, C/PZ/MM – the centre, the peripheral zone of the lymph node, and the mucous membrane

Location	Men n = 45		Women n = 34		p
	n	%	n	%	
PZ	0	0.0	1	2.9	0.2503
C	30	66.7	21	61.8	0.6521
MM	2	4.4	1	2.9	0.7284
C/PZ	5	11.1	3	8.8	0.7371
C/MM	5	11.1	8	23.5	0.1409
C/PZ/MM	1	2.2	0	0.0	0.3841

No statistically significant differences in the location of macrophages in women and men were noticed, either in the mucous membrane (MM), in the submucosa (SM) or in the lymph nodes ($p > 0.05$) (**Table 3**).

Table 3. Location of macrophages in women and men. MM- mucous membrane, MM/SM/lymph node – mucous membrane, submucosa, and whole lymph node

Location	Men n = 45		Women n = 34		p
	n	%	n	%	
MM	1	2.2	1	2.9	0.8436
MM/SM/LN	44	97.8	33	97.1	0.8436

Frequency of T lymphocytes, B lymphocytes and macrophages in age groups

Based on results of the Fisher-Snedecor distribution, statistically significant differences in the frequency of T lymphocytes were visible in the centre of the lymph nodes (C) ($p = 0.0401$). These cells were more frequent in persons aged 20–40 and 61–91 than in persons aged 41–60. The statistically significant difference was for the location both in the centre and in the peripheral zone (C/PZ) ($p = 0.0399$) in the group 41–60 years of age, compared to persons aged 20–40. In patients aged 61–91, T lymphocytes were not present in both locations simultaneously.

The significant difference in the frequency of B lymphocytes was confirmed with the Fisher-Snedecor F-test, observed in the mucous membrane (MM) ($p = 0.0443$) in the group 20–40 years of age (**Table 4**).

No statistically significant differences were observed with the help of the Fisher-Snedecor F-test in the number of macrophages, both in the mucous membrane (MM) and in the mucous membrane, submucosa and lymph nodes (MM/SM/LN) in any age groups ($p > 0.05$) (**Table 5**).

Table 4. Location of B lymphocytes in persons in various age groups. PZ- peripheral zone of the lymph node, C – centre of the lymph node, MM – mucous membrane, C/PZ – centre and peripheral zone, C/MM – centre of the lymph node, and mucous membrane, C/PZ/MM – centre, peripheral zone, and mucous membrane

Location	20–40 years of age n = 50		41–60 years of age n = 20		61–91 years of age n = 9		p
	n	%	n	%	n	%	
PZ	1	2.0	0	0.0	0	0.0	0.3589
C	32	64.0	12	60.0	7	77.8	0.5361
MM	3	6.0	0	0.0	0	0.0	**0.0443***
C/PZ	5	10.0	3	15.0	0	0.0	0.0670
C/MM	9	18.0	3	15.0	1	11.1	0.7679
C/PZ/MM	0	0.0	1	5.0	0	0.0	0.2079

* – statistically significant, $p < 0.05$

Table 5. Location of macrophages in various age groups and the Fisher-Snedecor F test for the k index of the structure. MM- the mucous membrane, MM/SM/LN – in the mucous membrane, submucosa and in the lymph node

Location	20–40 years of age n = 50		41–60 years of age n = 20		61–91 years of age n = 9		p
	n	%	n	%	n	%	
MM	1	2.0	1	5.0	0	0.0	0.4807
MM/SM/LN	49	98.0	19	95.0	9	100.0	0.4807

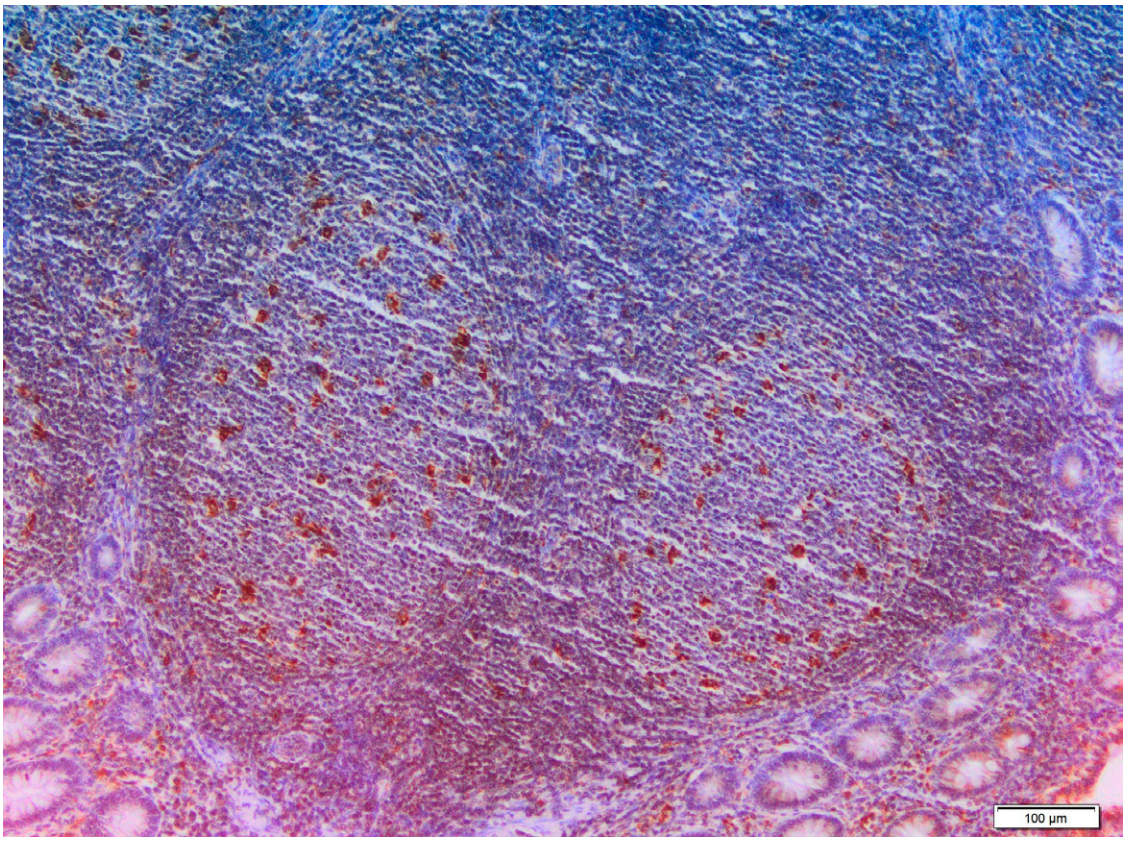

Figure 4. Macrophages in lymph nodes (CD68). Immunohistochemical staining. Magnification 100x

Number of cells of the immune system in lymph nodes

Based on results of the chi-squared test, a significant difference in the frequency of cells of the immune system in the lymph nodes ($p < 0.0001$) was observed. The most frequent were B lymphocytes (on average 3,441 cells per lymph node) — 59.3% of all cells. The second most frequent were T lymphocytes (on average 2,116 cells per lymph node), which constituted 36.5%. The least numerous were macrophages (on average 247 cells per lymph node) — 4.3% (**Figure 4**).

Location of B lymphocytes, T lymphocytes and macrophages

B lymphocytes were observed in the centre of the lymph node in 64.4% of cases (**Figure 5**). The chi-squared test showed a significant difference in the frequency of B lymphocytes ($p < 0.0001$). In 51 patients, they were observed in the centre of the lymph node (C). The lowest number was noted in the peripheral zone (PZ), and simultaneously in the centre, peripheral zone and in the mucous membrane (C/PZ/MM) (**Table 6**).

Table 6. Location of B lymphocytes. PZ — peripheral zone of the lymph node, C — centre, MM — mucous membrane, C/PZ — centre and peripheral zone, C/MM — centre of the lymph node, and mucous membrane, C/PZ/MM — centre, peripheral zone, and mucous membrane

Location	n	%	chi-squared test		
			χ^2	df	p
PZ	1	1.3	173.44	6	**< 0.0001***
C	51	64.6			
MM	3	3.8			
C/PZ	8	10.1			
C/MM	13	16.5			
C/PZ/MM	1	1.3			
No reaction	2	2.5			

* **statistically** significant, $p < 0.05$

Based on the chi-squared test, a statistically significant difference in the frequency of T lymphocytes ($p < 0.0001$) was observed. These cells were most frequently found in the peripheral zone (PZ) and in the centre of the lymph node (C): in 25 and 21 persons, respectively. T lymphocytes in the peripheral zone of the lymph node were observed in 31.6% of cases (**Figure 6**). They were least numerous in the mucous membrane (MM),

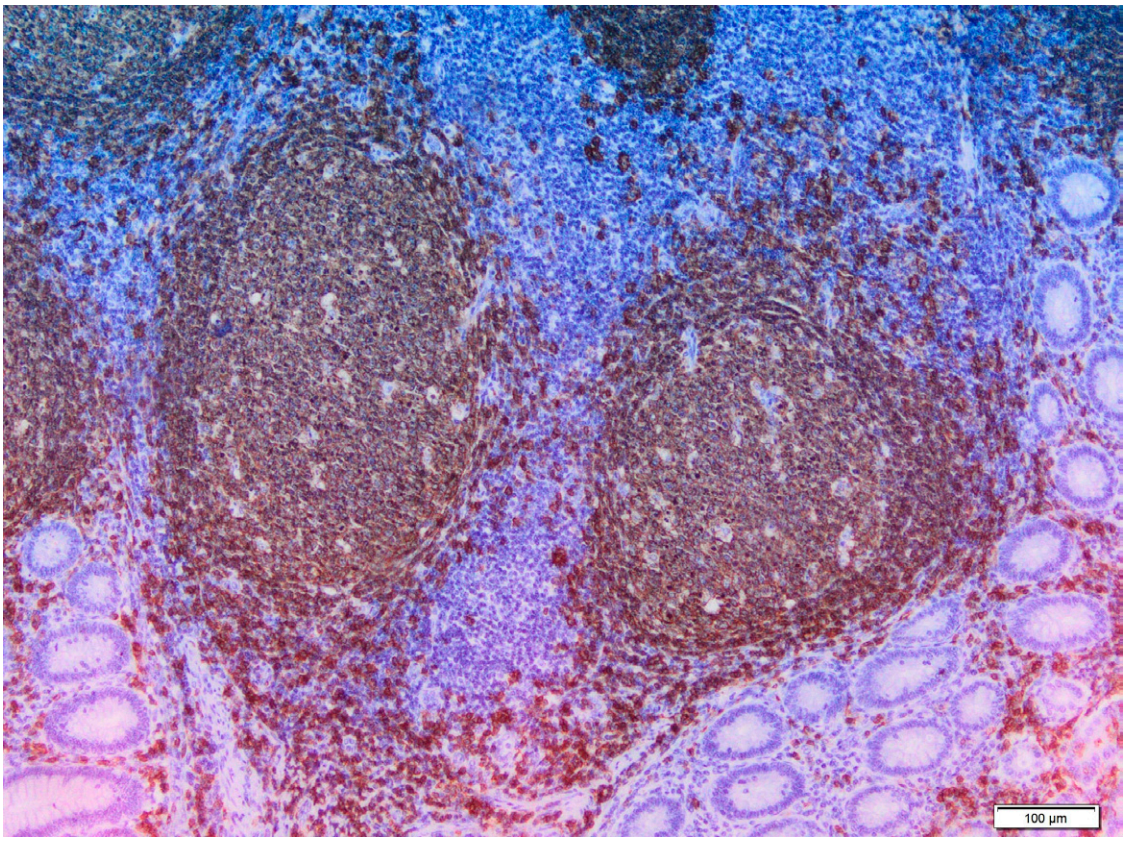

Figure 5. B lymphocytes in lymph nodes (CD20). Immunohistochemical staining. Magnification 100x

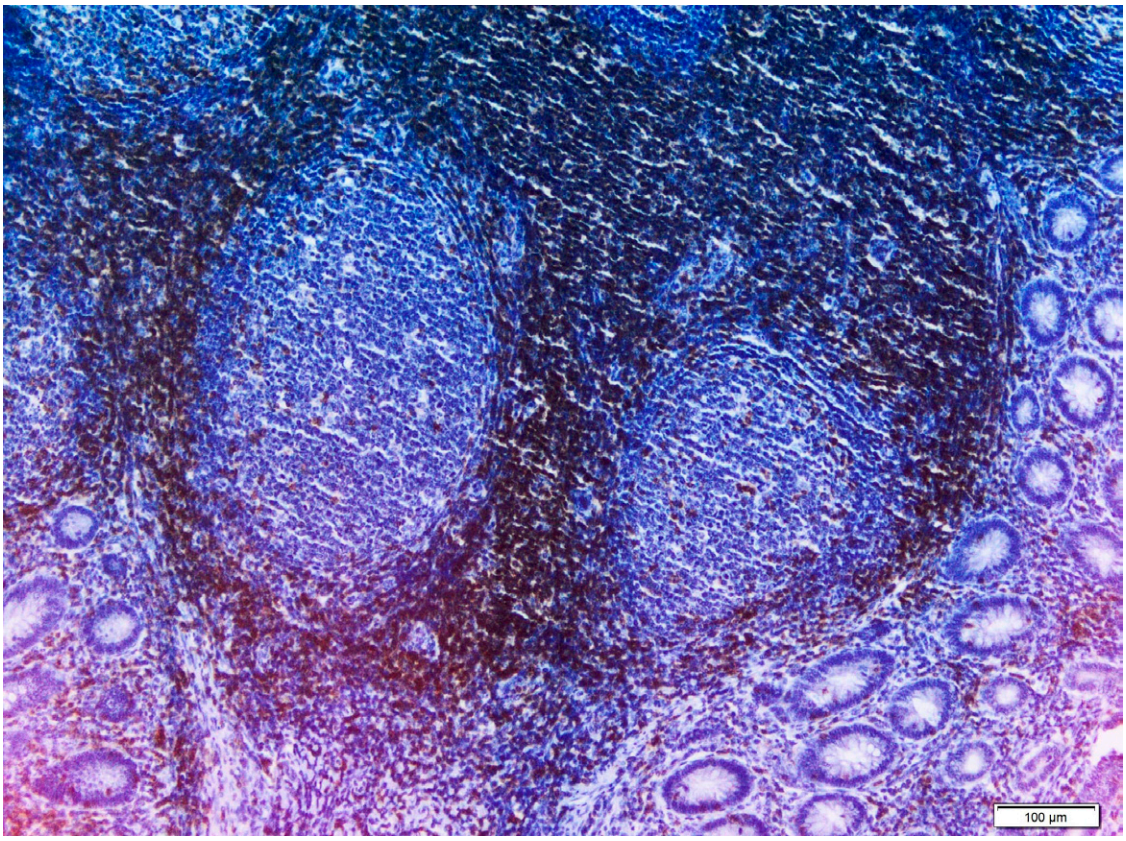

Figure 6. T lymphocytes in lymph nodes (CD3). Immunohistochemical staining. Magnification 100x

Table 7. Location of T lymphocytes. PZ — peripheral zone of the lymph node, C — centre, MM — mucous membrane, C/PZ — centre and peripheral zone, C/MM — centre of the lymph node, and in the mucous membrane

Location	n	%	chi-squared test		
			χ^2	df	p
PZ	25	31.6	42.30	6	<0.0001*
C	21	26.6			
MM	4	5.1			
C/PZ	9	11.4			
PZ/MM	13	16.5			
C/BMM	6	7.6			
No reaction	1	1.3			

* — statistically significant, $p < 0.05$

Table 8. Location of macrophages. MM — mucous membrane, MM/SM/LN — mucous membrane, submucosa and the whole lymph node

Location	n	%	chi-squared test		
			χ^2	df	p
MM	2	2.5	71.20	1	<0.0001*
MM/SM/LN	77	97.5			

* — statistically significant, $p < 0.05$

in four persons. In one case, no reaction with the CD 3 antibody was observed (**Table 7**).

The chi-squared test showed a significant difference in the frequency of their occurrence ($p < 0.0001$). In 77 patients, they were predominantly in the mucous membrane, submucosa and the whole surface of the lymph node simultaneously (MM/SM/LN). In two patients only, they were present solely in the mucous membrane (MM) (**Table 8**).

Discussion

The basic histopathological criterion for the diagnosis of acute appendicitis is neutrophil infiltration in the muscle membrane. In the samples examined, acute purulent appendicitis and acute gangrenous appendicitis were diagnosed histopathologically. Immunological reaction takes place in the lymph nodes located in the lamina propria of the mucous membrane and in the submucosa. It seems that the number of affected lymph nodes is individually variable. There are no published reliable data that would estimate an average number of activated lymph nodes per inflamed appendix. In the samples presented here, the number varied from 6 to 78. It is not, however, the total number of lymph nodes in the appendix, but only in the histological section. Independently of their number, in all cases, active nodes with a reactive centre and mantle zone were observed. Their size was dependent on the activity of centroblasts. The number of nodes was correlated with the age and gender of the patients, whereby the number decreased with age. The group examined comprised adults aged of 20 to 91 years. Our samples did not include children. Based on the data available in the literature for the children and on our own observations, we can safely assume that their number is much higher in that age group. For women, the number was smaller than for men. In the future, it would be of interest to correlate the higher number of activated lymph nodes found in men and the higher prevalence of the disease in this group.

In the cases of acute appendicitis, B lymphocytes, T lymphocytes and macrophages were observed in all tissue samples. Independently of the type of inflammation diagnosed, the cell composition of the lymph nodes was similar.

The pathogenesis of acute appendicitis is heterogeneous, and clinical symptoms are non-characteristic [3] without clear, unequivocal diagnostic criteria. The currently available literature is insufficient to determine the participation of immune cell populations during the course of acute appendicitis. Another problem is the subjectivity of the establishing of the final diagnosis by pathologists [4, 5]. Some authors consider appendectomy to be necessary. It is of significant importance in the case of neurogenic appendicitis. In the appendix, there may be an increased proliferation of cells, accompanied by secretions of vasoactive intestinal peptide and substance P. As a consequence, strong pain is present in the right lower iliac fossa. In these cases, no macroscopic or microscopic changes characteristic of acute inflammation are observed [6, 7]. Other authors advise against an appendectomy. According to them, the preservation of large quantities of lymphoid tissues in the appendix is important to the function of the immune system. [8]. After the appendectomy of a pain-free appendix, the patient may suffer from numerous post-operative complications including: wound infection, a hernia, bowel obstruction caused by post-operative adhesions, and even death [9].

A more thorough understanding of the course and location of immunological responses and the role of individual cells and their populations in relation to clinical presentation may help to establish reliable diagnostic criteria.

Conclusions

In the course of acute appendicitis, there is involvement of lymph node B lymphocytes, T lymphocytes and macrophages. The number of lymph nodes decreases with age and is gender dependent. A statistically significant difference in the number of the examined cells of the immunological system of the lymph nodes changed due to inflammation ($p < 0.001$) was observed. B and T lymphocytes in the lymph nodes and in the mucous membrane of the appendix differed depending on the sex. The presence of B lymphocytes in the mucous membrane was significantly higher in the group of 20–40 years of age. T lymphocytes were predominant in the centre of the lymph nodes in groups 20–40 and 61–91 years of age, and in the peripheral zone in the group of 41–60 years of age.

Acknowledgements

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

Funding sources
There are no sources of funding to declare.

References

1. Lamps LW. Infectious causes of appendicitis. Infect Dis Clin North Am. 2010;4:995–1018.
2. Kooij IA, Sahami S, Meijer SL, Buskens CJ, Te Velde AA. The immunology of the vermiform appendix: a review of the literature. Clin Exp Immunol. 2016;186:1–9.
3. Cartwright SL, Knudson MP. Evaluation of Acute Abdominal Pain in Adults. Am Fam Physician. 2008;77:971–978.
4. Herd ME, Cross PA, Dutt S. Histological audit of acute appendicitis. J Clin Pathol. 1992;45:456–458.
5. Benhamou G. Useless appendectomy, its diagnostic difficulties. Ann Gastroenterol Hepatol (Paris). 1986;22:339–340.
6. Güller U, Oertli D, Terracciano L, Harder F. Neurogenic appendicopathy: a frequent, almost unknown disease picture. Evaluation of 816 appendices and review of the literature. Chir Z Alle Geb Oper Medizen. 2001;72:684–689.
7. Di Sebastiano P, Fink T, di Mola FF, Weihe E, Innocenti P, Friess H, et al. Neuroimmune appendicitis. Lancet Lond Engl. 1999;354:461–466.
8. Gebbers JO, Laissue JA. Bacterial translocation in the normal human appendix parallels the development of the local immune system. Ann N Y Acad Sci. 2004;1029:337–343.
9. Guller U, Hervey S, Purves H, Muhlbaier LH, Peterson ED, Eubanks S, et al. Laparoscopic versus open appendectomy: outcomes comparison based on a large administrative database. Ann Surg. 2004;239:43–52.

Acceptance for editing: 2019-11-09
Acceptance for publication: 2019-12-30

REVIEW PAPER

DOI: https://doi.org/10.20883/medical.318

Neuropsychological deficits in depression – a challenge for cognitive-behavioral therapies

Bartosz Piasecki, Karolina Kabzińska*

Department of Clinical Psychology, Poznan University of Medical Sciences, Poland

* *Corresponding Autor:* Karolina Kabzińska, Department of Clinical Psychology, Poznan University of Medical Sciences, 70 Bukowska Street, 60-812 Poznań, Poland, phone: +48881966933, email: kabzinska.ump@gmail.com

[a] https://orcid.org/0000-0002-2567-682X [b] https://orcid.org/0000-0001-5515-8954

ABSTRACT

Neuropsychological deficits in depression are a significant therapeutic challenge. Their occurrence means poor therapeutic prospects, worse social and professional functioning after therapy, as well as a higher risk of relapse. Despite clinical improvement, they often remain even in a state of complete remission. Beck's model of depression does not include interventions directed at neuropsychological processes leading to neurocognitive mechanisms responsible for the development and maintenance of depression. More recent trends in cognitive-behavioral therapy seem to involve neuropsychological processes to a greater extent. This applies to Well's metacognitive model, which focuses on the meta-level of thinking. Therapeutic process involves various aspects of attention, as well as detached mindfulness. Available empirical studies indicate that this therapy model is more effective in reducing neuropsychological deficits than Beck's model. Acceptance and commitment therapy as well as mindfulness-based cognitivetherapy both focus on the development of skills that are related to the efficiency of executive functions and flexibility of attention, i.e. the cognitive processes whose deficits are characteristic of depression. However, research is needed to confirm their effectiveness in reducing neuropsychological deficiencies compared to other therapeutic models. Interventions in the field of cognitive remediationcan be used to enrich cognitive-behavioral therapies and increase their effectiveness. Until now, they have been used as a separate form of therapy, for example in anorexia.

Keywords: depression, neuropsychological deficits, cognitive-behavioral therapy.

Introduction

Depression is a frequent and recurrent disorder associated with a significant deterioration of quality of life and ability to perform social roles, as well as a greater risk of premature death due to suicide [1]. According to the World Health Organization (WHO), the total number of people suffering from depression is 322 million and its incidence is estimated at around 4.4% [2]. Diagnosis of a depressive episode according to ICD-10 requires the occurrence of at least two of the following symptoms: depressed mood, loss of interest and enjoyment, reduced energy or increased fatigability, as well as at least two consecutive symptoms: decrease in respect for or trust in yourself, excessive and unjustified sense of guilt or irrational remorse, recurrent thoughts of death, suicide or any suicidal behavior, reduced ability to concentrate and think, change in appetite, sleep disorders, changes in psychomotor activ-

ity. Symptoms must be present for most of the day almost every day for at least two weeks and cause significant suffering or impairment [3].

Neuropsychological deficits are a major challenge in the therapeutic process of depression. Their occurrence is associated with a negative impact on both the immediate results of therapy and the long-term functioning of patients, as well as a higher risk of relapse [4, 5]. Despite the clear overall improvement and positive response to therapy, neuropsychological symptoms often persist [6]. A meta-analysis of research on neuropsychological functioning of patients with recurrent depression showed significant deficits in attention, memory and executive functions [7]. Cognitive deficits have also been observed in patients with depression in remission [4, 8]. It is indicated that the neuropsychological dysfunctions are often insufficiently diagnosed and therapists focus primarily on the emotional and somatic health aspects and negative patterns of thinking [8, 11]. The results obtained by observation and self-report on cognitive functioning in depression often differ from neuropsychological tests results [10, 11].

Aim

The aim of this paper is to analyze the existing models of cognitive-behavioral psychotherapy (Beck's classic model, Wells's metacognitive therapy, acceptance and commitment therapy, and mindfulness-based Cognitive Therapy) in terms of their use in work on neuropsychological deficits in depression. In addition, possible directions of development of the analyzed therapies will be considered in the context of their potential for increased effectiveness of depressive patients treatment.

Beck's cognitive model of depression

The cognitive model of depression proposed by Beck [12–15] is still widely used by therapists. In this model, it is assumed that due to the experience of significant loss in the early stages of life, permanent cognitive structures are formed. The result is a greater vulnerability of the individual to depression in the situation of future losses. Those patterns can remain dormant and be activated by negative life events. Key beliefs and conditional assumptions that arose as a result of early experiences predispose to the occurrence of depressive symptoms in the case when events (e.g. next loss) activate these beliefs and violate hidden assumptions. Negative processes and thinking contents that are triggered lead to the consolidation of depressed mood and other depressive symptoms [12–15]. Beck and Bredemeier theorized that the above-described cognitive mechanism of depression is part of a natural, evolutionary mechanism (the so-called "depression program"), which in specific conditions is adaptive to people and allows the conservation of energy resources. Depressive beliefs play a crucial role of a mediator between information processing, autonomic nervous system, immune response and depressive symptoms. Too long activation of the "depression program" leads to the strengthening of depressive patterns, which increases the risk of relapse [16]. Beck assumed the existence of the so-called cognitive triad of depression consisting of distorted, negative beliefs about yourself, the world and the future. These beliefs usually appear in the present moment in the form of negative automatic thoughts (NATs). They maintain the symptoms of depression, namely they cause a depressed mood and a drop in energy and motivation that lead to a lower engagement in satisfying activities, which becomes evidence of negative beliefs [12, 14, 15]. For people in depression, cognitive distortions are also characteristic. Beck et al. [12] pointed to specific logical errors in patients with depression — primarily generalization, selective attention and dichotomous thinking. These processes increase access to information in accordance with negative beliefs and suppress information that is not consistent with them. This prevents verification of negative cognitive patterns and leads to their maintenance.

Cognitive-behavioral psychotherapy based on Beck's model is always adapted to the specifics of functioning of a given patient, but it is based on certain principles and assumptions. It is a directive-oriented therapy, limited in time, focused on the "here and now", structured and assuming constant cooperation between the therapist and the patient. The basic direction of

therapeutic work is the identification and reconstruction of cognitive distortions. For this purpose, a number of techniques are used, such as psychoeducation, Socratic dialogue, analysis of arguments for and against, down and up arrows, scaling, relaxation exercises, activation, imaginative exercises and behavioral experiments [13–15]. In therapeutic protocols based on Beck's model, four phases are usually specified in which emphasis is placed on different aspects of the disorder [12, 13, 17]. The first phase of therapy focuses on building a therapeutic relationship, completing conceptualization and defining goals, as well as on the education about the disorder and the cognitive-behavioral model, and finally on the behavioral activation of the patient. The second phase focuses on identifying emotions, negative automatic thoughts, cognitive distortions and then modifying them through the use of appropriate interventions. In the next phase, interventions are increasingly focused on the intermediary assumptions and key beliefs of the patient. The last phase of therapy is a summary and termination of the therapeutic process and prevention of relapse.

Cognitive-behavioral psychotherapy based on Beck's model has repeatedly proved its effectiveness while working with many disorders, including depression [13, 18, 19]. The severity of clinical symptoms of depression often fails to be directly related to the severity of neurocognitive dysfunctions, and full remission of clinical symptoms often does not lead to remission of neuropsychological deficits [4, 5, 8, 20]. In Beck's model, there are no interventions that would focus directly on neuropsychological deficits typical of depression and concerning executive functions or attention. Randomized studies conducted by Porter et al. [5] and Groves et al. [9] showed that in patients who participated in cognitive-behavioral psychotherapy based on this model, there were no significant changes in neuropsychological deficits despite significant clinical improvement. Those deficits involved executive functions, memory and verbal learning, as well as processing speed. These results require confirmation in further research, but they show possible limitations in Beck's therapeutic model. An interesting development of Beck's concept aimed at the neuropsychological sphere is the metacognitive model of depression proposed by Wells [21].

The metacognitive model

Wells's model assumes that the tendency to negative thinking and dysfunctional beliefs are important elements in the development and maintenance of mental disorders, including depression (which is consistent with Beck's model) [21]. Wells concentrates on the repetitive, unhelpful patterns of thinking, beliefs about thinking (metacognitive beliefs), and attentional bias forming the so-called 'cognitive attentional syndrome' (CAS) [21]. The CAS consists of the processes worrying, directing attention to the threat, and the use of unhelpful coping strategies that sustain negative thinking processes. In contrast to Beck's theory, in Wells's model, negative thoughts are considered a trigger factor activating dysfunctional ways of processing or thinking. These, on the other hand, play a key role in developing and sustaining symptoms. The metacognitive model does not focus directly on the content of negative thoughts (e.g. "I am worthless"), but on how the individual processes it (e.g. ruminates or worries about it). The metacognitive model of depression is composed of [21]:

- positive meta-beliefs about the need to ruminate as a way to reduce depressive symptoms or find a way to solve the problem,
- negative meta-beliefs about the inability to control ruminations, the risks associated with depression, and the individual's own vulnerability,
- reduced meta-ruminating awareness,
- CAS (ruminating, focusing on the threat, unhelpful coping strategies).

The metacognitive model of depression was presented in **Figure 1**.

The two depression therapies based either on the metacognitive model or on Beck's model have a lot in common. In both therapies, patients' activation, increasing their motivation, as well as changing their behavioral patterns that support the disorder are important elements. Also, both are aimed at the reconstruction of negative beliefs using similar therapeutic techniques. However, in the case of metacognitive therapy the focus is on identifying and changing the meta-beliefs, both negative and positive. An important difference in the therapeutic process, especially important in the deliberations on the impact of therapy on neuropsychological deficits, lies in attention

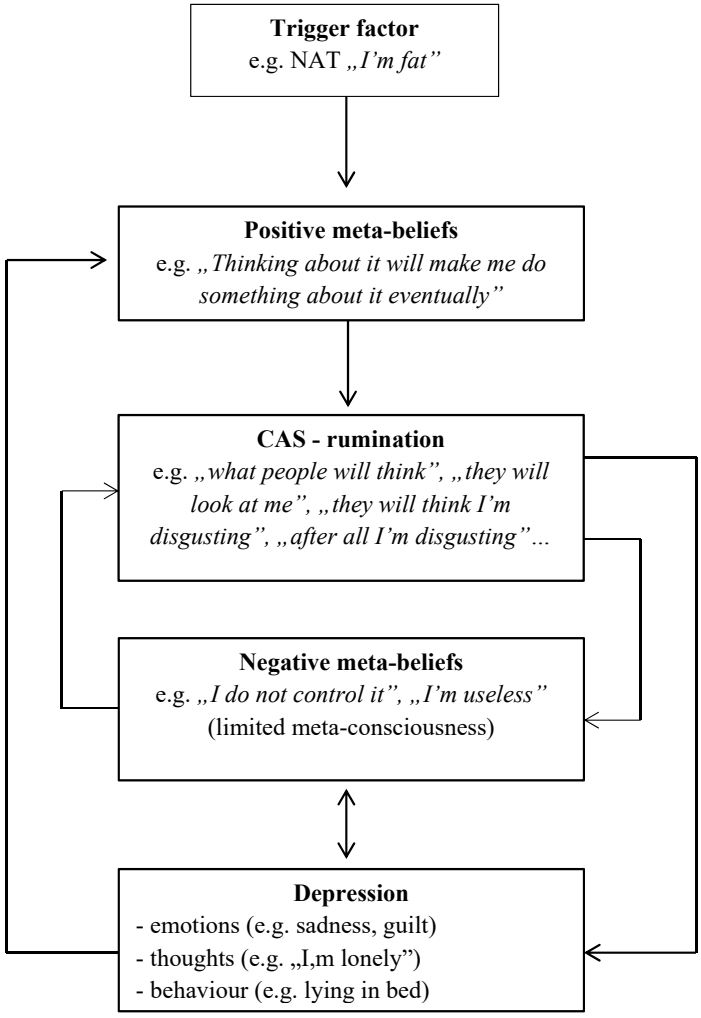

Figure 1. Metacognitive model of depression. Source: based on Wells (2009)

training techniques (ATT) and detached mindfulness, which is the central element of metacognitive therapy [21]. ATT consists of three elements that are practiced during the therapy:

- selective attention (keeping the patient's attention on specific sound stimuli and ignoring others),
- attention switching (shifting attention of the patient to different sound stimuli at an increasing rate),
- divisibility of attention (deepening and maintaining attention to various sound stimuli simultaneously).

The detached mindfulness is understood by Wells (2009) as a state of awareness of internal cognitive experiences (thoughts, memories, beliefs, imaginings, etc.) without engaging in their assessment, control, suppression, change or behavioral response to them. This experience is lived as independent of the whole experience of yourself, from the perspective of the observer. This skill develops during therapy through a series of exercises conducted during the session, as well as in the form of homework [21]. ATT and detached mindfulness are interventions whose aim is to increase self-awareness, minimize perseverative thinking patterns in the form of ruminations, engage in unhelpful behaviors and increase the flexibility of attention. From the neuropsychological perspective, these are elements that can be directly related to the operation of executive functions whose deficits are characteristic of depression. The executive functions are a complex system that supervises, controls and directs the cognitive activity of the individual [22, 23]. The meta-level of cognitive functioning, which is key in Wells's model, is to a large extent identical with the role of executive functions. ATT and detached mindfulness as an essential element of the therapeutic process

enable a direct impact on possible deficits in this area of neuropsychological functioning. Previous research seems to confirm this thesis. Metacognitive therapy has been shown to be effective in reducing both clinical symptoms of depression [24, 25] and improving neuropsychological deficits [26, 27]. Groves [9] compared in randomized studies the effectiveness of cognitive-behavioral psychotherapy according to both Beck's and Wells's models in reducing neuropsychological deficits. After the end of therapy, the group of patients who participated in Wells's metacognitive therapy was characterized by a significantly greater improvement in the scope of attention and executive functions in comparison with the group in therapy according to Beck's model. The metacognitive model, seems to have an advantage over Beck's model of therapy in improving neuropsychological deficits in patients with depression. According to the author's knowledge, these are the only studies so far comparing the effectiveness of various psychotherapeutic models in terms of changes in neuropsychological functioning in depression and further research in this area is necessary.

Mindfulness-based and acceptance and commitment therapies

Elements of metacognitive therapy are also present in approaches belonging to the so-called third wave of cognitive-behavioral psychotherapy, namely mindfulness-based cognitive therapy (MBCT) and acceptance and commitment therapy (ACT). Both approaches are used in psychotherapy of depression.

MBCT for depression is an extension of the stress relief program based on mindfulness created by Jon Kabat-Zinn [28, 29]. The MBCT program for depression is an eight-week training in the development of mindfulness through the regular and systematic use of meditation in many forms, both at sessions and at home [29, 30]. Mindfulness is understood as a special kind of attention, conscious, non-judgmental and directed at the present moment [28, 29]. All meditation exercises rely on the learning of a gentle, purposeful focus on the present and appreciating the perception of things as they are. In contrast to the detached mindfulness of Wells's model, in MCBT an attentive approach is practiced. It is about directing attention not only to the cognitive sphere but also to the experiences flowing from the body, emotions or the outside world [21]. Mindfulness training allows and individual to develop the ability of conscious, purposeful attention targeting, greater self-awareness of own thoughts and feelings, decentration by getting a distanced observer perspective for own cognitive or affective processes. As a result, the tendency to identify with them and automatically react to them decreases. This allows the individual to reduce inefficient thought processes (in particular ruminating and worrying) and unhelpful prevention strategies that are characteristic of depression [29, 30].

The acceptance and commitment therapy is a relatively new approach derived from the cognitive-behavioral trend. The primary goal of ACT is to increase psychological flexibility. This goal is achieved through the use of psychological interventions based on mindfulness (in the same sense as in MCBT) and acceptance combined with strategies of behavior modifications [31, 32]. Psychological flexibility is understood here as the ability to fully, consciously experience the present moment, "here and now", and behave in a way that allows achieving goals consistent with the values of the individual [31, 32]. It consists of:

- contact with the present moment (careful, conscious experience and getting involved in everything that is happening at the moment),
- acceptance (of painful feelings, all other feelings, desires, emotions as they are and making them a place in the field of our experience),
- I as a context (awareness of thoughts, feelings, experiences, actions, conscious observation of them from cognitive distance, taking the perspective of an observer),
- values (desirable characteristics of action, ideals of directing action),
- involved activity (effective action in accordance with professed values),
- cognitive defusion (distancing oneself from one's own cognitive processes, not identifying with them, not engaging automatically in them).

People with depression are characterized by a lack of psychological flexibility. They avoid experiencing negative emotions or thoughts. They are characterized by limited self-awareness and cognitive fusion with negative beliefs (typi-

cal of depression). In ACT, the immediate goal of working with a depressed patient is not to reduce symptoms, but to release and develop those elements that block the individual's psychological flexibility. Relief of depressive symptoms is treated as a desirable and highly probable side effect of increasing the individual's psychological flexibility [31].

Both ACT and MBCT in their therapeutic interventions focus on cognitive elements that have proved effective in the metacognitive model in working on neuropsychological deficits in depression. These models develop thinking at the meta-cognitive level, increase self-awareness and attentive, flexible and conscious directing of attention. They develop the ability to take a distanced, decentered perspective on experiences, as well as the ability to not react in an automatic manner in response to thoughts, impulses or emotional states that arise in the mind. Skills developed in these therapies are related to the efficiency of executive functions [22, 23]. However, according to the author's knowledge, there are no studies to date focusing on the effectiveness of ACT and MBCT in neuropsychological deficits reduction in depression. A meta-analysis [33] allows to formulate cautious predictions about a possible positive impact on this area also in people with depression — in particular on the selectivity of attention, working memory and executive functions.

Cognitive remediation

As indicated earlier, the problem of neuropsychological deficits is often overlooked during the therapeutic process, and psychological interventions focus on other areas of patient functioning. Cognitive-behavioral psychotherapy is a model that integrates various techniques and influences after empirically verifying their effectiveness [15]. It is reasonable to look for methods that could increase its effectiveness in working on neuropsychological dysfunctions. An idea worth considering is the introduction of the so-called cognitive remediation (CR) interventions into therapeutic protocols (like using ATT in Well's model). CR showed its effectiveness, among others, in work with schizophrenic patients, elderly patients and brain injuries [5, 22, 34].

CR should be treated as a general name for psychological interventions aimed at working with specific cognitive processes. The choice of methods should therefore be preceded by an appropriate diagnosis of existing neuropsychological deficits. In the light of current studies in depression, there are usually difficulties in the efficiency of executive functions, attention and working memory [5, 7]. Besides ATT, there are many interventions, both standardized and quality, which can be used to develop these neuropsychological functions [22]. There are also many CR protocols and computerized stimulation packages for neuropsychological functions that still require adequate empirical validation [35]. An example of such a protocol is the CR program for anorexia created by Tachanturia, Davies, Reeder and Wykes [27], whose effectiveness has been empirically confirmed [36]. After some modifications, this program could be adapted to the needs of patients with depression. Particularly noteworthy is the study by Priyamvada et al. [37], who focused on the effectiveness of the CR program in patients with depression lasting for a minimum of 2 years. The authors created a 15-session program for the rehabilitation of cognitive attention and memory in depressive patients, which significantly improved the efficiency of these functions in the study population [37]. Despite the promising results, the study had its limitations. Only 30 patients took part in it and there was no control group, which significantly limits the possibility of generalizing conclusions drawn from the results of the study. This program does not require any specialized hardware or software licenses and could be easily replicated or adapted to existing cognitive-behavioral depression protocols for further validation.

Conclusions

Neuropsychological deficits in depression are a significant therapeutic challenge. Despite their clinical improvement, they often remain even in a state of complete remission. Their occurrence is associated with poor therapeutic prospects, worse socio-occupational functioning after therapy, and a higher risk of relapse [4, 5, 38]. There is still a marked lack of research on neuropsychological functioning in mood disorders, and in particular on therapeutic (pharmacological and psycho-

therapeutic) impacts on this sphere of functioning. Beck's model of depression does not include interventions directed to neuropsychological processes, which would be a neuro-cognitive basis for the theorized mechanisms responsible for the development and maintenance of depression. Newer trends of cognitive-behavioral therapy involve more neuropsychological processes. This is especially true for the metacognitive model, which focuses on the meta-level of thinking and includes training of various aspects of attention (ATT), as well as training of detached mindfulness as an integral part of the psychotherapy process. Available empirical studies indicate that this model is more effective in reducing neuropsychological deficits than Beck's model [9]. ACT and MBCT therapies also focus on the development of skills that are related to the efficiency of executive functions and flexibility of attention, i.e. cognitive processes whose deficits are characteristic of depression. However, research is needed to confirm their effectiveness in reducing neuropsychological deficiencies compared to other therapeutic models. Methods that can be used to enrich cognitive-behavioral therapies and increase their effectiveness are interventions in the field of cognitive remediation, which until now have usually been used as a separate form of therapy. The metacognitive model by Wells with his ATT shows that these methods can be a complementary part of the classical psychotherapeutic intervention. However, before new models and therapeutic protocols are created and the availability of empirical research on the effectiveness of neuropsychological deficits therapy is increased, all cognitive-behavioral psychotherapists should pay more attention to this aspect of the patient's functioning. Specialists will be able to get a full picture of the patient's condition. Neuropsychological functioning evaluation using standardized tools is worth considering as a standard when working with depressed people. Future research on treating depression and preventing relapse should be oriented towards the neuropsychological sphere. Improving the cognitive functioning of patients will contribute to optimizing prognosis.

Acknowledgements

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

Funding sources
There are no sources of funding to declare.

References

1. Spijker J, Graaf R, Bijl RV, Beekman ATF, Ormel J, Nolen WA. Functional disability and depression in the general population. Results from the Netherlands Mental Health Survey and Incidence Study (NEMESIS). Acta Psychiatr Scand 2004;110:208–14. doi:10.1111/j.1600-0447.2004.00335.x.
2. WHO. Depression and Other Common Mental Disorders. Global Health Estimates 2017.
3. WHO. The ICD-10 Classification of Mental and Behavioural Disorders. 2009.
4. Bortolato B, Carvalho AF, McIntyre RS. Cognitive dysfunction in major depressive disorder: a state-of-the-art clinical review. CNS Neurol Disord Drug Targets 2014;13:1804–18.
5. Porter RJ, Bowie CR, Jordan J, Malhi GS. Cognitive remediation as a treatment for major depression: A rationale, review of evidence and recommendations for future research. Aust N Z J Psychiatry 2013;47:1165–75. doi:10.1177/0004867413502090.
6. Semkovska M, Quinlivan L, O'Grady T, Johnson R, Collins A, O'Connor J, et al. Cognitive function following a major depressive episode: a systematic review and meta-analysis. Lancet Psychiatry 2019;6:851–61. doi:10.1016/S2215-0366(19)30291-3.
7. Hasselbalch BJ, Knorr U, Kessing LV. Cognitive impairment in the remitted state of unipolar depressive disorder: a systematic review. J Affect Disord 2011;134:20–31. doi:10.1016/j.jad.2010.11.011.
8. Rock PL, Roiser JP, Riedel WJ, Blackwell AD. Cognitive impairment in depression: a systematic review and meta-analysis. Psychol Med 2014;44:2029–40. doi:10.1017/S0033291713002535.
9. Groves SJ, Porter RJ, Jordan J, Knight R, Carter JD, McIntosh VVW, et al. Changes in neuropsychological function after treatment with metacognitive therapy or cognitive behaviour therapy for depression. Depress Anxiety 2015;32:437–44.
10. Lahr D, Beblo T, Hartje W. Cognitive performance and subjective complaints before and after remission of major depression. Cognit Neuropsychiatry 2007;12:25–45. doi:10.1080/13546800600714791.
11. Rohling ML, Green P, Allen LM 3rd, Iverson GL. Depressive symptoms and neurocognitive test scores in patients passing symptom validity tests. Arch Clin Neuropsychol Off J Natl Acad Neuropsychol 2002;17:205–22.
12. Beck AT, Rush AJ, Shaw BF, Emery G. Cognitive therapy of depression. New York: Guilford Press; 1979.
13. Beck JS. Cognitive-behavior therapy. New York: Guilford Press; 2011.
14. Fennell M, Bennett-Levy J, Westbrook D. Depresja. Oksfordzki Podręcznik Eksp. Behawioralnych W Ter. Poznawczej, Gdynia: Alliance Press; 2004.
15. Popiel A, Pragłowska E. Psychoterapia poznawczo-behawioralna. Warszawa: Wydawnictwo Paradygmat.; 2008.
16. Beck AT, Bredemeier K. A Unified Model of Depression Integrating Clinical, Cognitive, Biological, and

Evolutionary Perspectives. Clin Psychol Sci 2016;4: 596–619.
17. Dobson KS. Cognitive Therapy for Depression. Cogn. Ther. Depress. Manag. Complex. Comorbidity, New York: Guilford Press; 2008, p. 3–33.
18. Driessen E, Hollon SD. Cognitive behavioral therapy for mood disorders: efficacy, moderators and mediators. Psychiatr Clin North Am 2010;33:537–55. doi:10.1016/j.psc.2010.04.005.
19. Hofmann SG, Asnaani A, Vonk IJJ, Sawyer AT, Fang A. The Efficacy of Cognitive Behavioral Therapy: A Review of Meta-analyses. Cogn Ther Res 2012;36:427–40. doi:10.1007/s10608-012-9476-1.
20. Bora E, Harrison BJ, Yucel M, Pantelis C. Cognitive impairment in euthymic major depressive disorder: a meta-analysis. Psychol Med 2013;43:2017–26. doi:10.1017/S0033291712002085.
21. Wells A. Metacognitive therapy for anxiety and depression. New York: Guilford Press; 2009.
22. Pąchalska M. Rehabilitacja Neuropsychologiczna. Lublin: Wydawnictwo Uniwersytetu Marii Curie-Skłodowskiej; 2008.
23. Pąchalska M. Neuropsychologia kliniczna. vol. 1. Warszawa: Wydawnictwo Naukowe PWN; 2007.
24. Dammen T, Papageorgiou C, Wells A. An open trial of group metacognitive therapy for depression in Norway. Nord J Psychiatry 2015;69:126–31. doi:10.3109/08039488.2014.936502.
25. Jordan J, Carter JD, McIntosh VVW, Fernando K, Frampton CMA, Porter RJ, et al. Metacognitive therapy versus cognitive behavioural therapy for depression: a randomized pilot study. Aust N Z J Psychiatry 2014;48:932–43. doi:10.1177/0004867414533015.
26. Siegle GJ, Ghinassi F, Thase ME. Neurobehavioral Therapies in the 21st Century: Summary of an Emerging Field and an Extended Example of Cognitive Control Training for Depression. Cogn Ther Res 2007;31:235–262.
27. Tchanturia K, Giombini L, Leppanen J, Kinnaird E. Evidence for Cognitive Remediation Therapy in Young People with Anorexia Nervosa: Systematic Review and Meta-analysis of the Literature. Eur Eat Disord Rev J Eat Disord Assoc 2017;25:227–36. doi:10.1002/erv.2522.
28. Kabat-Zinn J. Życie, piękna katastrofa. Warszawa: Wydawnictwo Czarna Owca; 2013.
29. Segal ZV, Williams MG, Teasdale JD. Mindfulness-Based Cognitive Therapy for Depression. New York: Guilford Press; 2013.
30. Teasdale JD, Williams M, Segal Z. Praktyka uważności. Ośmiotygodniowy program ćwiczeń pozwalający uwolnić się od depresji i napięcia emocjonalnego. Kraków: WUJ; 2016.
31. Harris R. Zrozumieć ACT. Terapia akceptacji i zaangażowania w praktyce. Sopot: GWP; 2018.
32. Hayes SC, Strosahl KD, Wilson KG. Acceptance and Commitment Therapy An Experiential Approach to Behavior Change. New York: Guilford Press; 2003.
33. Chiesa A, Calati R, Serretti A. Does mindfulness training improve cognitive abilities? A systematic review of neuropsychological findings. Clin Psychol Rev 2011;31:449–64. doi:10.1016/j.cpr.2010.11.003.
34. McGurk SR, Twamley EW, Sitzer DI, McHugo GJ, Mueser KT. A meta-analysis of cognitive remediation in schizophrenia. Am J Psychiatry 2007;164:1791–802. doi:10.1176/appi.ajp.2007.07060906.
35. Bowie CR, Gupta M, Holshausen K, Jokic R, Best M, Milev R. Cognitive remediation for treatment-resistant depression: effects on cognition and functioning and the role of online homework. J Nerv Ment Dis 2013;201:680–5. doi:10.1097/NMD.0b013e31829c5030.
36. Leppanen J, Adamson J, Tchanturia K. Impact of Cognitive Remediation Therapy on Neurocognitive Processing in Anorexia Nervosa. Front Psychiatry 2018;9:96. doi:10.3389/fpsyt.2018.00096.
37. Priyamvada R, Ranjan R, Chaudhury S. Cognitive rehabilitation of attention and memory in depression. Ind Psychiatry J 2015;24:48–53. doi:10.4103/0972-6748.160932.
38. Woo YS, Rosenblat JD, Kakar R, Bahk W-M, McIntyre RS. Cognitive Deficits as a Mediator of Poor Occupational Function in Remitted Major Depressive Disorder Patients. Clin Psychopharmacol Neurosci Off Sci J Korean Coll Neuropsychopharmacol 2016;14:1–16. doi:10.9758/cpn.2016.14.1.1.

Acceptance for editing: 2019-11-09
Acceptance for publication: 2019-12-30

REVIEW PAPER

DOI: https://doi.org/10.20883/medical.398

Education in Occupational Therapy: The Transition to the Academic Level. Changing the Professional Identity of Occupational Therapists in Switzerland

Ursula Gubler Thomann*

ZHAW Zürcher Hochschule für Angewandte Wissenschaften, Winterthur, Schweiz

* *Corresponding Autor:* email: ursula.gubler@zhaw.ch

ABSTRACT

The aim of the article is to summarise the development of the teaching and training programme for occupational therapy in the German part of Switzerland over the years 2006–2019. As the responsible program director and project manager in the transition from higher education to an academic level, the author of this article was strongly involved in changing the professional identity of occupational therapists in Switzerland. The following text presents her personal overview of this transition. The main focus lies on education, the change process and how academisation has gradually changed the curriculum in Switzerland.

Keywords: occupational therapy, Switzerland, Poland, education.

Introduction

The aim of the article is to summarise the development of the teaching and training programme for occupational therapy in the German part of Switzerland over the years 2006–2019. As the responsible program director and project manager in the transition from higher education to an academic level, the author of this article was strongly involved in changing the professional identity of occupational therapists in Switzerland. The following text presents her personal overview of this transition. The main focus lies on education, the change process and how academisation has gradually changed the curriculum in Switzerland.

Short presentation of Switzerland and a comparison with Poland

Switzerland lies in the heart of Europe and is a small country. With an area of 41,300 km^2, it is about 7.5 times smaller than Poland with its 312,700 km^2. In Switzerland, there are 26 cantons and four national languages including Swiss German, French, Italian and Romansh. Switzerland has 8.5 million inhabitants, while Poland has 38.4 million.

Poland has almost 3,000 occupational therapists (OTs) today, with about 900 of them possessing an academic degree; this includes about 30% of all OTs. In Switzerland, despite its size, the numbers are very similar. There are about 3,000

occupational therapists practicing, with 40–50% of them possessing an academic degree, and the rest, a diploma degree. The first national OT organisation in Poland was founded in 2015, while in Switzerland it was founded as early as 1956.

Brief historical review of relevant political decisions in connection with the academicisation of the occupational therapy profession

In order to gain a better understanding of career development in Switzerland, some political milestones relevant to occupational therapists are explained hereafter.

Until the 1990s, the education of non-university health professions at higher medical colleges was regulated at the cantonal level and led to a diploma degree. In 1998, with the federal decree to establish so-called "Universities of applied sciences", a change from cantonal sovereignty and legislation to national legislation took place. This means that the new regulations applied to the entire country. The aim of this new model was to open the educational system for people with an apprenticeship and to give them the opportunities to develop their competences at an academic level. The contemporary Swiss landscape of universities of applied sciences comprises seven public universities of applied sciences and a private one. The public universities of applied sciences are each supported by one or several cantons.

In 1999, the Bologna Declaration was signed. The Declaration paved the way for academicisation in health professions, including occupational therapy, physiotherapy, nursing, midwifery and other medical fields.

Universities in Switzerland and the relation to Occupational Therapy

Switzerland today has three different types of higher education institutions: Universities, the Swiss Federal Institute of Technology (ETH), and the Universities of Applied Sciences. In the canton of Zurich, where the ZHAW is situated, the options are as follows: The most well-known educational establishment is the Swiss Federal Institute of Technology (ETH), which is under federal authority and teaches 17,000 students. Then there is the University of Zurich, under cantonal authority, with 26,000 students. The Faculty of Medicine alone has about 50 institutes and clinics and teaches 2,000 students. The third type of higher education institution is called "Zürcher Fachhochschulen" (ZFH): It consists of three public institutes of higher education, the Zurich University of Teacher Education (PHZH), the Zurich University of the Arts (ZHdK), and the Zurich University of Applied Sciences (ZHAW).

Occupational Therapy is only offered at one German-speaking university of applied sciences in Switzerland, namely the ZHAW. There is another university of applied sciences in the French-speaking part of Switzerland and another one in the Italian-speaking part. Therefore, one can study the profession in three different universities of applied sciences in Switzerland. The ZHAW consists of eight departments with very different studies, for example, the School of Management and Law, the School of Architecture, Design and Civil Engineering, or the School of Applied Psychology. The School of Health Professions has existed since 2006, and consists of five institutes: The institutes for Occupational Therapy, Physiotherapy, Nursing, Midwifery, and Health Promotion and Prevention. All institutes have four areas of activity: The two areas that the government supports are the bachelor and masters degrees, while research and development are funded by third parties. The two other areas include consultancy and services as well as continuing education. The four areas of activity make one big difference in comparison to the previous system: research is situated close to education, with the idea being that the results of research are reincorporated into education.

Transition from OT education to university level and consequences for the "field"

Since 2006, occupational therapy in Switzerland has been taught exclusively at Universities of Applied Sciences. The decision for the academisation of the profession was instituted by

the government and justified with the high complexity of the professional activity, and the high responsibility (especially because occupational therapists can practise independently). The occupational therapists celebrated this decision as a success, and as recognition of the achievements of the profession. But the consequence for the two previous OT Schools in the German-speaking part was that they had to close in 2008/2009, and since then, two different levels of professional qualifications have existed simultaneously: The academic Bachelor of Science degree, and the previous diploma degree. In practice, this led to a division that caused uncertainties and major discussions about the job description, and what the political changes should bring about concretely in practice. This will be discussed in more detail later. For the teachers working at the previous OT schools, it was decided that they had to acquire a Masters degree if they wanted to apply for a position at ZHAW, therefore, the transition from OT education to university level brought along big changes for everyone involved.

In 2006, the different professional groups such as OT, PT, and Nurses jointly started their new Bachelor Degree Programmes — with new teams, at a new location, under new management and all professions consolidated. This was a huge change both in terms of team composition and location. For the students, it meant that everyone had to have a Higher School Certificate. Thus, the demands on both the trainers and the students increased at the same time. In practice, this led to many questions and uncertainties, as is often the case when change occurs. In 2006, the first Bachelor Degree Programme for Occupational Therapy at ZHAW included 72 students. After accreditation in 2008, the curriculum was refined and established in 2012. Currently, the third bachelor curriculum is being developed.

First phase of Curriculum Development in Occupational Therapy from 2005–2007: an overview of the challenges and opportunities

Hereafter, the changes and selected challenges and opportunities related to the process of academisation, the structures, and the contents will be outlined. Challenges and opportunities are often intertwined — therefore, both will be addressed in the following remarks.

Challenges and opportunities related to the developmental process from 2005–2007

The start of the curriculum development in the year 2005/2006 was especially demanding: A project team was put together to develop the curriculum, consisting of employees of the previous schools and new colleagues. The first appointments were made in the course of 2006. Some of the major points of discussion that emerged included:

«Academic degrees are expected from teachers»

At the previous OT schools, very few employees had an academic degree. They were all experienced practitioners with a further education in pedagogy. However, an academic degree was now required from teachers by the university of applied sciences. It was clear that a Master's degree was expected, but not exactly what kind of degree it had to be. Additionally, it was not yet possible to acquire such a degree in Switzerland. So the teachers went to Austria for a Master's degree in Neurology Sciences, or did an international Master's degree in OT or in education. The costs for a Master's degree amounted to approximately 20,000 to 25,000 Euros. The majority of these costs were born by the employees themselves. The graduation was one thing, but the question of which skills and which knowledge were required was unsettling because they were not defined in detail at that point.

«To develop the collaboration of a new team coming from different countries in a new organisation with a new head»

The project team included experienced teachers and members of the previous schools. This was of advantage on the one hand, since didactic knowledge and teaching experience was brought to the collaboration. But, most of the team members did not have an academic education or training, which on the other hand was a challenge. The developing of an academic socialisation, i.e. a common understanding of what it means to

teach at university level, was a challenge for the team in the beginning. Additionally, a new head of the Institute of Occupational Therapy was chosen from a neighbour country, and new colleagues from neighbour countries were employed, which meant a variety of other challenges concerning team building: Learning about the different cultural backgrounds, and at the same time defining processes and rules in a newly founded big organisation.

«To build collaborations with fieldwork practitioners»

Almost a quarter of the bachelor programme in Occupational Therapy takes place in the form of internships. Another challenge was therefore to recruit partners for the skill-trainings during the fieldwork. Most of the practice partners did not have an academic degree, and they were not used to scientifically working with OT-theories and models. In the beginning, some of them were critical of working together with ZHAW. Additionally, they feared that the students would be educated too much in theory and not in practical skills, and they were unsettled about what the future would bring for their own positions.

«To implement new structures because of the Bologna system»

Due to the annual university structure, there was less teaching time available and so-called modules with ECTS points had to be created, which was a new system for everyone. This meant that many pedagogical and didactic decisions had to be made, defining new forms of teaching and learning according to the Bologna System.

«To decide on new content such as evidence based practices and scientific research for the new curriculum as a team»

First of all, how the academic curriculum differed from the previous one was defined. The different (academic) backgrounds led to huge discussions on what was actually important. For example, the new head decided to install an American framework to structure the OT contents systematically. But this structure was new for the team and thus time was needed to develop a common understanding, and for internal trainings. An additional topic of discussion was the imparting of skills training in the different fields; now being justified not only with experience but also with scientific knowledge. It was therefore not sufficient to teach a procedure just because it had been taught before — the team had to create new contents and procedures in relation to teaching. Every content reform needed time. Additionally, the students of course knew immediately when teachers felt unsure, which led to other incriminating challenges in the everyday life of the teachers at that time. The alterations thus often led to uncertainty in the role of the teachers.

It was decided that one of the first things to be done was to define the competences the students should have at the end of their studies, which meant that interprofessional modules for the basics in scientific working and communication skills had to be offered. A starting point for the curriculum was thus to work together with other health professions. Thus, a common understanding of academic workings was developed for the health profession, which retrospectively made sense, because the imparting of knowledge about qualitative and quantitative research was a big addition content-wise to the curriculum.

«Offering a post graduate programme for OT practitioners without a Bachelor's degree»

It was clear from the beginning that a postgraduate programme for OTs that did not have a Bachelor's degree had to be offered. This was a governmental decision to bridge the gap between the previous and the new education. The postgraduate programme was a successful model for many years and was visited by many graduate occupational therapists. They also received the title "Ergotherapist BSc".

Visible academisation related to the first curriculum structure 2006

In 2006, the first structure of the curriculum contained 49 modules within a three year program, 11 of these modules being interprofessional ones, and three being fieldwork modules. "New" in the academic sense were the modules "Scientific Work", "Bachelor Thesis", and "Theories and Models of Occupation Therapy". The names of

the other modules were — from today's point of view — rather conservative: The students started with the basics in anatomy and physiology. The first academic curriculum was therefore a mix between the old curricula and theory concerning Occupational Therapy.

New and previous contents in the first academic OT curriculum 2006

The following decisions concerning the content of the curriculum were made: New modules containing basic scientific knowledge had to be created. For these modules, the students were put in interprofessional mixed groups for the first semesters. Then, OT theory and several different OT Models (MOHO, COPM, Bieler Modell (the only Swiss Model), KAWA etc.) were taught. Didactic decisions included to work case-based, theory-based, evidence-based, problem-based and fieldwork experience-based. A further challenge was that all teachers were expected to refer to theories, and if possible to research and evidence. Instead, because good evidence could not be found for all research fields, the lecturers often referred to their own experience in the beginning. The traditional focus was put on geriatrics, paediatrics, orthopedics, vocational rehabilitation, neurology and mental health, because these are the fieldworks in practice. In general, it was a mix between the core strength of the two German-speaking schools and other international curricula. Traditional western European module titles manifested the gaps between theory and practice: Titles such as «OT in geriatrics» and «OT Models and Theory» made that gap visible. The content that was decided on was in many ways a compromise of the new team.

Insights after the first phase 2006–2009

The challenges and solutions described above required a lot of flexibility and openness from everyone involved, which was demanding. From today's point of view, content alterations in the first curriculum were only partially possible, since team building and orientation within the new institution took a lot of time and energy. Therefore I conclude: too many challenges at the same time are difficult to handle. To become a team is very important, and you need to take enough time and energy for this process.

Furthermore, it is important to consider the characteristic of one's own country: adopting models and frameworks without adaptation from other universities may not be suitable for one's own situation. Occupational Therapy is also funded differently, depending on the country. In Switzerland, it is exclusively health insurance that pays for it; Occupational Therapy is prescribed solely by doctors and takes place mostly in a medical environment. For the curriculum, these circumstances need to be considered. To develop a common picture of the goal is also very important: What kind of scientific knowledge is expected for the Bachelor level, and why? What does evidence-based practice mean? What is our core task within our medical system? Additionally, the practitioners were not involved enough in the process of developing the curriculum. Looking back, this should have been done as early as possible.

Infrastructure as a base for good teaching

The start of the occupational therapy bachelor programme was a temporary arrangement: there were rooms available for offices and lessons in various buildings on the ZHAW campus. This was experienced as partially demanding, since a lot of time was needed to change rooms. And yet: the experts were allowed to teach and work in beautiful and well-equipped rooms, which was appreciated. In 2008, the health departments of ZHAW moved into a new big building created specifically for them. From then on, the collaboration was easier because everyone involved worked in the same place.

First accreditation in 2008 and a new national project for a common understanding of competences of the Bachelor programme in OT

In the same year, an external group of official experts conducted the accreditation of the occupational therapy bachelor programme. The recommendations they had were the following:

- the schedule for the OT students included too many contact hours: a bachelor programme should offer more space for self-studies and self-directed learning,
- because there were too many small modules, the OT students were expected to do too many examinations (since every module had to lead to an exam and a grade in the university system).

At the same time, a national project for all health professions started: Led by the national office for health professions, all Bachelor programmes were asked to define competences for the students at the end of their studies ("Abschlusskompetenzen"). For this cause, the author was asked to be project manager and to bring all three OT universities of applied sciences in Switzerland together to define these aims together. The idea was to work on a national law for health professions, which will be implemented in 2020. This project strengthened the professional identity by working together nationally. There were a lot of discussions in all three languages, which supported a common understanding and picture for the profession of Occupational Therapy in Switzerland for the future.

Second phase of Curriculum Development from 2008 to 2012: an overview of challenges and opportunities

Challenges and opportunities related to the process from 2008 to 2012

The next phase of the curriculum development began, triggered by accreditation and the defined new competencies. The most important points of this phase were the following:

«To implement a more occupation-based paradigm and new competences»

Motivated by the recommendations from the accreditation group, it was decided to create a more modern and more occupation-based OT curriculum. This meant to focus more on occupation and less on diagnoses and illnesses. Firstly, a detailed analysis of the actual curriculum was done and discussed with the whole team and external experts. Together, it was decided to create a new second curriculum and therefore to take the next steps in our professionalisation process, and to implement the new competences. For the team, this meant a lot of additional work, but most of them showed big motivation because of the participative process in which they were involved. For others, it was too much of a change and they unfortunately left.

«To create new didactic concepts, for example an OT-Skill-Concept and a big self-directed project to enable people in occupation»

Also, the didactic approaches were modernised. One decision for example was to create an «OT-Skill-Concept» (on how to teach the important occupation therapy skills) and to strengthen the fieldwork practice in the students' examinations. Another decision was to offer a new self-directed-project with the aim of enabling people in occupation. The students could choose to engage either in a medical or in an emerging OT-field.

«To learn the basic medical knowledge in a more self-directed way»

The discussion about the medical knowledge was challenging: What was the minimum that OTs need to know? And what was the appropriate form of teaching? It was decided that students had to learn the basics themselves, and that anatomy and pathology would not be taught in plenary sessions anymore. Simultaneously, lessons for questions and repetitions were offered.

«To change the role of the teachers: From a teaching to a supporting, accompanying role»

If the students were to study in a more self-directed way, and create their own projects, the role of the teacher changes as well: Accompaniment and consultation increase, while instructing decreases. The students are trusted and given more responsibility. This leads to a changing understanding of one's role as a teacher, which relies on reflection, and a conscious shaping of the changing role.

«To bridge the gap between education and practice»

The shift between theoretical and practical education and students' occupation in fieldwork grew within that time frame. The interventions in practice were not often occupation-based, but functional because of the medical system and specific backgrounds. Therefore, it was decided to sensitise both lecturers and practitioners to a more occupation-based occupational therapy. By encouraging

communication with practitioners, joint congresses were conducted and knowledge about each other and the different forms of work increased.

Visible academisation related to the second curriculum structure 2012

It was decided to create fewer modules, and bigger modules with more creditpoints per module instead. A new structure was chosen: Firstly, the modules were named more occupation-based, for example «occupation with children» or «enabling occupation with the elderly». Secondly, mutual occupation-based topics were taught independently of diagnoses, for example ADL. But, diagnoses such as apoplexy or rheumatoid arthritis were treated with different foci with children, adults or elderly people. The previous working fields remained, but were slightly less prominent. In addition, it was decided to offer the modules over a defined period of time, e.g. 2–4 weeks, and then conclude them with a certificate of achievement and no longer run all of the modules during the whole semester. Therefore, the examinations were distributed evenly at the end of the semester, which made it possible to work more topic-oriented and focus on one theme over a defined period of time.

New and previous contents in the second curriculum 2012

The second curriculum in 2012 was based on the new national competencies referred to as CANMED. It was also decided to work with the OTIPM Occupational Therapy Intervention Process Model developed by Anne Fisher. The idea was to teach the students occupation-based from the beginning. A different content structure was chosen: in the first year, the focus lied on the micro level (meaning on the client and the therapist), in the second year, on the meso level (the client, the therapist and the environment/organisation), and in the third year, on the macro level (the client in the society). Also, Occupational Science became more important as module content.

Some of the most important points were:

«New didactic concepts: more e-learning and blended-learning for medical knowledge and to teach more exemplarily»

We developed e-Learning tools for anatomy and pathology, and other basic medical content such as, for example developmental psychology, because of the conviction that learning the basics must be done by the students themselves, who are well-prepared and accompanied. Thus, the lessons were used more and more for the teaching of practical content, where e.g. presenting and explaining is indispensable (e.g. practicing a transfer, guiding a group, etc.).

«To implement a concept to accompany students and reflect how and what they learn together»

It was decided to give the students more control over their learning, while at the same time a student support concept was developed. Each student was assigned to a group of approximately seven persons and received a mentor for the entire period of study. Together with the mentor and the fellow students, a regular reflection on the acquisition of competence was stimulated. This took place individually and in groups, in writing as well as orally. Training reflective faculties as well as giving and receiving feedback are important competences that are demanded in everyday OT work, and that promote one's own professionality in the sense of reasoning.

«To discover emerging fields and experience the topic of "enabling occupation" in real life as much as possible»

It was also decided to create a new module called «Projektwerkstatt» (workshop) where students learned to plan and create their own projects to support clients in enabling occupations. Thereof came to be many different projects, for example «how to bring elderly people and children together to play and cook» or doing sports with people that cannot do it themselves. These projects took place in practice, and we thus expanded the cooperation with our practice trainers, which was experienced very positively. These new fields were in no way in competition with the existing ones. Moreover, it was about expanding the sphere of OT activity wherever possible and useful, to make the occupation more known.

Learnings after the second phase from 2008 to 2012

The biggest change of the second curriculum was conferring more importance to the occupation

focused attitude. At that time, the essentials of academisation, a common comprehension, and the new contents were already resolved. The team building had progressed, therefore new changes of the curriculum were possible. The decision to put the occupation in the foreground was probably the most important. Most teachers did not have their own practice with patients at the time, which made it a theoretical construct at first that needed time to be established. This focus proved to be a positive one, but needed time to be taught with head, heart and hand. The comprehension of learning had developed and thus reducing content and working more exemplarily was important to create space for new content (such as a project about self-learning) as well as trusting the students to connect related topics in the future.

Bringing together similar occupation-focused themes, for example «ADL», independent from diagnoses but focussed on the age of life, was another good decision to avoid duplication in class. In these years, research in OT grew throughout the whole world, including Switzerland. The teachers were now used to working with research knowledge from the OT profession much more. This was a big step in professionalisation and in working evidence-based. The teachers themselves were now also in a position to use the literature as a base for examples for the preparation of the skills-training instructions.

Outlook: Curriculum 2020

In 2018, the team started to develop a third curriculum. With an increase of students and an approaching relocation to a new and bigger building, the students should be able to study and practice in an even more personalised way. Specifically concerning the OTs, there is now a discussion about the WFOT Minimal Standards that focus on the macro level, and on social transformative activities (WFOT, 2016). Since there will be many more elderly people in the future, this will influence the daily occupations of people, and require projects or services to be developed with the people affected. The aim of the discussion is not only for personal development, but for wider social change (ENOTHE is supporting a project in this field as well).

In the future, there will not be enough OTs for the hospitals and other medical institutions. Now, the aim is to find a way that the current situation in the world and in our country can be thematised publicly without weakening the situation of the job market. Other points are: The interprofessional collaboration will be extended because it is becoming more highly valued in our hospitals. Therefore, it was decided to strengthen the focus of collaboration in our professions, and to work more cased-based and closer to the reality of the situation in the fieldwork. It is also planned to extend blended learning activities and self-directed learning because more students are allowed in the new building. Therefore, the time when students are all together in the building needs to be reduced, and more self-directed learning is offered — as can be seen, there is always a compromise between pedagogical thinking and reality.

Conclusions

After more than 13 years, it seems that professionalisation is a long-lasting process and includes much more team-development than expected. Teaching evidence and theories is very important and a big part of academisation. Occupational therapists must apply this knowledge, and work with it in practice. A challenge in the Swiss process was that OTs with the previous diplomas became scared that they were not doing a good job anymore — they lost self-confidence. Therefore, it is very important to bring together old and new knowledge and support one another, which necessitates not a competition between academics and practitioners, but teamwork. And if this is possible, the OT profession will be successful in the future as well. Research is another important part of academisation: at a university of applied sciences, it is important that the research takes as its point of departure questions which arise from practice, and that the results constitute feedback for practice. It would be ideal if the results could also impact teaching directly. This works especially well if teachers are involved in research activities. To date, this has only been possible to a limited extent at the ZHAW.

Professional identity is dependent on particular country, and on what is expected there from the professionals. It is not something theoretical, it is living in the everyday OT work. Every country has its own history. This influences the profes-

sional development, and therefore the compatriots can estimate best how the profession should develop further. Ideas from other countries are certainly helpful, but should not simply be adopted without taking the context into account. WFOT standards are supporting as stimulation, and ENOTHE is also a very good place to share and discuss these themes, for example at the annual meetings.

To conclude: the level of education of OT professionals should eventually be the same, with one system across the whole country. In this way, it will be easier to develop the profession together.

Acknowledgements

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

Funding sources
There are no sources of funding to declare.

References

1. Frank JR. (Ed.) The CanMEDS 2005 physician competency framework. Better standards. Better physicians. Better care. Ottawa: The Royal College of Physicians and Surgeons of Canada; 2005.
2. Gubler Thomann U. Partizipative Diagnose organisationskultureller Veränderungen im Rahmen eines Transformationsprozesses, Masterarbeit MAS Supervision und Organisationsberatung and der Pädagogischen Hochschule St. Gallen und Akademie für Erwachsenenbildung Schweiz. 2008.
3. Le Granse M, van Hartingsveldt M, Kinébanian A. (Hrsg.) Grundlagen der Ergotherapie. Stuttgart: Thieme; 2019.
4. Oevermann U. Theoretische Skizze einer revidierten Theorie professionalisierten Handelns. In: Combe & W. Helsper (Hrsg.). Pädagogische Professionalität, Untersuchungen zum Typus pädagogischen Handelns. Frankfurt a.M.: Suhrkamp; 1999. p. 70–182.
5. Internal Documents. BSC in Ergotherapie, Curriculum 2012 (Kneisner, M., Jakobs U., Hansen H.). 2012.
6. WFOT Minimal Standards, https://www.mailmens.nl/files/21072349/copyrighted+world+federation+of+occupational+therapists+minimum+standards+for+the+education+of+occupational+therapists+2016a.pdf.

Acceptance for editing: 2019-11-09
Acceptance for publication: 2019-12-30

REVIEW PAPER

DOI: https://doi.org/10.20883/medical.402

Effect of vitamin K supplementation on anthropometric parameters and adipokine levels – a systematic review

Małgorzata Jamka[a], Harald Walach[b], Magdalena Hołubiec[c], Maria Wasiewicz[d], Jarosław Walkowiak[e,*]

Department of Pediatric Gastroenterology and Metabolic Diseases, Poznan University of Medical Sciences, Poland

* *Corresponding Autor:* Jarosław Walkowiak, MD, PhD; Department of Paediatric Gastroenterology and Metabolic Diseases, Poznan University of Medical Sciences, 27/33 Szpitalna Street, 60-572 Poznan, Poland; phone: +48618491432; fax: +48618472685; email: jarwalk@ump.edu.pl

[a] https://orcid.org/0000-0002-0257-6180
[b] https://orcid.org/0000-0003-4603-7717
[c] https://orcid.org/0000-0001-7627-9157
[d] https://orcid.org/0000-0002-7084-663X
[e] https://orcid.org/0000-0001-5813-5707

ABSTRACT

Aim. The aim of this systematic review was to assess the effect of vitamin K supplementation on anthropometric parameters and adipokine levels in adults.
Material and Methods. Four databases (PubMed, Web of Sciences, Scopus and the Cochrane Library) were searched to select studies in which the effect of vitamin K supplementation on body weight, body mass index (BMI), fat mass, leptin and adiponectin levels were assessed.
Results. We identified nine studies that included a total of 542 subjects. Vitamin K supplementation did not influence body weight, BMI and percentage of fat mass. In addition, the effect of vitamin K supplementation on adipokines levels was equivocal.
Conclusions. Vitamin K supplementation did not affect anthropometric parameters and adipokines levels. Nevertheless, further studies are needed to clarify the effect of vitamin K supplementation on these parameters in adults.

Keywords: vitamin K, dietary supplements, body weight, leptin, adiponectin.

Introduction

Vitamin K is a fat-soluble vitamin that occurs in two forms: phylloquinone (vitamin K_1) and menaquinone (MK; vitamin K_2). Phylloquinone is synthesised by green vegetables such as spinach, broccoli, cabbage and brussels sprouts, whereas MK is produced by bacteria and occurs mainly in fermented food products like cheese and curd, as well as in animal products such as meat and eggs [1, 2].

Historically interest in vitamin K has focused on its role in haemostasis [3] and bone health [4]. Recently, much more attention was paid to the role of vitamin K in cardio-metabolic disorders [5, 6]. Several epidemiological studies have shown that higher vitamin K intake was associated with improved glycaemic status and insulin homeo-

stasis [7, 8]. However, these findings are in contrast to the results of randomised controlled trials (RCT) [9, 10]. On the other hand, a significant association between high vitamin K intake and a reduction in coronary heart disease was found [11, 12]. Limited evidence from human studies also suggests that vitamin K might improve blood lipid profile [13, 14]. Indeed, Braam et al. [13] observed that higher phylloquinone intake was associated with lower serum triglyceride (TG) concentrations. Moreover, Koitaya et al. [15] showed that MK-4 supplementation significantly decreased high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) levels. Additionally, the Framingham study observed that high serum vitamin K concentrations were associated with lower levels of inflammatory markers, which suggest a potential role of vitamin K in suppression of chronic inflammation [16], which is associated with the development of many metabolic disturbances, such as type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVD) [17, 18].

Recently, some evidence for a link between vitamin K, body mass and adipokine levels were found [19, 20]. Studies on an animal model showed that long-term supplementation of vitamin K_1 and MK-4 might reduce fat accumulation [19]. In humans, Knapen et al. [20] suggested a beneficial effect of vitamin K on fat metabolism. On the other hand, Shea et al. [21] did not find an association between vitamin K supplementation and changes in body weight and body composition.

Therefore, the aim of this systematic review was to assess the effect of vitamin K supplementation on anthropometric parameters and adipokine levels in adults.

Methods

Search strategy

PubMed, Web of Sciences, Scopus and the Cochrane Library databases were searched between October and November 2019 using the following medical subject headings terms (Mesh) and equivalent: *"vitamin K OR vitamin K_1 OR vitamin K_2 OR vitamin K_3" AND "dietary supplements" NOT "animals"*. No time limitations were applied in searching the databases. In addition, reference lists of retrieved articles were scanned for searching any missed studies that met the inclusion criteria. Before starting the review process, the systematic review protocol was prepared and registered with PROSPERO under the registration number: CRD42017079368 [22].

Study selection

Experimental studies: RTCs, non-randomized controlled trials and uncontrolled trials (UCT) in English and analysing the effects of vitamin K supplementation (as a single supplement) on anthropometric parameters and adipokine levels at least for 3 weeks in adults were included in this study. Eligible studies must have reported at least one of the following outcomes: body weight, body mass index (BMI), serum leptin and adiponectin concentrations. Any vitamin K or vitamin K analogue intervention was eligible because we were looking for a class effect. Co-administration with other dietary supplements was not allowed. We included studies that were conducted on healthy adults worldwide, as well as subjects with a history of cardio-metabolic diseases (e.g., hypertension, hyperlipidaemia, prediabetes, T2DM or previously diagnosed CVD (defined as documented myocardial infarction, coronary revascularisation, previous confirmed ischaemic stroke, or peripheral vascular disease defined as claudication symptoms with angiographically proven arterial stenosis or ankle-brachial pressure index of < 0.7)). There were no restrictions based on gender, body weight, the ethnicity of study participants, location of study or sample size.

Quality assessment

Two investigators (JW & MJ) evaluated each article independently in three main stages of the extraction process (**Figure 1**). First, the reviewers screened article titles, then abstracts and finally full texts for eligibility for inclusion in the systematic review. Disagreements were resolved by discussion between the reviewers until a consensus was reached. All reviewers agreed on the final decision of the studies to be included. Primary authors of relevant articles were contacted directly if the data sought were unavailable or published only in abstract form. For a quality assessment of included publications, the checklist derived from the *"Standard quality assessment criteria for evaluating primary research papers from a variety of field"* described by Kmet et al. [23] was used.

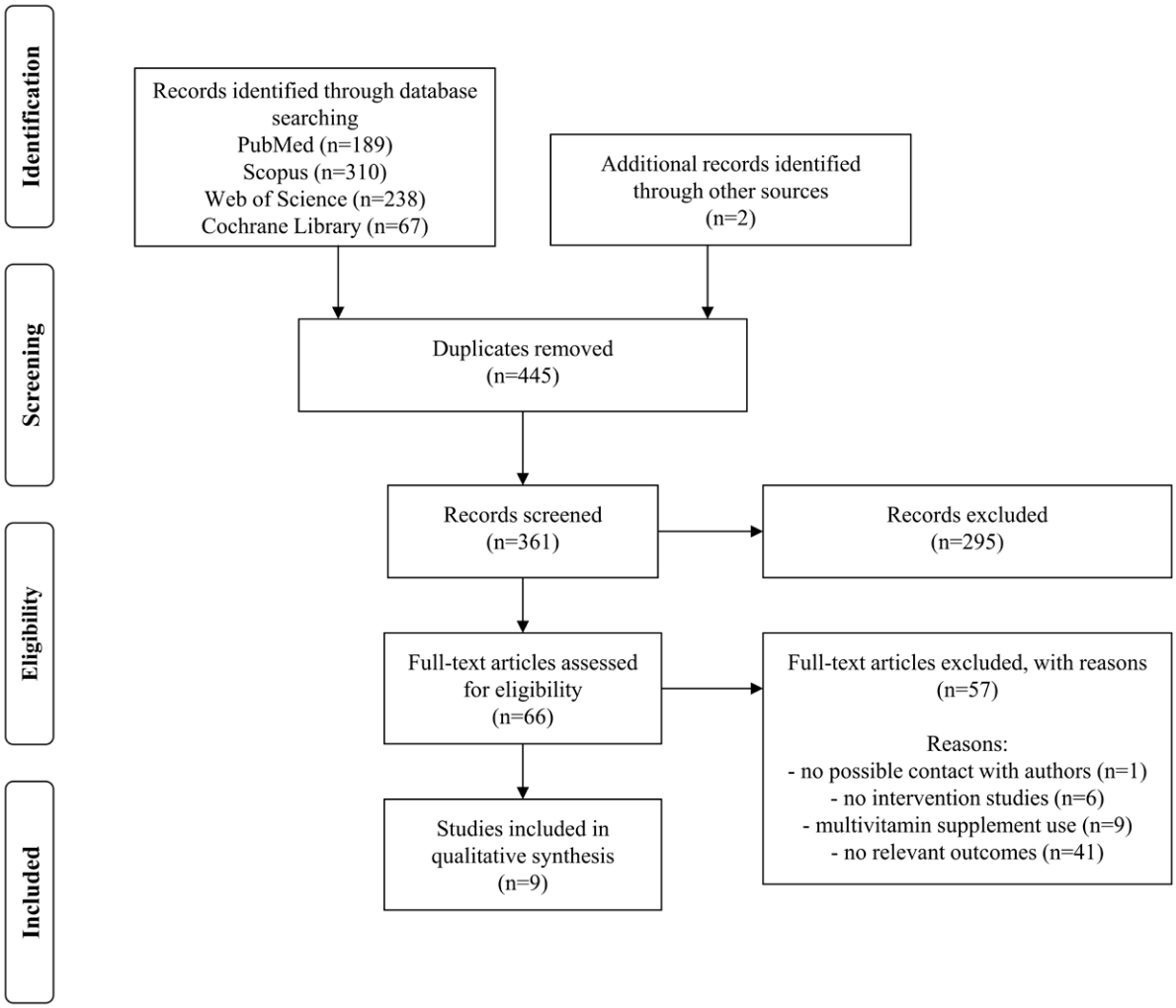

Figure 1. Process of the search

Data extraction

Eligible studies were reviewed and the following data were abstracted: 1) first author's name; 2) year of publication; 3) country; 4) study design and method of blinding; 5) type of intervention (form and dose of vitamin K, route of administration and duration of intervention); 6) number of participants; 6) characteristic of study participants (age, sex, origin and vitamin K intake); 7) baseline and post-intervention serum concentrations of vitamin K_1, menaquinone-4 (MK-4), menaquinone-7 (MK-7), uncarboxylated osteocalcin (ucOC); 8) outcomes: baseline and post-intervention body weight, BMI, serum leptin and adiponectin concentrations.

Statistical analysis

Data were presented as means ± standard deviations (SD). A p value less than 0.05 was considered to be statistically significant. Because of the high heterogeneity of the included studies, the synthesis in the form of meta-analysis was not performed. Results from individual studies were dealt with descriptively.

Results

Study selection

The search results are presented in Figure 1. We retrieved 189 records from the PubMed, 310 from the Scopus, 238 from the Web of Sciences and 67 from the Cochrane Library databases. Two additional references were identified from searching reference lists of the included papers. We identified and removed 445 duplications, leaving a total of 361 records. Initial screening of the title and abstract resulted in the exclusion of 295 references

leaving 66 articles to source in full text. After further inspection, we excluded 57 papers. The full text was not available for one study and contact with authors was not possible. Six papers were excluded because they were not intervention studies. Nine studies did not meet the inclusion criteria as they used a multivitamin supplement. The remaining 41 papers were excluded as they did not report relevant outcomes. Eventually, nine articles met the inclusion criteria and were further analysed [14, 15, 20, 24–29], one of which two references were related to the same population and the same intervention but reported on different outcomes [25, 26].

Study characteristics

The characteristics of the included studies are presented in **Table 1**. All papers were published between 2008 [29] and 2016 [24]. Eight studies were designed as RCTs [14, 15, 20, 24–26, 28, 29] and one paper was designed as UCT [27]. Five studies were conducted in Asia [14, 15, 25–27], three studies were performed in Europe [20, 24, 28] and one study was conducted in North America [29]. MK-4 supplementation was used in four studies [14, 15, 20, 27]. Doses of MK-4 ranged from 300 µg/d [27] to 45000 µg/d [20]. MK-7 was supplemented in three studies [20, 24, 28]. The dose of MK-7 ranged from 10 µg/d [20] to 360 µg/d [20, 28]. Vitamin K_1 was supplemented in two studies in dose from 600 µg/d [29] to 1000 µg/d [25, 26]. The time of intervention period in all included studies ranged from 4 weeks [25, 26] to 26 weeks [29].

Population characteristics

The baseline characteristics of the study populations are presented in **Table 2**. In all, 542 subjects were included in nine studies [14, 15, 20, 24–29]. The study size varied from 15 [27] to 164 [20] participants. The mean age of the study participants ranged between 25 years [27] and 76 years [24] in the intervention groups and varied from 37 years [29] to 77 years [24] in the control groups. The majority of the studies included only female participants [14, 15, 25, 26, 29], while one research was performed only in men [27]. Five papers included only Asian participants [14, 15, 25–27], while four studies were conducted in Caucasian subjects [20, 24, 28, 29]. At baseline, vitamin K intake was reported in seven papers [14, 15, 25–29]. In most studies [14, 15, 28, 29], vitamin K intake was higher than the recommended daily intake (RDI) in the adult population [30]. In the intervention groups, vitamin K intake ranged from 40.18 µg/d [25] to 243.20 µg/d [29], while similar values were observed in the control groups.

The effects of vitamin K supplementation on vitamin K status

The effect of vitamin K supplementation on vitamin K_1 levels was analysed in three studies [14, 15, 27]. One study reported that MK-4 supplementation significantly increased serum concentrations of vitamin K_1, however, there were no differences between the intervention group and the control group [15]. On the other hand, Nakamura et al. [27] observed a statistically signifi-

Table 1. Characteristics of included studies

Main author	Year	Country	Study design	Subjects (n)[a]	Intervention	Dose of vit. K (µg/d)	Time of intervention (week)
Fulton et al. [24]	2016	Scotland	RCT[b]	77	MK-7	100	26
Rasekhi et al. [25, 26]	2015	Iran	RCT[b]	82	Vit. K_1	1000	4
Koitaya et al. [14]	2014	Japan	RCT[b]	48	MK-4	1500	52
Nakamura et al. [27]	2014	Japan	UCT[c]	15	MK-4	0–1500[d]	5
Dalmeijer et al. [28]	2012	The Netherlands	RCT[b]	60	MK-7	180 or 360[e]	12
Knapen et al. [20]	2012	The Netherlands	RCT[b]	42	MK-7	0–360[f]	12
				164	MK-4	45000	156
Koitaya et al. [15]	2009	Japan	RCT[b]	40	MK-4	1500	4
Volpe et al. [29]	2008	USA	RCT[b]	14	Vit. K_1	600	26

[a] – Number of subjects who completed the study
[b] – Randomized Controlled Trial
[c] – Uncontrolled Trial
[d] – Subjects received MK-4 daily for 5 weeks at 0, 300, 600, 900 and 1500 µg/d in weeks 1, 2, 3, 4, and 5, respectively
[e] – Subjects received 180 µg/d or 360 µg/d MK-7 or placebo
[f] – All participants were randomised into seven groups of six subjects each. Each group received a daily supplement containing either 0, 10, 20, 45, 90, 180 or 360 µg/d MK-7 for 12 weeks

Table 2. Characteristics of subjects (n = 528)

Study	Group	Subjects (n)[a]	Age (years)		Sex (% of women)	Race / Ethnicity (%)	Vitamin K intake at baseline (μg/d). Mean ± SD
			Mean ± SD	Range			
Fulton et al. [24]	Intervention group[b] Control group	38 39	76 ± 4 77 ± 5	≥ 70	47% 42%	Caucasian — 100%	N/A
Rasekhi et al. [25, 26]	Intervention group[b] Control group	39 43	40 ± 5	22–45	100%	Asian — 100%	62.69 ± 15.45[25] 60.94 ± 14.13[26] 57.16 ± 19.03[25] 55.55 ± 17.73[26]
Koitaya et al. [14]	Intervention group[b] Control group	24 24	58 ± 4	50–65	100%	Asian — 100%	166.00 ± 81.00 182.00 ± 88.00
Nakamura et al. [27]	Intervention group[b]	15	25[c]	20–29	0%	Asian — 100%	40.18 (0.00–540.27)[c]
Dalmeijer et al. [28]	Intervention group[bd] Intervention group[be] Control group	22[d] 18[e] 20	59 ± 3[d] 60 ± 3[e] 59 ± 3	40–65	54%[d] 67%[e] 60%	Caucasian — 100%	179.00 ± 136.00[f] 24.70 ± 16.50[g] 191.00 ± 167.00[f] 23.50 ± 22.70[g] 203.00 ± 159.00[f] 26.00 ± 18.70[g]
Knapen et al. [20]	Intervention group[bh] Intervention group[bi] Intervention group[bj] Intervention group[bk] Intervention group[bd] Intervention group[be] Control group	6[h] 6[i] 6[j] 6[k] 6[d] 6[e] 6	28 ± 7[lm] 27 ± 7[no]	25–45	52%	Caucasian — 100%	N/A
	Intervention group[bp] Control group	89[n] 75	66 ± 6[p] 65 ± 6	55–75	100%		
Koitaya et al. [15]	Intervention group[b] Control group	20 20	60 ± 3 59 ± 4	53–65	100%	Asian — 100%	233.00 ± 114.00 285.00 ± 223.00
Volpe et al. [29]	Intervention group[b] Control group	8 6	35 ± 8 37 ± 10	25–50	100	Caucasian — 100%	243.20 ± 174.60 380.50 ± 200.20

[a] — Number of subjects who completed the study
[b] — Group receiving vit. K supplementation
[c] — Median (range)
[d] — Group received 180 μg/d MK-7
[e] — Group received 360 μg/d MK-7
[f] — Vit. K_1
[g] — Vit. K_2
[h] — Group received 10 μg/d MK-7
[i] — Group received 20 μg/d MK-7
[j] — Group received 45 μg/d MK-7
[k] — Group received 90 μg/d MK-7
[l] — Men
[m] — n = 20
[n] — Women
[o] — n = 22
[p] — Group received MK-4
N/A — not available

cant decrease of vitamin K_1 levels, and one other study showed no effect of MK-4 supplementation on serum vitamin K_1 concentrations [14].

Three studies analysed the effect of MK-4 supplementation on MK-4 levels. All studies noted that MK-4 supplementation increased MK-4 levels [14, 15, 27].

The effect of MK-4 supplementation on MK-7 levels was assessed in two studies, in which a non-significant decrease in serum MK-7 concentrations was found [14, 27].

Five studies analysed the effect of vitamin K supplementation on ucOC levels [14, 15, 20, 25, 27]. Rasekhi et al. [25] reported that vitamin K_1 supplementation significantly decreased ucOC concentrations, while no effect was observed in the control group. Similarly, a decrease in ucOC levels was shown after MK-4 supplementation [14, 15, 20, 27]. The same effect was noted by Knapen et al. [20] when MK-7 supplementation in dose from 90 μg/d to 360 μg/d was used (**Table 3**).

Table 3. Changes in serum concentrations of vit. K_1, MK-4, MK-7 and ucOC (ng/ml) during the intervention period in the intervention and the controls in selected studies

Study	Intervention (dose of vit. K (µg/d))	Group	n[a]	Vitamin K_1 (ng/ml) Mean ± SD		MK-4 (ng/ml) Mean ± SD		MK-7 (ng/ml) Mean ± SD		ucOC (ng/ml) Mean ± SD	
				Pre-intervention	Post-intervention	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
Rasekhi et al. [25]	Vit. K_1 (1000)	Intervention[b]	39	N/A		N/A		N/A		5.57 ± 2.34	2.47 ± 1.91[#*]
		Control	43							4.77 ± 2.49	4.79 ± 2.43
Koitaya et al. [14]	MK-4 (1500)	Intervention[b]	24	0.58 ± 0.54	0.35 ± 0.15[c] 0.40 ± 0.30[d]	0.10 ± 0.00	0.47 ± 1.03[c] 0.29 ± 0.18[d*]	4.08 ± 7.53	1.47 ± 2.02[c] 2.39 ± 2.71[d]	6.40 ± 2.70	2.80 ± 1.00[c*#] 3.60 ± 1.50[d*#]
		Control	24	0.45 ± 0.33	0.36 ± 0.29[c#] 0.39 ± 0.26[d]	0.10 ± 0.00	0.10 ± 0.00[c] 0.10 ± 0.00[d]	4.11 ± 5.05	3.87 ± 5.43[c] 3.24 ± 3.39[d]	5.70 ± 3.00	4.20 ± 2.10[c#] 4.90 ± 2.30[d#]
Nakamura et al. [27]	MK-4 (0–1500)	Intervention[b]	15	0.35 (0.22–0.85)[e]	0.20 (0.08–0.49)[e#]	0.14 (0.10–0.17)[e]	0.58 (0.33–1.78)[e#]	0.43 (0.10–7.92)[e]	0.25 (0.13–0.93)[e]	5.03 (2.20–23.03)[e]	6.77 (1.89–14.52)[ef] 4.82 (1.84–8.73)[eg#] 2.98 (1.27–6.90)[eh#] 3.92 (1.88–7.52)[ei#]
Dalmeijer et al. [28]	MK-7 (180)	Intervention[b]	22	N/A		N/A		N/A		2.40 ± 1.83	N/A
	MK-7 (360)	Intervention[b]	18							2.63 ± 1.64	
		Control	20							2.67 ± 1.40	
Knapen et al. [20]	MK-7 (10)	Intervention[b]	6	N/A		N/A		N/A		2.20[j]	3.40[j]
	MK-7 (20)	Intervention[b]	6							5.10[j]	5.20[j]
	MK-7 (45)	Intervention[b]	6							3.70[j]	3.80[j]
	MK-7 (90)	Intervention[b]	6							4.60[j]	3.10[j#]
	MK-7 (180)	Intervention[b]	6							6.30[j]	3.40[j#]
	MK-7 (360)	Intervention[b]	6							2.40[j]	1.00[j#]
		Control	6							4.00[j]	4.60[j]
	MK-4 (45000)	Intervention[b]	89	N/A		N/A		N/A		3.20 ± 1.90	-2.40 ± 1.60[k*]
		Control	75							3.00 ± 1.60	-0.04 ± 0.10[k]
Koitaya et al. [15]	MK-4 (1500)	Intervention[b]	20	0.60[j]	0.78[jl#] 0.70[jm]	0.10[j]	2.00[jl#] 1.10[jm*]	N/A		3.50[j]	2.20[jl*#] 2.20[jm*#]
		Control	20	0.70[j]	0.68[jl] 0.60[jm]	0.10[j]	0.10[jl] 0.10[jm]			5.10[j]	6.00[jl] 5.80[jm]

[a] – Number of subjects who completed the study; [b] – receiving vit. K supplementation; [c] – Data after 6 months of intervention; [d] – Data after 12 months of intervention; [e] – Median (range); [f] – Data after 15 days of intervention (0–7 days – 0 µg/d MK-4, 8–14 days – 300 µg/d MK-4); [g] – Data after 22 days of intervention (0–7 days – 0 µg/d MK-4, 8–14 days – 300 µg/d MK-4, 14–21 days – 600 µg/d MK-4); [h] – Data after 29 days of intervention (0–7 days – 0 µg/d MK-4, 8–14 days – 300 µg/d MK-4, 14–21 days – 600 µg/d MK-4, 22–28 days – 900 µg/d MK-4); [i] – Data after 36 days of intervention (0–7 days – 0 µg/d MK-4, 8–14 days – 300 µg/d MK-4, 14–21 days – 600 µg/d MK-4, 22–28 days – 900 µg/d MK-4, 29–36 days – 1500 µg/d MK-4); [j] – Data from figure; [k] – Changes from baseline; [l] – Data after 2 weeks of intervention; [m] – Data after 4 weeks of intervention; [o] – Mean ± standard error; [*] – p value (difference between intervention vs. controls) < 0.05

[#] – p value (baseline vs. intervention) < 0.05

N/A – not available

The effects of vitamin K supplementation on anthropometric parameters

Changes in body weight [14, 15, 20, 25–27, 29] and BMI [14, 15, 20, 25–27] after vitamin K supplementation were analysed in seven studies, while changes in fat mass were assessed in three studies [25, 26, 29] included in this systematic review. Average baseline BMI values in the intervention groups ranged from 21.10 kg/m^2 [15] to 28.34 kg/m^2 [25, 26]. Similar values were observed in the control groups. Following the intervention period, mean body weight, BMI and fat mass did not change in subjects who received vitamin K_1 supplementation [25, 26, 29]. Similar MK-4 or MK-7 supplementation did not affect body weight and BMI (**Table 4**) [14, 15, 20, 27, 28].

The effects of vitamin K supplementation on leptin and adiponectin levels

Changes in leptin levels after vitamin K supplementation were measured in two studies [14, 26]. At baseline, in the intervention groups, the mean serum leptin concentrations varied from 6.20 ng/ml [14] to 28.59 ng/ml [26]. Similar results were observed in the control groups. Rasekhi et al. [26] observed that vitamin K_1 supplementation did not change the leptin levels. On the other hand, Koitaya et al. [14] reported that MK-4 supplementation increased serum leptin concentrations in the intervention group, but a similar effect was observed in the control group (**Table 5**).

The effect of vitamin K supplementation on adiponectin levels was assessed in three stud-

Table 4. Body weight (kg) and BMI (kg/m^2) changes during the intervention period in the intervention and the control groups in selected studies

Study	Intervention (dose of vit. K (µg/d))	Group	n[a]	Body weight (kg) Mean ± SD		BMI (kg/m^2) Mean ± SD		Fat mass (%) Mean ± SD	
				Pre-intervention	Post-intervention	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
Fulton et al. [24]	MK-7 (100)	Intervention[b] Control	40 40	79.00 ±15.00 77.00 ± 13.00	N/A	N/A		N/A	
Rasekhi et al. [25, 26]	Vit. K_1 (1000)	Intervention[b] Control	39 43	71.21 ± 6.47 71.09 ± 6.59	70.72 ± 6.39 71.00 ± 6.76	28.34 ± 1.72 27.93 ± 1.53	28.19 ± 1.80 27.91 ± 1.61	38.77 ± 3.86 38.55 ± 3.99	38.46 ± 4.05 38.57 ± 4.10
Koitaya et al. [14]	MK-4 (1500)	Intervention[b] Control	24 24	52.20 ± 5.60 51.90 ± 4.70	52.90 ± 5.80[c] 52.50 ± 5.80[d] 52.20 ± 5.20[c] 52.00 ± 4.90[d]	22.00 ± 1.80 21.80 ± 2.20	22.30 ± 1.80[c] 22.10 ± 1.90[d] 21.90 ± 2.20[c] 21.70 ± 2.10[d]	N/A	
Nakamura et al. [27]	MK-4 (0–1500)	Intervention[b]	15	58.55 (50.55–68.25)[e]	57.95 (50.50–66.90)[e]	20.60 (18.50–24.10)[e]	20.10 (18.30–23.70)[e]	N/A	
Dalmeijer et al. [28]	MK-7 (180) MK-7 (360)	Intervention[b] Intervention[b] Control	22 18 20	N/A		24.90 ± 3.00 23.70 ± 1.90 24.40 ± 2.50	0.14 (0.50[f])[g] 0.18 (0.80[f])[g] -0.06 (-0.20[f])[g]	N/A	
Knapen et al. [20]	MK-7 (0–360)	Intervention[b]	20[a] 22[b]	81.30 ± 11.50[a*] 67.60 ± 7.40[b]	N/A	24.00 ± 2.50[a] 23.20 ± 2.70[b]	N/A	N/A	
	MK-4 (45000)	Intervention[b] Control	89 75	66.60 ± 8.10 66.70 ± 8.20	66.60 ± 8.90[*] 67.60 ± 8.70[#]	25.40 ± 2.80 25.50 ± 2.40	25.50 ± 3.00[*] 26.00 ± 2.50[#]		
Koitaya et al. [15]	MK-4 (1500)	Intervention[b] Control	20 20	50.20 ± 5.80 54.20 ± 7.80	50.00 ± 5.80 53.70 ± 7.50[#]	21.10 ± 2.20[*] 22.70 ± 2.60	21.00 ± 2.20 22.50 ± 2.50[#]	N/A	
Volpe et al. [29]	Vit. K_1 (600)	Intervention[b] Control	8 6	60.73 ± 9.78 63.00 ± 6.82	-0.14 ± 1.35[g] 0.50 ± 1.97[g]	22.86 ± 4.01 22.81 ± 2.95	N/A	25.04 ± 9.72 28.33 ± 6.94	1.66 ± 1.28[g] 1.50 ± 1.92[g]

[a] – Number of subjects who completed the study
[b] – Group receiving vit. K supplementation
[c] – Data after 6 months of intervention
[d] – Data after 12 months of intervention
[e] – Median (range)
[f] – %
[g] – Changes from baseline
[*] – p value (difference between intervention vs. control groups) < 0.05
[#] – p value (baseline vs. intervention) < 0.05
N/A – not available

ies [14, 20, 26]. In the vitamin K groups, the mean serum adiponectin concentrations ranged from 6.20 µg/ml [20] to 14.30 µg/ml [14] and similar values were noted in the control groups. The mean adiponectin levels increased after vitamin K_1 supplementation. In addition, significant differences between groups were observed [26]. Contrary, Knapen et al. [20] showed no effect of MK-4 supplementation on adiponectin levels, while Koitaya et al. [14] found a significant increase in adiponectin levels after 12 months of intervention. However, a similar effect was observed in the control group. MK-7 supplementation had no effect on serum adiponectin concentrations. Only for a dose of 180 µg/d a significant decrease in adiponectin levels was noted (**Table 5**) [20].

Discussion

Here we present the effect of vitamin K supplementation on changes in anthropometric parameters and adipokines levels in adults. While the results of the considered studies were equivocal, the findings of this systematic review showed no effect of vitamin K supplementation on body weight, BMI, leptin and adiponectin levels.

Osteocalcin (OC) is an abundant noncollagenous protein, which is synthesized by osteoblasts during bone formation and undergoes a posttranslational vitamin K dependent modification, in which 3 glutamic acid residues are carboxylated, which thereby allows the protein to bind calcium. The circulating measure of total OC, which includes both carboxylated osteocalcin (cOC) and ucOC forms, is used as a biomarker of bone formation, whereas serum ucOC concentrations are used as a marker of the vitamin K status [31]. Previous studies have shown that ucOC levels increase in response to vitamin K depletion and decrease after vitamin K supplementation [27, 32, 33]. These results are consistent with our finding showing that vitamin K supplementation might significantly reduce ucOC levels [15, 20, 25–27].

Studies in animal models have shown that OC might be the mediator of energy metabolism in the bone, pancreas and adipose tissue [24, 35]. It was also demonstrated that subjects with a high degree of cOC were leaner and had less body fat than those with lower OC carboxylation. These findings suggest that vitamin K status might be related to subjects' nutritional status [20]. Indeed, Shea et al. [36] showed that women with the high-

Table 5. Changes serum concentrations of leptin (ng/ml) and adiponectin (µg/ml) during the intervention period in the intervention and the control groups in selected studies

Study	Intervention (dose of vit. K (µg/d))	Group	n[a]	Leptin (ng/ml) Mean ± SD		Adiponectin (µg/ml) Mean ± SD	
				Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
Rasekhi et al. [26]	Vit. K_1 (1000)	Intervention[b]	39	28.59 ± 9.61	28.29 ± 9.86	9.19 ± 1.80	10.44 ± 1.20[*#]
		Control	43	26.78 ± 10.33	25.62 ± 10.21	8.81 ± 1.54	8.54 ± 1.87
Koitaya et al. [14]	MK-4 (1500)	Intervention[b]	24	6.20 ± 3.60	7.20 ± 4.10[c#] 7.80 ± 4.90[d#]	14.30 ± 6.70	14.30 ± 7.70[c] 14.40 ± 7.70[d#]
		Control	24	5.20 ± 2.80	6.00 ± 2.40[c] 7.60 ± 3.40[d#]	14.30 ± 5.90	14.10 ± 6.10[c] 14.80 ± 6.40[d]
Knapen et al. [20]	MK-7 (10)	Intervention[b]	6	N/A		7.70[e]	7.60[e]
	MK-7 (20)	Intervention[b]	6			6.20[e]	6.30[e]
	MK-7 (45)	Intervention[b]	6			8.30[e]	8.30[e]
	MK-7 (90)	Intervention[b]	6			7.00[e]	7.40[e]
	MK-7 (180)	Intervention[b]	6			9.20[e]	7.80[e#]
	MK-7 (360)	Intervention[b]	6			8.10[e]	8.10[e]
		Control	6			8.40[e]	8.20[e]
	MK-4 (45000)	Intervention[b]	89	N/A		14.20 ± 9.70	-1.10 ± 3.80[f]
		Control	75			14.30 ± 9.50	- 1.10 ± 5.00[fh]

[a] — Number of subjects who completed the study
[b] — Group receiving vit. K supplementation
[c] — Data after 6 months of intervention
[d] — Data after 12 months of intervention
[e] — Data from figure
[f] — Change from baseline
[*] — p value (difference between intervention vs. control groups) < 0.05
[#] — p value (baseline vs. intervention) < 0.05
N/A — not available

est percentage of body fat had lower serum vitamin K concentrations and a poorer vitamin K status. In addition, Takeuchi et al. [37] presented evidence that MK-4 but not phylloquinone inhibited adipogenesis *in vitro*. However, here we did not show a significant effect of vitamin K supplementation on body weight, BMI and fat mass. Nevertheless, it is plausible that the unhealthy lifestyle of study participants might have attenuated the beneficial effect of vitamin K supplementation on body weight reduction.

It has been shown that adipokines levels might be associated with serum OC concentrations [38, 39] suggesting that vitamin K might also have an effect on adipokine levels. In addition, Kanazawa et al. [40] found that the ucOC/OC ratio positively correlated with serum adiponectin levels in men. On the other hand, Reinehr et al. [41] studied obese children and observed no significant relationship between serum adiponectin and OC concentrations. In addition, a recent meta-analysis did not demonstrate an effect of vitamin K supplementation on leptin and adiponectin levels. However, in their meta-analysis authors did not compare the effect of a different forms of vitamin K on adipokines levels [42]. Results obtained in this systematic review assessing the effect of vitamin K supplementation on adiponectin and leptin concentrations were equivocal. Vitamin K_1 supplementation did not change the leptin levels, but a significant increase in adiponectin levels was noted [26]. Koitaya et al. [14] reported that MK-4 supplementation increased serum leptin concentrations, but a similar effect was observed in the control group. On the other hand, MK-4 supplementation had no effect on adiponectin levels [14, 20], while MK-7 supplementation in a dose of 180 µg/d significantly reduced serum adiponectin concentrations [14]. The inconsistencies between studies might be related to variations in study design and intervention.

In this systematic review, we noted that the various vitamin K supplements seem to have partially antagonistic effects. Unfortunately, previous systematic reviews did not analyse a class effect of vitamin K supplements on anthropometric parameters and adipokines levels [42, 43]. Nevertheless, Takeuchi et al. [37] reported that MK-4, but not vitamin K_1, inhibited adipogenesis and stimulated osteoblastic differentiation *in vitro*. This is in line with a body of evidence that MK-4 has direct effects on a variety of metabolic and cellular processes by activating the steroid and xenobiotic receptors on the nuclear membrane [44]. On the other hand, Schurgers et al. [45] reported that MK-4 had a short serum half-life and a small area under the curve compared to vitamin K_1, whereas MK-9 displayed a long serum half-life compared to vitamin K_1 or MK-4. Sato et al. [46] also demonstrated that a nutritional dose of MK-7 was well absorbed in humans, and significantly increased serum MK-7 levels, whereas MK-4 had no effect on serum MK-4 concentrations. Therefore, the nutritional values of vitamin K homologues might be differentiated with regard to bioavailability and efficacy. In addition, several studies showed that the effects of long chain MK such as MK-7 on blood coagulation are greater and longer than vitamin K_1 and MK-4 [47, 48]. There is also evidence that MK-7 was much more effective than vitamin K_1 in increasing the degree of OC carboxylation [49].

There are several potential explanations as to why no significant effects of vitamin K supplementation on anthropometric parameters and adipokines levels were seen. In all studies included in this systematic review, a daily dose of vitamin K was above the RDI for vitamin K [14, 15, 20, 24–29], which in the United States of America is currently set at 90 µg/d for women and 120 µg/d for men [30]. However, it should be noted that the current RDI for vitamin K is based on saturation of the coagulation system [30] and a larger amount of vitamin K may be required to produce the significant effect on anthropometric parameters and adipokine levels [42, 43, 50]. In the United Kingdom, the Department for Health suggests that taking 1 mg or less of vitamin K supplements a day is unlikely to cause any harm in healthy individuals associated with intake of the recommended dose [51]. In addition, some epidemiological studies have suggested that recommended vitamin K levels required for maintaining health might vary according to age [50, 52]. Tsugawa et al. [52] found that the concentration of circulating vitamin K should be maintained at a higher level in the elderly than in young people. Moreover, no tolerable upper limit for vitamin K has been set with no known toxicity [30], which suggests that for most people, vitamin K supplementation is safe and had no side effects [14, 15,

20, 25–27]. Among studies included in this systematic review, only Fulton et al. [24] noted an excess of falls and gastrointestinal side effects in the vitamin K group compared to placebo, but no difference in serious adverse events or deaths was found. On the other hand, it is also probable that the lack effect of vitamin K supplementation on analysed parameters may be potentially due to the short supplementation period. It is also probable that vitamin K is not acting on pathways that improve anthropometric parameters and adipokine levels [42, 43]. Moreover, a possible explanation lack of response to vitamin K supplementation is that unhealthy diet and lifestyle of study participants attenuated any beneficial effect of vitamin K supplementation.

Several limitations should be listed regarding the study. Firstly, the number of studies that were included in this systematic review was relatively small. In addition, analysed studies had different designs, used different methods of exposure measurement and reported different outcomes. Moreover, our findings were limited to Caucasian and Asian descent and it is not clear if these results generalize to other ethnicities. In addition, we could not always analyse the reported outcomes of interest because information regarding variance was not always reported or provided by authors after attempts at contact. Eventually, despite a thorough search strategy, including grey literature and different databases, unavailable studies may exist, which have not been included.

Finally, the divergence between the outcomes of epidemiological and experimental supplementation studies should be noted. While epidemiological studies have long observation periods and use vitamin K rather as a proxy for certain lifestyles, for instance, healthy nutrition rich in vegetables and good sources of protein, supplementation studies use vitamin K as isolated agents. It might well be the case that it is the synergy of vitamin K with other substances that produce health effects that cannot be gleaned from isolated supplementation. This can only be clarified by long term supplementation studies in comparison with natural cohorts [53].

On the other hand, the strength of this systematic review includes details on the characteristics of the studies and study populations. Moreover, this is the first systematic review that assessed the effect of vitamin K supplementation on anthropometric parameters. Recent meta-analysis measured the effect of vitamin K supplementation on the cardiometabolic risk factor but did not take into account the effect of vitamin K supplementation on body weight, BMI and fat mass [54].

Conclusion

Currently available data showed no effect of vitamin K supplementation (K_1, MK-4 or MK-7) on body weight, BMI, fat mass, leptin and adiponectin levels. Nevertheless, further studies are needed to evaluate the role of vitamin K on nutrition status and adipokines levels.

Acknowledgements

Author contributions: M.J. searched databases, performed the selection of studies, analysed the data and wrote the manuscript. H.W. commented the manuscript. J.W. designed the study, searched databases, analysed the data and edited the manuscript. All authors reviewed and approved the final manuscript.

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

Funding sources
There are no sources of funding to declare.

References

1. Schurgers LJ, Vermeer C. Determination of phylloquinone and menaquinones in food. Effect of food matrix on circulating vitamin K concentrations. Haemostasis. 2000 Nov-Dec;30(6):298–307.
2. Bolton-Smith C, Price RJ, Fenton ST, Harrington DJ, Shearer MJ. Compilation of a provisional UK database for the phylloquinone (vitamin K1) content of foods. Br J Nutr. 2000 Apr;83(4):389–399.
3. Cranenburg ECM, Schurgers LJ, Vermeer C. Vitamin K: the coagulation vitamin that became omnipotent. Thromb Haemost. 2007 Jul;98(1):120–125.
4. Booth SL, Broe KE, Gagnon DR, Tucker KL, Hannan MT, McLean RR, et al. Vitamin K intake and bone mineral density in women and men. Am J Clin Nutr. 2003 Feb;77(2):512–516.
5. Dam V, Dalmeijer GW, Vermeer C, Drummen NE, Knapen MH, van der Schouw YT, et al. Association between vitamin K and the metabolic syndrome: a 10-year follow-up study in adults. J Clin Endocrinol Metab. 2015 Jun;100(6):2472–2479.
6. Shea MK, Booth SL, Weiner DE, Brinkley TE, Kanaya AM, Murphy RA, et al. Circulating vitamin K is inversely associated with incident cardiovascular disease risk among those treated for hypertension in the

health, aging, and body composition study (Health ABC). J Nutr. 2017 May;147(5):888–895.
7. Sakamoto N, Nishiike T, Iguchi H, Sakamoto K. Relationship between acute insulin response and vitamin K intake in healthy young male volunteers. Diabetes Nutr Metab. 1999 Feb;12(1):37–41.
8. Yoshida M, Booth SL, Meigs JB, Saltzman E, Jacques PF. Phylloquinone intake, insulin sensitivity, and glycemic status in men and women. Am J Clin Nutr. 2008 Jul;88(1):210–215.
9. Yoshida M, Jacques PF, Meigs JB, Saltzman E, Shea MK, Gundberg C, et al. Effect of vitamin K supplementation on insulin resistance in older men and women. Diabetes Care. 2008 Nov;31(11):2092–2096.
10. Mazzanti L, Battino M, Nanetti L, Raffaelli F, Alidori A, Sforza G, et al. Effect of 1-year dietary supplementation with vitaminized olive oil on markers of bone turnover and oxidative stress in healthy post-menopausal women. Endocrine. 2015 Nov;50(2):326–334.
11. Erkkilä AT, Booth SL, Hu FB, Jacques PF, Manson JE, Rexrode KM, et al. Phylloquinone intake as a marker for coronary heart disease risk but not stroke in women. Eur J Clin Nutr. 2005 Feb;59(2):196–204.
12. Geleijnse JM, Vermeer C, Grobbee DE, Schurgers LJ, Knapen MHJ, van der Meeret IM, al. Dietary intake of menaquinone is associated with a reduced risk of coronary heart disease: the Rotterdam Study. J Nutr. 2004 Nov;134(11):3100–3105.
13. Braam L, McKeown N, Jacques P, Lichtenstein A, Vermeer C, Wilson P, et al. Dietary phylloquinone intake as a potential marker for a heart-healthy dietary pattern in the Framingham Offspring cohort. J Am Diet Assoc. 2004 Sep;104(9):1410–1414.
14. Koitaya N, Sekiguchi M, Tousen Y, Nishide Y, Morita A, Yamauchi J, et al. Low-dose vitamin K2 (MK-4) supplementation for 12 months improves bone metabolism and prevents forearm bone loss in postmenopausal Japanese women. J Bone Miner Metab. 2014 Mar;32(2):142–150.
15. Koitaya N, Ezaki J, Nishimuta M, Yamauchi J, Hashizume E, Morishita K, et al. Effect of low dose vitamin K2 (MK-4) supplementation on bio-indices in postmenopausal Japanese women. J Nutr Sci Vitaminol (Tokyo). 2009 Feb;55(1):15–21.
16. Shea MK, Booth SL, Massaro JM, Jacques PF, D'Agostino RB, Dawson-Hughes B, et al. Vitamin K and vitamin D status: associations with inflammatory markers in the Framingham Offspring Study. Am J Epidemiol. 2008 Feb;167(3):313–320.
17. Lee B-C, Lee J. Cellular and molecular players in adipose tissue inflammation in the development of obesity-induced insulin resistance. Biochim Biophys Acta. 2014 Mar;1842(3):446–462.
18. Lumeng CN, Saltiel AR. Inflammatory links between obesity and metabolic disease. J Clin Invest. 2011 Jun;121(6):2111–2117.
19. Sogabe N, Maruyama R, Baba O, Hosoi T, Goseki-Sone M. Effects of long-term vitamin K(1) (phylloquinone) or vitamin K(2) (menaquinone-4) supplementation on body composition and serum parameters in rats. Bone. 2011 May;48(5):1036–1042.
20. Knapen MHJ, Schurgers LJ, Shearer MJ, Newman P, Theuwissen E, Vermeer C. Association of vitamin K status with adiponectin and body composition in healthy subjects: uncarboxylated osteocalcin is not associated with fat mass and body weight. Br J Nutr. 2012 Sep;108(6):1017–1024.
21. Shea MK, Dawson-Hughes B, Gundberg CM, Booth SL. Reducing undercarboxylated osteocalcin with vitamin K supplementation does not promote lean tissue Loss or fat gain over 3 years in older women and men: a randomized controlled trial. J Bone Miner Res. 2017 Feb;32(2):243–249.
22. Jamka M, Walach H, Walkowiak J. Effect of vitamin K supplementation on cardio-metabolic parameters in adults. Prospero: CRD42017079368. https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID = 79368. Published 2017. Accessed November 28, 2017.
23. Kmet LM, Lee RC, Cook LS. Standard quality assessment criteria for evaluating primary research papers from a variety of fields. Edmonton: Alberta Heritage Foundation for Medical Research; 2004.
24. Fulton RL, McMurdo MET, Hill A, Abboud RJ, Arnold GP, Struthers AD, et al. Effect of vitamin K on vascular health and physical function in older people with vascular disease — a randomised controlled trial. J Nutr Health Aging. 2016 Mar;20(3):325–333.
25. Rasekhi H, Karandish M, Jalali MT, et al. The effect of vitamin K1 supplementation on sensitivity and insulin resistance via osteocalcin in prediabetic women: a double-blind randomized controlled clinical trial. Eur J Clin Nutr. 2015 Aug;69(8):891–895.
26. Rasekhi H, Karandish M, Jalali M-T, Mohammad-Shahi M, Zarei M, Saki A, et al. Phylloquinone supplementation improves glycemic status independent of the effects of adiponectin levels in premonopause women with prediabetes: a double-blind randomized controlled clinical trial. J Diabetes Metab Disord. 2015 Aug;14(1):1.
27. Nakamura E, Aoki M, Watanabe F, Kamimura A. Low-dose menaquinone-4 improves γ-carboxylation of osteocalcin in young males: a non-placebo-controlled dose-response study. Nutr J. 2014 Aug;13(1):85.
28. Dalmeijer GW, van der Schouw YT, Magdeleyns E, Ahmed N, Vermeer C, Beulens JWJ. The effect of menaquinone-7 supplementation on circulating species of matrix Gla protein. Atherosclerosis. 2012 Dec;225(2):397–402.
29. Volpe SL, Leung MM, Giordano H. Vitamin K supplementation does not significantly impact bone mineral density and biochemical markers of bone in pre- and perimenopausal women. Nutr Res. 2008 Sep;28(9):577–582.
30. Food and Nutrition Board. Dietary Reference Intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. Washington (DC): National Academies Press (US); 2001.
31. Booth SL, Lichtenstein AH, O'Brien-Morse M, McKeown NM, Wood RJ, Saltzman E, et al. Effects of a hydrogenated form of vitamin K on bone formation

and resorption. Am J Clin Nutr. 2001 Dec;74(6):783–790.
32. Je SH, Joo N-S, Choi B, Kim K-M, Kim B-T, Park S-B, et al. Vitamin K supplement along with vitamin D and calcium reduced serum concentration of undercarboxylated osteocalcin while increasing bone mineral density in Korean postmenopausal women over sixty-years-old. J Korean Med Sci. 2011 Aug;26(8):1093–1098.
33. Binkley NC, Krueger DC, Engelke JA, Foley AL, Suttie JW. Vitamin K supplementation reduces serum concentrations of under-gamma-carboxylated osteocalcin in healthy young and elderly adults. Am J Clin Nutr. 2000 Dec;72(6):1523–1528.
34. Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, et al. Endocrine regulation of energy metabolism by the skeleton. Cell. 2007 Aug;130(3):456–469.
35. Ferron M, Hinoi E, Karsenty G, Ducy P. Osteocalcin differentially regulates beta cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. Proc Natl Acad Sci U S A. 2008 Apr;105(13):5266–5270.
36. Shea MK, Benjamin EJ, Dupuis J, Massaro JM, Jacques PF, D'Agostino Sr RB, et al. Genetic and non-genetic correlates of vitamins K and D. Eur J Clin Nutr. 2009 Apr;63:458–464.
37. Takeuchi Y, Suzawa M, Fukumoto S, Fujita T. Vitamin K(2) inhibits adipogenesis, osteoclastogenesis, and ODF/RANK ligand expression in murine bone marrow cell cultures. Bone. 2000 Dec;27(6):769–776.
38. Fisher A, Srikusalanukul W, Davis M, Smith P. Interactions between serum adipokines and osteocalcin in older patients with hip fracture. Int J Endocrinol. 2012;2012:684323.
39. Kindblom JM, Ohlsson C, Ljunggren Ö, Karlsson MK, Tivesten A, Smith U, et al. Plasma osteocalcin is inversely related to fat mass and plasma glucose in elderly Swedish men. J Bone Miner Res. 2009 May;24(5):785–791.
40. Kanazawa I, Yamaguchi T, Yamamoto M, Yamauchi M, Kurioka S, Yano S, et al. Serum osteocalcin level is associated with glucose metabolism and atherosclerosis parameters in type 2 diabetes mellitus. J Clin Endocrinol Metab. 2009 Jan;94(1):45–49.
41. Reinehr T, Roth CL. A new link between skeleton, obesity and insulin resistance: relationships between osteocalcin, leptin and insulin resistance in obese children before and after weight loss. Int J Obes (Lond). 2010 May;34(5):852–858.
42. Suksomboon N, Poolsup N, Darli Ko Ko H. Effect of vitamin K supplementation on insulin sensitivity: a meta-analysis. Diabetes, Metab Syndr Obes Targets Ther. 2017 May;10:169–177.
43. Rees K, Guraewal S, Wong YL, Majanbu DL, Mavrodaris A, Stranges S, et al. Is vitamin K consumption associated with cardio-metabolic disorders? A systematic review. Maturitas. 2010 Oct;67(2):121–128.
44. Shearer MJ, Newman P. Metabolism and cell biology of vitamin K. Thromb Haemost. 2008 Oct;100(4):530–547.
45. Schurgers LJ, Vermeer C. Differential lipoprotein transport pathways of K-vitamins in healthy subjects. Biochim Biophys Acta. 2002 Feb;1570(1):27–32.
46. Sato T, Schurgers LJ, Uenishi K. Comparison of menaquinone-4 and menaquinone-7 bioavailability in healthy women. Nutr J. 2012 Nov;11(1):93.
47. Groenen-van Dooren MM, Ronden JE, Soute BA, Vermeer C. Bioavailability of phylloquinone and menaquinones after oral and colorectal administration in vitamin K-deficient rats. Biochem Pharmacol. 1995 Sep;50(6):797–801.
48. Sato T, Ohtani Y, Yamada Y, Saitoh S, Harada H. Difference in the metabolism of vitamin K between liver and bone in vitamin K-deficient rats. Br J Nutr. 2002 Apr;87(4):307–314.
49. Schurgers LJ, Teunissen KJF, Hamulyák K, Knapen MHJ, Vik H, Vermeer C. Vitamin K-containing dietary supplements: comparison of synthetic vitamin K1 and natto-derived menaquinone-7. Blood. 2007 Apr;109(8):3279–3283.
50. Booth SL, Martini L, Peterson JW, Saltzman E, Dallal GE, Wood RJ. Dietary phylloquinone depletion and repletion in older women. J Nutr. 2003 Aug;133(8):2565–2569.
51. Vitamin K — NHS Choices. http://www.nhs.uk/Conditions/vitamins-minerals/Pages/Vitamin-K.aspx. Accessed August 12, 2017.
52. Tsugawa N, Shiraki M, Suhara Y, Kamao M, Tanaka K, Okano T. Vitamin K status of healthy Japanese women: age-related vitamin K requirement for gamma-carboxylation of osteocalcin. Am J Clin Nutr. 2006 Feb;83(2):380–386.
53. Walach H, Loef M. Using a matrix-analytical approach to synthesizing evidence solved incompatibility problem in the hierarchy of evidence. J Clin Epidemiol. 2015 Nov;68(11):1251–1260.
54. Verma H, Garg R. Effect of vitamin K supplementation on cardiometabolic risk factors: a systematic review and meta-analysis. Endocr Metab Immune Disord Drug Targets. 2019;19(1):13–25.

Acceptance for editing: 2019-11-09
Acceptance for publication: 2019-12-30

THOUSAND WORDS ABOUT...

DOI: https://doi.org/10.20883/medical.377

Technological advances in free-circulating tumour-derived DNA methylation analysis

Marcin Skalski[a], Jarosław Paluszczak*, [b]

Department of Pharmaceutical Biochemistry, Poznan University of Medical Sciences, Poland

* *Corresponding Autor:* Jarosław Paluszczak, Department of Pharmaceutical Biochemistry, Poznan University of Medical Sciences, 4 Święcickiego Street, 60-781 Poznań, Poland, phone: +48618546624, fax: +48618546620, email: paluszcz@ump.edu.pl

[a] https://orcid.org/0000-0003-3377-8313 [b] https://orcid.org/0000-0002-6187-7549

ABSTRACT

Cancers still constitute an important health burden, due to their high incidence and mortality. The discovery and clinical implementation of novel sensitive diagnostic markers could improve treatment outcomes. Circulating cell-free DNA (ccfDNA) offers great promise for the development of new molecular biomarkers. However, the analysis of ccfDNA methylation poses significant challenges due to ccfDNA fragmentation and low concentration in the blood. Techniques which have recently been developed for liquid biopsy studies have been adapted to the detection of trace amounts of ccfDNA. This mini-review focuses on recent technological advances which allow the discovery of new sensitive and specific liquid biopsy cancer biomarkers based on DNA methylation detection.

Keywords: cancer; biomarkers; liquid biopsy; circulating cell-free DNA; cfDNA; ctDNA; DNA methylation.

Introduction

Cancers are one of the main health burdens, with about 17 million new cases and 9.6 million deaths worldwide in 2018 [1]. Due to their high incidence and mortality, there is an urgent need for new diagnostic methods. The gold standards in cancer management, thus far, are tissue biopsy and protein-based biomarkers, but recently, rising enthusiasm for the development of so called „liquid biopsies" has been noted [2]. A liquid biopsy avoids invasive sampling because it relies on the analysis of cancer-derived components which circulate in the bloodstream, e.g. circulating tumour cells, exosomes or tumour-derived DNA, or which are present in other biological fluids, e.g. the saliva or urine. Importantly, a liquid biopsy is expected to overcome issues regarding tumour heterogeneity, since it should reflect the characteristics of virtually all cancer cell subclones, in contrast to tissue biopsies. This article focuses on recent methodological advances in the detection of DNA methylation in a liquid biopsy, which may potentially bring it closer to wider clinical use.

Characteristics of ctDNA

The field of liquid biopsy studies was significantly stimulated by the discovery of the presence of circulating cell-free DNA (ccfDNA) in the blood. ccfDNA is typically 80—200 bp in length, but ± 147 bp-long fragments are most prevalent, since this

is the average length of DNA participating in nucleosome formation. ccfDNA is released into biological fluids mainly as the result of apoptotic or necrotic cell death, but other mechanisms are also possible, e.g. active release. It can be found not only in the blood, but also in the urine, cerebrospinal fluid and saliva of healthy and cancer subjects. Physiologically, its blood concentration is low, but it can be elevated, e.g., in inflammatory diseases, after intensive physical training or during pregnancy [3]. Many studies investigated the applicability of the assessment of free-circulating tumour-derived DNA (ctDNA) in cancer diagnostics [4,5]. It is widely accepted that the blood concentration of cell-free DNA is significantly higher in cancer patients in comparison to healthy controls, and it depends on the cancer stage (tumour size and vasculature). The total concentration of ctDNA in the plasma ranges from undetectable amounts up to ~1000 ng/ml in advanced cancers [7]. Much is already known in terms of ctDNA isolation, storage and handling [3]. Apart from simple quantitation, ctDNA can be used in the clinic to detect cancer-specific mutations or assess ctDNA methylation pattern changes [2, 6, 7]. The analysis of the ctDNA methylation profile shows the greatest promise, because it allows for early detection and is considered to be tissue-specific in many cancers [8].

Methods of ctDNA methylation analysis

There is extensive data concerning methylation-based biomarkers in different cancers [4,8], but the number of commercially available tests is limited to just one, namely the FDA-approved Epi proColon® 2.0 CE (Epigenomics, Germany). It utilises the HeavyMethyl technique to detect the methylation of *SEPT9* in serum. The analysis of *SEPT9* methylation showed high sensitivity and specificity in the detection of colorectal cancer, although it did not outperform other available tests (e.g. FIT) when used in asymptomatic patients [9]. Nevertheless, the advantage of this type of assay lies in its non-invasive nature in comparison to troublesome faecal analysis or colonoscopy. Thus, blood-based liquid biopsies are preferable because of potentially high patient compliance.

The amount of ctDNA in the plasma is usually insufficient to analyse the methylation of more than a single gene per sample when using standard methods. However, reliable detection of most types of cancer usually requires the assessment of a panel of several genes. Thus, methods suitable for DNA methylation-based liquid biopsy should not only detect trace amounts of DNA, but also allow for the analysis of highly fragmented ctDNA in multiplexing formats. This means that as little as 7–10 pg of methylated DNA can be detected. Moreover, an amplicon length of up to 100 bp long is preferable in assays analysing short DNA fragments. ctDNA is always accompanied by some ccfDNA shed by other types of cells, thus the epigenetic background stemming especially from blood cells is always a possible problem. To minimise this risk, only genes whose methylation has been confirmed to be cancer-specific should be included in diagnostic panels. All of these factors need to be taken into account when designing assay conditions.

Several improved techniques for the sensitive detection of ctDNA methylation have been described recently (**Table 1**). They have been successfully implemented in the search for cancer biomarkers (**Table 2**). Due to the low concentrations of ctDNA in blood samples, the enrichment of target sequences, e.g. by the addition of carrier nucleic acid and/or by nested PCR, is frequently necessary in the first steps of the analysis. In this regard, the use of whole genome amplification of bisulfite-converted ctDNA does not seem to yield satisfactory results.

PCR-based methods focus on site-specific analysis of one or several DNA regions [10,11]. One of the most interesting variants of quantitative methylation-specific PCR (qMSP) is cMethDNA. This modification of multiplex qMSP uses gene-specific standards and a two-step PCR procedure — enrichment of the target regions by multiplex-nested PCR followed by probe-based signal detection. It is relatively simple, but very robust — it allows for the sensitive detection of 1 methylated in 100,000 unmethylated copies. The examination of a panel of 10 differentially methylated genes using this approach allowed for highly sensitive and specific detection and monitoring of metastatic breast cancer [12]. On the other hand, next generation sequencing (NGS)-based methods simultaneously analyse hundreds or thousands

Table 1. Comparison of different DNA methylation-based techniques used in liquid biopsy studies

Method	Advantages	Disadvantages	Additional information	Amount of sample required	Number of analyzed regions per sample	Reference
ddMSP	High sensitivity High throughput	Multiplexing is challenging Relatively high cost	Allows for absolute quantitation	0.9 ml plasma	12 genes and 4 internal controls	[18]
cMethDNA	High sensitivity High throughput Relatively low cost	Complex assay design	Modification of qMSP, two sequential PCR reactions Multiplex reaction in preamplification step External control added	0.3 ml serum	10 genes	[12]
MCTA-seq	Very high number of analyzed genes Low sequencing depth required	Low throughput High cost	Low potential clinical usefulness due to complex analysis	2 ml plasma	Thousands of promoters adjacent to CpG tandems	[15]
qMSP	High throughput Relatively low cost	Limited number of multiplexed genes Challenging optimization of PCR conditions	One tetraplex reaction (3 test genes + reference gene on one sample)	35–70 ml urine	Up to 4 genes	[11]
			Several multiplex reactions on one sample	2-3 ml plasma	9 genes	[19]
QM-MSP	High throughput Relatively low cost	Complex assay design	Similar to cMethDNA but without external standards Results based on detected ratio of methylated vs unmethylated DNA	0.3 ml serum	Up to 12 genes	[20]

of regions [13, 14]. MCTA-Seq is a very interesting technique combining the sensitivity of PCR and the high throughput of NGS. The key idea of this technique is to use primers flanking CpG tandem repeats, which are found in many gene promoters, amplify them, and perform genome-wide analysis of the products. Such a workflow overcomes the problem of high DNA input required for NGS and the low ctDNA quantity in the bodily fluids [15]. However, the implementation of NGS-based techniques in diagnostic laboratories is problematic due to the high cost, thus this assay will rather be used for scientific investigations only.

Conclusions

We are currently experiencing a significant increase in the number of publications concerning ctDNA analysis [16]. Technological advances have made it possible to sensitively detect methylation in trace amounts of ctDNA. Unfortunately, knowledge about differentially methylated genes, coming from studies in large cohorts of patients, is still lacking. This problem can potentially be solved in the coming years thanks to the GRAIL consortium initiative [17]. Its aim is to create a Cell-free Genome Atlas, and thus fill almost all of the gaps in our understanding of ctDNA biology. Such data would be invaluable for any future applications. If successful, it will be a major breakthrough in the field of blood biomarker testing in precision medicine.

Acknowledgements

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

Funding sources
This paper was supported by a grant from The National Centre for Research and Development (POIR.04.01.04-00-0003/17-00).

Table 2. Recent examples of liquid biopsy biomarkers based on the analysis of ctDNA methylation

Method	Sensitivity/ specificity	Cancer type	Genes analyzed	Clinical utility	Reference
qMSP	52–59%/ 95–96%	HNSCC	*SHOX2* *SEPT9*	Detection Prognosis (survival) Monitoring (recurrence)	[21]
qMSP	78%/84% (1 in 3 replicates) 73%/96% (2 in 3 replicates)	Colorectal cancer	*SEPT9*	Early detection	[9]
qMSP	63%/86%	Lung cancer	*CDO1* *TAC1* *SOX17*	Detection	[22]
qMSP, quasi-digital PCR	49–65%/ 88–94%	HNSCC	*SHOX2* *SEPT9*	Detection	[23]
Multiplex qMSP	72%/74%	Breast Colorectal Lung	*APC, FOXA1, RASSF1A*	Detection	[19]
cMethDNA	91%/96%	Breast	*AKR1B1, COL6A2, GPX7, HIST1H3C, HOXB4, RASGRF2, TM6SF1, ARHGEF7 TMEFF2, RASSF1*	Dectection Treatment response	[12]
methylBEAMing	NA	Glio-blastoma	*MGMT*	Treatment response	[24]
methylBEAMing	NA	Colorectal cancer	*EYA4, GRIA4, ITGA4, MAP3K14-AS1, MSC*	Prediction of response to regorafenib	[25]
Bisulfite sequencing	79.5–92.7%/ 85.2–92.8%	Lung cancer	9 regions	Detection	[26]
Targeted bisulfite sequencing	NA	Hepato-cellular carcinoma	10 markers 8 markers	Prognosis (survival) Treatment response	[27]
MCTA-seq	94%/89%	Hepato-cellular carcinoma	*RGS10 ST8SIA6 RUNX2 VIM* and 15 regions	Early detection	[15]
cfMeDIP-Seq	NA	Pancreatic cancer	Thousands of differentially methylated CpGs	Early detection	[28]

HNSCC – Head and Neck squamous cell carcinoma, MSP – methylation-specific PCR, NA – data not available, sensitivity – proportion of cancer case subjects who test positive for the biomarker, specificity – proportion of control subjects who test negative for the biomarker

References

1. Worldwide cancer statistics [Internet]. Cancer Research UK. 2019 [cited 2019 Jul 15]. Available from: https://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer.
2. De Rubis G, Krishnan SR, Bebawy M. Circulating tumor DNA — Current state of play and future perspectives. Pharmacol Res. 2018;136:35–44.
3. Bronkhorst AJ, Ungerer V, Holdenrieder S. The emerging role of cell-free DNA as a molecular marker for cancer management. Biomol Detect Quantif. 2019 Mar 1;17:100087.
4. Wang J, Han X, Sun Y. DNA methylation signatures in circulating cell-free DNA as biomarkers for the early detection of cancer. Sci China Life Sci. 2017 Apr;60(4):356–62.
5. Vlassov VV, Laktionov PP, Rykova EY. Circulating nucleic acids as a potential source for cancer biomarkers. Curr Mol Med. 2010 Mar;10(2):142–65.
6. Gai W, Sun K. Epigenetic Biomarkers in Cell-Free DNA and Applications in Liquid Biopsy. Genes. 2019 Jan;10(1):32.
7. Elazezy M, Joosse SA. Techniques of using circulating tumor DNA as a liquid biopsy component in cancer management. Comput Struct Biotechnol J. 2018 Oct 9;16:370–8.
8. Zeng H, He B, Yi C, Peng J. Liquid biopsies: DNA methylation analyses in circulating cell-free DNA. J Genet Genomics. 2018 Apr 20;45(4):185–92.
9. Song L, Jia J, Peng X, Xiao W, Li Y. The performance of the SEPT9 gene methylation assay and a comparison with other CRC screening tests: A meta-analysis. Sci Rep. 2017 Jun 8;7(1):1–12.
10. Guzzetta AA, Pisanic TR, Sharma P, Yi JM, Stark A, Wang T-H, et al. The promise of methylation on beads for cancer detection and treatment. Expert Rev Mol Diagn. 2014 Sep;14(7):845–52.
11. Olkhov-Mitsel E, Zdravic D, Kron K, van der Kwast T, Fleshner N, Bapat B. Novel multiplex MethyLight protocol for detection of DNA methylation in patient tissues and bodily fluids. Sci Rep. 2014 Mar 21;4:4432.
12. Fackler MJ, Bujanda ZL, Umbricht C, Teo WW, Cho S, Zhang Z, et al. Novel Methylated Biomarkers and a Robust Assay to Detect Circulating Tumor DNA

in Metastatic Breast Cancer. Cancer Res. 2014 Apr 15;74(8):2160–70.
13. Han X, Wang J, Sun Y. Circulating Tumor DNA as Biomarkers for Cancer Detection. Genomics Proteomics Bioinformatics. 2017 Apr 1;15(2):59–72.
14. Volik S, Alcaide M, Morin RD, Collins C. Cell-free DNA (cfDNA): Clinical Significance and Utility in Cancer Shaped By Emerging Technologies. Mol Cancer Res. 2016 Oct 1;14(10):898–908.
15. Wen L, Li J, Guo H, Liu X, Zheng S, Zhang D, et al. Genome-scale detection of hypermethylated CpG islands in circulating cell-free DNA of hepatocellular carcinoma patients. Cell Res. 2015 Nov;25(11):1250–64.
16. Trigg RM, Martinson LJ, Parpart-Li S, Shaw JA. Factors that influence quality and yield of circulating-free DNA: A systematic review of the methodology literature. Heliyon. 2018 Jul 1;4(7):e00699.
17. Morrison C. Search for liquid biopsy grail points the way to drug discovery and development gems. Nat Rev Drug Discov. 2017 Jun;16(6):373–4.
18. Uehiro N, Sato F, Pu F, Tanaka S, Kawashima M, Kawaguchi K, et al. Circulating cell-free DNA-based epigenetic assay can detect early breast cancer. Breast Cancer Res BCR. 2016 19;18(1):129.
19. Nunes SP, Moreira-Barbosa C, Salta S, Palma de Sousa S, Pousa I, Oliveira J, et al. Cell-Free DNA Methylation of Selected Genes Allows for Early Detection of the Major Cancers in Women. Cancers. 2018 Oct;10(10):357.
20. Fackler MJ, Sukumar S. Quantitation of DNA Methylation by Quantitative Multiplex Methylation-Specific PCR (QM-MSP) Assay. Methods Mol Biol Clifton NJ. 2018;1708:473–96.
21. Schröck A, Leisse A, Vos L de, Gevensleben H, Dröge F, Franzen A, et al. Free-Circulating Methylated DNA in Blood for Diagnosis, Staging, Prognosis, and Monitoring of Head and Neck Squamous Cell Carcinoma Patients: An Observational Prospective Cohort Study. Clin Chem. 2017 Jul 1;63(7):1288–96.
22. Hulbert A, Jusue-Torres I, Stark A, Chen C, Rodgers K, Lee B, et al. Early Detection of Lung Cancer using DNA Promoter Hypermethylation in Plasma and Sputum. Clin Cancer Res Off J Am Assoc Cancer Res. 2017 Apr 15;23(8):1998–2005.
23. de Vos L, Gevensleben H, Schröck A, Franzen A, Kristiansen G, Bootz F, et al. Comparison of quantification algorithms for circulating cell-free DNA methylation biomarkers in blood plasma from cancer patients. Clin Epigenetics. 2017 Dec 1;9(1):125.
24. Barault L, Amatu A, Bleeker FE, Moutinho C, Falcomatà C, Fiano V, et al. Digital PCR quantification of MGMT methylation refines prediction of clinical benefit from alkylating agents in glioblastoma and metastatic colorectal cancer. Ann Oncol. 2015 Sep 1;26(9):1994–9.
25. Amatu A, Schirripa M, Tosi F, Lonardi S, Bencardino K, Bonazzina E, et al. High Circulating Methylated DNA Is a Negative Predictive and Prognostic Marker in Metastatic Colorectal Cancer Patients Treated With Regorafenib. Front Oncol. 2019;9:622.
26. Liang W, Zhao Y, Huang W, Gao Y, Xu W, Tao J, et al. Non-invasive diagnosis of early-stage lung cancer using high-throughput targeted DNA methylation sequencing of circulating tumor DNA (ctDNA). Theranostics. 2019 Apr 6;9(7):2056–70.
27. Xu R, Wei W, Krawczyk M, Wang W, Luo H, Flagg K, et al. Circulating tumour DNA methylation markers for diagnosis and prognosis of hepatocellular carcinoma. Nat Mater. 2017 Nov;16(11):1155–61.
28. Shen SY, Singhania R, Fehringer G, Chakravarthy A, Roehrl MHA, Chadwick D, et al. Sensitive tumour detection and classification using plasma cell-free DNA methylomes. Nature. 2018 Nov;563(7732):579–83.

Acceptance for editing: 2019-11-09
Acceptance for publication: 2019-12-30

DOI: https://doi.org/10.20883/medical.379

Recovery from bone loss, diminished mineral density and strength in mice after treatment with steroidal and nonsteroidal anti-inflammatory drugs by injection of exosomes enriched with agomir miRNAs

Tomasz P. Lehmann*[1, a], Ewa Pruszyńska-Oszmałek[2, b], Paweł Kołodziejski[2, c], Magdalena Wojtków[3, d], Celina Pezowicz[3, e], Mirosław Szybowicz[4, f], Paweł Jagodzinski[1, g], Marek Nowicki[5, h], Aleksandra Trzaskowska[6, i], Sławomir Mielcarek[6, j], Maciej Głowacki[7, k]

[1] Department of Biochemistry and Molecular Biology, Poznan University of Medical Sciences, Poland
[2] Department of Animal Physiology and Biochemistry, Poznan University of Life Sciences, Poland
[3] Department of Biomedical Engineering, Mechatronics and Theory of Mechanisms, Wroclaw University of Science and Technology, Poland
[4] Institute of Materials Research and Quantum Engineering, Faculty of Technical Physics, Poznan University of Technology, Poland
[5] Wielkopolska Centre for Advanced Technologies, Poznań, Poland
[6] Crystal Physics Division, Faculty of Physics, Adam Mickiewicz University, Poznań, Poland
[7] Department of Paediatric Orthopaedics and Traumatology, Poznan University of Medical Sciences, Poland

* *Corresponding Autor:* Tomasz P. Lehmann, Department of Biochemistry and Molecular Biology, Poznan University of Medical Sciences, 6 Swiecickiego Street, 60-781 Poznań, Poland email: tlehmann@ump.edu.pl

[a] https://orcid.org/0000-0001-8445-0970
[b] https://orcid.org/0000-0002-7182-6905
[c] https://orcid.org/0000-0002-7941-0955
[d] https://orcid.org/0000-0003-0082-0386
[e] https://orcid.org/0000-0002-3516-4764
[f] https://orcid.org/0000-0001-8933-571X
[g] https://orcid.org/0000-0002-9046-6802
[h] https://orcid.org/0000-0002-0305-6881
[i] https://orcid.org/0000-0003-0451-7093
[j] https://orcid.org/0000-0002-0215-3313
[k] https://orcid.org/0000-0002-9932-670X

ABSTRACT

The project "Recovery from bone loss, diminished mineral density and strength in mice after treatment with steroidal and nonsteroidal anti-inflammatory drugs by injection of exosomes enriched with agomir miRNAs" is an animal experiment project, and an attempt to apply small RNA strands to reverse the harmful effects of anti-inflammatory drugs. Patients chronically treated with an anti-inflammatory drug suffer from musculoskeletal side effects, including reversed mineralisation and disabled bone fracture healing. The aims of the study are to measure changes in bone mineral density and bone strength in mice treated with methylprednisolone or diclofenac in combination with treadmill exercise. The reversal of the negative effects of these drugs will be assayed using modified miRNA agomir. Bones obtained from the treated mice will be analysed using micro-CT, dynamic mechanical analysis (DMA), nanoindentation, Raman spectroscopy and gene expression. We expect to find specific miRNA counteracting the demineralisation of the mice bones caused by methylprednisolone or diclofenac.

Keywords: methylprednisolone, diclofenac, miRNA, bone, exercise, bone strenght.

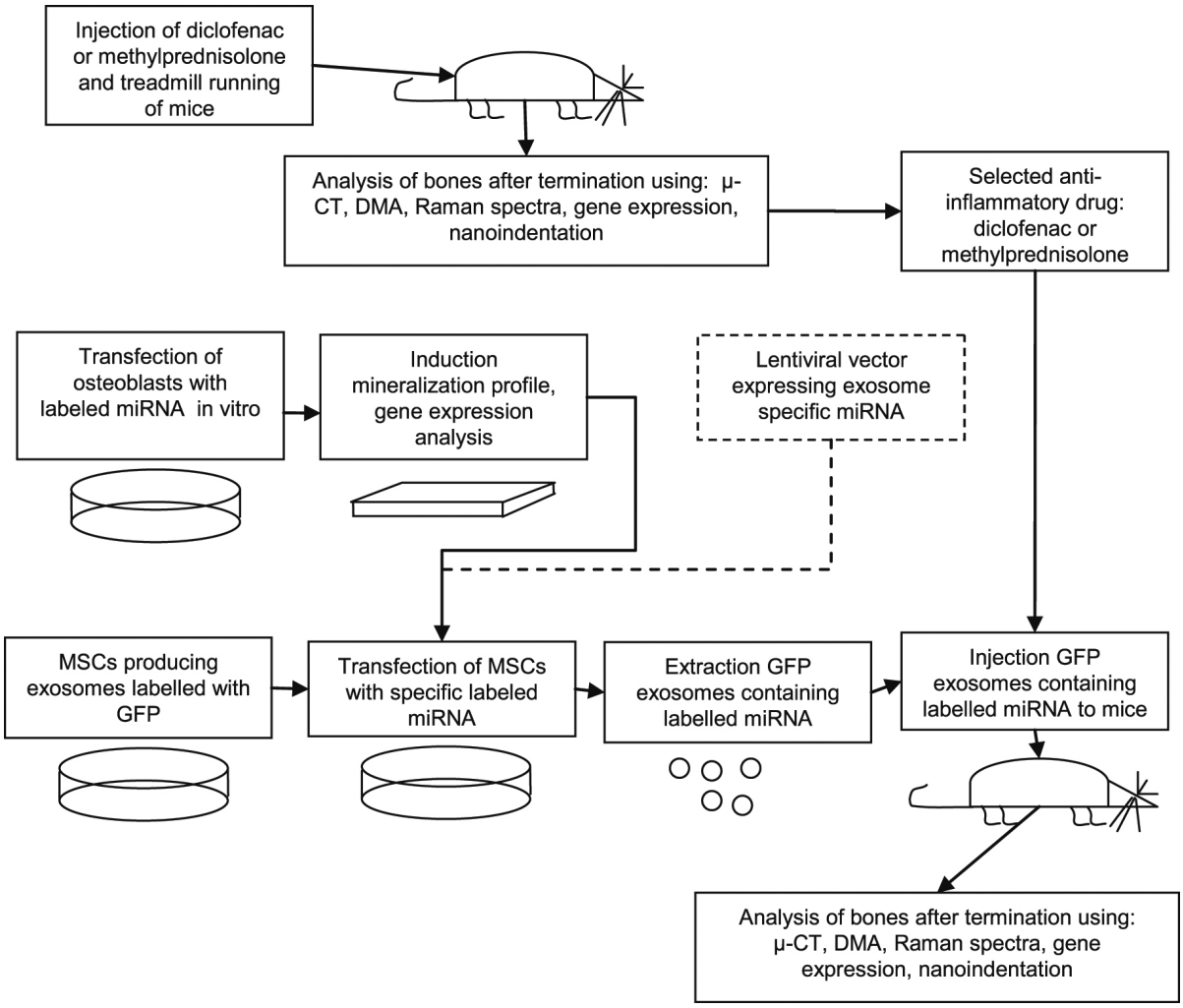

Figure 1. A general overview of the project

General information

The project "Recovery from bone loss, diminished mineral density and strength in mice after treatment with steroidal **and nonsteroidal anti-inflam**matory drugs by injection of exosomes enriched with agomir miRNAs" was awarded by the Polish National Science Center (NCN) under project number: 2016/21/B/NZ7/02748 (OPUS 11 competition). The duration of the grant is 36 months, from 3rd March 2017 to 2nd March 2020. The contract between NCN and Poznan University of Medical Sciences (PUMS), Poland, was signed on 3 March 2017.

Management

The Principal Investigator of the grant is professor Maciej Głowacki, Department of Paediatric Orthopaedics and Traumatology, Poznan University of Medical Sciences.

The main Co-Investigator of the grant is dr Tomasz Lehmann Department of Biochemistry and Molecular Biology, Poznan University of Medical Sciences. In addition, **the Co-In**vestigators in alphabetical order are: prof. dr hab. Paweł Jagodzinski, dr Paweł Kołodziejski, prof. dr hab. Sławomir Mielcarek, dr Marek Nowicki, prof. dr hab. Celina Pezowicz, dr Ewa Pruszyńska-Oszmałek, prof. dr hab. Sławomir Szybowicz, dr hab. Aleksandra Trzaskowska, and dr Magdalena Wojtków.

Ethics

The experiments were approved by the Local Ethics Commission for Investigation on Animals, Poznań University of Life Sciences, 14th July 2017.

Finance

The total amount of grant funding is PLN 620,750 (about 145,000 Euro). The amount of funding in the first year is PLN 243,750 (about 56,937 Euro). Grant funds were designed to purchase animals, equipment, and reagents, and also to cover the personnel costs of the project participants, and the dissemination of the research results, such as publications in peer reviewed journals.

Research Project Objectives

Rehabilitation after hip replacement, bone fracture healing, and other orthopedic operations require the application of steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs). In bones, glucocorticoids (GC) act on osteoblast cells reversing the mineralisation of the extracellular matrix [1]. When treated with these steroids, patients suffer from musculoskeletal side effects; mainly accelerated bone loss [2]. Consequently, fractures of the femur and spine are a frequent consequence of GC treatment in 30–50% of patients. This medication causes apoptosis of osteoblasts and osteocytes and depresses osteoblastic function, simultaneously acting on the expression of many genes in the bones [3].

Chronic inflammation triggers bone loss as a result of fractures, infection, non-unions, arthritis, osteoporosis, metabolic bone disease, tumours and post-operative complications [4]. The production of pro-inflammatory prostaglandins are a required component of normal fracture-healing, implicating that cyclooxygenase inhibitors such as non-steroidal anti-inflammatory drugs (NSAIDs) may directly influence osseous repair [5]. NSAIDs interfere in the rehabilitation of post-traumatic bone fractures and post-operative healing of bones after hip replacement and other types of orthopedic surgery.

Mineralisation is the main factor of bone strength and mechanical resistance, the application of anti-inflammatory drugs diminishes the mechanical properties of bone [6, 7].

Several strategies have been proposed to assist in the recovery of the adverse effects of analgesic treatment on the skeletal system [8]. Calcium, vitamin D supplementation and bisphosphonates treatment are the most appropriate strategies to prevent glucocorticoid-induced bone-loss in patients [9]. Another method to diminish the side effects is hormone replacement therapy for women (HRT) applied to diminish the osteoporosis outcome [10]. The long-lasting strategy that can delay the onset of GC-induced osteoporosis is exercise. Osteogenesis is induced by exercise and promotes bone anabolism. However, there are no current data comparing training with concomitant analgesic pharmacological intervention [11, 12]. There is little data on the impact of the composed effect of NSAIDs or GC on trained mice, and ambiguous results of studies showing the impact of physical exercises on bone strength.

New experimental approaches take advantage of knowledge covering gene expression and the role of small nucleic acid oligonucleotides (miRNAs) in the homeostasis of the bone microenvironment [13]. As a result of these studies, several miRNA were proposed as potential anti-bone-loss agents.

In our project, we intend to follow this research using microcomputer tomography (μ-CT) for detailed examination of the bone structure *in situ*. We also want to extend this approach by a complementary study of bones using dynamic mechanical analysis (DMA), gene expression, nanoindentation, and Raman spectroscopy. Such an approach to *ex vivo* studies has so far rarely been applied to mice treated with GC s, and NSAIDs in combination with exercise. There is also a small number of papers describing μ-CT, mechanical and histological methods applied to assess bone strength with miRNA treatment.

Aim

The aims of the study are a) to treat mice with methylprednisolone or diclofenac and measure changes in bone mineral density and bone strength, b) to produce exosomes containing the modified miRNA agomir by the synthesis of modified miRNA agomir (miR-29a or miR216a), and the transfection of mouse mesenchymal stem cells, and c) by injecting the exosomes into mice, to promote bone re-mineralisation and increase the bone strength.

Research Plan and Basic Concept

Step I

Estimation of bone mineralisation and mechanical strength in mice treated with diclofenac, and methylprednisolone in combination with exercise

- Mice treatment with diclofenac or methylprednisolone for four weeks in combination with enforced treadmill exercise.
- Mice termination, analysis of bones by micro-CT dynamic mechanical analysis (DMA), nanoindentation, Raman spectroscopy and gene expression (**Figure 1**).

Step II

Selection of miRNA with the highest remineralisation potential using mouse osteoblasts in culture

- Synthesis of oligonucleotides miR-29a, miR-216a, and miR-150.
- Transfection of osteoblast cell line MC3T3-E1 by oligonucleotides. After four days of incubation, cells will be harvested and the mRNA will be extracted.
- Analysis of mRNA profile for 10 genes involved in mineralisation, including genes regulated by miR-29a and miR-216a.
- Specific miRNA which strongly reverses the effect of the anti-inflammatory drug will be selected for further studies.
- Selected miRNA will be labelled with Cy5 and transfected into MC3T3-E1 cells.
- Extraction of exosomes and miRNA from exosomes. Measurement of selected miRNA in exosomes by RT-PCR.

Step III

Production of exosomes with selected miRNA in bone marrow mesenchymal stem cells

- Selected miRNA will be labelled with Cy5 and transfected into MSCs.
- Transfection of MSCs with the plasmid system introducing GFP to the exosomes.
- Extraction of labelled exosomes and miRNA from the exosomes. Measurement of the selected miRNA in the exosomes by RT-PCR.
- Treatment of MC3T3 with the labelled exosomes with miRNA, analysis of gene expression.

Step IV

Injection of exosomes into mice treated with anti-inflammatory drug

- Injection of labelled exosomes into mice treated with the anti-inflammatory drug.
- Treatment of the mice with diclofenac or methylprednisolone for 4 weeks.
- Mouse termination, and analysis of bones with fluorescent microscope, by μ-CT dynamic mechanical analysis (DMA), nanoindentation, Raman spectroscopy, and gene expression.

Step V

Vector-driven expression of selected miRNA in bone marrow mesenchymal stem cells and injection into mice treated with anti-inflammatory drug

- Construction of plasmid lentiviral vector bearing the selected miRNA.
- Transduction of MSCs with lentivirus encoding the selected miRNA and extraction of exosomes.
- Injection of the labelled exosomes with the selected miRNA into mice and treatment of the mice with the anti-inflammatory drug for 4 weeks.
- Mouse termination, analysis of bones by μ-CT, DMA, nanoindentation, Raman spectroscopy, and gene expression.
- Analysis of the mRNA profile in the bones for 10 key genes involved in mineralisation.
- Incubation of human femur explants with osteoporosis treatment with the labelled exosomes. Analysis of the mRNA profile in the bones.

Research Methodology

1. Animals. 8-week-old female mice will be randomly divided into six treatment groups (n = 10). The mice will be intraperitoneally injected with 5 mg/kg/day of methylprednisolone or diclofenac. After four weeks, these mice will be terminated by an overdose of Pentothal. The bones (femurs and tibias) will be obtained.

2. Enforced treadmill exercises. The animals will be subjected to a running procedure using a rodent horizontal treadmill at 12 m/min. for 30 min./day (5% gradient).

3. Dynamic Mechanical Analysis (DMA) is a physical method used to measure the mechanical properties of materials. DMA makes it possible to specify a storage modulus, a loss modulus

and a composite modulus. These moduli enable a bone sample's the ability to retain energy to be determined along with the ratio of its absorption.

4. Micro-computed tomography (μ-CT) To measure the microstructure of the bone, the bone mineral density**, and other parameters, the samples** will be analysed using a μ-CT scanner and associated analysis software.

5. Raman spectroscopic analysis of mouse tibia chemical composition. In order to determine the changes in the mineral and organic structure of the compacted tissue of the tibial bones of the mice, Raman light scattering studies will be conducted using a confocal Raman microscope. It will allow the index of bone mineralisation MI (*mineralisation index*) to be determined. In order to investigate the quantitative changes in a mineral component, the ratio of integral intensities of these bands (CO_3^{2-}/PO_4^{3-}), the CI (*carbonate index*) will be used.

6. Nanoindenter micromechanical analysis. Optical measurements will be performed using a laser confocal measuring microscope. The indenter registers the force versus depth curve by making an indent. During the test, the force applied to the indenter tip oscillates sinusoidally, which allows a quasi-continuous determination of the hardness value and bone elasticity module. The measurement will be carried out for a depth of indentation between 400 and 2400 nm from the cross-sectional area. The hardness and modulus of elasticity will be determined at 10–15 points in the middle of the cross-section of the bone wall on each bone.

7. Cell lines. A multipotent immortalised mouse mesenchymal cell line will be cultured **in mesenchymal stem cell culture media** according to the protocol of the cell line supplier.

The MC3T3-E1 mouse osteoblast cell line will be cultured in a mineralisation medium. The cultures **will be induced to differentiate by transferring** the cells, after detachment, into a culture medium supplemented with L-ascorbic acid and glycerol phosphate at final concentrations of 50 mg/ml and 10 mM, respectively.

8. Oligonucleotide synthesis, labelling Cy5. Synthetic oligonucleotides, agomirs, antagomirs and their Cy5-labelled counterparts will be ordered from a high quality external service as siRNA.

9. mRNA extraction. The bone material designated for molecular testing will be stored at -80°C. Prior to RNA isolation, the bones will be mechanically powdered in liquid nitrogen. Total RNA obtained from the bones and also from the cultured cells will be extracted, quantified and stored at -80°C. Real time RT-PCR will be used to measure gene expression and **the levels of specific** miRNA in the bones and MC3T3-E1 cells.

10. GFP labelling of exosomes and extraction. A lentivector-based method expressing CD63, CD9 or CD81 fused to GFP will be applied to the staining of the exosomes. These vectors will be used for transfections of MSCs to produce green exosomes. The green exosomes will be extracted with an Exosome Isolation Kit.

Other lentivirus system vectors will be used to clone selected miRNA, which will be used in the constitutive production of agomirs and antagomirs. The vectors will be used for transfection, viron production in 293T cell line packing cells and the transduction of MSCs.

11. Fluorescent microscopy, confocal microscopy. Examination of the fluorescently labelled cells/exosomes will be carried out using fluorescence and confocal microscopy. Flow cytometry finds particular application for testing exosome purity and the presence of GFP and Cy5.

12. Matrix mineralisation will be detected by alizarin red S (ARS) staining. The cells will be stained with calcium alizarin red staining kits **and quantified** by spectrophotometer. The results of Alizarin Red staining will be expressed as ng/mg protein.

Measurable Effects and Expected Results

In step I the interference of methylprednisolone and exercise, or diclofenac and exercise, on the mice's **bone structure, composition and mechanical strength** will be determined.

In step II an anti-demineralisation miRNA will be selected in osteoblast cells in culture.

In step III exosomes containing selected anti-demineralisation miRNA will be produced.

In step IV exosomes will be injected into mice to achieve transient stimulation of bone rebuilding.

In step V exosomes **will be efficiently produced** in vector-transduced cell lines.

Acknowledgements

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

Funding sources
There are no sources of funding to declare.

References

1. Siebler T, Robson H, Shalet SM, Williams GR. Dexamethasone inhibits and thyroid hormone promotes differentiation of mouse chondrogenic ATDC5 cells. Bone. 2002;31(4):457–64.
2. Hant FN, Bolster MB. Drugs that may harm bone: Mitigating the risk. Cleve Clin J Med. 2016;83(4):281–8.
3. Hartmann K, Koenen M, Schauer S, Wittig-Blaich S, Ahmad M, Baschant U, et al. Molecular Actions of Glucocorticoids in Cartilage and Bone During Health, Disease, and Steroid Therapy. Physiol Rev. 2016;96(2):409–47.
4. Loi F, Cordova LA, Pajarinen J, Lin TH, Yao Z, Goodman SB. Inflammation, fracture and bone repair. Bone. 2016;86:119–30.
5. Kurmis AP, Kurmis TP, O'Brien JX, Dalen T. The effect of nonsteroidal anti-inflammatory drug administration on acute phase fracture-healing: a review. J Bone Joint Surg Am. 2012;94(9):815–23.
6. Zimmermann EA, Gludovatz B, Schaible E, Busse B, Ritchie RO. Fracture resistance of human cortical bone across multiple length-scales at physiological strain rates. Biomaterials. 2014;35(21):5472–81.
7. Nair AK, Gautieri A, Chang SW, Buehler MJ. Molecular mechanics of mineralized collagen fibrils in bone. Nat Commun. 2013;4:1724.
8. Derry S, Conaghan P, Da Silva JA, Wiffen PJ, Moore RA. Topical NSAIDs for chronic musculoskeletal pain in adults. Cochrane Database Syst Rev. 2016;4:CD007400.
9. Moghadam-Kia S, Werth VP. Prevention and treatment of systemic glucocorticoid side effects. Int J Dermatol. 2010;49(3):239–48.
10. Benkhadra K, Mohammed K, Al Nofal A, Carranza Leon BG, Alahdab F, Faubion S, et al. Menopausal Hormone Therapy and Mortality: A Systematic Review and Meta-Analysis. J Clin Endocrinol Metab. 2015;100(11):4021–8.
11. Russo CR. The effects of exercise on bone. Basic concepts and implications for the prevention of fractures. Clin Cases Miner Bone Metab. 2009;6(3):223–8.
12. Santos L, Elliott-Sale KJ, Sale C. Exercise and bone health across the lifespan. Biogerontology. 2017;18(6):931–946.
13. Jing D, Hao J, Shen Y, Tang G, Li ML, Huang SH, et al. The role of microRNAs in bone remodeling. Int J Oral Sci. 2015;7(3):131–43.

Acceptance for editing: 2019-11-09
Acceptance for publication: 2019-12-30

DOI: https://doi.org/10.20883/medical.000

Comparison of the effects of endurance and endurance-strength training programmes on the level of endothelial dysfunction in women with abdominal obesity: study protocol for a randomised controlled trial

Małgorzata Jamka[1, a], Paweł Bogdański[2, b], Patrycja Krzyżanowska-Jankowska[1, c], Joanna Karolkiewicz[3, d], Radosław Mądry[4, e], Aleksandra Lisowska[1, f], Jarosław Walkowiak[1, g, *], Edyta Mądry[5, h]

[1] Department of Pediatric Gastroenterology and Metabolic Diseases, Poznan University of Medical Sciences, Poland
[2] Department of Treatment of Obesity, Metabolic Disorders and Clinical Dietetics, Poznan University of Medical Sciences, Poland
[3] Department of Physiology, Biochemistry and Hygiene, Poznań University School of Physical Education, Poland
[4] Department of Oncology, Poznan University of Medical Sciences, Poland
[5] Department of Physiology, Poznan University of Medical Sciences, Poland

* *Corresponding Autor:* Jarosław Walkowiak, MD, PhD; Department of Paediatric Gastroenterology and Metabolic Diseases, Poznan University of Medical Sciences, 27/33 Szpitalna Street, 60-572 Poznań, Poland; phone: +48618491432; fax: +48618472685; email: jarwalk@ump.edu.pl

[a] https://orcid.org/0000-0002-0257-6180
[b] https://orcid.org/0000-0002-0563-1624
[c] https://orcid.org/0000-0001-8676-9803
[d] https://orcid.org/0000-0002-7176-5540
[e] https://orcid.org/0000-0003-0738-2788
[f] https://orcid.org/0000-0002-5453-4716
[g] https://orcid.org/0000-0001-5813-5707
[h] https://orcid.org/0000-0002-0081-6558

ABSTRACT

The primary objective of the study is to compare the effect of endurance and endurance-strength training on endothelial function in women with abdominal obesity. The secondary objectives include the assessment of the effect of both types of training on anthropometric, densitometric and biochemical parameters. In total, at least 100 women will be recruited for the study. The study population will be randomly divided into two groups according to the type of training: endurance and endurance-strength training. During the 3-month of intervention, both groups will be performed three times a week training of an equal exercise volume and duration of 60 minutes. Before and after the intervention selected anthropometric and densitometric parameters will be measured and body composition will be analysed. In addition, biochemical parameters related to glucose and insulin homeostasis, lipid metabolism, antioxidant status, oxidative stress, inflammatory markers and endothelial function will be assessed.

Keywords: endurance training; endurance-strength training; endothelial function; cardiovascular risk.

Research Project Objectives

The primary aim of the study is to assess the effect of endurance and endurance-strength training on endothelial function in women with abdominal obesity. The secondary aims include the assessment of the effect of both types of training on anthropometric parameters, body composition,

densitometric parameters, glucose and insulin homeostasis, lipid metabolism, oxidative stress, antioxidant status and inflammatory markers.

Research Plan and Basic Concept

Basic Concept

It is well known that excessive body weight increases the progression of atherosclerosis [1]. One of the indicators of atherosclerosis is endothelial dysfunction which is also an independent risk factor for cardiovascular diseases [2, 3]. Several, albeit not all, studies have shown that regular physical activity may reduce the risk of atherosclerosis by the improvement of endothelial function [4, 5]. Regular training has also been shown to have other health-promoting properties such as: weight reduction, lowering blood pressure, improving lipid profile and glucose-insulin metabolism [4].

Current guidelines suggest that endurance training should be recommended for obese subjects [6–9]. It has been shown that this type of exercise has a beneficial effect on the reduction of body weight and improves cardiometabolic parameters [10]. On the other hand, strength training also contributes to the reduction in body weight, has a positive effect on body composition and reduces the risk of metabolic abnormalities related to obesity [10, 11]. Therefore, we suppose that the implementation of strength components for endurance training might intensify the beneficial effects of physical activity. However, results of studies comparing the effect of endurance and endurance-strength training on cardiovascular risk and endothelial function parameters are equivocal [10–12]. Therefore, randomised controlled trials are needed, which would compare the effects of endurance and endurance-strength training in obese subjects.

Study population

Adult women with abdominal obesity will be recruited to the study. The inclusion and exclusion criteria are presented in **Table 1**. The study population will be informed that participation is voluntary and that each participant can withdraw at any time without providing reasons, as well as all the subjects will receive the detailed information about the study protocol. Written informed consent will be obtained from all participants. The present study will be conducted according to the guidelines of the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Poznan University of Medical Sciences (refs. 1077/12 with supplement 753/13).

Study design

The study is designed as a prospective randomised trial. Subjects will be randomly divided into two groups: endurance and endurance-strength training, using a randomisation

Table 1. The inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
– Age 50–60 years – Obesity (body mass index (BMI) ≥ 30 kg/m^2) – Waist circumference > 80 cm – A percentage of body fat assessed by bioimpedance ≥ 33% – Stable body weight in the month prior to the trial	– Secondary form of obesity – Secondary form of hypertension – Type 2 diabetes mellitus – History of coronary artery disease – Stroke – Congestive heart failure, clinically significant arrhythmias or conduction disorders – Malignancy – History of use of any dietary supplements within 3 months before the study – Poorly controlled hypertension (mean systolic blood pressure > 140 mmHg and/or mean diastolic blood pressure > 90 mmHg) during the month prior to the trial and/or necessity to modify antihypertensive treatment in the last 3 months – Lipid disorders requiring the implementation of drug treatment in the last 3 months – Abnormal liver, kidney, or thyroid gland function – Clinically significant acute or chronic inflammatory process within the respiratory, digestive or genitourinary tract or in the oral cavity, pharynx or paranasal sinuses or connective tissue disease or arthritis – History of infection in the month – Nicotine, alcohol or drug abuse – Pregnancy or childbirth at enrolment or in the 3 months before enrolment, breast-feeding in the 3 months prior to enrolment – Any other condition that would make participation not in the best interest of the subject, or could prevent, limit or confound the efficacy of the study

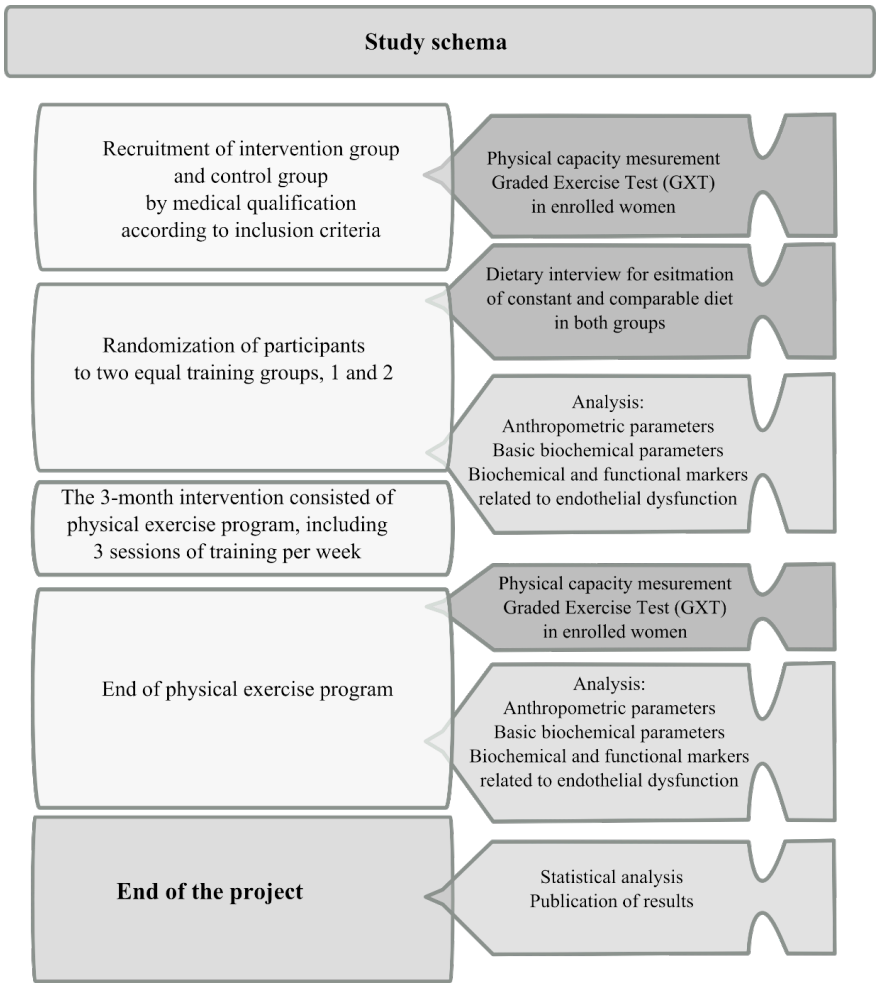

Figure 1. Scheme of the study

list. Both groups will perform 3-month of physical training which will vary only in the nature of the effort but have a comparable exercise volume. Aside from the training, all subjects will be instructed to maintain the physical activity and they dietary habits as they have previously led. At the baseline and after the 3-month of intervention anthropometric parameters, body composition, densitometric parameters, markers of endothelial function, oxidative stress, antioxidant status and inflammatory markers, as well as glucose and insulin homeostasis and lipid metabolism will be assessed. The scheme of the study is presented in **Figure 1**.

Intervention

The 3-month intervention will consist of a physical exercise programme, including three sessions of training per week. The training will be performed under the supervision of a qualified and certified fitness instructor and medical supervision. A single workout will last 60 minutes The endurance group will undergo training on cycle ergometers (Schwinn Evolution, Schwinn Bicycle Company, Boulder, Colorado, USA). Training sessions will consist of 5 minutes of warm-up, 45 minutes of training at an intensity between 50–70% of maximum heart rate (HR), 5 minutes of cycling without load and 5 minutes of closing stretching and breathing exercises. Similarly, endurance-strength training will consist of 5 minutes of warm-up, 20 minutes of strength exercises at 50–60% of one repetition maximum, 25 minutes of endurance exercises on cycle ergometers (Schwinn Evolution, Schwinn Bicycle Company, Boulder, Colorado, USA) of intensity between 50–70% of maximum HR, 5 minutes of cycling without load and 5 minutes of closing exercises. The strength component will involve exercises with a neck barbell and a gymnastic ball. The general scheme of the training plan is presented in **Figure 2**.

Research Methodology

Graded Exercise Test (GXT)

To determine the subjects' physical capacity, GXT will be performed at the beginning of the intervention on an electronically braked cycle ergometer (Kettler DX1 Pro, Ense-Parsit, Germany). GXT will begin at a work rate of 25 W. The work rate will be incremented by 25 W every 2 minutes until the subject could no longer maintain the required pedal cadence. Expired gases and minute ventilation will be monitored continuously with an automated system (Oxycon Mobile; Viasys Healthcare, Hoechberg, Germany). Oxygen intake (VO_2) and carbon dioxide output (VCO_2) will be measured. VO_2 peak, HR peak, time to exhaustion and maximal work rate will be assessed. To determine the ventilatory threshold, the V-slope method and the ventilatory equivalent method will be used.

Physiological parameters and markers related to the function of endothelium

Blood pressure will be measured at baseline and after the intervention period according to guidelines of the European Society of Hypertension [13]. Artery flow mediated dilatation will be assessed. Pulse wave analysis will be performed by SphygmoCor system (EINST Technology Pte Ltd., Singapore).

Anthropometry parameters

Anthropometry parameters (body weight, body height, waist and hip circumferences) will be measured [14] and BMI will be calculated before and after the intervention period [15]. In this study, abdominal obesity will be recognised according to the International Diabetes Federation criteria with a waist circumference exceeding 80 cm in women [16].

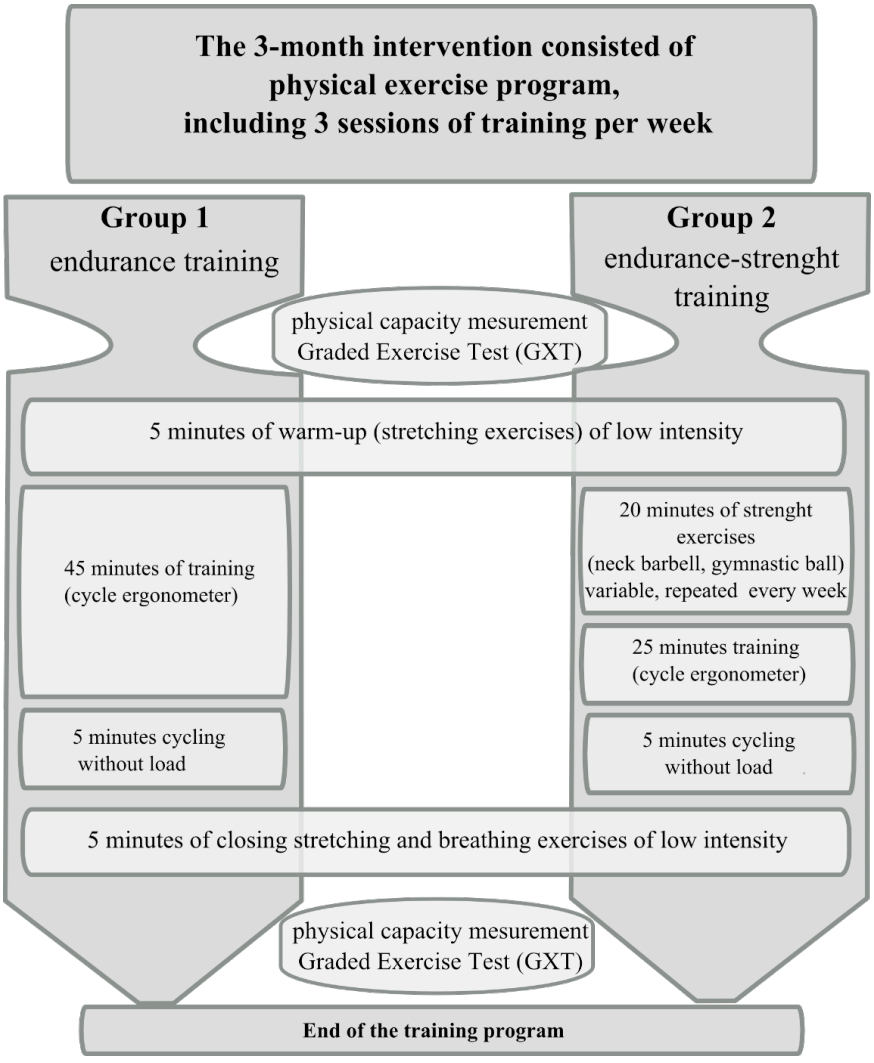

Figure 2. Scheme of a single training session

Body composition

Body composition will be assessed by bioelectrical impedance analysis with InBody 370 analyser (InBody Co. Ltd., Seoul, South Korea) during the recruitment process to check if subjects meet the inclusion criteria [17].

Densitometry

At baseline and after the intervention period densitometric measurement will be performed using Dual X-Ray Absorptiometry (Hologic Discovery QDR). The assessment will be carried out in accordance with the methodology recommended by the International Society for Clinical Densitometry [18]. Body composition (fat mass and free-fat mass) will be assessed for total body and separately for each part of the body. Visceral adipose tissue will be measured. Appendicular lean mass index and lean mass index will be calculated. Bone mineral content and bone mineral density will be assessed for the whole skeleton and additionally at the lumbar (L1-L4) spine and hip regions.

Biochemical measurements

Fasting blood samples will be collected from all study participants before and after the intervention period. Markers of endothelial function, glucose and insulin homeostasis, lipid metabolism. oxidative stress, antioxidant status and inflammation will be analysed by standard clinical chemical assays (see **Supplementary Table 1**).

Measurable Effects

Data from this study will potentially provide additional information that allows more efficient and precise planning of training regimes for obese subjects and may result in, beyond body weight reduction, prevention of endothelial dysfunction and improvement of cardiovascular health.

Expected Results

Previous studies have shown that exercise may decrease the risk of cardiovascular disease by preventing endothelial dysfunction [19–21]. However, it is supposed that the beneficial effect of physical activity may depend on the intensity of training. While strenuous exercise increases oxidative stress, regular and moderate physical activity promotes an antioxidant state and preserves endothelial function [22]. It is also suggested that the effect of physical activity on endothelial function may also depend on the type of training. However, results of studies comparing the effects of endurance and strength training on endothelial function are conflicting. Schjerve et al. [10] observed that both types of training significantly improve endothelial function in obese subjects after 12 weeks of intervention. However, high-intensity aerobic training was more effective in the improvement of endothelial function compared with strength training and moderate-intensity groups. On the other hand, Rakobowchuk et al. [23] noted that 12 weeks of resistance training in healthy young men did not change endothelial function, but the increased arterial diameter and, hence, blood flow. Østergård et al. [24] also reported no changes in endothelial function after 10-week of aerobic training in obese subjects with type 2 diabetes.

We suppose that the implementation of strength components for endurance training might intensify the beneficial effects of physical activity. Comparison of the effects of endurance and endurance-strength training programmes on endothelial function and other metabolic parameters in obese women will allow to verify the hypothesis and will answer the question of which type of training is more effective in the improvement of endothelial function and cardio-metabolic parameters. In addition, the results of this study should give a better insight into the effect of endurance and endurance strength training on human health. The expected findings may enable to construct first special physical activity guidelines for obese subjects to prevent endothelial dysfunction.

Acknowledgements

E.M., M.J. & J.W. wrote the manuscript. E.M., P.B., J.K. & J.W. designed the study. J.K., P.B., R.M., A.L. & P.K-J. edited the manuscript. All authors reviewed and approved the final manuscript.

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

Funding sources
This study was supported by the National Science Centre (JW — UMO-2014/13/B/NZ7/02209). Principal Investigator: Professor Jarosław Walkowiak, MD, PhD. Main Co-investigators: Edyta Mądry, MD, PhD., Professor Paweł Bogdański, MD, PhD; Joanna Karolkiewicz, MD, PhD.

Supplementary Table 1. List of biochemical parameters that will be analysed in the study

Parameter	Method of analysis
Endothelial function	
Asymmetric dimethylarginine (ADMA)	Immunoenzymatic method (SunRed Human (ADMA) ELISA Kit, China)
Endothelial nitric oxide synthase (eNOS)	Immunoenzymatic method (MyBioSource Human Endothelial Nitric Oxide Synthase ELISA kit, US)A
Homocysteine (Hcy)	Immunoenzymatic method (Axis Homocysteine EIA kit, United Kingdom)
NO_2	Method described by Tsikas[1]
NO_3	Method described by Tsikas[1]
Plasminogen activator inhibitor-1 (PAI-1)	Immunoenzymatic method (Human Total Serpin E1/PAI-1 Quantikine ELISA, R&D Systems a biotechne brand, USA)
Vascular endothelial growth factor (VEGF)	Immunoenzymatic method (Human VEGF, Quantikine ELISA, R&D Systems a biotechne brand, USA)
Glucose and insulin homeostasis	
Glucose	Enzymatic method with hexokinase
Insulin	Electrochemiluminescence method
Glycated haemoglobin (HbA1c)	Turbidimetric immunoinhibitory method in hemolysate prepared from blood
Insulin-like growth factor (IGF-1)	Immunoenzymatic method (IGF-1 600 ELISA kit, DRG Intruments GmbH, Germany)
Lipid metabolism	
Total cholesterol (TC)	Enzymatic colorimetric method
Low-density lipoprotein cholesterol (LDL-C)	Friedewald formula: LDL-C = TC — (HDL-C + TG/5)
High-density lipoprotein cholesterol (HDL-C)	Homogeneous enzymatic colorimetric method
Triglycerides (TG)	Enzymatic colorimetric method
Oxidized low-density lipoprotein (ox-LDL)	Immunoenzymatic method (Human ox-LDL ELISA kit, SunRed, China)
Apolipoprotein A1 (ApoA1)	Nephelometric method
Apolipoprotein B (ApoB)	Nephelometric method
Apolipoprotein E (ApoE)	Immunoenzymatic method (Human Apolipoprotein E ELISA Kit, Assaypro, USA)
Oxidative stress	
Advanced glycation end products (AGEs)	Immunoenzymatic method (Human AGEs ELISA Kit, MyBiosource, USA)
Antioxidant status	
Glutathione (GSH)	Immunoenzymatic method (Human Reduced GSH), ELISA Kit, MyBiosource, USA)
Superoxide dismutase (SOD)	Colorimetric method (SOD Assay Kit, Cayman Chemical, USA)
Total antioxidant status (TAS)	Immunoenzymatic method (Human TAS ELISA kit, Qayee-bio, China)
Paraoxonases (PON)	Immunoenzymatic method (Human PON ELISA Kit, MyBiosource, USA)
Inflammatory markers	
High-sensitivity C reactive protein (hs-CRP)	Latex enhanced turbidimetric immunoassay method
Interleukin-6 (IL-6)	Immunoenzymatic method (Human IL-6 Immunoassay, Quantikine HS ELISA, R&D Systems a biotechne brand, USA)
Interleukin-8 (IL-8)	Immunoenzymatic method (Human CXCL8/IL-8 Immunoassay, Quantikine HS ELISA, R&D Systems a biotechne brand, USA)
Monocyte chemoattractant protein 1 (MCP-1)	Immunoenzymatic method (MCP-1 human ELISA, DRG Intruments GmbH, Germany)
Matrix metalloproteinase-2 (MMP-2)	Immunoenzymatic method (Total MMP-2 Immunoassay, Quantikine ELISA, R&D Systems a biotechne brand, USA)
Matrix metalloproteinase-9 (MMP-9)	Immunoenzymatic method (Human MMP-9 Immunoassay, Quantikine ELISA, R&D Systems a biotechne brand, USA)
Tumor necrosis factor-α (TNF-α)	Immunoenzymatic method (Human tumor necrosis factor alfa, ELISA kit, Qayee-bio, China)

[1] Tsikas D. Simultaneous derivatization and quantification of the nitric oxide metabolites nitrite and nitrate in biological fluids by gas chromatography/mass spectrometry. Anal Chem. 2000 Sep;72(17):4064–4072.

References

1. Lee S-Y, Chang H-J, Sung J, Kim KJ, Shin S, Cho I-J, et al. The impact of obesity on subclinical coronary atherosclerosis according to the risk of cardiovascular disease. Obesity. 2014 Jul;22(7):1762–1768.
2. Vanhoutte PM, Shimokawa H, Tang EHC, Feletou M. Endothelial dysfunction and vascular disease. Acta Physiol. 2009 Jun;196(2):193–222.
3. Matsuzawa Y, Lerman A. Endothelial dysfunction and coronary artery disease: assessment, prognosis, and treatment. Coron Artery Dis. 2014 Dec;25(8):713–724.
4. Lippincott MF, Desai A, Zalos G, Carlow A, De Jesus J, Blum A, et al. Predictors of endothelial function in employees with sedentary occupations in a worksite exercise program. Am J Cardiol. 2008 Oct;102(7):820–824.
5. Duncker DJ, Bache RJ. Regulation of coronary blood flow during exercise. Physiol Rev. 2008 Jul;88(3):1009–1086.
6. Jensen MD, Ryan DH, Apovian CM, Ard JD, Comuzzie AG, Donato KA, et al. 2013 AHA/ACC/TOS guideline for the management of overweight and obesity in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and The Obesity Society. Circulation. 2014 Jun;129(25 Suppl 2):S102-S138.
7. Donnelly JE, Blair SN, Jakicic JM, Manore MM, Rankin JW, Smith BK, et al. Appropriate physical activity intervention strategies for weight loss and prevention of weight regain for adults. Med Sci Sport Exerc. 2009 Feb;41(2):459–471.
8. Jakicic JM, Clark K, Coleman E, Donnelly JE, Foreyt J, Melanson E, et al. American College of Sports Medicine position stand. Appropriate intervention strategies for weight loss and prevention of weight regain for adults. Med Sci Sports Exerc. 2001 Dec;33(12):2145–2156.
9. Fogelholm M, Stallknecht B, Van Baak M. ECSS position statement: exercise and obesity. Eur J Sport Sci. 2006 Aug;6(1):15–24.
10. Schjerve IE, Tyldum GA, Tjønna AE, Stølen T, Loennechen JP, Hansen HEM, et al. Both aerobic endurance and strength training programmes improve cardiovascular health in obese adults. Clin Sci. 2008 Nov;115(9):283–293.
11. Ho SS, Dhaliwal SS, Hills AP, Pal S. The effect of 12 weeks of aerobic, resistance or combination exercise training on cardiovascular risk factors in the overweight and obese in a randomized trial. BMC Public Health. 2012 Aug;12(1):704.
12. Azarbayjani M, Abedi B, Peeri M, Stannard SR. Effects of combined aerobic and resistant training on lipid profile and glycemic control in sedentary men. Int Med J. 2014 Apr;21(2):132–136.
13. Parati G, Stergiou GS, Asmar R, Bilo G, de Leeuw P, Imai Y, et al. European Society of Hypertension guidelines for blood pressure monitoring at home: a summary report of the Second International Consensus Conference on Home Blood Pressure Monitoring. J Hypertens. 2008 Aug;26(8):1505–26.
14. National Health and Nutrition Examination Survey (NHANES). Anthropometry procedures manual. 2007. Accessed: 15.09.2019. Online: https://www.cdc.gov/nchs/data/nhanes/nhanes_07_08/manual_an.pdf.
15. World Health Organization. Global database on body mass index. World Health Organization; 2006. Accessed: 15 Dec 2018. Online: https://www.who.int/nutrition/databases/bmi/en/.
16. International Diabetes Federation. The IDF consensus worldwide definition of the metabolic syndrome. Belgium: International Diabetes Federation; 2006.
17. Kyle U, Bosaeus I, De Lorenzo AD, Deurenberg P, Elia M, Gómez JM, et al. Bioelectrical impedance analysis? Part I: review of principles and methods. Clin Nutr. 2004 Oct;23(5):1226–1243.
18. Hangartner TN, Warner S, Braillon P, Jankowski L, Shepherd J. The official positions of the International Society for Clinical Densitometry: acquisition of dual-energy X-ray absorptiometry body composition and considerations regarding analysis and repeatability of measures. J Clin Densitom. 2013 Oct-Dec;16(4):520–536.
19. Edwards DG, Schofield RS, Lennon SL, Pierce GL, Nichols WW, Braith RW. Effect of exercise training on endothelial function in men with coronary artery disease. Am J Cardiol. 2004 Mar;93(5):617–620.
20. Cohen ND, Dunstan DW, Robinson C, Vulikh E, Zimmet PZ, Shaw JE. Improved endothelial function following a 14-month resistance exercise training program in adults with type 2 diabetes. Diabetes Res Clin Pract. 2008 Mar;79(3):405–411.
21. Beck DT, Martin JS, Casey DP, Braith RW. Exercise training improves endothelial function in resistance arteries of young prehypertensives. J Hum Hypertens. 2014 May;28(5):303–309.
22. Di Francescomarino S, Sciartilli A, Di Valerio V, Di Baldassarre A, Gallina S. The effect of physical exercise on endothelial function. Sport Med. 2009 Oct;39(10):797–812.
23. Rakobowchuk M, McGowan CL, de Groot PC, Hartman JW, Phillips SM, MacDonald MJ. Endothelial function of young healthy males following whole body resistance training. J Appl Physiol. 2005 Jun;98(6):2185–2190.
24. Østergård T, Nyholm B, Hansen TK, Rasmussen LM, Ingerslev J, Sørensen KE, et al. Endothelial function and biochemical vascular markers in first-degree relatives of type 2 diabetic patients: the effect of exercise training. Metabolism. 2006 Nov;55(11):1508–1515.

Acceptance for editing: 2019-11-09
Acceptance for publication: 2019-12-30

Journal of Medical Science
Instructions for Authors

Journal of Medical Science (JMS) is a PEER-REVIEWED, OPEN ACCESS journal that publishes original research articles and reviews which cover all aspects of clinical and basic science research. The journal particularly encourages submissions on the latest achievements of world medicine and related disciplines. JMS is published quarterly by Poznan University of Medical Sciences.

ONLINE SUBMISSION:
Manuscripts should be submitted to the Editorial Office by an e-mail attachment: nowinylekarskie@ump.edu.pl. You do not need to mail any paper copies of your manuscript.

All submissions should be prepared with the following files:
- Cover Letter
- Manuscript
- Tables
- Figures
- Supplementary Online Material

COVER LETTER: *Manuscripts* must be accompanied by a *cover letter* from the author who will be responsible for correspondence regarding the manuscript as well as for communications among authors regarding revisions and approval of proofs. The cover letter should contain the following elements: (1) the full title of the manuscript, (2) the category of the manuscript being submitted (e.g. Original Article, Brief Report), (3) the statement that the manuscript has not been published and is not under consideration for publication in any other journal, (4) the statement that all authors approved the manuscript and its submission to the journal, and (5) a list of at least two referees.

MANUSCRIPT: Journal of Medical Science publishes Original Articles, Brief Reports, Review articles, Mini-Reviews, Images in Clinical Medicine and The Rationale and Design and Methods of New Studies. From 2014, only articles in English will be considered for publication. They should be organized as follows: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Conflict of Interest, References and Figure Legends. All manuscripts should be typed in Arial or Times New Roman font and double spaced with a 2,5 cm (1 inch) margin on all sides. They should be saved in DOC, DOCX, ODT, RTF or TXT format. Pages should be numbered consecutively, beginning with the title page.

Ethical Guidelines
Authors should follow the principles outlined in the Declaration of Helsinki of the World Medical Association (www.wma.net). The manuscript should contain a statement that the work has been approved by the relevant institutional review boards or ethics committees and that all human participants gave informed consent to the work. This statement should appear in the Material and Methods section. Identifying information, including patients' names, initials, or hospital numbers, should not be published in written descriptions, illustrations, and pedigrees. Studies involving experiments with animals must be conducted with approval by the local animal care committee and state that their care was in accordance with institution and international guidelines.

Authorship:
According to the International Committee on Medical Journal Ethics (ICMJE), an author is defined as one who has made substantial contributions to the conception and development of a manuscript. Authorship should be based on all of the following: 1) substantial contributions to conception and design, data analysis and interpretation; 2) article drafting or critical advice for important intellectual content; and 3) final approval of the version to be published. All other contributors should be listed as acknowledgments. All submissions are expected to comply with the above definition.

Conflict of Interest
The manuscript should contain a conflict of interest statement from each author. Authors should disclose all financial and personal relationships that could influence their work or declare the absence of any conflict of interest. Author's conflict of interest should be included under Acknowledgements section.

Abbreviations
Abbreviations should be defined at first mention, by putting abbreviation between brackets after the full text. Ensure consistency of abbreviations throughout the article. Avoid using them in the title and abstract. Abbreviations may be used in tables and figures if they are defined in the table footnotes and figure legends.

Trade names
For products used in experiments or methods (particularly those referred to by a trade name), give the manufacturer's full name and location (in parentheses). When possible, use generic names of drugs.

Title page
The first page of the manuscript should contain the title of the article, authors' full names without degrees or titles, authors' institutional affiliations including city and country and a running title, not exceeding 40 letters and spaces. The first page should also include the full postal address, e-mail address, and telephone and fax numbers of the corresponding author.

Abstract
The abstract should not exceed 250 words and should be structured into separate sections: Background, Methods, Results and Conclusions. It should concisely state the significant findings without reference to the rest of the paper. The abstract should be followed by a list of 3 to 6 Key words. They should reflect the central topic of the article (avoid words already used in the title).

The following categories of articles can be proposed to the Journal of Medical Science:

ORIGINAL RESEARCH

Original articles: Manuscripts in this category describe the results of original research conducted in the broad area of life science and medicine. The manuscript should be presented in the format of Abstract (250-word limit), Keywords, Introduction, Material and Methods, Results, Discussion, Perspectives, Acknowledgments and References. In the Discussion section, statements regarding the importance and *novelty of the study* should be presented. In addition, the limitations of the study should be articulated. The abstract must be structured and include: Objectives, Material and Methods, Results and Conclusions. Manuscripts cannot exceed 3500 words in length (excluding title page, abstract and references) and contain no more than a combination of 8 tables and/or figures. The number of references should not exceed 45.

Brief Reports: Manuscripts in this category may present results of studies involving small sample sizes, introduce new methodologies, describe preliminary findings or replication studies. The manuscript must follow the same format requirements as full length manuscripts. Brief reports should be up to 2000 words (excluding title page, abstract and references) and can include up to 3 tables and/or figures. The number of references should not exceed 25.

REVIEW ARTICLES

Review articles: These articles should describe recent advances in areas within the Journal's scope. Review articles cannot exceed 5000 words length (excluding title page, abstract and references) and contain no more than a combination of 10 tables and/or figures. Authors are encouraged to restrict figures and tables to essential data that cannot be described in the text. The number of references should not exceed 80.

A THOUSAND WORDS ABOUT... is a form of Mini-Reviews. Manuscripts in this category should focus on *latest achievements of life science and medicine.* Manuscripts should be up to 1000 words in length (excluding title page, abstract and references) and contain up to 5 tables and/or figures and up to 25 most relevant references. The number of authors is limited to no more than 3.

OTHER SUBMISSIONS

Invited Editorials: Editorials are authoritative commentaries on topics of current interest or that relate to articles published in the same issue. Manuscripts should be up to 1500 words in length. The number of references should not exceed 10. The number of authors is limited to no more than 2.

Images in Clinical Medicine: Manuscripts in this category should contain one distinct image from life science or medicine. Only original and high-quality images are considered for publication. The description of the image (up to 250 words) should present relevant information like short description of the patient's history, clinical findings and course, imaging techniques or molecular biology techniques (e.g. blotting techniques or immunostaining). All labeled structures in the image should be described and explained in the legend. The number of references should not exceed 5. The number of authors is limited to no more than 5.

The Rationale, Design and Methods of New Studies: Manuscripts in this category should provide information regarding the grants awarded by different founding agencies, e.g. National Health Institute, European Union, National Science Center or National Center for Research and Development. The manuscript should be presented in the format of Research Project Objectives, Research Plan and Basic Concept, Research Methodology, Measurable Effects and Expected Results. The article should also contain general information about the grant: grant title, keywords (up to five), name of the principal investigator and co-investigators, founding source with the grant number, *Ethical Committee permission number,* code in clinical trials (if applicable). Only grant projects in the amount over 100,000 Euro can be presented. Manuscripts should be up to 2000 words in length (excluding references) and can include up to 5 tables and/or figures. The abstract should not exceed 150 words. The number of authors is limited to the Principal Investigator and Co-investigators.

Acknowledgements

Under acknowledgements please specify contributors to the article other than the authors accredited. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.). Also acknowledge all sources of support (grants from government agencies, private foundations, etc.). The names of funding organizations should be written in full.

References

All manuscripts should use the 'Vancouver' style for references. References should be numbered consecutively in the order in which they appear in the text **and listed at the end of the paper.** References cited only in Figures/Tables should be listed in the end. Reference citations in the text should be identified by Arabic numbers in square brackets. Some examples:

This result was later contradicted by Smith and Murray [3].
Smith [8] has argued that...
Multiple clinical trials [4–6, 9] show...

List all authors if there are six or fewer; if there are seven or more, list first six follower by "et al.". Journal names should be abbreviated according to Index Medicus.

Some examples

Standard journal articles

1. Fassone E, Rahman S. Complex I deficiency: clinical features, biochemistry and molecular genetics. J Med Genet. 2012 Sep;49(9):578–590.

2. Pugh TJ, Morozova O, Attiyeh EF, Asgharzadeh S, Wei JS, Auclair D et al. The genetic landscape of high-risk neuroblastoma. Nat Genet. 2013 Mar;45(3):279–284.

Books

Personal author(s)

1. Rang HP, Dale MM, Ritter JM, Moore PK. Pharmacology. 5th ed. Edinburgh: Churchill Livingstone; 2003.

Editor(s) or compiler(s) as authors

2. Beers MH, Porter RS, Jones TV, Kaplan JL, Berkwits M (editors). The Merck manual of diagnosis and therapy. 18th ed. Whitehouse Station (NJ): Merck Research Laboratories; 2006.

Chapter in the book

1. Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995. p. 465–478.

TABLES: Tables should be typed on sheets separate from the text (each table on a separate sheet). They should be numbered consecutively with Arabic numerals. Tables should always be cited in text (e.g. table 2) in consecutive numerical order. Each table should include a compulsory, concise explanatory title and an explanatory legend. Footnotes to tables should be typed below the table body and referred to by superscript lowercase letters. No vertical rules should be used. Tables should not duplicate results presented elsewhere in the manuscript (e.g. in figures).

FIGURES: All illustrations, graphs, drawings, or photographs are referred to as figures and must be uploaded as separate files when submitting a manuscript. Figures should be numbered in sequence with Arabic numerals. They should always be cited in text (e.g. figure 3) in consecutive numerical order. Figures for publication must only be submitted in high-resolution TIFF or EPS format (*minimum 300 dpi resolution).* Each figure should be self-explanatory without reference to the text and have a concise but descriptive legend. All symbols and abbreviations used in the figure must be defined, unless they are common abbreviations or have already been defined in the text. Figure Legends must be included after the reference section of the Main Text.

Color figures: Figures and photographs will be reproduced in full colour in the online edition of the journal. In the paper edition, all figures and photographs will be reproduced as black-and-white.

SUPPLEMENTARY ONLINE MATERIAL: Authors may submit supplementary material for their articles to be posted in the electronic version of the journal. To be accepted for posting, supplementary materials must be essential to the scientific integrity and excellence of the paper. The supplementary material is subject to the same editorial standards and peer-review procedures as the print publication.

Review Process

All manuscripts are reviewed by the Editor-in-Chief or one of the members of the Editorial Board, who may decide to reject the paper or send it for external peer review. Manuscripts accepted for peer review will be blind reviewed by at least two experts in the field. After peer review, the Editor-in-Chief will study the paper together with reviewer comments to make one of the following decisions: accept, accept pending minor revision, accept pending major revision, or reject. Authors will receive comments on the manuscript regardless of the decision. In the event that a manuscript is accepted pending revision, the author will be responsible for completing the revision within 60 days.

Copyright