Volume 1, Number 1, April 2017.
ISSN 2459-9484
http://seemedj.mefos.unios.hr

SEEMEDJ SOUTHEASTERN EUROPEAN MEDICAL JOURNAL

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Southeastern European Medical Journal (SEEMEDJ)

Published by

University Josip Juraj Strossmayer Osijek

Faculty of Medicine Osijek

Secretary: Mirko Pešić, PhD

English Language Proofreaders: AdHoc

Cover: minimal.com.hr

Technical Editors: minimal.com.hr

Web page: minimal.com.hr

Published online:

http://seemedj.mefos.unios.hr

ISSN 2459-9484

Implementation of the N - terminal proB-type Natriuretic Peptide Test in National Guidelines for Diagnosis of Heart Failure in Croatia

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Abstract

Aim: Our aim was to implement N - terminal proB-type natriuretic peptide (NT-proBNP) testing at the Department of Medical Biochemistry and Laboratory Medicine (DMBLM), accredited according to the HRN EN ISO 15189 standard, for clinical use at the Emergency Unit of the Internal Medicine Department in Merkur University Hospital.

Methods: Electro-chemiluminescence immunoassay (ECLIA) is a sandwich principle test with two monoclonal NT-proBNP-specific antibodies. PreciControl Cardiac II level 1 and level 2 were analyzed using the ECLIA on Roche Cobas e411, in triplicate, for five consecutive days, for the purpose of calculating within-laboratory precision, according to the Clinical and Laboratory Standards Institute (CLSI) protocol. We prospectively studied 87 Emergency Department (ED) patients with symptoms of decompensated heart failure (HF) during one month, measuring their NT-proBNP levels.

Results: According to the CLSI protocol, we calculated standard deviation and coefficient of variation for repeatability, intermediate precision and within-laboratory precision from control results. Calculated coefficient of variation for the overall laboratory precision for level 1 and level 2 was within the desirable biological criteria for precision, and within the manufacturer's criteria for overall laboratory precision. We assessed the association between the new NT-proBNP method and the outcome in HF patients during one month, and showed the distribution of NT-proBNP values in our ED patients.

Conclusion: Results indicate that the NT-proBNP test met all the set criteria and it has been implemented at the DMBLM for clinical use in Merkur University Hospital, according to Croatian national guidelines for diagnosis of heart failure.

(Barbir R, Zoric M, Perkov S, Pocanic D, Svagusa T, Prkacin, I. Implementation of the N - terminal proB-type natriuretic peptide test in national guidelines for diagnosis of heart failure in Croatia. SEEMEDJ 2017;1(1);1-4)

Received: March 5, 2017; revised version accepted: April 19, 2017; published: April 24. 2017

KEYWORDS: new natriuretic peptide test, laboratory precision

Introduction

Croatian guidelines have been prepared in accordance with the new 2016 European Society of Cardiology (ESC) Guidelines (1) and they note the use of N - terminal proB-type natriuretic peptide (NT-proBNP) biomarker as the first line of diagnosis of heart failure (HF), which enables rapid diagnosis and proper cardiac monitoring. The Guidelines were launched on November 3, 2016, the first day of the 11th Congress of the Croatian Cardiac Society. The guidelines were accepted with great enthusiasm by the participants – both primary care physicians and cardiology specialists.

Our aim was to implement the NT-proBNP test at the Department of Medical Biochemistry and Laboratory Medicine (DMBLM), accredited according to the HRN EN ISO 15189 standard, for clinical use at the Emergency Unit of the Internal Medicine Department in Merkur University Hospital.

Materials and methods

ECLIA is a sandwich principle test with two monoclonal NT-proBNP-specific antibodies. In the first incubation antigen in the sample, a biotinylated monoclonal NT-proBNP-specific antibody and a monoclonal NT-proBNP-specific antibody, labeled with ruthenium complex, form a sandwich complex. After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. After addition, tripropylamine voltage is applied to the electrode, which induces chemiluminescent emission (2). Accreditation requirements include the need to verify the method performance prior to using it on patient samples (3). During verification, lyophilized control samples were stored at 2-8˚C and stabilized at 20-25˚C before measurement. PreciControl Cardiac II level 1 and level 2 with values of 150 ng/L and 4930 ng/L were analyzed by the ECLIA on Roche Cobas e411, in triplicate, for five consecutive days, in order to calculate within-laboratory precision, according to the Clinical and Laboratory Standards Institute (CLSI) protocol: User Verification of Performance for Precision and Trueness, EP15-A2 (4). According to the protocol, we calculated arithmetic mean and standard deviation for each day of measurement, from which we determined the repeatability for each day, as well as the standard deviation for intermediate precision for all five days. Standard deviation for within-laboratory precision was calculated from the square root of the sum of standard deviation for repeatability and intermediate precision.

We prospectively studied the Emergency Department (ED) patients with symptoms of decompensated heart failure (HF) during one month, and measured their NT-proBNP levels. We included 87 consecutive patients in whom HF was determined based on clinical symptoms and congestive HF symptoms were determined based on admission chest X-Ray. Patients without decompensated heart failure were excluded. Patients' characteristics were: females/males = 41/47 (45%/55%), age: females 77 (range 52 – 92 years) and males: 65 (range 44 – 86 years).

The study was approved by the local Ethics Committee and written informed consents to participate were obtained.

Results

We implemented the NT-proBNP test according to the CLSI protocol and calculated standard deviation (SD) and coefficient of variation (CV) for repeatability, intermediate precision and within-laboratory precision from the control results. CV calculated for within-laboratory precision was 4,48% for level 1 and 4,15% for level 2 (Table 1)..

Calculated coefficients of variation for within-laboratory precision for two control samples were compared with coefficients of variation for the two set criteria, desirable biological criteria for precision and manufacturer's criteria for within-laboratory precision (Table 2).

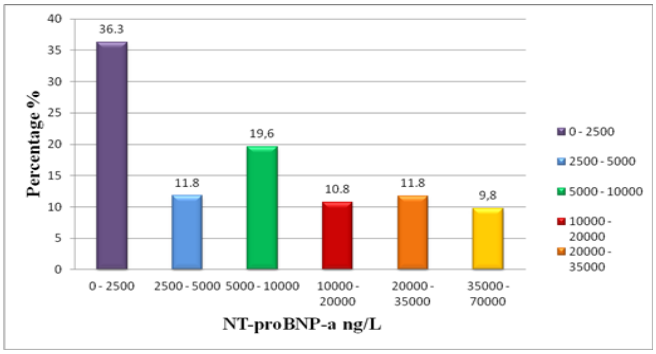
Table 1. *Calculated standard deviation (SD) and coefficient of variation (CV) for repeatability, intermediate precision and within-laboratory precision. (n – number of measurements per day, D – number of days)*

Control samples (n = 3, D = 5)	Repeatability		Intermediate precision		Within-laboratory precision	
Preci Control Cardiac II	SD (ng/L)	CV (%)	SD (ng/L)	CV (%)	SD (ng/L)	CV (%)
Level 1 (149 ng/L)	4,04	2,66	5,97	3,92	6,82	4,48
Level 2 (4920 ng/L)	83,96	1,77	189,98	3,89	197,27	4,15

Table 2. *Achieved coefficients of variation for within-laboratory precision and set criteria*

Preci Control Cardiac II	Manufacturers criteria for the overall laboratory precision	Achieved within-laboratory precision	Desirable biological criteria for precision
	CV (%)	CV (%)	Precision 0,5 CVintra (%)
Level 1 (149 ng/L)	5,00	4,48	5,00
Level 2 (4920 ng/L)	5,00	4,15	5,00

Figure 1. *Distribution of 105 NT-proBNP values (min. 8,29 ng/L-max. 70000 ng/L)*



From January 5 to February 9, the concentration of NT-proBNP was measured in 87 patients, with repeated measurements for 18 patients. 20 – 30% increase in concentration was observed in three patients (3/87, 1F/2M) in the period of ten days after admission, with a fatal end (short-term mortality in 1 month). The highest NT-proBNP value was detected in a man with cardiac shock (>70000n/L). Results of the NT-proBNP levels are shown in Figure 1.

Discussion

Despite very good curative cardiology, Croatia is still among the countries with high cardiovascular risk and mortality. Therefore, Croatian Guidelines for HF Diagnosis were developed according to the ESC Guidelines. NT-pro BNP is a proven diagnostic and prognostic biomarker for acute and chronic HF. It is highly recommended by the ESC guidelines (1). Natriuretic peptides predict cardiovascular events in patients and are associated with prognosis in decompensated heart failure (5). Implementation of the NT-proBNP test was conducted according to the CLSI protocol – calculated CV for the within-laboratory precision was 4,48% for level 1 and 4,15% for level 2, which was compared with the set criteria. Desirable biological criteria for precision was 5,00%, according to Ricos C. and colleagues (6), and manufacturer's criteria for within-laboratory precision was also 5,00 %, which indicates that

the achieved results were within the set criteria, desirable biological criteria for precision and manufacturer's criteria for within-laboratory precision, respectively (7). Concentration of NT-proBNP in samples obtained from the ED patients indicate good negative predictive value of the test, whereas high values were correlated with a higher mortality risk (8). In our paper, elevated NT-proBNP values were strongly predictive of adverse outcomes and rising values identified a rising risk, but lowering of NT-proBNP denoted improved outcomes. Thus, the direction of change is important.

Very high NT-proBNP concentration is an independent predictor of adverse outcomes in patients (9). Serial NT-proBNP testing provides very important clinical information. Monitoring of HF with NT-proBNP serial testing provides more clinical information than single testing (1). Implementation of the NT-proBNP test could lower the number of repetitive referrals to ED, help improve the quality of service and reduce unnecessary waste of resources.

Conclusion

Results indicate that the NT-proBNP test met all the set criteria and it has been implemented at the DMBLM since January 5, 2017, for clinical use at the Emergency Unit of the Internal Medicine Department in Merkur University Hospital, in accordance with Croatian national guidelines for diagnosis of HF.

Acknowledgement. None.

Disclosure

Funding. No specific funding was received for this study.

Competing interests. None to declare.

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Dirty Croatian Money: How Big is the Threat?

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Abstract

Aim: The objective of this study was to determine the extent of bacterial contamination, expressed as colony forming units (CFU), on individual banknotes and coins of Croatian Kuna (HRK). The purpose of the study was to define if the fear of money-transmitted diseases is founded.
Methods: One-hundred twenty pieces of banknotes and coins were collected for the experiment, 10 bills of 10, 20, 50, 100, 200 and 500 HRK and 10 coins of 10, 20 and 50 Croatian Lipa and 1, 2 and 5 HRK. At the Department of Microbiology and Parasitology, Faculty of Medicine, University of Osijek, swabs were taken from money, moistened in saline, planted on blood agar and incubated for 24 hours under ambient conditions at 37 ˚C. After growing the bacteria, CFU were counted and replanted for further identification, which was performed in accordance with the microbiological professional standards.
Results: In total, 739 bacterial CFU were grown and six bacterial species have been identified: *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus viridans*, *Bacillus sp.*, *Klebsiella sp.*, *Neisseria sp.* Almost 30% of the money was bacteriologically clean. There were no statistical differences between the prevalence of bacterial contamination of banknotes and coins. The most common bacteria isolated was *S. epidermidis* (86.33%) with statistical significance both on banknotes and coins ($p<0.0001$).
Conclusion: The identified bacterial species are mostly part of the normal human flora. Pathogenic, and potentially pathogenic bacterial species were not found on Croatian banknotes and coins in a respect for one colony of *Klebsiella sp*.

(Belić D, Ilinić M, Burian S, Talapko J, Drenjančević, D. Dirty Croatian Money: How Big is the Threat? SEEMEDJ 2017;1(1);5-10)

Received: March 14, 2017; revised version accepted: March 29, 2017; published: April 24. 2017

KEYWORDS: bacterial contamination, normal bacterial flora, banknotes, coins

Introduction

The conventional wisdom is that the money is dirty and contaminated with microorganisms that cause diseases. Generally, money keeps circulating from person to person, from device to device and probably is the most frequently exchanged article between human beings. The microorganisms present on money are part of the normal human flora or pathogens such as bacteria, viruses and fungi. Microbial contaminants may be transmitted directly, through hand-to-hand contact, or indirectly, via food or other inanimate objects. Cause for special concern is the possibility of transmission of nosocomial pathogens that represent one of the most important global threats of our time, such as the spread of multi-resistant bacteria. For example, methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most resistant nosocomial pathogens. This pathogen can survive on banknotes and coins (1). Medical staff and patients who do not observe prescribed protection measures (hand washing and disinfection) before and after a direct contact with an infected person are the most common carriers of MRSA. There is no person that did not suffer from diarrhea at least once in their life. Diarrhea is mainly caused by enteric pathogens such as enterotoxigenic *E. coli*, *Vibrio Cholerae*, *Salmonella*. It has been proven in well-designed experimental research that bacteria mentioned above can survive on money surface (1, 2). Although this is an important issue, currently there is limited literature available. In this research, we are trying to answer some important questions regarding the hygienic safety of Croatian money and its role in transmission of infectious diseases and to clarify some urban legends and fears regarding the impurity of money. Indeed, what kind of money is dirty?

Methods

We collected 120 pieces of banknotes and coins for the experiment, 10 pieces (paper money) of 10, 20, 50, 100, 200 and 500 Croatian Kuna (HRK) and 10 coins of 10, 20 and 50 Croatian Lipa and 1, 2 and 5 HRK. These different denominations of HRK were circulating between people and were collected during everyday activities: in various shops and a bank. In the laboratory of the Department of Microbiology and Parasitology, Faculty of Medicine, University of Osijek, plain sterile swabs (Copan Italia S.p.A, Brescia, Italy) were taken for complete notes and coins (on both sides). Swabs were moistened in saline, plated on blood agar plate (BD, Sparks, USA) and incubated for 24 hours under ambient conditions at 37 ˚C. After growing the bacteria, colony forming units (CFU) were counted and replanted to a new solid growth medium for further identification. Identification of genera and species of bacteria was performed in accordance with the microbiological professional standards. Moreover, chromogenic UriSelect agar (BioRad, Redmond, USA), Gram staining and additional enzymatic and biochemical tests have been performed, such as coagulase, catalase, bacitracin test, optochin test, novobiocin test for identifications of Gram-positive bacteria and oxidase, sugar fermentation with acid and gas production (triple sugar iron), test for indole production, H2S production, citrate utilization and urease test for identification of Gram-negative bacteria.

Statistical Analysis

Statistical analysis was performed using the SPSS software (IBM SPSS Statistics ver. 16, IBM Corporation, Chicago, IL) and Microsoft Excel. Proportions were compared by chi-square test and Fisher's exact test. P values <0.05 were considered to be statistically significant.

Results

In total, 739 bacterial CFU were grown with the mean value of 6.16 colonies in six species of bacteria: *Staphylococcus epidermidis*, *Staphylococcus saprophyticus, Streptococcus viridans, Bacillus sp., Klebsiella sp., Neisseria sp*. Altogether, 36 samples (30%), 14 coins and 22 banknotes, were without the growth of bacteria, as shown in Figure 1.

On the coins of 1 and 2 HRK, bacteria were detected in 90% of cases (9/10), as opposed to 5

HRK coins, where bacteria were detected in 60% of cases (6/10).

With regard to banknotes, on 500 HRK notes bacteria were detected in 80% of cases (8/10), as opposed to 20 HRK notes, where bacteria were detected in 40% of cases (4/10) (Figure 2).

All the banknotes and coins were taken from everyday life: there were nine banknotes which appeared almost "brand new" with the assumption that a minimum number of people touched them, as well as two coins and three banknotes that appeared extremely dirty. In total, there were 571 colonies on 38 coins, and 168 colonies on 46 banknotes isolated, which shows that some coins or banknotes had multiple colonies isolated. On the 10 Lipa, 2 HRK and 5 HRK coins, S. epidermidis and Bacillus sp. were isolated. Along with the above listed bacteria, on the coin of 50 Lipa, *Neisseria sp*. was also isolated. *S. epidermidis* and *S. viridans* were found on the 20 Lipa coin. It should be noted that on a single 20 Lipa coin there were 201 colonies isolated, which was the maximum number of colonies found on a single coin. Money with the most bacteria grown was 1 HRK, *S. epidermidis*, *S. viridans, Bacillus sp., Neisseria sp.* Among banknotes, the most common bacterial species was *S. epidermidis*, found on notes of 10, 20, 50, 100, 200 and 500 HRK. *Bacillus sp.* was also found on all of the listed notes, except 10 and 20 HRK. On the banknotes of 10 HRK, beside *S. epidermidis, Klebsiella sp., S. saprophyticus* were also isolated. There is no statistical significance between the prevalence of bacterial contamination of banknotes and coins. The identified bacterial species are mostly part of the normal human flora. Clinically relevant pathogenic, and potentially pathogenic bacterial species were not found on Croatian banknotes and coins except forone colony of *Klebsiella sp*.

Discussion

Six species of bacteria were grown in 739 bacterial CFU. Almost 30% of the money was bacteriologically clean. There were no statistical differences between the prevalence of bacterial contamination of banknotes and coins. The most common bacteria isolated was *S. epidermidis* (86.33%) with statistical significance both on banknotes and coins.

Despite the expectation that money is highly contaminated with bacteria and fungi (3), in this experiment we have shown that 30% of Croatian money was bacteriologically clean, which is in accordance with observations from an experimental bacterial survival study conducted in the Netherlands (4). It was also expected that the banknotes or coins with lower denomination will have more contaminants compared to those with higher denomination, but that was not proven in our study. There is no statistical significance between the prevalence of bacterial contamination of banknotes and coins. Identified bacterial species are mostly part of normal human flora. Some studies have shown that copper seems to be a limiting factor for bacterial survival on coins (1, 5, 6, 7), but in our study no statistical significance was found between banknotes and coins. Also, it was expected that lower denomination banknotes are used more frequently in daily life and would be more contaminated, as shown in a Bangladesh study (8), but in our study no statistical significance was found between

Figure 2. *The frequency of banknotes and coins with cultured bacteria (60 banknotes and 60 coins).*

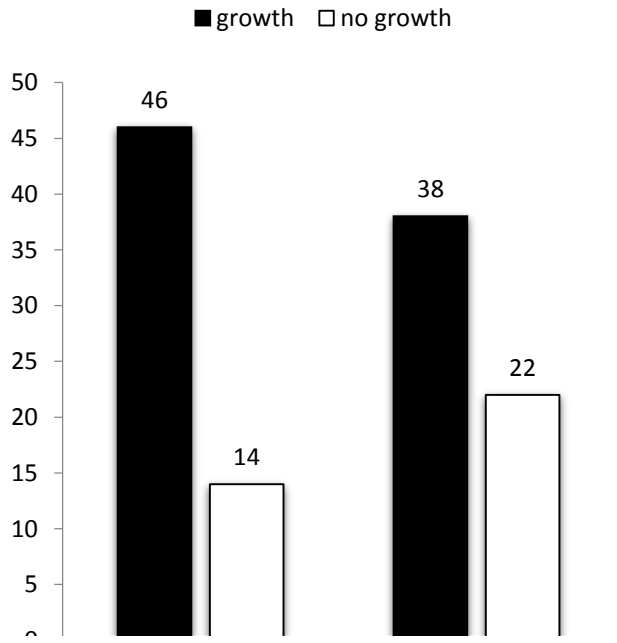
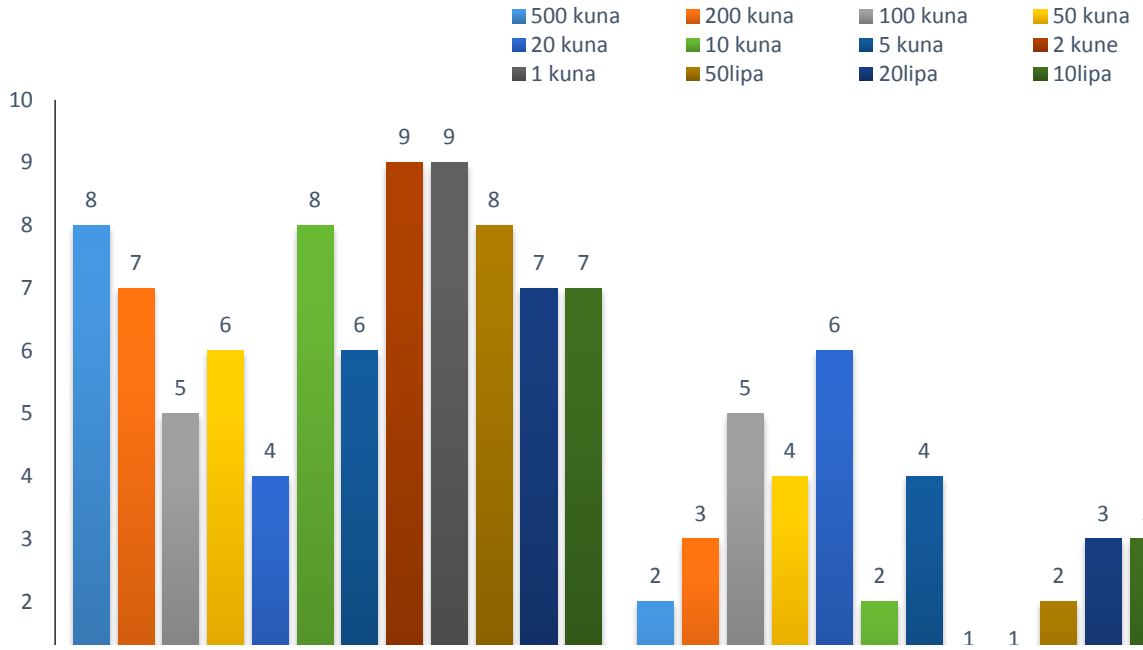


Table 3. *Number of colony forming units (CFU) on banknotes and coins. There were 120 pieces of banknotes and coins in total.*

Bacterial species	Total CFU	Coins with growth	Banknotes with growth	Total positive (percentage)
S. epidermidis	638	41	38	79 (66%)
Bacillus sp.	33	9	7	16 (13%)
S. saprophyticus	6	0	1	1 (1%)
S. viridans	41	2	0	2 (2%)
Neisseria sp.	20	2	0	2 (2%)
Klebsiella sp.	1	0	1	1 (1%)
total	739	54	47	101 (84%)

Figure 4. *Distribution of bacteriologically positive and negative findings per denominations in HRK (10 per each banknote/coin, total 120)*



higher and lower denominations of banknotes. In recent, well designed experimental research, whose objective was to ascertain the survival rates of some of the most important bacterial species, including nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), extended spectrum beta-lactamase (ESBL) producing *Escherichia coli*, and *Vancomycin-Resistant Enterococci* (VRE), it has been shown that Croatian Kuna was the cleanest currency among those examined (4). Furthermore, the Croatian Kuna was found to inhibit the growth of all of the multi-drug resistant bacteria tested, which was completely unexpected (4). Other currencies had a tendency to grow colonies of different pathogens: cultures of the Romanian Leu yielded MRSA, VRE and ESBL producing *E. coli*, the Canadian and US Dollar only yielded MRSA; the Euro only ESBL-producing *E. coli*, the Indian Rupee only VRE, and

the Croatian Kuna did not yield any of the 3 microorganisms (4). This can be the result of the material of which banknotes are made, as polymer-based banknotes presented lower bacterial counts than cotton-based bank-notes (1,3). Other studies have shown that 100% of currency notes from India (9, 10, 11), Bangladesh (8,9), Iraq (9,11), and Ghana (9,11,12) were found to carry pathogenic or potentially pathogenic bacteria. In 1972, a study was conducted by Abrams and Waterman and it was found that 42% of paper money and 13% of coins collected from laboratory workers was contaminated by potential pathogens, such as *S. aureus, E. coli, Klebsiella sp., P. aeruginosa, and Proteus mirabilis* (6).

Studies which showed significant contamination of banknotes and coins of different currencies with pathogenic and potentially pathogenic bacteria have been conducted previously (3, 13). It was also shown that money can be a vector for the spreading of the most significant clinical bacterial isolates and also have an impact on the emergence of hospital infections (1).

Conclusion

The identified bacterial species are mostly part of the normal human flora. Pathogenic, and potentially pathogenic bacterial species were not found on Croatian banknotes and coins, except for one colony of *Klebsiella sp*. Despite the resulting information from our research, hand hygiene should be implemented after each contact with money.

Acknowledgement

Results have been presented as a poster during a student scientific meeting held in Sarajevo in February 2016 (SaMed 2016).

Disclosure

Funding. No specific funding was received for this study.

Competing interests. None to declare.

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Hepatitis C Treatment: A Review and Update

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Abstract

Hepatitis C virus infection (HCV) infects approximately 185 million individuals worldwide. It is a leading cause of chronic liver disease and the primary reason for liver transplantation. The main aim of antiviral treatment is to achieve a sustained virologic response, which means eradication of the virus. The combination of pegylated-interferon and ribavirin was the standard of care for over a decade, despite the long treatment duration and severe adverse effects. The introduction of direct-acting antivirals with pan-genomic properties and excellent tolerance increased rates of SVR and shortened the duration of the therapy. Furthermore, it allowed clinicians to customize HCV therapy according to important clinical parameters such as HCV-genotype and liver fibrosis stage.

(Jakab J, Žulj M, Volarić N, Tepeš B, Včev A. Hepatitis C Treatment: A Review and Update. SEEMEDJ 2017;1(1);11-18)

Introduction

Hepatitis C virus infection (HCV) represents a significant public health burden and infects approximately 185 million individuals worldwide. It is a leading cause of chronic liver disease, which can evolve to cirrhosis in 15% to 20% of those infected within 20 years, resulting in severe outcomes such as end-stage liver disease and hepatocellular carcinoma (1). It is the primary reason for liver transplantation in Europe and the United States (2). There are seven HCV genotypes with marked differences in global geographic distribution and susceptibility to antiviral therapy (3). Since the discovery of HCV in 1989, there have been numerous advances in medical therapies available for the treatment of HCV infection. The main aim of antiviral treatment is to eradicate the virus, defined as a sustained virologic response (SVR), which means undetectable levels of plasma HCV RNA for 12 to 24 weeks after completion of therapy (4). SVR is associated with decreases in all-cause mortality, liver-related death rates and complications, and hepatocellular cancer rates (5, 6). The first agents available for treatment of HCV were the alfa interferons which resulted in SVR rates of approximately 15% (7). The

Received: March 15, 2017; revised version accepted: April 12, 2017; published: April 24. 2017

KEYWORDS: hepatitis C infection, treatment, direct-acting antivirals

combination of interferon (IFN) and ribavirin (RBV) improved SVR rates to 41% for 48-week treatment regimens (8). With the introduction of pegylated-interferon (Peg-IFN), SVR rates increased to 50% (9). Besides its limited effectiveness, the combination of Peg-IFN and ribavirin was associated with long treatment duration and frequent and severe adverse effects, but remained the standard of care for over a decade. The introduction of direct-acting antivirals (DAAs) has revolutionized therapeutic options for HCV infection (10). The first two approved drugs were boceprevir and telaprevir, NS3/4A protease inhibitors, used in combination with traditional dual therapy (11, 12). This strategy managed to increase the rates of SVR to 70% (10-12), but with greater toxicity, an increased pill burden, drug-drug interactions, low barriers to resistance, and notable side effects (13-15). Since 2013, and the introduction of new, more effective DAAs with pan-genomic properties and excellent tolerance, the treatment of HCV has had a new scenario: increased rates of SVR (even up to 100%), shorter therapy duration and less toxicity (16).

Direct-acting antivirals

Currently available DAAs are classified into four categories based on their mechanism of action: non-structural 3/4A serine protease inhibitors, non-structural 5A inhibitors, nucleotide analogue inhibitors of the non-structural 5B polymerase and non-nucleoside inhibitors of the non-structural 5B polymerase (17, 18). These drugs target the replication, polyprotein processing, packaging in nucleocapsid, assembly, and release of HCV infectious particles (3). Mutations at different positions in the NS3 protease, NS5B5 polymerase and NS5A protein affect viral susceptibility and the outcome of DAA-based therapies. However, the high specificity of DAAs makes them sensitive to small changes in the viral sequence responsible for antiviral resistance (10). Different currently approved interferon-free combination therapies with DAAs provide synergistic antiviral potency and prevent the development of DAAs resistance (19).

First-generation NS3/4A protease inhibitors

The NS3/4A protease is involved in post-translational processing and releasing of the NS3, NS4A, NS4B, NS5A and NS5B from the HCV polyprotein (20), and it is critical for replication of the virus (21). In 2011, the first generation of NS3/4A inhibitors (boceprevir and telaprevir) was specific for the treatment of chronic hepatitis C, genotype 1, and was used in combination with PegINF and RBV. The mechanism of action is the forming of a reversible covalent bond with the NS3/4A active site. Limitations to the triple therapy for CHC included adverse effects such as gastrointestinal issues, skin reactions and bone marrow toxicity, and low barriers to resistance (22). Also, boceprevir and telaprevir are potent cytochrome p-150 inhibitors, resulting in significant drug-drug interaction (12).

Second-generation protease inhibitors

Second-wave protease inhibitors offer several advantages over the first generation: improved pharmacokinetics, which allows a once-a-day dosing schedule, more tolerable side-effects, and fewer drug-drug interactions (23).

NS5A inhibitors

Because of its critical involvement in viral replication and assembly (27), NS5A has been identified as a target for viral inhibition. Inhibition of NS5A at picomolar concentrations has been associated with significant reductions in HCV RNA levels in cell culture-based models (28, 29). NS5A inhibitors have pan-genotypic activity. Synergistic inhibition of viral production and an increased barrier to resistance (30) have been accomplished with the use of multiple DAAs, including an NS5A inhibitor, in cell culture. The exact mechanism of antiviral action of NS5A inhibitors is unknown, and its detailed function remains unclear. Available evidence suggests that they have multiple effects, interfering with several functions of NS5A in the HCV life cycle, and disrupt the establishment of replication sites (31). NS5A inhibitors are daclatasvir, ombitasvir, ledipasvir, elbasvir, and velpatasvir.

Table 1. *The list of currently available DAAs*

Medicine	Brand name	DAA class	Dosing	Genotypes
Sofosbuvir	Sovaldi	Nucleotide NS5B polymerase inhibitor	400 mg (one pill) per day	Genotypes 1, 2, 3, 4
Simeprevir	Olysio	NS3/4A protease inhibitor	150 mg (one capsule) per day	Genotype 1
Daclatasvir	Daklinza	NS5A protein inhibitor	60 mg (two pills) once a day	Genotypes 1, 3 and 4 [9]
Sofosbuvir + ledipasvir	Harvoni	NS5A inhibitor	Sofosbuvir (400 mg) plus ledipasvir (90 mg), in a single pill taken once per day	Genotype 1 [10]
Ombitasvir-Paritaprevir/ Ritonavir and dasabuvir	Viekira	NS5A inhibitor + NS3/4A protease inhibitor + CYP3A4 enzyme inhibitor + non-nucleoside NS5B polymeraseinhibitor	12.5-75-250-50 mg	Genotype 1
Daclatasvir in combination with sofosbuvir +/ RBV		NS5A inhibitor + nucleotide polymerase NS5B inhibitor	60 mg once daily + sofosbuvir 400 mg once daily ± weight-based ribavirin	Genotypes 1-4
Velpatasvir in combinationwith sofosbuvir	Epclusa	NS5A inhibitor + nucleotide NS5B polymerase inhibitor	400 mg of sofosbuvir and 100 mg of velpatasvir once daily	Genotypes 1-6

Polymerase NS5B inhibitors

NS5B is the RNA-dependent RNA polymerase, and it is next reasonable target for the treatment of HCV. NS5B inhibitors can be classified into non-nucleotide inhibitors (NNIs) and nucleotide inhibitors (NIs) (32), both of them binding to the NS5B polymerase to terminate replication of the virus. NS5B utilizes the HCV RNA genome as a template for RNA synthesis. Nucleoside analogue inhibitors incorporate into the HCV RNA chain leading to chain termination. Because of that, NS5B inhibitors commonly show pan-genotypic activity with a high barrier to resistance. They are commonly used in combination with other classes with higher barriers to resistance. The most used NS5B inhibitors are sofosbuvir, as a nucleoside analogue inhibitor (33), and dasabuvir, as an example of a non-nucleoside inhibitor.

Currently available DAAs and treatment strategies

A number of highly effective DAAs allow clinicians to customize HCV therapy according to HCV-genotype, liver fibrosis stage and previous treatment experience (34, 35). Commercialized DAAs approved by the United States Food and Drug Administration (FDA) (4) are listed in Table 1.

Several factors must be taken into account when selecting the suitable therapeutic regimen. Clinically, it is necessary to evaluate

Table 2. *Treatment strategies in Croatia*

Clinical parameters	**Treatment option**
Genotype 1 naive patients	
Mild fibrosis	Peg IFN +RBV for 24-48 weeks.
Advanced fibrosis	Simeprevir/sofosbuvir and PeglFN + ribavirin.
Significant fibrosis, contraindications to IFN therapy, presence of extrahepatic manifestations, HIV-Coinfection, transplanted patients	IFN-free regimens for 12 weeks: Ombitasvir, ritornavir-boosted paritaprevir, dasabuvir + ribavirin; Sofosbuvir and ledipasvir ± ribavirin; Sofosbuvir and simeprevir ± ribavirin.
Decompensated cirrhosis	Sofosbuvir and ledipasvir ± ribavirin for 24 weeks.
Genotype 1 experienced patients	
Relapse or partial responders with F1-F3	PegIFN-α + RBV and simeprevir or sofosbuvir for 12 weeks.
Non-response (regardless of fibrosis); F4 fibrosis (regardless of type of response); TT IL-28B genotype; contraindications to IFN therapy; extrahepatic manifestations, HIV-coinfected and transplanted patients	IFN-free regimens for 12 weeks: Ombitasvir, paritaprevir and ritonavir, dasabuvir ± ribavirin; Sofosbuvir and ledipasvir ± ribavirin; Sofosbuvir and simeprevir ± ribavirin.
Decompensated cirrhosis	Sofosbuvir and ledipasvir with ribavirin for 12 weeks, or 24 weeks without ribavirin.
Previously treated with PegIFN-α + RBV + PI	Sofosbuvir + ledipasvir ± ribavirin for 12 weeks.
Genotype 4	The same recommendations as for genotype 1, with the exception of fixed combination of ombitasvir, paritaprevir and ritonavir, which is used without dasabuvir.
Genotype 2	
Treatment naive with F1-F3 fibrosis	PegIFN-α and RBV for 24 weeks.
Treatment naive with F4 fibrosis, treatment experienced regardless of fibrosis stage, contraindications to IFN, extrahepatic manifestations or HIV coinfected patients, transplanted patients	Sofosbuvir and ribavirin for 12-20 weeks (depending on cirrhosis).
Genotype 3	
Treatment naive with F1-F3 fibrosis	Peg IFN and ribavirin for 24 weeks.
Treatment naive with F4 fibrosis, treatment experienced	PegIFN-α, RBV and sofosbuvir for 12 weeks.
F1-F3 fibrosis, contraindication to IFN	Sofosbuvir and ribavirin.
F4 fibrosis, contraindication to IFN therapy	Sofosbuvir, ledipasvir and ribavirin for 24 weeks or sofosbuvir, daclatasvir and ribavirin for 24 weeks.

disease, the degree of hepatic fibrosis, the presence of decompensation in patients with cirrhosis, renal function, the presence of extrahepatic manifestations of HCV, and the other medications the patient is taking. Virologically, it is necessary to know the genotype and its subtype, as well as to monitor the viral load at 12 to 24 weeks after therapy is completed and, in some cases, to determine the IL28B polymorphism or Q80K mutation (36, 37).

The highest priority for treatment should be given to the patients with advanced fibrosis, compensated cirrhosis, severe extrahepatic hepatitis, as well as those on the active liver transplant waiting list, and liver transplant patients with recurrence of the infection. Patients with high priority for treatment are the non-responders to triple therapy or patients with extrahepatic manifestations of HCV. Independently of the disease status, treatment should also be considered for certain subgroups of HCV patients, such as patients at high risk of transmission or women of child-bearing age (34, 35).

HCV treatment in Croatia

The prevalence of HCV in Croatia in the general population is low, with the estimated number of HCV patients around 39,000 (38, 39) and higher prevalence rates in prison populations, HIV patients, individuals with high-risk sexual behavior and alcohol abusers. Management of HCV infection in Croatia is following the European Association for the Study of the Liver (EASL) guidelines, Croatian Guidelines, and the recommendations of the Croatian Health Insurance Fund (HZZO). According to the recommendations, the highest priority is given to the patients with significant fibrosis or cirrhosis, extrahepatic manifestations, in patients with HCV recurrence after liver transplantation and in HBV/HCV and HIV/HCV-coinfected patients. Priority is also given to individuals at risk of transmitting HCV (injection drug users, people with high-risk sexual practices, women of child-bearing age, haemodialysis patients). Treatment can be postponed in patients with no or mild liver fibrosis with no clinically significant extrahepatic manifestations. On the other hand, treatment is not recommended in patients with limited life expectancy (35-37, 40, 41). Available drugs covered directly by the Croatian Health Insurance Fund in 2016 are Peg IFN; ribavirin; simeprevir; sofosbuvir, ombitasvir + ritonavir-boosted paritaprevir +/- dasabuvir; and sofosbuvir + ledipasvir. Treatment strategies for patients with different clinical parameters are listed in Table 2.

Conclusion

The appearance of direct-acting antiviral drugs has brought significant advances in the treatment of hepatitis C – high tolerability and convenient dosing of an all-oral regimen, reduced progression to cirrhosis and lower incidence of complications. With continuous screening and education, as well as timely detection, diagnose and treatment of HCV, reducing the prevalence and final eradication of the disease appears to be promising.

Acknowledgement. None.

Disclosure

Funding. No specific funding was received for this study.

Competing interests. None to declare.

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Functional Carotid Ultrasound Markers of Subclinical Atherosclerosis in Men With Cardiovascular Risk Factors

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Abstract

Aim: The aim of this study was to compare the most commonly used ultrasound parameters of elasticity and stiffness of the arterial wall in detection of carotid subclinical atherosclerosis (SA) in vascular healthy men with one of the most prominent cardiovascular risk factors (CVRF).

Methods: Total of 120 vascular healthy men between 33 and 59 years of age ($\bar{x}$(SD) = 49.7(6.93)) were allocated into hypertension, diabetes, smokers and control group of respondents without CVRF. Ultrasound examination of carotid arteries was used to measure intima-media thickness and maximal and minimal lumen diameter. Along with the blood pressure of all the respondents, the following markers of elasticity/stiffness of arterial wall were calculated: distensibility coefficient (DC), compliance coefficient (CC), Young's elasticity modulus (YEM) and β stiffness index (β).

Results: DC, CC and β indicated significantly lower elasticity and higher stiffness of arterial wall in hypertension and diabetic groups of respondents without CVRF (multiple comparison, $p<0.001$). There were significant changes in functional characteristics of carotid arteries present between respondents-smokers and the control group detected by DC and β (multiple comparison, $p<0.05$). There were 94 respondents (78%) with subclinical carotid atherosclerosis criteria. ROC analysis indicated that DC, β and CC (AUC 0.811, 0.810, 0.799) are good markers for SA.

Conclusions: In conclusion, it is possible to use an ultrasound in detection of changes of elasticity/stiffness in arterial wall caused by the major CVRF in vascular healthy men. DC and β seem to be the best indicators of the presence/absence of subclinical carotid atherosclerosis.

(Buljan K, Benšić M, Buljan V, Vladetić M, Butković Soldo S. Functional carotid ultrasound markers of subclinical atherosclerosis in men with cardiovascular risk factors. SEEMEDJ 2017;1(1);19-26)

Received: March 8, 2017; revised version accepted: April 13, 2017; published: April 24. 2017

KEYWORDS: ultrasonography; carotid atherosclerosis; risk factors; arterial stiffness

Introduction

Subclinical atherosclerosis (SA) is a latent precursor of cardiovascular disease, but the complete prevalence remains unfamiliar (1). There is a high level of clinical interest in detecting vascular changes by non-invasive methods, as it could contribute to identifying individuals at high risk of cardiovascular incident, who might also be in need of more aggressive approach to risk factors (2). SA indicates the cumulative effect of all known and unknown risk factors together, thus providing better data than the data evaluation of only known risk factors (3). Different types of ultrasound tests of subclinical atherosclerosis bear different prognostic features. There was an independent correlation recognized between the changes of the elastic properties of the carotid arteries and cardiovascular outcome (4) as well as ischemic stroke (5). The aim of this study was to compare the most commonly used ultrasound parameters of elasticity and stiffness of the arterial wall in the detection of carotid SA in vascular healthy men with one of the most prominent cardiovascular risk factors (CVRF).

Patients and methods

The research was conducted from October 2011 to June 2014 and it included 120 men from eastern Croatia (Osijek and 40 km radius) between 33 and 59 years of age ($\bar{x}$(SD)=49.7 (6.93)), who had never had a vascular disease. The respondents were patients referred to diagnostic procedures or specialist examinations at the Clinical Hospital Center Osijek; they were diabetic patients hospitalized at the Department of Endocrinology at the Clinical Hospital Center Osijek and volunteers (who were mostly part of the control group). Three risk factor criteria including hypertension, diabetes type II and smoking were indicators in forming three groups; the risk factors being present over the last five years. The control group consisted of respondents without CVRF.

To eliminate the possibility of arterial hypertension (according to the American Heart Association), each respondent's blood pressure was set at <140/<90 (6). To exclude the diabetes, fasting blood glucose (FBG) level was set at <7.0 mmol/L and the value of glycated hemoglobin (HbA1c) was set at ≤6.5% (according to the American Diabetes Association) (7). Respondents smokers were men who smoked for longer than five years and more than 10 cigarettes a day on average, while non-smokers were identified as men who have not actively been smoking for the last five years, and in time prior to that no more than 10 cigarettes per day in a period of one year (8). Excluding factors were the presence of another CVRF, i.e. anamnestic or medical history data of arterial disease, both present or past (i.e. myocardial infarction, angina pectoris, ischemic cardiomyopathy, cerebral infarction, transient cerebral ischemic attack, peripheral arterial disease, abdominal aortic aneurysm and cardiac arrhythmia) or previous therapeutic procedure of arterial disease (i.e. percutaneous transluminal angioplasty, endarterectomy, intra-arterial stent implantation, arterial bypass).

The carotid arteries ultrasound scan of all respondents has been performed in B-mode by the same examiner (KB), by linear probe 7.5 MHz on Aloka Pro Sound 5000, Tokyo, Japan. Carotid atherosclerotic plaque was defined by *Mannheim*carotid intima-media thickness and plaque*consensus (*9, 10) and it was observed in the segment including 30 mm distal common carotid arteries (CCA), carotid bulb and proximal 20 mm internal carotid arteries on both sides of the neck. Intima-media thickness was measured by the standard protocol on the far wall of the distal segment of both CCA, 10 mm proximal of the starting point of bifurcation (9), with multiangle insonation (anterolateral, lateral and posterolateral) (11), in diastolic blood flow phase with maximum magnification of the image. The mean of carotid intima-media thickness (cIMT) is set for the right and left side for each respondent. Ultrasound criteria for defining SA were the presence of atherosclerotic plaque on carotid arteries and/or cIMT values ≥75th percentile of the control group (11, 12). The measurement of the minimal diastolic diameter of CCA between two lines of adventitia-media was performed 10mm proximal of bifurcation

(13), three times on each side, and the lowest value was taken as the referential one. The maximum systolic and minimum diastolic lumen diameter of common carotid arteries in intima-media area of close and far arterial wall was measured in maximum systolic expansion of the artery and minimal lumen width, during the relaxation of the artery at the end of diastole (14). Measuring was performed four or five times on each artery, with the maximum image magnification, along with the examination of previously recorded and stored images over three to five cardiac cycles (15). The results of the maximum and minimum diameter were an average of two maximal systolic and an average of two minimal diastolic lumen diameters for each respondent.

Just before the first and during the carotid arteries diameter measurement, the blood pressure was also measured on the upper arm side that corresponded to the test of the current carotid artery. It was taken by automatic electronic sphygmomanometer Omron M6 Comfort, Kyoto, Japan, which was validated according to the international protocol of the European Society of Hypertension (16). The conversion factor of the measured blood pressure from mmHg to kPa is 0.13.

Ultrasound elasticity markers of the carotid wall - distensibility coefficient (DC) and compliance coefficient (CC) were calculated according to the following:

$$DC = \frac{\left(\frac{2\Delta D}{D_D}\right)}{\Delta P}[kPa^{-1}]$$ (15, 17),

$$CC = \frac{\pi D_D \Delta D}{2\Delta P}[m^2 kPa^{-1}]$$ (17, 18).

Stiffness markers of arterial wall – Young's elasticity modulus (YEM) and beta stiffness index (β) were calculated according to the following:

$YEM = \frac{E_P D_D}{2CIMT}[kPa]$, where $E_P = \frac{\Delta P D_D}{\Delta D}[kPa]$ (18, 19) and

$E_P = \frac{\Delta P D_D}{\Delta D}[kPa]$ where $STRAIN = \frac{\Delta D}{D_D}[\%]$ (18,20).

In the above expressions, D_D is CCA lumen diameter at the end of diastole, ΔD is pulsatile diameter change, i.e. the difference in systolic and diastolic diameter of the CCA lumen, ΔP is pulse pressure, i.e. the difference in systolic and diastolic pressure readings, E_P is Peterson's (elastic) modulus, ln is natural logarithm, and strain is the change in lumen diameter of the CCA during cardiac cycle (expressed in percentage).

Total cholesterol was quantitatively measured by enzymatic spectrophotometric method, while hsCRP was measured by turbidimetry (Beckman Coulter AU 680 analyzer). Blood glucose was measured quantitatively by UV enzymatic test (hexokinase method) on Beckman Coulter AU 680 analyzer. HbA1c was quantitatively measured by turbidimetric inhibition immunoassay (TINIA) principle on Dimension clinical chemistry system RxL, Siemens.

Statistical analysis

Wilcoxon's signed-rank test, when applied to 17 respondents, did not indicate statistically significant difference in the first and second (performed within three months) ultrasound parameters of the following: cIMT, diameter, ΔD. Furthermore, statistically significant difference was not found in the number of subjects with or without carotid atherosclerotic plaque.

Figure 3. *Distensibility coefficient of the respondents with the cardiovascular risk factor.*

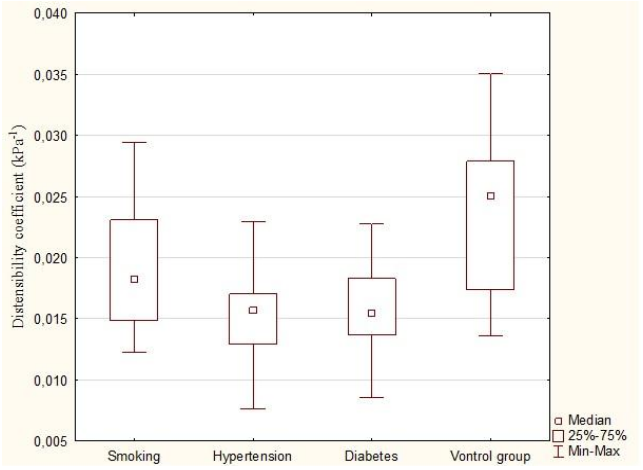

Figure 2. *ROC curve of functional ultrasound tests of the carotid arteries with subclinical atherosclerosis prediction.*

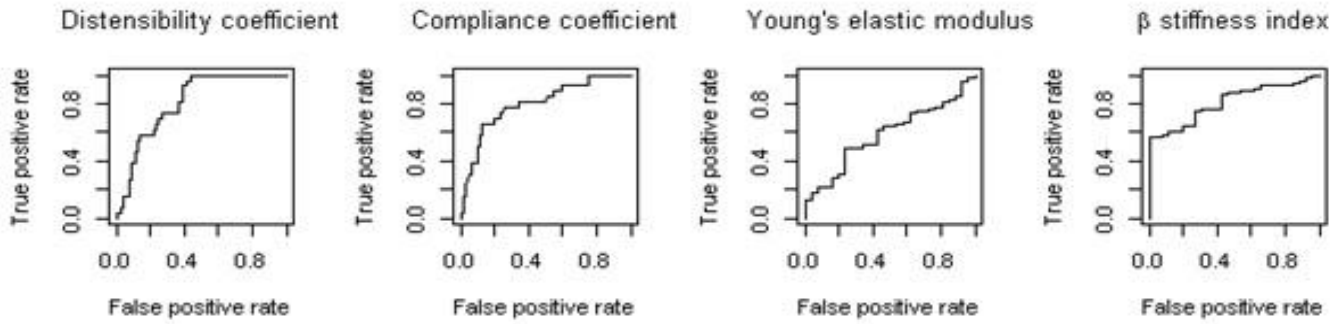

Table 1. *Average value (±SD) of hemodynamic and metabolic variables of respondents with respect to cardiovascular risk factor.*

MEN (N=120)	CARDIOVASCULAR RISK FACTOR			Control group (N=30)	p
	Hypertension (N=28)	Diabetes (N=30)	Smoking (N=32)		
	x̄(SD)				
Age, years	49,7(8,16)	50,9(5,69)	49,1(6,94)	49,1(6,53)	0,741‡
Duration of the risk factor, years	8,2(3,72)	10,8(4,32)	26,1(6,79)	-	<0,001§
Systolic blood pressure, mmHg	145,1(16,46)	128,8(9,38)	127(10,32)	126(12,18)	<0,001§
Diastolic blood pressure, mmHg	90,6(10,84)	81,7(5,37)	81,6(7,15)	80,3(8,17)	<0,001§
Total cholesterol, mmol/L	5,73(0,874)	5,81(2,415)	5,95(1,024)	5,69(0,97)	0,904‡
HsCRP*, mg/L	2,012(1,371)	3,018(2,579)	3,52(4,964)	1,096(0,701)	<0,001‖
Fasting plasma glucose, mmol/L	5,25(0,692)	9,99(2,658)	5,24(0,745)	4,96(0,419)	<0,001§
HbA1c†, %	5,62(0,375)	9,21(2,02)	5,68(0,437)	5,52(0,301)	<0,001§

In order to verify the difference in the expected numerical values of arterial walls, depending on their groups, T-test, i.e. ANOVA test was used. In some cases, in order to verify the difference in data distribution, it was necessary to use Kruskal-Wallis test, Mann-Whitney U test for independent samples, or Wilcoxon's signed-rank test for paired samples. The effectiveness of the numerical variable in subclinical atherosclerosis risk assessment is discussed because of its sensitivity and specificity, but also because of the analysis of the ROC curve results. Significance level was set at 0.05 in all of the tests. Statistical analysis was carried out in the data analysis program R (https://cran.r-project.org, package ROCR and pROC) and Statistica (StatSoft, version 11, http//www.statsoft.com/company).

Results

Table 1 presents basic hemodynamic and metabolic variables of respondents with respect to CVRF. Statistically significant difference in age and levels of total cholesterol has not been found between the control group and risk factor groups (p=0.741, i.e. p=0.904). The hypertension group indicated the highest values of blood

Table 2. *Ultrasound carotid arteries' parameters of elasticity and stiffness with respect to the risk factor.*

MEN (N=120) **Functional ultrasonic parameter**	**CARDIOVASCULAR RISK FACTOR**			**Control group (N=30)**	**p¶**
	Hypertension (N=28)	Diabetes (N=30)	Smoking (N=32)		
	$\bar{x}$(SD)				
DC*, kPa^{-1}	0,0157 (0,0129-0,017)	0,0154 (0,0136-0,0183)	0,0182 (0,0148-0,0231)	0,0251 (0,0173-0,0279)	<0,001
CC‡, $10^{-7}m^2kPa^{-1}$	4,81 (4,15-5,56)	4,26 (3,65-5,24)	5,21 (4,7-6,49)	6,46 (5,42-7,27)	<0,001
YEM‡, kPa	623,2 (553-717,3)	529,9 (428,4-616,6)	533,2 (394,7-615,5)	444,2 (368,6-547,4)	<0,001
β§	4,86 (4,78-5,05)	4,87 (4,72-4,99)	4,71 (4,46-4,92)	4,38 (4,27-4,75)	<0,001

**distensibility coefficient; ‡compliance coefficient; ǂYoung's elasticity modulus; §beta stiffness index; ǁinterquartile range; ¶Kruskal-Wallis test*

pressure, while FBG and HbA1c were significantly higher in diabetic respondents (Table 1). Smokers indicated statistically significant higher values of hsCRP when compared to other risk factor groups and control group (p<0.001). Ultrasound markers of carotid arteries' elasticity – DC and CC – indicated differences between the control group and groups of respondents with hypertension and diabetes (multiple comparison, p<0.001), while indicating the difference of smokers in DC, but not in CC (multiple comparison, p=0.033, i.e. p=0.055) (Table 2, Figure1).

The analysis of β stiffness index indicated significantly higher arterial stiffness in respondents belonging to the hypertension and diabetic groups (multiple comparison, p<0.001), as well as smokers (multiple comparison, p=0.028) in comparison with the control group. YEM values, as the second arterial stiffness indicator, proved to be the highest in the hypertension group, i.e. they were significantly higher than those in the control group (p<0.001) (Table 2). Pearson's correlation coefficient indicated a significant positive correlation of age and β (0.507; p<0.001), and negative correlation of age and DC (-0.496; p<0.001), independent of CVRF presence/absence. ROC analysis of functional vascular ultrasound parameters with carotid SA prediction was done with respect to the SA ultrasound criteria – cIMT values ≥75th percentile in the control group and/or presence of the atherosclerotic plaque in carotid arteries (Figure2, Table 3). There were 94 out of 120 respondents (78%) with one or two subclinical carotid atherosclerosis criteria and 26 (22%) without any of the mentioned criteria.

Of all the functional ultrasound tests in predicting SA, the following seem to be the best indicators: DC (AUC 0.811), ß (AUC 0.810) and CC (AUC 0.799). The borderline value of DC to discriminate between the respondents with and without SA was set at ≥0.0231 kPa^{-1} (sensitivity 0.872, specificity 0.538), and for ß≤4.47 (sensitivity 0.872, specificity 0.538). ROC analysis of the ultrasound tests of carotid arteries has been lower in participants with CVRF (N=90, group+ =78, group- =12). According to AUC data, DC, CC, YEM and ß were lower (0.806, 0.784, 0.513 and 0.798) than in ROC analysis, which also included the respondents from the control group.

Discussion

Given that numerous research has already reported the correlation of age and arterial elasticity/stiffness indicators (18, 21-23), which has been corroborated in our research as well, the absence of significant age difference has been a key prerequisite of this research. As expected, the highest difference in blood pressure values was found in the hypertension

group, while FBG and HbA1c were found in respondents with diabetes. HsCRP was significantly higher in smokers than in non-smokers ($p<0.001$), similar to previous research (24, 25).

In this research, DC, CC and ß index indicated statistically significant lower elasticity and higher stiffness of the carotid arterial wall in respondents with diabetes, hypertension and smokers when compared to healthy participants without CVFR. These findings corroborate the results found in previous research (21, 26-28). Similar to our results, Sharret et al. found higher arterial wall stiffness in respondents with diabetes, but they have also found the correlation between smoking and higher elasticity of the arteries (29). By contrast, our research found ß and DC to be the ultrasound parameters that indicate statistically lower elasticity and higher stiffness of the arterial wall in smokers in comparison with the control group ($p<0.05$). The previous literature did not account for the comparison of values of different functional ultrasound parameters in detecting subclinical carotid atherosclerosis. ROC analysis indicated that DC, ß, and CC seem to be valid predictors of the subclinical carotid atherosclerosis in vascular healthy men, independently of the presence/absence of the CVRF. YEM, when compared to other functional ultrasound tests, did not indicate significant differences in determining the presence/absence of the SA of the observed groups of men.

Automatic measurement of the oscillation of carotid arteries' lumen width was not performed in this research due to technical malfunction of necessary appliances. According to Prado et al., some recent research used standardized manual approach, which could be as precise as automatic measuring (18). Cuadrado Godia et al. have reported good reproducibility of CCA diameters measured with B/M-mode sonography in their results (30).

Conclusions

To conclude, the results of this research indicate the possibility of ultrasound detection of early arteriosclerotic changes in elasticity and stiffness of carotid arteries in vascular asymptomatic hypertensive and diabetic respondents, as well as smokers. DC and ß seem to be the most prominent ultrasound markers, which could differentiate between male respondents with or without SA better than CC and YEM.

Acknowledgement. None.

Disclosure

Funding. No specific funding was received for this study.

Competing interests. None to declare.

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Pathophysiological Mechanisms of Takotsubo Cardiomyopathy - a Systematic Review

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Abstract

Takotsubo cardiomyopathy, also referred to as stress-induced cardiomyopathy, is an acute condition associated with transient left ventricular dysfunction. Since it can be induced by increased emotional stress (such as losing a loved one or constant anxiety) it is also called the broken heart syndrome. This type of cardiomyopathy occurs in all age groups and both sexes, but it is most common in postmenopausal women. There are several clinical manifestations such as chest pain, sometimes with heart failure, and often with ST-segment changes that may present as acute coronary syndrome. It is characterized by absence of coronary artery obstruction, with transient regional wall motion abnormalities and minimal elevation of cardiac enzyme levels. Although wall motion abnormalities are reversible in almost all cases, and long-term prognosis is excellent, this condition is important because in the acute phase it may cause sudden cardiac death. Mechanisms and cause of this disease still remain unclear. Some possible causes of the disorder include: 1) coronary artery vasospasm, 2) microcirculatory dysfunction, 3) transient obstruction of the left ventricular outflow tract, and 4) excessive release of catecholamine, which seems to have the most important role. The aim of this review is to summarize the most important pathophysiological mechanisms that may be responsible for the development of this type of cardiomyopathy.

(Mlinarevic D, Roguljic H, Juric I, Zebic Mihic P, Ivandic M, Stupin M. Pathophysiological Mechanisms of Takotsubo Cardiomyopathy - a Systematic Review. SEEMEDJ 2017;1(1);27-39)

Introduction

Takotsubo cardiomyopathy (TTC), also known as stress-induced cardiomyopathy, is a condition with left ventricular (LV) dysfunction, most commonly apical ballooning and less frequently midventricular or basal dysfunction. It was first described by Sato et al. in the early 1990s, who

Received: March 29, 2017; revised version accepted: April 13, 2017; published: April 24. 2017

KEYWORDS: Takotsubo Cardiomyopathy, stress-induced, left ventricular dysfunction, catecholamines

named it Takotsubo because the appearance of the LV is reminiscent of the octopus trap (1). The exact cause of this disease is still unclear. TTC clinically manifests with sudden chest pain and dyspnea preceded by emotional or physical stress, which are similar to those in acute coronary syndrome (ACS). Besides the onset of chest pain, ST-segment elevation and increase in creatine kinase and troponin are very common, and it is necessary to exclude obstructive coronary artery disease (CAD) (2).

Different types of LV wall motion abnormalities have been reported – apical, mid-ventricular or basal hypokinesia, dyskinesia or akinesia. Beside the LV, right ventricle may also be affected and it is associated with more severe LV dysfunction (3). Any form of dysfunction is reversible with resolution achieved in several days or weeks. Overall prognosis is favorable, but acute phase can be accompanied with acute heart failure and cardiogenic shock, rupture of the LV, malignant arrhythmias and in worst cases sudden cardiac death (4). For TTC diagnosis, four diagnostic criteria are suggested: 1) new electrocardiogram (ECG) abnormalities (convex ST-segment elevation); 2) transient apical dyskinesia or akinesia detected by echocardiography (ECHO); 3) non-obstructive CAD at angiography: 4) absence of myocarditis, pheochromocytoma, head trauma and intracranial hemorrhage or hypertrophic cardiomyopathy (5).

The actual incidence of TTC is unknown, but it is considered that prevalence among patients with ACS symptoms is 0.7-2.5%, and it is found most commonly in postmenopausal women (6). Since no large studies have confirmed the etiology of stress cardiomyopathy, determination of underlying cause is not possible, but it is almost always preceded by exaggerated emotional, physical or mental stress. Sudden death of a loved one, traffic accidents, various types of abuse, business failure, endoscopy, sexual intercourse and other are described as potential triggers of TTC. So far, several possible pathological mechanisms have been proposed, including coronary artery vasospasm, coronary microcirculatory dysfunction, myocarditis, obstruction of the left ventricular outflow tract (LVOT), abnormal metabolism of free fatty acids in the cardiac apex and catecholamine overload (7). TTC does not require specific treatment; management is primarily empirical and needs to be individualized for each patient (8).

This review will explain recent findings about the pathophysiological mechanisms in this type of cardiomyopathy.

Clinical Presentation, Diagnosis and Prognosis

Since TTC patients present with chest pain, dyspnea and syncope, it is difficult to differentiate it from ACS based on ECG and laboratory findings. Last findings suggest that approximately 1-2 % of patients presenting as ACS are ultimately identified as TTC (3, 9). There is no age group or sex that cannot be affected, but there is female predominance (more than 80% of patients are postmenopausal women) and it affects older adults more frequently (10-16). Similar to ACS, TTC patients also have cardiovascular comorbidities such as smoking, hypertension and dyslipidemia (17).

TTC is often, but not always, triggered by emotional or physical stress such as receiving bad news, unexpected death of relatives, dissatisfaction with relationships, devastating financial loss or acute medical illness. Some of the physical stressors include major surgeries like trauma surgery, infections or neurologic conditions. Patients with psychiatric or neurologic disorders may be predisposed to stress cardiomyopathy. Virtually any stressor, evena minor one, can be a precipitant of stress cardiomyopathy. It is worth mentioning that no stressor is identified in up to one-third of patients.

Physical findings are nonspecific and often normal, but the patient may present with signs of ACS or acute congestive heart failure. Other symptoms include nausea, vomiting and palpitations. Cardiac bradyarrhythmias and tachyarrhythmias, including ventricular tachycardia and ventricular fibrillation, may develop. (10, 11, 18-20). Some patients may develop signs of heart failure, and

approximately 10% of patients may develop cardiogenic shock (21-23). Left ventricular outflow tract obstruction caused by left ventricular basal hyperkinesis produces late-peaking systolic murmur and can contribute to severe mitral regurgitation, hypotension and shock (24).

Like any patient in whom ACS is suspected, ECG should be the initial test obtained. ECG abnormalities are common in patients with TTC (25). Most common abnormalities on initial ECG are ST-segment elevation and T-wave inversion. Studies found that ST-segment elevations involve the precordial leads and are maximal in leads V2-V3 (26). Patients with TTC have a significantly lower amplitude of ST segment elevation compared to STEMI from LAD occlusion. ST depression is a less common finding in patients with TTC. Kosuge et al. found that combination of absent abnormal Q waves, absent reciprocal changes, lack of ST-segment elevation in lead V1, and the presence of ST-segment elevation in lead aVR had 91% sensitivity and 96% specificity for TTC. Other possible findings include QT interval prolongation and non-specific ECG abnormalities. However, all these criteria have imperfect diagnostic accuracy and are not reliable for differentiation between the two conditions in the emergency setting to guide their management (e.g. decision to undergo emergency coronary angiography) (27).

Serum cardiac troponin I levels (TnI) are elevated in 90% of patients with TTC, while creatine kinase levels are generally normal or slightly elevated. The brain natriuretic peptide (BNP) orN-terminal pro-BNP are elevated in most patients with TTC (25). In patients with TTC, mean TnI level at the time of admission has been reported as moderately elevated. Nascimento et al. used this finding to create a criterion for differentiating between STEMI and TTC (28). The troponin ejection fraction product (TEFP) is the product of the peak troponin I level and the echocardiographically acquired ejection fraction. A TEFP ≥250 had an overall accuracy of 91% for STEMI identification. Budnik et al. found that the NTproBNP / TnI ratio was capable of distinguishing between TTC and STEMI. In this study, the concentration of NTproBNP was greater in patients with TTC than in ones with STEMI, while the concentration of TnI and CKMB mass was higher in the STEMI group than in the TTC group (29). Several studies analyzed the levels of circulating catecholamines in the acute phase and found that 75% of TTC patients had higher levels than patients with STEMI, but their role in diagnosing TTC is unclear (30,31).

Wall motion abnormalities are best identified by echocardiography or left ventriculography. Trans-thoracic echocardiography (TTE) is used as a quick method of diagnosing wall-motion abnormalities typical for TCC (32), such as hypokinesis or akinesis of the mid-segment and apical segment of the LV, which is present in 81.7% of patients (25). Crucially, these wall-motion abnormalities extend beyond the distribution of any single coronary artery. LV ejection fraction was found to be 20-49% on admission. The resolution of TTC usually occurs within four weeks with LVEF improving to 59-76%.

TTC diagnosis is usually confirmed by coronary angiography. Acute presentation with ST-segment elevation and symptoms suggestive of ACS mandate immediate evaluation with coronary angiography to exclude coronary occlusion.

The prognosis in TCM is excellent in most cases, with nearly 95% of patients experiencing complete recovery within 4-8 weeks (13). Mortality estimates range from 1% to 5.9%. Complications occur in around 20% of patients and include LV outflow obstruction, heart failure, ventricular arrhythmias, mitral regurgitation, LV mural thrombus formation or death. International Takotsubo Registry reported 5.9% mortality after 30 days (33). The rate of mortality during long-term follow-up was 5.6% per patient-year. A Swedish registry study found a 30-day mortality of 4.1% in 302 patients with TTC (34). These trials compared mortality in TTC with matched cohorts of patients with acute myocardial infarction or acute coronary syndrome and found a similar risk of death.

Coronary vasospasm and microcirculatory dysfunction

Coronary vasospasm was the first pathophysiological process considered as a cause TTC in the original article by Sato et al. in 1991 (35). Since TTC usually presents with transient wall motion abnormalities ("stunned myocardium") covering the irrigation territories of several coronary arteries (36), a multivessel coronary vasospasm could be the potential cause. However, according to current literature, coronary vasospasm is not a likely cause, because spontaneous vasospasm is rare in these patients (37) and cannot be induced in all the patients during angiography (38). Finally, TTC has a specific histological phenotype which differs from stunning associated with coronary artery disease (39).

On the other hand, there is an increasing body of evidence that microvascular dysfunction is one of potential pathophysiological mechanisms of disease. Diffuse microcirculatory dysfunction could explain wall motion abnormalities (WMAs) in several myocardial regions. Microcirculatory dysfunction may be primary or secondary, i.e. caused by excess of circulating cateholamines (37). The consequence of microcirculatory dysfunction is coronary slow flow (CSF), which in turn causes myocardial WMAs (40). Several methods have been used for evaluating CSF – Doppler guidewire during angiography (41), TIMI (Thrombolysis in myocardial infarction) myocardial perfusion grade (TMPG) (42, 43) and TIMI frame count (TFC) (11,44). The most commonly used method is TFC which is defined as "the number of frames required for the contrast material to travel from coronary ostium to the standardized distal landmark" (44). Several studies have demonstrated an increased TFC (i.e. slower coronary flow) in TTC patients (45-47), which supports the role of microcirculatory dysfunction. A study by Martin et al. in 2013, using peripheral arterial tonometry, demonstrated increased vascular reactivity and decreased endothelial function in response to acute mental stress in patients with previous TTC (48).

Although most of the available literature supports the role of either primary or secondary microcirculatory dysfunction in TTC, it is important to acknowledge that the results are not always uniform. A recent retrospective study by Khalid et al. found the TFC to be higher in left anterior descending (LAD) coronary artery of TTC patients, but no difference was found in TFC in right (RCA) or circumflex (CX) coronary artery (45). This anatomical distribution could explain the most common form of TTC involving the apex and the midventricular subtype, but not the less frequent form which involves the basal myocardium. Sharkey et al., in 2008, found a modest increase in TFC in TTC patients compared to controls (acute anterior STEMI with LAD occlusion), which was statistically significant in LAD and CX, but not in the RCA (30). Abe et al. (2003) found no coronary slow flow, no abnormalities using Doppler guidewire technique and no evidence of viral myocarditis in a series of 17 patients (49). Collste et al. (2015) investigated coronary flow reserve (CFR) by dobutamine stress echocardiography and the authors could not induce microcirculatory dysfunction, but found CFR at low-dose dobutamine was significantly lower in patients with TSC compared to controls (50).

Metabolic Disturbance

One possible hypothesis is that TTC may be considered a metabolic form of cardiomyopathy with disturbed cardiomyocyte metabolism. Several studies that were researching metabolic changes in stunned myocardium found alterations in glucose and fatty acid uptake. Those alterations may be the result of primary metabolic disturbance in cardiomyocyte or due to mitochondrial disturbance (51, 52). The metabolic disturbance was likely linked to the sudden preceding stress and resulted in corresponding perfusion abnormalities.

Yoshida et al. describe abnormalities in coronary perfusion and severe myocardial metabolic disorder in patients with TTC based on the results of thallium-201 myocardial single-photon emission computed tomography (SPECT) and F-18 fluorodeoxyglucose (F-18 FDG) myocardial

positron emission tomography (PET). They noticed markedly decreased uptake of F-18 FDGon PET at the apical region while thallium 201 images showed only mildly reduced uptake. Reason for decreased uptake may be due to increased density of beta receptors noted in apex (53).Several studies have reported prolonged reduction and reduced uptake of F-18 FDG in patients subjected to multiple cycles of ischemia and reperfusion (54, 55). Still, the precise mechanism for reduced glucose uptake in stunned myocardium remains unknown.

LVOT obstruction & Myocarditis

According to earlier studies, LVOT obstruction is registered in 15-25% of patients with TTC (10, 56) and Kawaji et al detected it in 33% of patients (57). Although it was proposed as a possible pathophysiological mechanism of the disease, it remains uncertain whether it is a consequence rather than a cause of stress cardiomyopathy (37, 58).

Some researchers claim that there is much evidence indicating this relation actually exists. Transient dynamic LVOT gradient was detected at initial evaluation in a substantial proportion of patients described by Tsuchihashi et al. (1) and other investigators (24, 59). At least in some patients, a possible mechanism for TTC could be a dynamic LVOT obstruction preceding the ischemic event. Some of those patients, primarily women, may have geometric predisposition to dynamic LVOT obstruction, such as sigmoid or bulging interventricular septum (60, 61), reduced left ventricular volume (62-64) or abnormal mitro-aortic and septo-aortic angles (65), which may manifest only in the setting of intense adrenergic stimulation or hypovolemia (37). Elderly women have a higher tendency to develop hypertrophy of the basal anterior septum. The angle of the septum may cause increase in the speed in the outflow tract which simulate a hypertrophic cardiomyopathy (66). It is also associated with an abnormal orientation of the mitral valve due to flaccidity, deformity of valve, false chordae, disturbances of the papillary muscles, or systolic anterior movement (67-69) with mitral regurgitation. It is known that even in a normal heart, exposure to an exogenous catecholamine, such as dobutamine infusion, can precipitate dynamic LVOT obstruction (70).

If present, the dynamic obstruction increases apical LV wall stress and LV filling pressure, increasing myocardial oxygen demand at the mid-to-apical cavity. If this persists, apical hypoperfusion and ischemia may occur, with regional wall motion abnormality and stunning. Increased adrenergic tone might produce primary LVOT obstruction leading to secondary ischemia and focal wall-motion abnormalities. Physical or emotional stress could be the trigger of acute development of LVOT obstruction, which could produce severe apical ischemia. Identification of acute dynamic LVOT obstruction as the possible initial mechanism in some of the patients with stress cardiomyopathy may have important clinical and therapeutic implications (71).

Previously suggested possible role of a transient dynamic LVOT obstruction in the pathogenesis of this syndrome is not strongly supported by other investigators. According to Ishihara, it is unlikely that LVOT obstruction is the cause of TTC because most of these patients do not have LVOT obstruction. It is known that this condition is characterized not only by reduced apical LV wall motion, but also hyperkinesis of the basal LV wall, and that the combination possibly causes the LVOT obstruction (57, 72, 73). LVOT obstruction is not a prerequisite, but can contribute in a deteriorating clinical course of TTC (71, 73).

Takotsubo cardiomyopathy is characterized not only by reduced apical LV wall motion, but also hyperkinesis of the basal LV wall. This combination causes the LVOT obstruction in TTC.

The suggested possibility that myocarditis leads to transient LV dysfunction and Takotsubo cardiomyopathy is not well supported by the data. Arguments to rule out myocarditis include absence of typical clinical signs, unspecific findings on myocardial biopsy and negative results on serum tests for viral serology. Some studies used cardiac magnetic resonance

imaging, which has shown no evidence of myocarditis (35, 74-77).

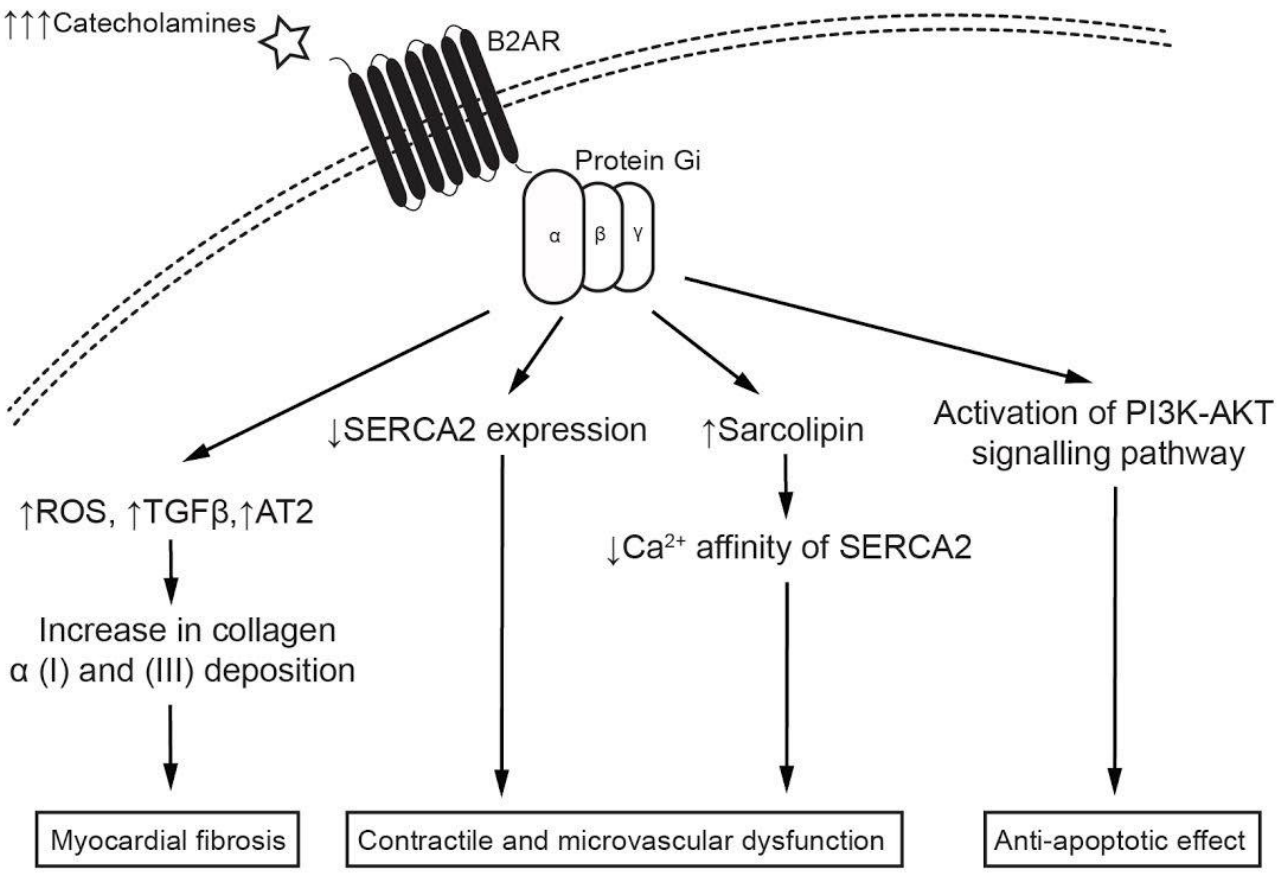
Catecholamines – pathophysiological hallmark of stress cardiomyopathy

Exposure to high intensity stress conditions, whether physical or emotional, has been associated with most cases of TTC (37). Furthermore, patients with pheochromocytoma, a catecholamine-producing tumor, are prone to develop a similar form of cardiomyopathy (78). In the animal model of subarachnoid hemorrhage, a condition with heightened catecholamine levels, a correlation between the extent of myocardial damage and sympathetic discharge was reported (79). Those observations suggest increased sympathetic activity and catecholamine mediated effect as a crucial factor for development of the broken heart syndrome. Although apart from catecholamine-mediated effect other theories about pathophysiology of stress cardiomyopathy coexist, the current Mayo Clinic criteria require catecholamine-producing tumor to be ruled out for establishing a TTC diagnosis (80).

Relationship between high levels of serum catecholamines and stress in patients suffering from cardiomyopathy was first shown in 2003 (76). Abraham et al. reported the emergence of all morphologic forms of TTC in patients exposed to catecholamines and other beta-receptor agonists used routinely during procedures and diagnostic tests (81). Study of Wittstein et al. reported that levels of catecholamines and dopamine are approximately two to three times higher in patients with TTC in comparison with cardiomyopathy caused by acute myocardial infarction (77). Interestingly, the correlation between morphological changes of the left ventricle in TTC and distribution of adrenoreceptors has been observed. A majority of β2 receptors with negative inotropic effect is in the apex of the left ventricle where ballooning process takes place which is consistent with a theory of catecholaminergic stress (39). Moreover, increased release of the catecholamines from the hearts of the patients affected with TTC has been reported (82). Some of the nuclear imaging studies also stressed the influence of the sympathetic nervous system in development of TTC. In eight patients with TTC, a decreased 123i-metaiodobenzylguanidine uptake within left ventricle was registered, indicating the existence of cardiac sympathetic hyperactivity as pathophysiological pathway (83). Moreover, a concordance between regional wall motion abnormalities and reduced uptake of the glucose and free fatty acid has been shown (84). Although this impairment of metabolism is not fully understood, catecholamine induced injury of cardiomyocytes is probably the cause of a metabolic stunned myocardium. Experiments in animals provided further evidence regarding the role of the catecholamines in disease patho-genesis. Iatrogenic administration of β2 agonists or immobilization stress in animals can result in reversible left ventricular apical ballooning. This adverse effect could be mitigated by α- and β-receptor blocking agents (85, 86).

Stress cardiomyopathy is characterized by similar molecular manifestations as the other catecholamine-mediated cardiomyopathies. Those morphological alterations caused by catecholamine overload include: extracellular matrix overproduction, contraction band necrosis and mononuclear cell infiltration (37). Catecholamine induced accumulation of collagen α-1 (I) chain in extracellular matrix results in large and rapid increase in fibrosis. High levels of catecholamine may result in high levels of profibrotic mediators (angiotensin II and free oxygen radicals), which can activate stimulating connective tissue growth factor, transforming growth factor β and the profibrotic osteopontin (87). Catecholamine overload stimulates β-adrenoreceptors and alters the expression of calcium-regulatory protein genes which cause alteration of the calcium regulatory system (88). Sarcolipin (SLN) and Phospholamban (PLN) are proteins of sarcoplasmic reticulum (SR) which regulate cardiac contractility. SLN regulates the sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) by lowering its affinity for

Figure 1. *Schematic overview of pathophysiological mechanisms involved in catecholamine-mediated Takotsubo cardiomyopathy.*



calcium. In acute phase of TTC, ventricular expression of SLN is raised and could contribute to contractile dysfunction (89).

At physiological conditions, binding norepinephrine on β1 – adrenoreceptors (β1AR) and epinephrine on β2 adrenoreceptors (β2AR) in cardiomyocytes results in positive inotropic response. In normal human ventricular myocardium, there are four times more β1AR than β2AR (90). Positive inotropic response is the result of β1AR or β2AR activating stimulatory G protein (Gs) family, which activates protein kinase A (PKA) pathway reflected as an increased contractile response (91). Supraphysiological levels of catecholamines result in β2-coupling from Gs to inhibitory G protein (Gi), which is reflected as a negative inotropic effect. This process is also called stimulus trafficking (92). The density of β2AR is the highest in cardial apex, so there is the greatest negative inotropic effect (91). The β2AR -Gi protein pathway can activate the p38 mitogen-activated protein kinase (MAPK) alteration of myofilament sensitivity. β2AR–Gi protein has a favorable outcome on stress cardiomioypathy by stimulating the Pi3K–aKt-signaling pathway, which activates antiapoptotic genes (NFκB1 and BCL2) (37). This is a physiological balance because β1AR–Gs protein pathway has the proapoptotic effect (93). It is cardioprotective because it minimalizes catecholaminergic stimulation. After epinephrine levels are normalized, β2AR–Gi switch to β2AR–Gs or are degraded, which results in recovery of cardiomyocyte contractile function (91).

Although pathophysiological pathway of TTC is still unclear, it is certain that catecholamine overload presents a common denominator in development of the broken heart syndrome, as presented in Figure 1. Despite plentiful clinical findings, further research is obligatory to complete the puzzle of this rare but potentially severe disease.

Conclusion

Takotsubo cardiomyopathy is an important type of acute heart failure with transient left ventricular wall motion abnormalities. The symptomatology, echocardiographic and electrocardiographic features frequently mimic acute coronary syndrome, which is why TTC must be considered in a differential diagnosis of patients with acute chest pain. According to contemporary literature, TTC is most likely caused by supraphysiological levels of catecholamines due to acute mental stress. Elevated catecholamine levels induce myocardial fibrosis, and contractile and microvascular dysfunction. While the manifestation of TTC can be clinically dramatic and potentially life-threatening, the prognosis is usually excellent – 95% of patients fully recover within one to two months.

Acknowledgement. None.

Disclosure

Funding. No specific funding was received for this study.

Competing interests. None to declare.

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The Correlation of Ultrasonographic and Pathophysiologic Measurements of Umbilical Vessels in Gestational Diabetes

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Abstract

Aim: The resistance of placental blood vessels might be increased in diabetic pregnancies. This increased resistance can affect uteroplacental blood flow and influence the oxygen and nutrient supply of the fetus and fetal growth. Our aim was to compare the ultrasonographic, pathomorphologic data and vasoreactivity of umbilical and placental vessels of gestational diabetic newborns with that of normal pregnancy newborns.

Methods: In this case-control study the placental vascularization of pregnant women was determined by 3D power Doppler ultrasound technique. We calculated the vascularization index (VI), flow index (FI) and vascularization flow index (VFI). We performed a tissue bath experiment (treatment with oxytocin and desmopressin) on umbilical vessels and collected pathomorphologic data according to the Royal College of Pathologists' 2011 protocol.

Results: The placental vascularization index and the umbilical artery S/D were significantly lower in the case group. The mean VI was 6.21% (±2.69 SD) in control versus 3.5% (±2.97 SD) ($p<0.05$) in GDM. The mean value of the umbilical artery S/D was 2.27 (±0.22 SD) and 2.18 (±0.45 SD) ($p<0.05$) respectively. In an isolated tissue bath experiment, oxytocin and desmopressin did not elicit significant contraction on umbilical cord vessels.

Conclusion: Our results suggest that umbilical vessels might have a different receptor pathway regulation that can compensate for the rheological changes in the pregnant woman's blood flow and gives opportunity for selective therapy to fetuses more vulnerable to hypoxia.

(Jakó M, Surányi A, Kaizer L, Domokos D, Bártfai G. The Correlation of Ultrasonographic and Pathophysiologic Measurements of Umbilical Vessels in Gestational Diabetes. SEEMEDJ 2017;1(1):40-49)

Received: March 7, 2017; revised version accepted: April 20, 2017; published: April 24. 2017

KEYWORDS: Gestational Diabetes, 3D Power Doppler, Histopathology, Umbilical cord

Introduction

Gestational diabetes mellitus (GDM) affects 14% of pregnancies, or 135,000 women a year in the USÄ, although its incidence varies nationwide. The mother's risk for conversion to type 2 diabetes ranges from 2.6 to 70% over a period from 6 weeks to 28 years postpartum (1). There are many, noncorresponding data regarding the prevalence of GDM in Hungary. In a population-based screening program, 75 g OGTTs were offered to all pregnant women between 24–28 weeks of gestation and evaluated according to WHO criteria. In that study 8.7% of pregnant women were diagnosed with GDM, and the risk increased linearly with maternal age. Women with the highest BMI (≥ 29.2 kg/m2) had decreased risk compared to women with a BMI of 26.1–29.1 kg/m2 ($p<0.05$). These results suggest that pre-pregnancy BMI and previous deliveries elevate the risk of GDM only to a certain level, above which the risk decreases (2).

Postprandial glucose concentrations steadily increase as tissue sensitivity to insulin decreases. To maintain proper blood glucose value along pregnancy, the pancreatic gland of the mother increases insulin secretion to compensate. Insulin resistance and impaired insulin secretion by the pancreas cause the development of GDM, especially during the third trimester of pregnancy (3). Pregnancy is a complex metabolic state that involves dramatic alterations in the hormonal milieu, changes in adipokines and inflammatory cytokines. There are high levels of estrogen, progesterone, prolactin, cortisol, human chorionic gonadotropin, placental growth hormone, human placental lactogen, leptin, TNF α, and oxidative stress biomarkers. In addition, decreases in adiponectin worsen maternal insulin resistance in the second trimester (4). The placental and fetal demands for glucose are considerable and approach the equivalent of ~150 grams per day of glucose in the third trimester. Glucose transport to the fetus occurs in direct proportion to maternal glucose levels, and is augmented by a five-fold increase in a placental glucose transporter, (GLUT-1) which increases transplacental glucose flux (5). Fetuses exposed to a high glucose environment usually have medical complications after delivery, including infant respiratory distress syndrome, cardiomyopathy, hypoglycemia, hypocalcemia, hypomagnesaemia, polycythemia, and hyperviscosity. The hyperglycemia and adverse pregnancy outcome (HAPO) study, which investigated about 25,000 pregnant women in 15 centers, found that even in subclinical hyperglycemia, higher maternal BMI was associated (odds ratio [95% confidence interval] with an increased frequency of birthweight >90th percentile (3.52 [2.48–5.00]) and percentage of body fat >90th percentile (3.28 [2.28–4.71]), caesarean section (2.23 [1.66–2.99]), and cord C-peptide >90th percentile (2.33 [1.58–3.43]) (6). The first line of management of women with gestational diabetes is medical nutrition therapy and a given minimum of exercise. Patients who fail to maintain normal glycemic values via diet and exercise therapy receive insulin (7).

As umbilical cord vessels represent a suitable model for the study of vascular alterations brought about by GDM, the aim of the present work was to compare the ultrasonographic vascular flow measurements to pathological microvascular changes, and also to test the vasoreactivity of the vessels. The chosen agents were oxytocin, which is present naturally at the time of pregnancy and can pass through the placenta (8), and vasopressin, an oxytocin receptor agonist. Oxytocin and vasopressin are both peptide hormones, and oxytocin is widely used to promote labor contractions in clinical practice. The effects of these peptides are mediated via transmembrane receptors. Both the oxytocin receptor (OTR) and the V1a vasopressin receptor (V1aR) are expressed in human myometrium. The expression of OTR is significantly higher in gravid uterus while V1aR expression is not significantly elevated compared to non-gravid uterus (9). Oxytocin gene and receptor expression have also been shown in human chorion, deciduas and amnion epithelial cells with the onset of labor. The structural similarity of the two peptides (approximately 80%), as well as the homology of the receptors, causes some cross-reactivity

between peptides and receptors (10).The placental presence of V1aR mRNA is known in sheep but not in humans. The lack of AVP mRNA suggests the fetal origin of the ligand (11). Pharmacological studies suggest the existence of vascular endothelial AVP/OT receptors that mediate a vasodilatory effect. However, the nature of the receptor subtype(s) involved in this vasodilatory response remains controversial. RT-PCR experiments with total RNA extracted from a human umbilical vein endothel amplified the OT receptor sequence only, but various vascular beds display different sensitivities to AVP (12). Other studies suggest that the vasopressine receptor type 2 is responsible for vasodilation via nitrogen-monoxide production (13,14). To the best of our knowledge, no receptor subtypes have been investigated in umbilical arteries, and the fetal production of the ligands is a hypothesis.

We hypothesize that during myometrial contraction the above mentioned vascular relaxation can maintain fetoplacental circulation. The aim of this study was to investigate whether the vasodilatory effect of AVP/OT receptor pathways is present in fetoplacental circulation. We also wanted to investigate whether the diabetic vasculopathy and vessel calcification affects this vasodilation.

Materials and Methods

Ultrasound screening

We collected samples of umbilical cords from the Department of Obstetrics and Gynecology at the University of Szeged from October, 2013 until June, 2014. Focusing on the well-known risk factors, a detailed medical history was taken, and relying on the results of routine ultrasound screening, fetal biometry and anamnesis, we set up a case group of gestational diabetic pregnancies (GDM fetuses) (n = 5) and compared them to a control group (n = 22).

Gestational diabetes was diagnosed at gestational weeks 24-28 as hyperglycemia, first detected in pregnancies that met the WHO's guide criteria (15). Each of the patients with gestational diabetes was treated with dietary restrictions and monitoring. No insulin treated patients were included. The control group consisted of patients without diabetes or other endocrinologic, vascular or pulmonary disease. They delivered normal weight, healthy newborns. Those who did not fit any of the above criteria were excluded from this study.

Gestational age was determined based on the first day of the last menstrual period and on ultrasound biometry (crown-rump length [CRL] and biparietal diameter [BPD]) at the 10th week of pregnancy. All patients were scanned in a semirecumbent position. The factorial default setting "Obstetrics/2-3 trimester" was used in 2-D mode. The examination was followed by a fetal biometry to assess BPD, head circumference (HC), abdominal circumference (AC) and femur length (FL). Fetal weight was calculated by the formula B of Hadlock (16). A conventional color Doppler study of umbilical arteries was also performed and the systolic velocity/diastolic velocity(S/D) ratio, the resistance index (RI) and pulsatility index (PI) were read from the ultrasound report display.

The images used for the determination of placental volume and three-dimensional power Doppler (3DPD) indices were obtained at the time of the visit. All 3-D scans and the 2-D ultrasound measurements were performed. Voluson 730 Expert ultrasound machines equipped with a multifrequency probe (2-5 MHz) were used to acquire all images (GE Medical Systems, Kretztechnik GmbH&Co OHG, Austria). Each sample was examined using a 3-D rendering mode, in which the color and gray value information was processed and combined to produce 3-D images (mode cent; smooth: 4/5; FRQ: low; quality: 16; density: 6; enhance: 16; balance: 150; filter: 2; actual power: 2 dB; pulse repetition frequency: 0.9). A power Doppler window (pulse repetition frequency at 900 Hz and wall filter of 50 Hz) was placed over the placenta mapping the vascular tree from the basal to chorionic plates. We used fast low resolution acquisition to avoid any kind of artifact. The 3-D static volume box was placed over the highest villous vascular density zone at umbilical cord insertion (17-19). During gestation we recorded one sample from each patient. The

stored volumes were further analyzed using the Virtual Organ Computer-aided Analysis (VOCAL) program pertaining to the computer software 4D View (GE Medical Systems, Austria, version 10.4) by an expert in 3-D analysis (18).The power Doppler technique shows high sensitivity, because it is based on amplitude instead of mean frequencies to depict the vascular tree (19). Moreover, the color mapping is independent from the angle of insonation and does not show "aliasing". The placental vascular indices from sonobiopsy are a valid alternative for evaluation of the placental vascular tree when visualization of the entire placenta is not feasible. Based on the ultrasonographic technique vascularization index (VI, the perfusion of the tissue, the percentage of blood flowing through), the flow index (FI, the amount of red blood cells flowing through a given area in a given time) and their combination, the vascularization flow index (VFI) can be determined (20). The spherical sample volume was consistently 28ml. The VOCAL program calculated automatically gray and color scale values in a histogram from the acquired spherical sample volume in each case (18-19).

The delivery mode and duration with gestational age were recorded in the delivery room along with the body weight and body length of the newborn, of which Rhohner's ponderal index could be calculated with a normal value between 2.2 and 2.9. For statistical reasons, the gender and 1-5 minute Apgar-scores were also registered.

Tissue Bath Study

At the placental insertion two 10 cm long segments of the umbilical cord were removed immediately and the one proximal to the placenta was stored at 4C, in pH 7.4 Krebs-Henseleit solution (118 mM NaCl; 4.7 mM KCl; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4.7H_2O$; 2.5 mM $CaCl_2$ $2H_2O$; 25 mM $NaHCO_3$, 11.7 mM dextrose). The samples were processed and stored in an ice-lined stainless steel thermos, and the solution was freshly prepared on a weekly basis and stored in a refrigerator until used. The vessels were dissected from Wharton's jelly within 24 hours to maintain their reactivity (11).The arteries were cut into two 3-5 mm long rings, and 4 similar rings were prepared from the vein. They were suspended on stainless steel hooks and placed in a tissue bath containing a Krebs-Henseleit solution at 37˚C, exposed to 2g initial tension and bubbled with carbogen (95% O_2, 5% CO_2). During the sixty-minute equilibration time we washed the system throughout with fresh solution every 15 minutes and let the spontaneous basal tone of the vessels to develop. Oxytocin and desmopressin were added to the vessels in the concentration range of 10-9-1x10-5M and 10-11-10-7M, respectively. The change in the vascular tone was detected by isometric sensors and recorded and analyzed by ISOSYS S.P.E.L. advanced computer software (Experimetria Ltd, Budapest, Hungary). The results are interpreted as the percentage of the basal, spontaneous vascular tone represented as 100% in value. The functionality of the vessel rings was tested with serotonin, which caused vasoconstriction, proving the viability of the vessels.

Pathological Examination

The placenta, along with the other segment of the cord, was stored in formalin for the morphological and histological examinations. The macroscopic evaluation of the samples followed the protocol published in 2011 by the Royal Society of Pathologists (22). From the length of the sample and the number of 360 turns by the vessels, we calculated the umbilical coiling index (UCI), with the standard normal value of one. In addition, with two scalpels fixed in the distance of two cm, three sections of the cord were cut. We stained their ends with ink and made prints on a millimeter paper, allowing them to be pushed to the paper by their natural weight. All the samples were handled by the same person. The area of the cross-sectional surface was measured using Image J 1.47v (2013) computer software on 3x6 prints in each case. For the preparation and evaluation of the histological sections of the samples, we used the remaining cord and placental tissue. These data fundamentally determined the subsequent evaluation of the results. Two-way ANOVA

statistical analysis was performed via Prism 6 (c 1992-2012 GraphPad Software Inc.).

Results

All together 27 placentas and cords (22 control and 5 GDM) were examined. The mean age of the pregnant women in the control group was 32.09 (±5.66 SD) versus 34.20 (±4.97 SD) in the GDM group. The number of previous gestations was 1.46 in the control and 2.8 in the GDM group. We recorded two previous intrauterine growth restricted newborns in the diabetic group, and one macrosomic newborn in the control group. Pregnancy has long been known to be diabetogenic in the sense of the progressive metabolic changes. Proportional regression analysis revealed that the relative risk of developing non-insulin-dependent diabetes mellitus postpartum was 1.95 (95% CI, 1.63-2.33) for each 4.5 kg of weight gained during follow-up (23).

In the control group, medications taken during pregnancy were either one of the available over-the-counter pregnancy multivitamins or none at all. In the GDM group, methyldopa, magnesium, vitamin B6, verapamil-hydrochloride, calcium-dobezilat and multivitamins were take none month prior to giving birth. The clinical data of the pregnant women and their newborns are summarized in Table 1.

All three vessels were present in each case along the entire length of the three to seven days old formalin-fixed samples. The pathomorphological data are shown in Table 2. The coiling of the cords is typically counterclockwise, twisted to the left, but twisting to the right occurred in 4 cases too; in the healthy control group (n = 3) and in the GDM group (n = 1). The UCI was calculated as the number of full turns made by the vessels within five centimeters. Perivascular hematoma and one hemangioma cavernosum were observed in the control, while two cases of multiple false knots, a slight funisitis and edematous cord were found in the GDM group. True knots, abnormal vascular division, stenosis, striction, meconium staining, maceration, thrombosis, calcification around the vessels, or other differences, discoloration, or odor were not observed. The cross-sectional surface area of the formalin-fixed tissue was compared to the measurements made on the ultrasonographic images. The cross-sectional surface area of the GDM fetuses' umbilical cords is roughly 65% of control cords measured in utero, and 76% after formalin fixation. The mean cord cross-sectional areas measured by 2D ultrasound were 344.00 mm2 (±0.46 SD) in the control group and 224.50 mm2 (±0.74 SD) in the diabetic group ($p<0.05$). After formalin fixation for three to seven days the cross-sectional areas were 124.00mm2 (±35.47 SD) and 93.75mm2 (±9.86 SD) respectively ($p<0.05$).

Measured with the ultrasonographic VOCAL technique, we found that the mean FI was 34.45% (±4.59 SD) in the control group and 29.80% (±3.68 SD) in the GDM group (ns); the mean VI was 6.21% (±2.69 SD) and 3.5% (±2.97 SD) ($p<0.05$), and the mean VFI was 2.31% (±1.23SD) and 0.95% (±1.20 SD) (ns) respectively. The mean value of the umbilical artery S/D was 2.27 (±0.22 SD) in controls and 2.18 (±0.45 SD) ($p<0.05$) in diabetic pregnancies.

Oxytocin did not elicit significant change in the vascular tone in the arteries and the veins, and the responsiveness of the vessels was not different significantly in the two groups (Figure 1). Desmopressin was also added to the vessels in logarithmic noncumulative concentrations (24) as shown in Figure 2, but there was again no response.

Discussion

In our study, umbilical 3D ultrasound examination showed that, compared to their gestational age, GDM fetuses have the normal S/D velocity curve. The VI is lower than in normal pregnancies because the development of blood vessels is not proportional to the growth of the placenta. In addition, they are damaged by the high serum glucose level. The

Table 1. *Clinical and biometric data of the pregnants and newborns.*

	Control (n=22)			GDM (n=5)			
	Mean	SD	SEM	Mean	SD	SEM	p
BMI before pregnancy	21.68	3.18	1.20	24.42	5.58	2.79	0.3968
BMI after pregnancy	25.85	1.85	0.58	30.83	4.44	1.98	0.0425*
previous pregnancies	1.46	1.97	0.59	2.80	0.84	0.37	0.0226*
systolic blood pressure (Hgmm)	123.20	25.86	7.80	124.00	19.49	8.72	0.9185
diastolic blood pressure (Hgmm)	78.00	10.63	3.21	83.00	13.04	5.83	0.4395
gestational age (weeks)	39.20	1.23	0.39	38.22	2.54	0.85	0.2812
1 minute Apgar	9.46	0.52	0.16	8.20	1.1	0.49	0.0213*
5 minute Apgar	10.00	0.00	0.00	9.00	1.23	0.55	0.1419
Rhohner's ponderal index	2.69	0.25	0.08	2.85	0.35	0.16	0.1867
BMI of newborn	13.16	0.96	0.32	14.10	1.99	0.89	0.3518
weight (g)	3221	266.1	94.08	3627	363.5	148.40	0.0411*
AC (mm)	290.5	34.65	24.50	383.80	29.26	14.63	0.6981
HC (mm)	303.50	9.19	6.50	318.50	16.30	8.15	0.1533
AC/HC	1.04	0.07	0.04	1.28	0.08	0.04	0.1238
BPD (mm)	88.00	4.69	2.34	92.50	3.78	2.18	0.1750
FOD (mm)	109.50	5.92	3.42	115.00	9.01	4.50	0.3458
FL (mm)	65.50	6.61	3.30	73.00	4.00	2.31	0.0832
FL/BPD	0.74	0.07	0.04	0.79	0.03	0.02	0.0980
BMI before pregnancy	21.68	3.18	1.20	24.42	5.58	2.79	0.3968

**p<0.05*

BMI= body mass index, AC= abdominal circumference, HC= head circumference, BPD= biparietal diameter, FOD= frontooccipital diameter, FL= femur length.

increased entry of glucose into vascular smooth muscle cells via GLUT1 transporter mediated by IL1 β activates the pentose phosphate pathway, thus permitting some of the excess glucose to be metabolized via this route. This in turn led to an over activation NADPH oxidase, resulting in increased generation of free radicals and the subsequent downstream proinflammatory signaling. Free radicals, inflammation and oxidative stress lead to atherosclerosis (25).

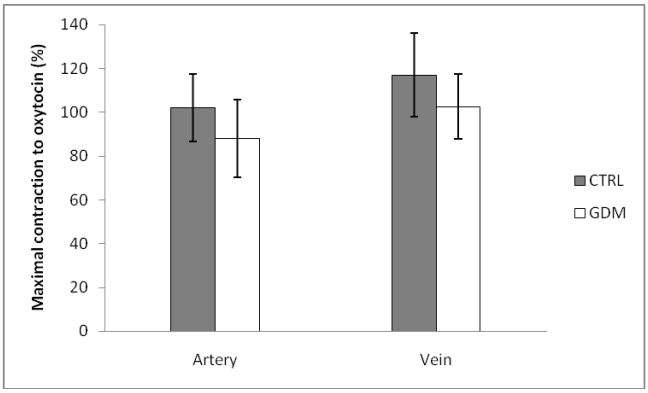
The uteroplacental circulation can be detected by the flowmetry of the umbilical and uterine arteries. The wave and shape of the flow, both in umbilical and uterine arteries, depends on the

Table 2. *The pathomorphological and histopathologic data of the samples.*

	Control (n=22)			GDM (n=5)			
	Mean	SD	SEM	Mean	SD	SEM	p
cord cross-sectional area (mm2)	124.00	35.47	11.22	93.75	9.86	4.41	0.0027*
cord length (cm)	58.00	9.15	2.89	59.60	7.13	3.19	0.6400
placental weight (g)	463.80	56.77	17.12	535.40	78.53	35.12	0.1083
fetal weight/placental weight	7.38	0.76	0.23	6.29	0.56	0.25	0.0134*
placenta thickness (mm)	27.73	4.10	1.24	28.00	4.47	2.00	0.8880
placenta longest diameter (mm)	160.90	13.75	4.15	164.00	5.48	2.45	0.1778
placenta shortest diameter (mm)	139.10	17.00	5.13	158.00	8.37	3.74	0.0071
umbilical coiling index (UCI)	1.43	0.77	0.25	2.00	1.00	0.45	0.0891

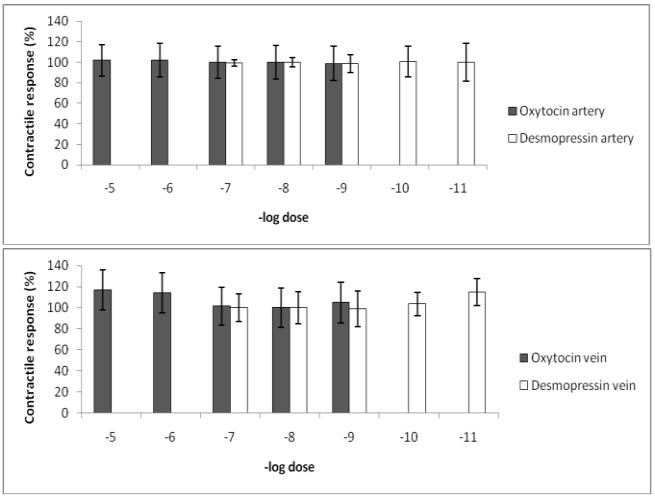
**p<0.05*

Figure 1. *Maximum change in vascular tone after oxytocin dosage.*



Although the mean value for the case group is lower, the difference was not significant; p=0.208 in case of the arteries and p=0.396 in case of the veins. CTRL= control group, GDM= gestational diabetic group.

Figure 2. *Vascular tone after oxytocin and desmopressin dosage*



We considered the spontaneously developing basal tone as 100%.24

resistance in placental circulation. In diabetes, the increased blood flow resistance of the umbilical artery is just a late pathognomic sign of ischemic vascular changes in the placenta related to a direct ischemic effect on vascularization. These ischemic-hypoxic alterations can be seen in histopathological samples as syncytial nodes, hypervascularized villi and excessive interstitial calcification (26).We would like to emphasize the importance of the placental-umbilical cord unit in pathologic pregnancies.

According to our results, the umbilical cord in the case of a gestational diabetic patient can be described as a thin cord with normal length, hypercoiled or normally coiled rope-like vessels. The incidence of multiple false knots is higher, and they rather tend to have a marginal placental insertion.

On the placental perfusion model, introducing vasopressin to the maternal side (30pg/ml-60 000pg/ml) and increasing the dosage until the vasopressin reaches its maximum concentration on the fetal side, they received 3110 pg/ml as the maximal agent transported through the placenta. This concentration is equivalent with 5.74x10-9M, while our highest concentration was 10-7M. This suggests that treating pregnant women with vasopressin would not influence the fetoplacental circulation. Vasopressin or oxytocin concentration still can be increased in vivo by fetal production (27).

As described by Holcberg G., et.al (2002) oxytocin does have a vasoactive effect on meconium impregnated placentas, but no effect on normal placentas. Maternal oxytocin can pass through the placenta and reach the fetal brain, and induce the hyperpolarization of GABAergic neurons in the fetal hippocampus and neocortex during delivery. Reduction of GABA-mediated excitation induced by oxytocin has been demonstrated and completely eliminated by Atosiban. Since hypoxic brain damage is the leading cause of fetal death, the important conclusion is that oxytocin has an inhibitory cortical and hippocampal neuronal effect by which it reduces fetal brain oxygen and nutrient requirements, and therefore it is less sensitive to hypoxia (4). Therefore we assume that in cases of placentas without meconium impregnation, oxytocin does not bind to the receptors to elicit vasoconstriction in the placenta, and it can pass to the umbilical cord, where there is no active receptor. Thus, it can exert a greater hyperpolarization in the fetal brain. In term pregnancies during delivery, if there is an acute hypoxia, the oxytocin produced by the mother can protect the fetal brain. In contrast, it seems that in cases of the presence of meconium for several hours, it is important to note the increase of vascular tone in the placenta.

The macroscopic evaluation of the formalin fixed tissues showed that the volume had reduced, but the ratios between the control and case groups remained the same, so these data correlated with the clinical measurements. In cases of diabetic pregnancies, we found an increased umbilical coiling index (number of 360° turns within 5cm long segment at the placental end). The clinical aspect of the coiling pattern and coiling index is that a thin and hypocoiled cord is more vulnerable to strangulation, striction and other mechanical events. The thin cord that is not well- protected by Wharton's jelly is prone to striction and mechanical injuries too. There was no significant difference in the case of birth weight and placental weight either, but we did find significant difference in their ratios. The shortest diameter of the GDM placentas was significantly longer than the control ones, while there was no significant difference in the longer diameter or thickness measured at the cord insertion point. Therefore, we might observe the diabetic patients' placentas as more round and less oval shaped, heavier, and exhibiting a pale calcification.

We suppose that umbilical vessels have a unique receptor profile to regulate vascular tone though these vessels may injure easily because of mechanical insult. Regarding the importance of the umbilical vascular tone in fetal development, the investigation of the exact biochemical mechanisms could be crucial in the prevention of any developmental disturbances since the onset of gestational diabetes is within the time of pregnancy, resulting in a decreased possibility of awareness of the illness by the pregnant woman. By taking into consideration all the receptor changes of fetoplacental vascularization, we may be able to protect the fetus from an adverse outcome by specific treatment.

Acknowledgement. None.

Disclosure

Funding. No specific funding was received for this study.

Competing interests. None to declare..

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Weight Status and Body Composition in Freshman Students at the College of Applied Sciences "Lavoslav Ruzicka" in Vukovar, From 2008 to 2016

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Abstract

Aim: The aim of the study is to analyze the common occurrence of different weight categories among first-year students at Lavoslav Ruzicka College for Applied Sciences in the city of Vukovar, as well as to make an assessment of their body composition.

Methods: During the period from 2008 to 2016 there were 710 first-year students (461 women and 249 men) whose height and weight were measured, and body composition assessed, by bio-electric impedance analysis.

Results: Most of the subjects were of normal weight (74.5%), while the ratio between the weight status categories of men has proven to be significantly different from that of women. It was established that 8.9% of women as opposed to 2% of men were below normal weight, while there were more obese individuals among men (25.7%) than there were among women (15.4%). However, the percentage of obesity was similar in both sexes, being 5.4% in women and 5.2% in men. There was no significant difference in the ratio between the weight categories during the measurement period.

Conclusion: Most of the students at the College for Applied Sciences fall in the normal weight category. There are more obese individuals among men, while among women there are more of those with lower average body weight. The dominance of obesity and the overall ratio between the weight categories and the body composition in the category of first-year students have not changed significantly during the period between 2008 and 2016.

(Nešić N, Davidović Cvetko E, Šeper V. Weight Status and Body Composition in Freshman Students at the College of Applied Sciences "Lavoslav Ruzicka" in Vukovar, from 2008 to 2016. SEEMEDJ 2017;1(1):50-58)

Received: March 24, 2017; revised version accepted: April 23, 2017; published: April 24. 2017

KEYWORDS: obesity, body fat percentage, waist to hip ratio, body mass index

Introduction

Morphological characteristics are often the subject of research due to the great impact they have on health and disease development. Obesity and fat accumulation are intertwined with hypertension, hyperlipidemia and hypercholesterolemia, as well as low levels of high-density cholesterol and hyperglycemia – all of which, among many other factors, are associated with cardiovascular problems (1). Of all the previously mentioned conditions, obesity has by far the most important influence on the quality of life (2,3).

In the USA, over one third of adults and around 17% of children are obese (4), while studies on European nations showed that around 17% of adults are obese, with some variations among countries (5). As for studies on students and younger adults, some data show that there are around 22% overweight students (6), while 50% of people aged 18-24 are subject to at least one risk factor for the development of cardiovascular diseases (7).

It has been proven that being overweight and obese during pregnancy are conditions which increase the risk for the child itself to become overweight or to develop obesity. On the other hand, girls who are overweight during their adolescence are more prone to gaining weight in pregnancy, demonstrating a vicious cycle (8).

The consequences of overweight and obesity not only affect overweight and obese persons, but society in global terms. The socioeconomic results of obesity include higher expenses for treating the diseases resulting from being obese (9-11). Finally, obesity greatly increases the risk of mortality (12,13). Therefore, preventive actions are very important, including education about diet, physical exercise and a healthy way of life.

Anthropometrical research conducted during the Croatian Adult Health Survey in 2003 (14) show that 58.5% of the adult population (over the age of 18) is overweight, whereas 20.4% is obese. Because of such widespread adult obesity, there are some predictions that by the year 2030 37% of men and 48% of women will be obese—if the same trends towards increased obesity continue (14).

The aim of this research is to analyze the common occurrence of different weight categories among first-year students at the Lavoslav Ruzicka College of Applied Sciences in the city of Vukovar, and to assess their body constitution. Additional goals of the study are the following: (1) the analysis of the weight status and the body constitution of students who began their studies within this institution between 2008 and 2016 in order to determine if there have been some significant changes; and (2) the examination of occurring variations in the weight status and body composition between the sexes.

Methods and Materials

Study design

During the period from 2008 to 2016, 710 first year students (461 women and 249 men) volunteered to participate in the study. Every participant was measured once, during their first academic year. The average age at the time of the measurement was 19 (ranging from 18 to 26).

Measurements

The subjects' height was measured using standard techniques and equipment.

Weight and body composition were measured and assessed by bio-electric impedance analysis using the body composition analyzer GAIA 359 (Jawon Medical, Korea). This was performed in the morning hours after overnight fasting by a trained technician, following the device manufacturer's directions. Measurement results included weight, body fat mass (BFM), body fat percentage (BF%) and soft lean mass (SLM). The Body Mass Index (BMI) was calculated by the following equation: weight (in kilograms) divided by height (in square meters). WHR (Waist to Hip Ratio) was calculated by the following equation: waist circumference divided by hip circumference.

Weight status was defined following current recommendations from the WHO, according to which a BMI between 18.5 and 24.9 represents normal weight, lower values of BMI are considered being underweight, while a BMI over 25 is considered being overweight and a BMI of 30 or higher is considered obesity (15).

Statistical analysis

Statistical analysis was carried out using IBM SPSS statistics 22. Statistical significance was set at $p<0.05$. Since all variables failed in the Kolmogorov-Smirnov test of normal distribution, they are presented as median and 95% confidence interval (CI) for median. The ratio between the weight status categories between sexes and during the year of measurement was tested by the chi-square test. The Mann-Whitney U test was used for testing the differences in the values of measured variables between sexes. Spearman's coefficient of correlation was used to calculate the correlation between BF% and BMI, and between BF% and WHR.

Results

BMI and body composition, as well as WHR, for the total sample and by sex, are provided in Table 1. The difference between the sexes was statistically significant in all variables (Table 1). Men had a higher value of BMI compared to women, but the range of results for BMI in women was wider than in men, so that the highest BMI measured was as follows: For women, it was 44.1 kg/m2, and for men it was 36.3 kg/m2; the lowest measured BMI was similar for both sexes (Table 1). WHR was also higher in men than in women, but BF% showed that women have more fat content compared to men. The range of BF% showed that fat content in both sexes ranges from very low (4.3%) to over 37% for men and over 45% for women.

There was also a significant difference between sexes in the distribution along the weight status categories ($p<0.0001$). The difference between sexes in distribution along the weight status categories are shown in Figure 1. The highest predominance was in the category of normal weight for both sexes, with around three quarters of participants (75.7% of women and 72.3% of men). In the underweight category, the women prevailed with 8.9%, whereas only 2%of men were in this category. A higher predominance of men compared to women was registered in the category of a BMI value over 25 (overweight and obese), with 25% of men vs. 15% of women. Since similar proportions of men and women were obese (5.4% of women and 5.2% of men), the difference in the proportion of subjects with a BMI higher than 25 is consequent to the predominance of overweight subjects rather than obese ones.

Distribution along the weight status categories through the years is presented in Figure 2. A chi-square test did not show any significant difference between the different measurement years ($p=0.9$). Distribution through the weight status categories was similar during the entire period of the study, with some small changes, mostly regarding the prevalence of terminal categories: category obese, and category underweight.

Secular trends for men and women in BMI, WHR and BF% are shown in Figures 3-5. While there were no significant differences during the years of measurements for any of the variables, distinctive differences between the sexes remained present throughout all the measurement years.

Spearman's correlation yielded a positive correlation of 0.532 ($p<0.001$) between BF% and BMI, and a positive correlation of 0.582 between BF% and WHR. When calculated separately by sex, the correlation between BF% and BMI for men was 0,803 ($p<0.001$), and for women 0.924 ($p<0.001$). The correlation between BF% and WHR for men was 0,983 ($p<0.001$) and for women 0.996 ($p<0.001$).

Discussion

The main finding of the present study is that the distribution across the weight status categories is significantly different between sexes (Table 2). Although there was a similar proportion for the subjects of both sexes of normal weight, there

are more overweight individuals among men, while among women there are more of those with lower average body weight. It has also been found that the prevalence of obesity among those overweighted is similar in both sexes (Table 2). The result of the similar prevalence of normal weighted among both sexes presented here complies with the results of other similar studies made throughout the student populations in India, Saudi Arabia and the USA, with the exception of the published results in listed countries showing lower portions of subjects of normal weight in their samples (16-18). The portion of subjects with normal weight was around 20% lower than in the present study. A higher prevalence of underweight women as opposed to men was also reported in the study made by Zhang et al. (19) and Sira and Pavlak (18). These results are similar to the results of studies performed at some other high schools or universities (16, 17,20). Kritsotakis et al. (21) also reported lower values of BMI in women than in men. In the paper published by Kantanista et al. (22), the authors concluded that under weighted adolescent girls are more satisfied with their body than those who are of normal weight and/or overweight. Yatsuya et al. (23) reported a higher prevalence of obesity in men than in women in Europe, which complies with the results of our study. In their study, the prevalence of obesity was assessed in different world regions, and the second highest rate was noted in Europe. The results of research done in India (16), Saudi Arabia (17), USA (18) and Brazil (20) show the prevalence of obese individuals among students of different races and ethnicities of about 6-15%, while the study of prevalence of obesity in 22 developing countries reached results similar to our study, which are 5-6% (24). Great differences in the prevalence of obesity throughout Europe were reported in comparisons made by Borsika et al.(25). They reported that the highest obesity prevalence is present in Hungary (19.5%), the Russian Federation (15.2%) and Romania (14.7%), while the lowest obesity rates are in Norway (6.2%), France (7%) and Italy (7%). Similar results were reported in the study by Van Viet Ostapchouk et al. (5). From the study by Fišter et al. (26), it could be concluded that the number of obese individuals rises later in life, while according to Musić Milanović et al. (27) the critical years when people are starting to become obese are up to 35, when most changes from overweight to obesity are indeed recorded. Huang et al. (28) reported obesity prevalence rates at the University of Kansas to be similar to those in our results. However, some recent studies reported lower proportions of obesity among university students in Croatia of up to 3% (29-31). Similar reports of overweight/obesity predominance from neighboring countries (32-34) showed that there are great variations between and within the countries of our region: At the University of Novi Sad overweight rates reported are 33.5% in men and 7.5% in women (32), while at the University of Niš overweight prevalence is 38.18% in men and 7.95% in women (33). Rašeta et al. reported 22.4% of overweight students at the University of Banja Luka (34). It would be interesting, in our opinion, to compare the weight status and body composition of students of medical science and kinesiology as opposed to those who study social or natural sciences. Since medical students and students of kinesiology should be more aware of the risks and potential dangers of unhealthy weight, it would be interesting to see if their weight status is different in comparison to others. Something similar was done by Rašeta et al. at the University of Banja Luka (34), where obesity rates among students of three different faculties were compared: the Faculty of Physical Education and Sport, the Faculty of Economics and the Faculty of Medicine. Results showed that the students of the Faculty of Economics had the highest value of BMI and BF%, while the medical students had the highest WHR.

Other very important values for weight status assessment are WHR and BF%, which are considered more accurate measurements of body weight type and body composition than BMI, because BMI cannot distinguish between weight associated with fat and weight associated with fat-free mass (35). Most of the subjects in the present study had values of WHR and BF% within the recommended ranges for health maintenance (Figures 4 and 5). Since those measures are predictive factors for

Table 1. *Descriptive statistics.*

	Total (N=710)		Women (N=461)		Men (N=249)		
	Median (95% CI)	Min-Max	Median (95% CI)	Min-Max	Median (95% CI)	Min-Max	P*
BMI (kg/m2)	22.10 (21.9-22.5)	15.9-44.1	21.5 (21.1-21.8)	15.9-44.1	23.4 (22.9-23.9)	16.3-36.3	<0.0001
WHR	0.75 (0.74-0.76)	0.64-0.98	0.73 (0.72-0.74)	0.66-0.95	0.78 (0.78-0.80)	0.64-0.98	<0.0001
BF%	22.7 (22.2-23.5)	4.3-45.4	25.1 (24.5-25.7)	4.3-45.4	17.4 (16.5-18.4)	4.3-37.0	<0.0001

P - p value obtained by performing Mann-Whitney test for difference between sexes*

BMI - body mass index; WHR - waist to hip ratio; BF% - body fat percentage

Figure 1. *Difference between sexes in distribution along the weight status categories.*
Absolute frequencies in each category are presented below the graph. Chi-square test showed significant difference between men and women (p<0.0001)

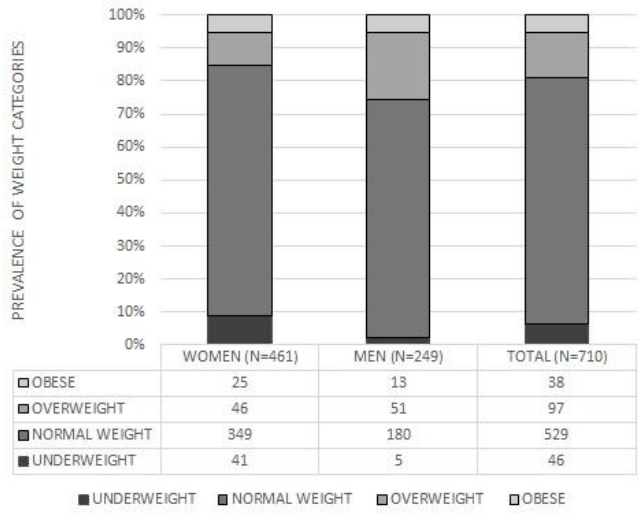


Figure 2. *Distribution along the weight status categories through the years.*
Absolute frequencies in categories are presented below the graph. Chi-square test did not show any significant difference between the different measurement years (p=0.9)

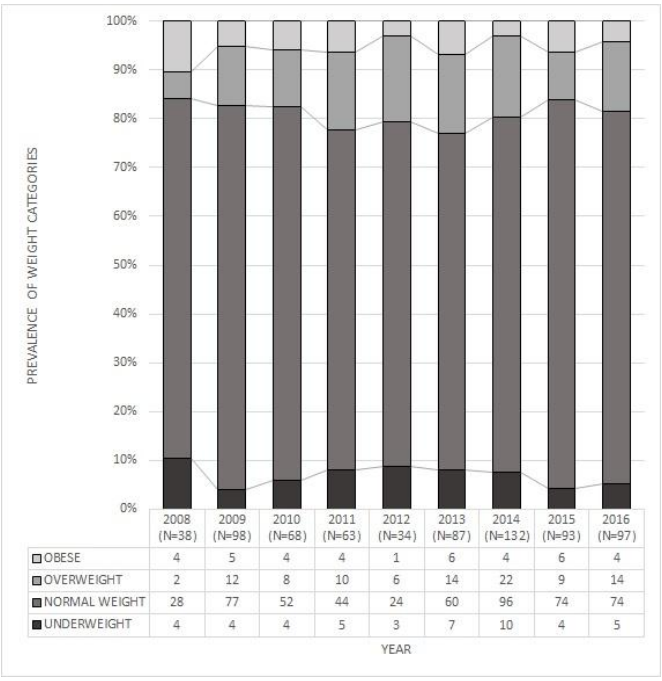
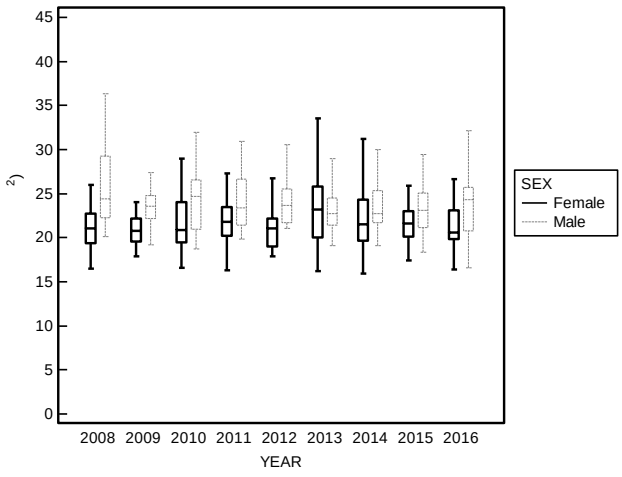


Figure 3. *Secular trend for men and women in BMI.*
Figure represents differences between sexes in BMI from 2008 to 2016 expressed in median (with 95% CI for median) values.



cardiovascular problems (35), it is very important that the values were within healthy limits. Some researches show that, similar to BMI, values for PBF and WHR rise as people age (36).

The body composition (presented in Table 1) of the subjects shows that they have appropriate mass of fat and muscles, and their body composition is in accordance to weight status based on BMI value. In other words, the prevalence of obesity based on BMI distribution would probably be similar if they were distributed according to the BF% or SLM. Results reported by Grygiel-Gorniak et al. (37) show similar values of BMI, WHR and BF% with the results of the present study, as well as the similar differences between sexes. Our findings regarding differences in BMI and BF% between men and women showed that men had higher BMI, but lower BF% compared to women. This could be ascribed to the greater muscle mass in men. However, the correlation between BF% and BMI showed a statistically significant high positive value, indicating a strong connection between those two variables. Correlation was slightly lower in men than in women. There was also high correlation between BF% and WHR in both sexes. Collins et al., in their study of association of BMI and BF% among BMI-defined non-obese middle-aged individuals, found that the BMI category was not concordant with the %BF classification for 30% of the population. The greatest discordance between %BF and BMI was observed among %BF-defined overweight/obese women (38). A strong correlation of BMI and BF% in young women was reported in the study of Bakir et al. (39). They obtained correlation coefficients between BMI and BF% of 0.74 for women aged 18-30 years.

Proportions of weight categories were not significantly different over the years in which measurements were made. Students that choose to attend the College of Applied Sciences in Vukovar are similar in weight status throughout the years. This result is different compared to predictions of increase in obesity prevalence, and shows a steady state in the weight status of first-year students during the years examined, without any increase of obesity. It is possible, however, that it might have been too short a period for potential trends to reveal themselves.

Based on the presented results, the conclusion could be made that most of the freshman students at the College of Applied Sciences fall in the category of normal weight, with an overweight prevalence of around 19-20%, including around 5% obese persons among them. There are also 6.5% of those who are underweight. There is a higher tendency toward the prevalence of overweight persons among men, while among women there is a higher tendency for underweight prevalence. The prevalence of obesity and the overall distribution across the weight categories, as well as the body composition of the first-year students have not changed during the period from 2008 to 2016.

Acknowledgement. None.

Disclosure

Funding. No specific funding was received for this study.

Competing interests. None to declare.

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The Relationship of Saliva Microcrystalline Characterization and Contractile Duration of Skeletal Muscle in Medical Students

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Abstract

Aim: The aim of the study was to analyze the possibility of the diagnostic use of saliva investigation in relation to medical conditions among medical students, and provide a background of non-invasive physiological-based preventive measures for their health outcomes.

Methods: The study was conducted among 70 students from Danylo Halytsky Lviv National Medical University (Ukraine), who were asked for general information and interviewed about the subjects of lifestyle risk factors related to physical inactivity, circadian rhythmicity, the use of technical gadgets, and the presence of functional gastrointestinal disorders. Their saliva secretion and microcrystallization were evaluated. For detection of the microcrystallines of non-organic origin in saliva, X-Ray diffractional powder analysis was used.

Results: The results of the study indicated that 70% of students have decreased daily skeletal muscle contractile duration, circadian dysfunction and extended time of using gadgets all of which leads to changes in saliva secretion and the microcrystallization of biogeneous substances. About 30% of participants have signs of functional disorders of the upper gastrointestinal tract.

Conclusion: An integrative view on saliva microcrystalline changes could be a novel diagnostic tool for detection of early health disorders, and maintaining regular skeletal muscle contractile activity and normal circadian rhythmicity is a promising physiological approach to improve health outcomes in young people.

(Zvir M, Beliak A, Pohoretska Y, Savytska M, Zayachkivska, O. The Relationship of Saliva Microcrystalline Characterization and Contractile Duration of Skeletal Muscle in Medical Students. SEEMEDJ 2017;1(1);59-66)

Introduction

The modern lifestyle is characterized by an increasingly sedentary lifestyle, chronic stress overload, and sleep rhythm disorders as a result of intensive usage of various modern gadgets (tablets, smartphones, computer games, etc.), which cause circadian disruption (1,2,3). All these factors lead to additional negative impacts on health, early manifestations of lifestyle diseases, osteoporosis, metabolic disorders, obesity, and early aging. Prolonged sitting duration and the

Received: March 14, 2017; revised version accepted: April 23, 2017; published: April 24. 2017

KEYWORDS: lifestyle, saliva, microcrystalline, X-ray diffractional powder analysis

rapid progress of information technology lead to a sedentary lifestyle of young people, which adversely affects their health and subsequently leads to pathological processes in the body (4,5). The use of modern technological gadgets leads to the desynchronization of circadian rhythms, accompanied by sleep and appetite disturbance, worsening of mood, and initial mental disorders, autonomic nervous system (ANS) imbalance, which often stimulates different functional and organic diseases (3).

Young people are very sensitive to changes of lifestyle and have a tendency to an increased number of mental disorders as cognitive functions decrease, leading to a decline of natural resistance of the organism and initiating a wide range of mental and medical problems, including depression and suicide. All of these lifestyle factors have also been suggested as possible risk factors for upper functional gastrointestinal (GI) disorders (FGID), which are tightly linked to brain-gut interaction (6,7). To prevent health problems in young people and guarantee active aging, the need for the detection of these early risk factors attracted our attention.

Modern views on saliva show that it can be an important source for the early diagnosis of many diseases and the functional state of our body. Moreover, it is known that inorganic and organic components of saliva actively influence the cytoprotection of the esophagogastric region. The effects of normal salivation provide sufficient moisture of the mucous membrane of upper GI part that contains numerous growth factors, like the epidermal growth factor, EGF, which provides physiological regeneration, mucin – the formation of mucous-bicarbonate barrier, and specific and non-specific defensive bioregulators, which are crucial for the integrity of the GI epithelial barrier and a general resistance to extreme factors (8).

Noninvasiveness and simplicity make a saliva-sampling method attractive for microcrystalline investigations in terms of biogeneous mineral substances. The salivary biogeneous substances were studied using the X-ray diffractional powder analysis (XDPA) technique to determine all mineral phases of biogeneous minerals. Recently published data suggests the importance of the inorganic components of saliva, and the quantitative and qualitative state of saliva bioregulators, as determining factors of solid textures that are formed after dehydration (9,10,11).

Thus, we formed a hypothesis that the investigation of saliva as a non-invasive simple diagnostic tool could be used for detection of the early changes, associated with ANS imbalance, caused by physical inactivity (12,13).

In the present study, we aimed to provide an integrative view of saliva investigation, the screening of lifestyle risk factors, and the skeletal muscle contractility duration that occurs in healthy medical students.

Material and Methods

The study was conducted on a group of 70 participants who were second-year students of the medical faculty from Danylo Halytsky Lviv National Medical University (mean age 18.5 years, 38 women and 32 men) with 16 months of an academic lifestyle (during studying). Selected students were healthy—without any previous chronic diseases and addictions, including caffeine abuse or family-ancestral diseases—and also possessed good oral health—without dentures or dental restorations—according to the reports of the student's dental and orofacial examinations by dentists. Their daily fluid intake did not exceed 2.5 L. They were eligible to participate in this study. The exclusion criteria were medical students who refused to participate. The students who agreed to participate signed a consent form. The design of the study and the test procedures were approved by the ethics committee of Lviv National Medical University (15.02.2016; N2).

The subjective and objective evaluation of the students was divided into 3 parts. The first part of the study concerned general information: gender, previous general illness, administered medication, addictions, and an anthropometric examination. Anthropometric data included height, weight, body mass index (BMI), and age.

Height was measured with a regular stadiometer, while weight and body composition, using the bioelectric impedance method, were evaluated with electronic scales (OMRON Corporation, Kyoto, Japan). BMI was calculated using the following parameters: fat content (% of body weight), visceral fat (%), and muscle mass (%).

The second part of the study was based on questionnaires: the International Physical Activity Questionnaire (IPAQ) (14), the Pittsburgh Sleep Quality Index (PSQI) (15), 10 closed questions on the usage of technological gadgets, and a determination of the daily time of physical inactivity (sitting time more than 60 minutes without a break), estimated as a mean value after evaluation every 5 days, and finally the assessment of the symptom manifestations of upper FGIDs through a standard self-report questionnaire, included in the Rome III consensus (7).

The third part of study concerned the saliva investigation by the examination of samples of saliva that were collected at the same time, not less than 2-3 hours after eating in the morning (between 9 and 11 a.m.), with a sterile pipette from the bottom of the oral cavity, in the volume of 2 ml into dry sterile test tubes. All women were examined in the estrogen phase of their menstrual cycle. The level of stimulated and non-stimulated saliva secretion was measured. Non-stimulated saliva was collected from students in the beginning. Later, students were asked to chew gum without sugar for 5 minutes and stimulated saliva was carried out in a similar manner into test tubes. Both deposition time and volume of deposited saliva were measured. The collected data made it possible to determine the rate of saliva secretion (ml/min). The morphological type of saliva crystallization was estimated by the dehydration of drops of mixed saliva in the air at room temperature for 24 hours on a sterile glass slide using light microscopic investigation with a Leica DM 750/4 microscope and Leica DFC 420 digital photo camera, both manufactured in Germany (Figure 1), and by phase contrast imaging for the evaluation of saliva samples without fixing and staining with Leica DM-2500 (Switzerland) microscopy and camera Leica DFT 450 with software application SUIT version 4.4 microscope Leica DM-2500 (Switzerland) (Figure 2). The evaluation of saliva crystallization was performed blind by two independent investigators and the estimation of the type was based on grading four main types according to Shatohina S.N., 2004 (16). The first (1st) type is characterized by clear crystal tread-prismatic structures connected between themselves in a fern leaf and evenly placed; the second (2nd) type is characterized by certain tree-crystal or small single crystals of various shapes placed evenly on the field of view in a grid; the third (3rd) type is characterized by isometric-placed irregularly shaped structures; and the fourth (4th) type is characterized by the absence of crystals. The rest of saliva was taken for further tests.

For detection of the microcrystallines of non-organic origin in saliva, X-Ray diffractional powder analysis was used. For this samples of saliva were selected with the 4th type of crystallization. The samples were applied on the polymer coat and remained there until drying. The coat with dry substance was fixed and covered with the second coat. Experimental intensities and angles reflected from samples were obtained by an automatic diffractometer STOE STADI P ("STOE & Cie GmbH", Germany) with a linear position-precision detector. Primary processing of the experimental diffraction, the calculation of the theoretical diffraction, and the indexing parameters of unit cells were performed using the software package STOE WinXPOW and PowderCell with the method of comparing X-ray diffraction profiles (18,19).

Statistical data analysis was performed by calculating means with their standard errors and proportions. Differences in means were estimated by the Mann-Whitney U-test. All data was processed using the statistical package Statistica 10.0 (Statsoft, Tulsa, Oklahoma, USA).

Results

Among the 70 medical students of Lviv National Medical University who participated in the study

Table 4. *Study group's general information characteristics (n=70).*

Variable	Results
Gender	Male: 32 (45.7%) Female: 38 (54.3%)
Age	Mean: 18.5 years, range 17-19 years
Height	Mean: 171±9 cm, range 156-190 cm
Weight	Mean: 63±9 kg, range 47-82 kg
BMI	Mean: 21.5±1.4 kg/m^2, range 18-24.5 kg/m^2
Body composition: fat content visceral fat muscle mass	 Mean: 25.1±6.8%, range 14.2–36.6% Mean: 6.14±1.2%, range 3.94–8.73% Mean: 29.7±6.8%, range 24.2–44.2%
Current smoker	Total: 13 (18.6%)

Table 2. *Types of physical activity among medical students (mean values in MET min/week).*

Type of activity	Gender		Total
	male	female	
moderate	748.1	545.6	632.4
intensive	938.7	456.5	618.2
walking	984.2	1024.1	992.2
total	2,671.0	2,026.2	2,242.8

Figure 1*. Light microscopy of samples of saliva by the type of microcrystallines in medical students (data from study group); x 120: A – 1st type; B – 2nd type; C – 3rd time; D – 4th type.*

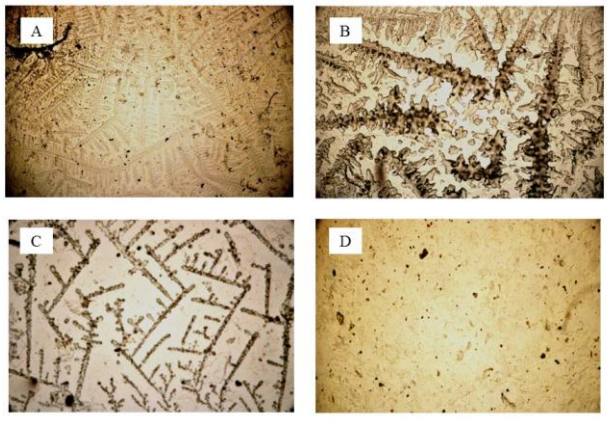


Table 3*. Characterization and duration of daily physical inactivity among medical students.*

Daily sitting time	
6-8 hours/day	48 (68.6%)
>8 hours/day	22 (31.4%)
Total usage of gadgets	
2-3 hours per day	24 (34.2%)
4-5 hours per day	31 (44.3%)
>5 hours per day	15 (21.5%)
Usage of gadgets for more than 2 hours without a break	
Never	8 (11.6%)
Only occasionally	12 (17.1%)
A few times per month	18 (25.6%)
A few times per week	21 (30.0%)
Almost every day	11 (15.7%)

Table 4. *Differences between distributions of types of saliva between groups of students with and without functional gastrointestinal disorders is statistically significant (p<0.001).*

Type of saliva microcrystalli-nes	Symptoms of upper functional gastrointestinal disorders			
	no		yes	
	n	%	n	%
1	0	0	12	24.5
2	1	4.8	24	49.0
3	8	38.1	11	22.4
4	12	57.1	2	4.1
All	21	100.0	49	100.0

Figure 2*. Micro photos of saliva samples from medical students by phase contrast imaging, ranging to four microcrystallines types: A – 1st type; x200; B – 2nd x200; C – 2nd x400; D – 3rd x200; E – 4th x200; F – 4th x400 (data from study group).*

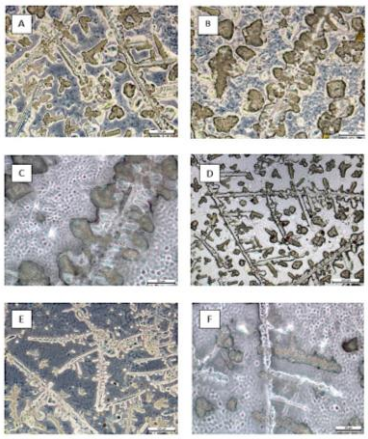
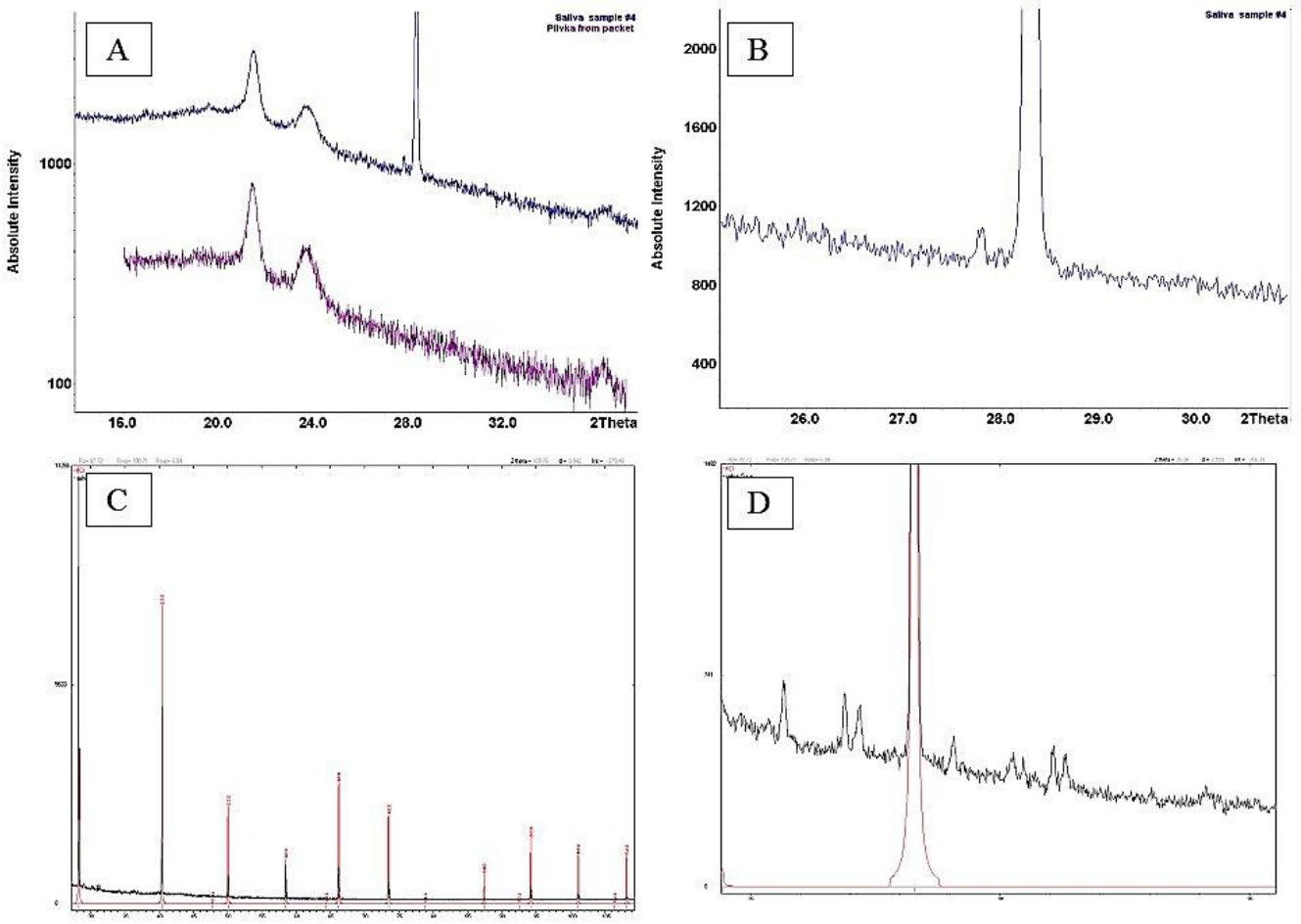


***Figure 3**. Results of investigation of saliva bio compounds by X-ray powder diffraction analysis: A – tested comparison of packet film and investigated dry precipitate of saliva samples; B – detection of two-phase KCl microcrystallines and undetected compound; C – standardization with theoretical diffractogram KCl; D – KCl detection with small traces of sodium.*



a mean age of 18.5. Table 1 presents the data for all participants, including age, gender, administered addictions, and anthropometric examination. Anthropometric data involved height, weight, BMI, body composition and general characteristics of study group represented in Table 1.

The physical activity of medical students involved in the study was estimated by IPAQ, expressed by arithmetic mean (MET min/week), to have reached the value of 2,242.8, with higher values observed in males (2,671.0) than in females (2,026.2). We also estimated the type of activity: moderate, intense, and walking. A larger number of participants confirmed walking as the dominant type of physical activity. Walking was the dominant type of physical activity in females (1024.1 vs. 984.2 in males) (Table 2).

The study assessed the level of physical activity that was calculated from the total physical activity and categorized it into three ranks: low, moderate, and high. A significant variation was observed at each level according to the gender, and the larger differences were in high level of physical activity in males (43.8%) vs. females (23.7%). Moderate physical activity was better obtained in females (50.0%), compared to males (34.4%).

It was observed that 78.5% of medical students use gadgets 3.6±0.5 hours/day and 21.5% of them spend more than 5 hours/day with technical gadgets. There were no significant differences in gender distribution in the collected data. More than half of participants do not take any breaks when they use computers, leading to an increased daily sitting time

characterized as a physically inactive lifestyle (Table 3).

We also screened sleeping patterns by PSQI and figured out that poor sleep quality was in 67.2% of medical students with low physical activity vs. 25.7% with moderate or high physical activity. The study indicated that lost sleep because of late night computer use was very common among young men and women: almost every day – 11%, a few times per week – 15%, or a few times per month – 37% of participants.

To determine the prevalence and clinical-pathological characteristics of upper FGID, records of questionnaires were evaluated. The most commonly presented complaints were heartburn, indigestion, epigastric pain/burning, excess throat mucous, or a lump in throat etc. These symptoms were absent in 30% of enrolled participants, but among the students who fulfilled the criteria for FGID, were more common in males (65.5%), than in females (34.5%). Smoking causes a high risk for the prevalence of upper FGID.

The data of the microscopic analysis of human saliva, namely samples of non-stimulated saliva microcrystallines, detects different types of distribution according to FGID (Table 4). There were not any significant differences in saliva microcrystallines between the genders.

The 1st first type of saliva microcrystallines was dominant among participants with symptoms of upper functional GID, because increased gastric acidity entailed the increase of saliva crystallization. Both 2nd and 3rd crystallization types were the most common among the participants. It has been established that the 4th type of saliva microcrystallines is prevalent among medical students, who displayed both circadian disruption and physical inactivity. A growing amount of evidence argues that saliva could be a diagnostic tool for ANS imbalance, which is represented by decrease of general adaptive reserve (20-22). Microscopically, a large number of irregular crystal structures are seen in the 4th type of saliva crystallization (Figure 1D, and Figure 2E, F). Investigation of stimulated saliva secretion didn't show any significant differences in volumes and pH. The average pH of saliva was about 7.9, which is in the normal range, considered to be 5.6 to 7.9, and this keeps local balance in the oral cavity.

Figure 3 represents the crystal structure of the bio compounds in samples of saliva microcrystallines, which were determined using X-ray powder diffraction analysis according to standard procedure. At the beginning, the comparable characteristics of packet film and the dry precipitate of saliva samples with the 4th type of microcrystalline were tested in comparison for the elimination of wrong signals (Figure 3A). Phase analysis by X-ray diffraction confirmed that the microcrystalline samples were two-phase KCl (structural type NaCl, with group *Fm–3m*), parameter of unit cubic cell a = 6.3015(3) Å, its volume V = 250.227 (18) $Å^3$ (Figure 3B) with another compound with un-identified small reflex for 2 θ = 27,78°. After standardization with theoretical diffractogram KCl (parameter of unit cubic cell *a* = 6.3060(4) Å, value *hkl* for first reflex 200 (Figure 3C) were a set of mild reflexes for the second phase (Figure 3D). Regarding several well-known data, parameter *a* for KCl could be from 6.26 to 6.3163 Å; for $K_{1-x}Na_xCl$, x= 0-1, a=6.2916–5.6400 Å. Thus, the main bio compound in dry microcrystalline is pure KCl. The traces of sodium are very small (x ~ 0.01 in $K_{1-x}Na_xCl$).

Discussion

This study confirmed that the intensive lifestyle of medical students related to their demanding educational process, modern behaviors of young people, and the lack of contractile duration of skeletal muscles, is quite common and could be a specific type of stress which causes imbalance in capacities for adaptation, leading to functional disorders in the very stress-sensitive gastrointestinal tract. Numerous data suggests impact dysfunction of brain-gut axis in induction FGID (23-26). Regarding the final data, saliva represents a potential fluid for diagnostics of various diseases (13, 16, 17) and our results have shown its microcrystallines could be a diagnostic tool for stress-related gastrointestinal disorders. It is well known that epigenetic modifications play a crucial role in

maladaptation and chronic stress-associated health outcomes. Physiologically-based therapeutic implications of the student lifestyle should be included to recommendations of prevention and the treatment of FGID.

In conclusion, the above observations underline the obtained results in saliva microcrystalline samples and suggest that an integrative view of saliva investigation could be novel very promising diagnostic tool for the early detection of changes in the human body. Considering the obtained data about medical students' modern lifestyle, the disturbance of daytime/nighttime rhythmic changes, and the prevalence of risk factors, we can conclude that these factors are involved in the changes in saliva microcrystalline formation. The obvious changes in saliva microcrystallines in persons with higher physical inactivity and circadian disruption may present external stimuli for the induction of upper functional GI disorders. A physiologically based program of increased duration of skeletal muscle contractility will be helpful in the prevention of functional digestive disorders and their health outcomes.

Acknowledgement
The authors are grateful to Pavlo Demchenko and Roman Gladyshevskii from the Department of Inorganic Chemistry of Ivan Franko National University of Lviv, Lviv, Ukraine for performing X-ray diffraction methods on saliva samples.

Disclosure

Funding. No specific funding was received for this study.
Competing interests. None to declare.

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Evaluation of Antibacterial Activity of Two Different Honeys against Clinical Isolates of β-hemolytic Streptococci Group A

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Abstract

Introduction: The aim of this investigation was to evaluate *in vitro* antibacterial activity of horse chestnut honey and acacia honey at different concentrations against clinical isolates of the β-hemolytic streptococci group A (BHS-A). Honey's active compounds have multiple therapeutic effects and it is used as traditional medicine for treatment and prevention of various illnesses.

Materials and methods: The antimicrobial effects of two honeys were tested on forty-four strains of BHS-A, isolated from the throat by a modified disk diffusion method. A bacterial suspension of BHS-A strains was plated onto *Müller-Hinton agar with 5% defibrinated horse blood.* Using a sterile 8 mm diameter cork borer, wells were cut in the agar and into each was introduced 100μL of the different concentrations of the honey solution (25 %v/v, 50 %v/v, 75% v/v and 100%v/v). A disk of penicillin was added as the positive control. The plates were incubated aerobically for18-24 hours at 36 (± 1)˚C and zones of inhibition were measured.

Results: The average diameter of the inhibition zones of acacia honey (100 %v/v) was 12.48 mm ± 1.73 mm, for acacia honey (75 %v/v) it was 11.06 mm±1.24 mm and for horse chestnut honey (100 %v/v) it was 11.08 mm ± 1.02 mm. The positive control showed the average diameter of 30.45 mm ± 3.21 mm. Statistical significance ($p < 0.05$) was observed comparing the zone diameters of the acacia honey (100%) and antibiotic penicillin, and between the horse chestnut honey (100%) and antibiotic penicillin.

Conclusion: Acacia and horse chestnut honeys exhibit limited but effective antibacterial activity upon clinical isolates of BHS-A.

(Kotris I, Talapko J, Drenjančević D. Evaluation of Antibacterial Activity of Two Different Honeys against Clinical Isolates of β-hemolytic Streptococci Group A. SEEMEDJ 2017;1(1);67-73)

Received: March 15, 2017; revised version accepted: April 19, 2017; published: April 24. 2017

KEYWORDS: antibacterial activity, acacia honey, horse chestnut honey, beta-hemolytic streptococci group A

Introduction

β-hemolytic streptococci group A (BHS-A) are the most common bacterial cause of tonsillopharyngitis. This microorganism can also cause acute otitis media, pneumonia, skin and soft-tissue infections; cardiovascular, musculoskeletal, and lymphatic infections, bacteremia and meningitis (1). Classic streptococcal tonsillopharyngitis has an acute onset; produces concurrent dysphagia, headache, and upon examination is characterized by intense tonsillopharyngeal erythema, white-yellow exudates, and tender/enlarged palatine tonsils. Patients with acute streptococcal tonsillopharyngitis should receive therapy with an antimicrobial agent in a dose and for a duration that is likely to eradicate the infecting organism from the pharynx. Most oral antibiotic therapy must be administered for the conventional 10 days to achieve maximal rates of pharyngeal eradication of BHS-A (2-3).

Over the past decade, several research groups have focused their attention to the use of bee honey as a supplement therapy (4-6). The use of bee honey as a traditional remedy for microbial infections dates back to ancient times (7). Aristotle, c.350 BC recommended that bee honey collected in specific regions and seasons (and therefore presumably from different floral sources) can be used for the treatment of different ailments (8). Certain types of honey exhibit broad-spectrum antimicrobial activity and are effective against antibiotic resistant bacterial pathogens (9-13). The present study aimed to evaluate the antibacterial activity of two bee honey varieties (horse chestnut honey and acacia honey) onto forty-four clinical isolates of BHS-A.

Materials and Methods

Bacterial strain

Forty-four clinical isolates of beta-hemolytic streptococci group A (BHS-A) were evaluated. All these clinical isolates were donated by the Microbiology Department of the Public Health Institute of the County Brodsko-Posavska. Isolates of BHS-A used in our study were collected from throat swabs and were positive to BHS-A. They were identified on the basis of zone diameter around a bacitracin disk (10 µg/disk) as well as on susceptibility testing and by a latex agglutination slide test for the grouping of Streptococci of the Lancefield groups A, B, C, D, F and G from culture plates as recommended by CLSI (14).

Honey samples and dilution of honey

Two honey varieties were used: horse chestnut honey and acacia honey, which were purchased from the local market from local beekeepers. Because of the intensified viscosity of acacia honey, different concentrations were made in sterile distilled water: 25 % v/v, 50 % v/v, 75 % v/v. This was done by dissolving the respective volumes: 8.3 ml, 16.6 ml and 25 ml of acacia honey, into corresponding volumes of sterile distilled water to give a 33.33 ml preparation. These were the tested different concentrations in addition to non-diluted horse chestnut honey, acacia honey and penicillin disk as the control.

Susceptibility testing of honey

A bacterial suspension of BHS-A strains, equal to the McFarland standard of 0.5 was prepared in saline. With sterile swabs (Copan Italia S.p.A, Brescia, Italy -plain swab sterile), the suspension was coated onto a Müller-Hinton agar with 5% defibrinated horse blood. Using a sterile 8 mm diameter cork borer, five wells were cut in the agar and into each was introduced 100µL of the different concentrations of the honey solution. Into the place of the sixth well, a disk of penicillin was added as the positive control. The plates were incubated aerobically for18-24 hours at 36 (± 1)°C and examined for zones of inhibition. The zones of inhibition were measured (in millimeters) and recorded.

Table 1. *Inhibition zones of acacia honey and horse chestnut honey against beta-hemolytic streptococci group A (BHS-A) isolates.*

Beta-hemolytic streptococci group A isolates	Acacia honey (concentration of honey)				Horse chestnut honey 100%	Antibiotic-penicillin
	100%	75% v/v	50% v/v	25% v/v		
BHS-A1	12	11	0	0	10	28
BHS-A2	14	12	0	0	12	28
BHS-A3	11	10	0	0	10	30
BHS-A4	12	11	0	0	11	23
BHS-A5	16	14	11	0	12	25
BHS-A6	15	12	0	0	10	33
BHS-A7	13	10	0	0	11	33
BHS-A8	14	12	0	0	11	30
BHS-A9	15	12	9	0	12	33
BHS-A10	12	11	0	0	11	28
BHS-A11	13	9	0	0	0	28
BHS-A12	12	11	0	0	0	34
BHS-A13	12	12	0	0	11	31
BHS-A14	18	13	10	0	13	33
BHS-A15	12	11	0	0	11	32
BHS-A16	15	11	0	0	0	27
BHS-A17	15	14	0	0	12	31
BHS-A18	11	11	0	0	11	32
BHS-A19	0	0	0	0	0	25
BHS-A20	10	0	0	0	0	28
BHS-A21	12	10	0	0	0	26
BHS-A22	12	11	0	0	10	32
BHS-A23	12	12	0	0	10	29
BHS-A24	0	9	0	0	0	27
BHS-A25	11	0	0	0	11	31

BHS-A26	11	11	0	0	0	33
BHS-A27	11	0	0	0	0	28
BHS-A28	15	13	0	0	13	31
BHS-A29	11	0	0	0	10	32
BHS-A30	12	0	0	0	10	30
BHS-A31	11	10	0	0	0	32
BHS-A32	12	9	0	0	0	33
BHS-A33	13	10	0	0	0	26
BHS-A34	12	0	0	0	0	34
BHS-A35	12	10	0	0	0	32
BHS-A36	12	11	0	0	0	29
BHS-A37	13	11	0	0	0	40
BHS-A38	10	10	0	0	0	32
BHS-A39	10	0	0	0	0	33
BHS-A40	12	10	0	0	13	31
BHS-A41	11	10	0	0	10	28
BHS-A42	13	12	0	0	11	36
BHS-A43	11	11	0	0	0	30
BHS-A44	13	11	0	0	0	33

**The zones of inhibition were measured in millimeters*

Statistical analysis

Statistical analysis was performed using the program SigmaPlot Version 12.0. The differences observed between the groups were tested by multiple comparison procedures (Turkey test). The statistical significance level was confirmed at $p < 0.05$.

Results

The activity of acacia honey and horse chestnut honey against BHS-A isolates and the inhibitions zones are shown in Table 1. The average diameter of the inhibition zones of acacia honey (100 % v/v) on strains that were sensitive to the honey was 12.48 mm (standard deviation - SD = 1.73 mm). The average diameter of the inhibition zones of acacia honey (75 % v/v) on strains that were sensitive to the honey was 11.06 mm (SD = 1.24mm). Finally, the average diameter of the inhibition zones of horse chestnut honey (100 % v/v) on strains that were sensitive to the honey was 11.08 mm (SD = 1.02 mm). Antibiotic penicillin showed the average diameter of the inhibition zones to be 30.45 mm (SD = 3.21mm). Comparing the zone of inhibitions, by multiple comparison procedures, of different concentrations of

honeys and penicillin (positive control), astatistically significant difference was observed with 100% acacia honey and100 % horse chestnut honey ($p < 0.05$).

Discussion

Honey has several properties that contribute to its antimicrobial activity. Low pH and high osmolarity are combined with the enzymatic production of hydrogen peroxide that exerts an antimicrobial effect (15-16). Hydrogen peroxide is the major contributor to the antimicrobial activity of honey (17). The different concentrations of this compound in different honeys result in their varying antimicrobial effects (7).

In our research, we evaluated the antimicrobial activity of honey on BHS-A because, if in the case of positive antibacterial activity of honey on the BHS-A, it can be used primarily in the supportive therapy for e.g. tonsillopharyngitis. The use of honey is extremely uncomplicated and easily applicable.

Researchers who evaluated the antimicrobial effect of honey showed that certain microorganisms have better sensitivity to it. Mittal et al. reported greater honey activity in vitro against *Staphylococcus aureus,* and *Escherichia coli* than on *Pseudomonas aeruginosa* (18). Cooper et al. reported honey activity on catalase negative, gram positive cocci (5); while Hannan et al. reported honey activity on typhoidal Salmonellae.

When observing the antimicrobial activity of honey, the key element of effectiveness is the type of honey. Certain types of honey, such as Manuka honey, exhibit extensive antimicrobial activity. A group of researchers from Ireland and Australia reported that Manuka honey has clinical potential and a greater antimicrobial effect in vitro against *Staphylococcus aureus,* and *Escherichia coli* than on *Pseudomonas aeruginosa* (19, 20). A group of researchers from Cameroon focused on the antimicrobial activity of honeys on clinical isolates of *Helicobacter pylori* (6, 21, 22). The results of such research can provide alternative therapies against certain bacteria.

In our research, it was observed that 100% of acacia honey and horse chestnut honey show maximum antimicrobial activity as shown in Table 1. When the concentrations of honey were 50% v/v and 25% v/v there was not an antimicrobial effect. This conclusion of our study is inconsistent with the conclusion of other authors (6). It is assumed that such differences are due to the various kinds of honeys.

In this study, in order to evaluate the antibacterial activity of two honey varieties (horse chestnut honey and acacia honey) upon clinical isolates of BHS-A, we concluded that honeys exhibit limited, but effective antibacterial activity. Such results support the use of honey as a supplement therapy for BHS-A infections e.g. tonsillopharyngitis. Further research is required to determine the *in vivo* activity of horse chestnut honey and acacia honey onto BHS-A.

Acknowledgements

The authors would like to thank Maja Tomić Paradžik MSc, MD, from the Microbiology Department of Public Health Institute of the County Brodsko-Posavska, for donating clinical isolates of BHS-A strains.

This research was presented as a poster/abstract at the 9th International Scientific and Professional Conference - WITH FOOD TO HEALTH, on the 13th of October, 2016 in Osijek, Croatia.

Disclosure

Funding. No specific funding was received for this study.

Competing interests. None to declare.

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The Dual Nature of the Antiepileptic Drug Valproic Acid, with Possible Beneficial Effects in Alzheimer's Disease

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Abstract

Valproic acid (VPA) is a short fatty acid with strong anticonvulsant properties. It has diverse effects in different tissues with opposing mechanisms of physiological action. Due to the effects on energy, fatty acid, and cholesterol metabolism, it may be a risk factor for the development of diabetes with its associated complications of atherosclerosis, weight gain, hypertension, insulin resistance and other complications. Its negative effects on the endocrine system can have severe health consequences, especially in the female population. VPA produces proinflammatory and proapoptotic effects in the liver and anti-inflammatory and antiapoptotic effects in the central nervous system. It also causes abnormalities in lipid and cholesterol transport in the liver and the reproductive organs, while in neural stem cells it decreases cholesterol accumulation and helps neural growth and differentiation. However, in the CNS it has some beneficial effects, which are proposed to be important in Alzheimer's disease (AD). In AD mouse models, VPA exerted antiapoptotic effects and the expression of transcription factors that promote neurite growth. Most of the adverse pathogenic actions or beneficial molecular effects are not fully understood. We present an overview and comparison of the different properties of VPA and their effects on estrogen and cholesterol metabolism, lipid transport, Alzheimer's disease, and on the physiology of the liver, reproductive organs, and neurons from in vitro and in vivo (in animal models and patients) studies.

(Đikić D, Jutrić D, Dominko K. The Dual Nature of the Antiepileptic Drug Valproic Acid, with Possible Beneficial Effects in Alzheimer's Disease. SEEMEDJ 2017;1(1):74-89)

Introduction

Valproic acid (VPA) is a short, branched-chain fatty acid with anticonvulsant properties and one of the most widely prescribed drugs for many types of epilepsy in children and adults. Typically, it is applied as the sodium salt form, the sodium valproate and VPA, or mixture of both compounds. The therapeutic concentration varies between 50 and 150mg/l in the serum (1-

Received: March 15, 2017; revised version accepted: April 24, 2017; published: April 24. 2017

KEYWORDS: hepatotoxicity, metabolic disorders, lipid transport, steroidogenesis, Alzheimer's disease

Table 1. *The results of literature survey on two search engines using the key word Valproate.*

Search engine	Total No. of references (1971-2017)	Year 2017 (first trimester)	Year 2016	Year 2015	Year 2014	Year 2013	Year 2012
SCOPUS	15050	86	437	519	596	598	626
PUBMED	17146	203	782	747	805	788	762

3). The LC-MS/MS or chemiluminescent microparticle immunoassay are used for determination of the VPA level in plasma (4). At serum concentrations 200mg/l and above, VPA can have adverse drug reactions, and it is necessary to lower the dosage of the drug to avoid the acute overdose toxic effects that may include hyperthermia/hypothermia, tachycardia, hypotension (with severe overdose), serious respiratory depression necessitating airway assistance, and cardiac arrest (with severe overdose). The central nervous system (CNS) findings in cases of VPA overdose may include: coma, confusion, somnolence, worsened seizure control, dizziness, hallucinations, irritability, headache, ataxia, and cerebral edema. Death by overdose and death associated with chronic complications in adults are recorded as well as death among children (1, 5).

Although VPA is very efficient in treating epilepsy, its chronic therapeutic application is associated with many unwanted adverse drug reactions in certain prone patients (6).Adverse drug reactions, which are described within this manuscript from cited literature sources, include: weight gain, atherosclerosis, hypertension, nonalcoholic fatty liver disease (NAFLD), alterations of adipocytokine homeostasis, increased oxidative stress, insulin resistance, severe liver toxicity, death, abnormalities in estrogen and androgen metabolism, and PCOS (polycystic ovary syndrome) (Figure 1).

On the other hand, experimental data, which are described in manuscripts from cited literature sources, suggest that VPA also has effects which seem to be beneficial regarding the physiology of neural tissue (Figure 2), especially in Alzheimer's disease. The effects of VPA in the CNS seem to be completely opposite of those occurring in the liver and other tissues.

By survey of the literature in Table 1, major search engines yield between 15000 and 17000 hits when using the key word Valproate. In the last 6 years, there have been 2862 (Scopus) and 4087 (PubMed) published papers on the subject of VPA (Table 1).

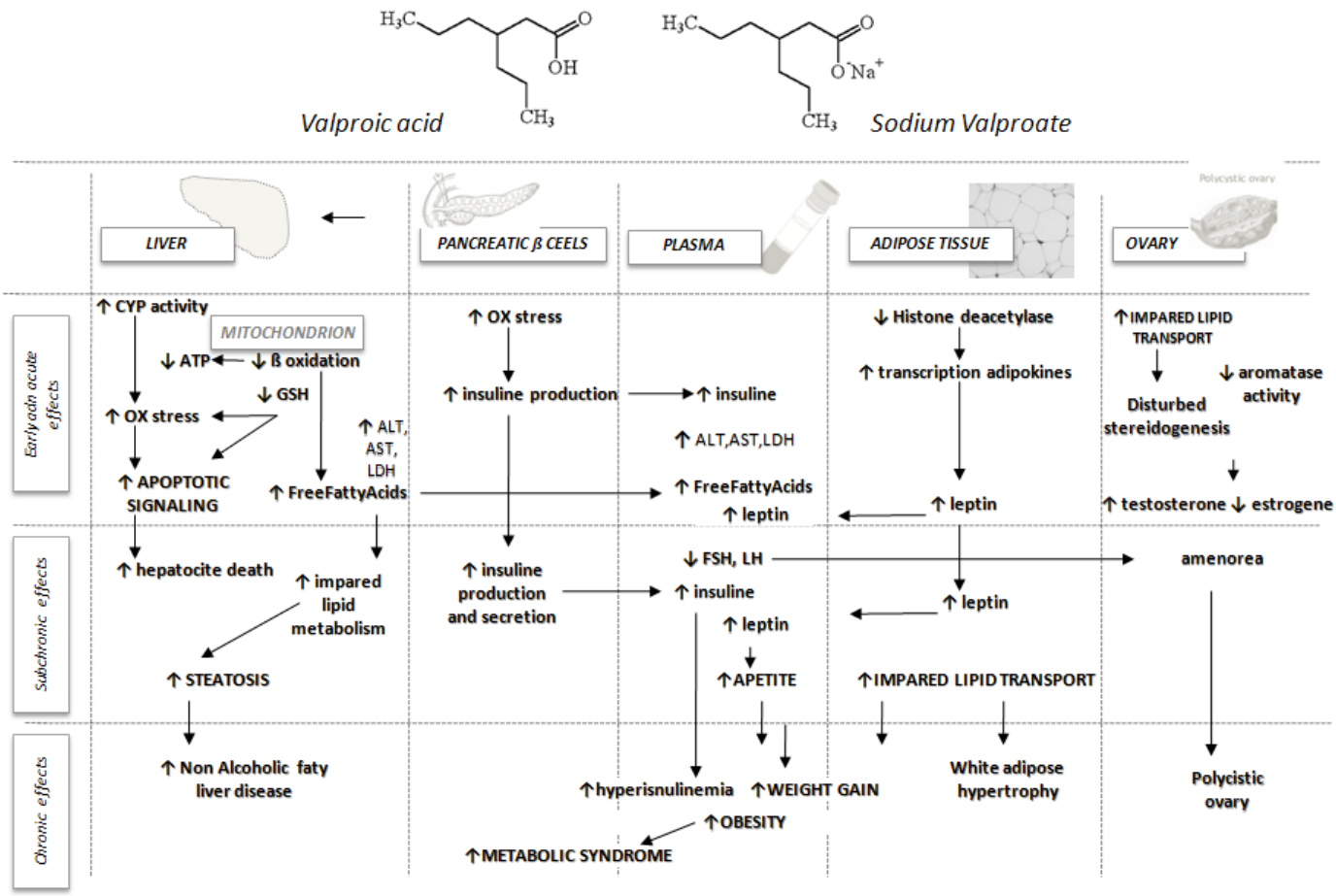
This review has been written to summarize the latest evidence of VPA's effects in living organisms. Novel physiological roles are being discovered, which we point to in the last chapter of the review.

Metabolism, physiological and molecular effects

Absorption, distribution, metabolism and elimination (ADME) of Valproic acid

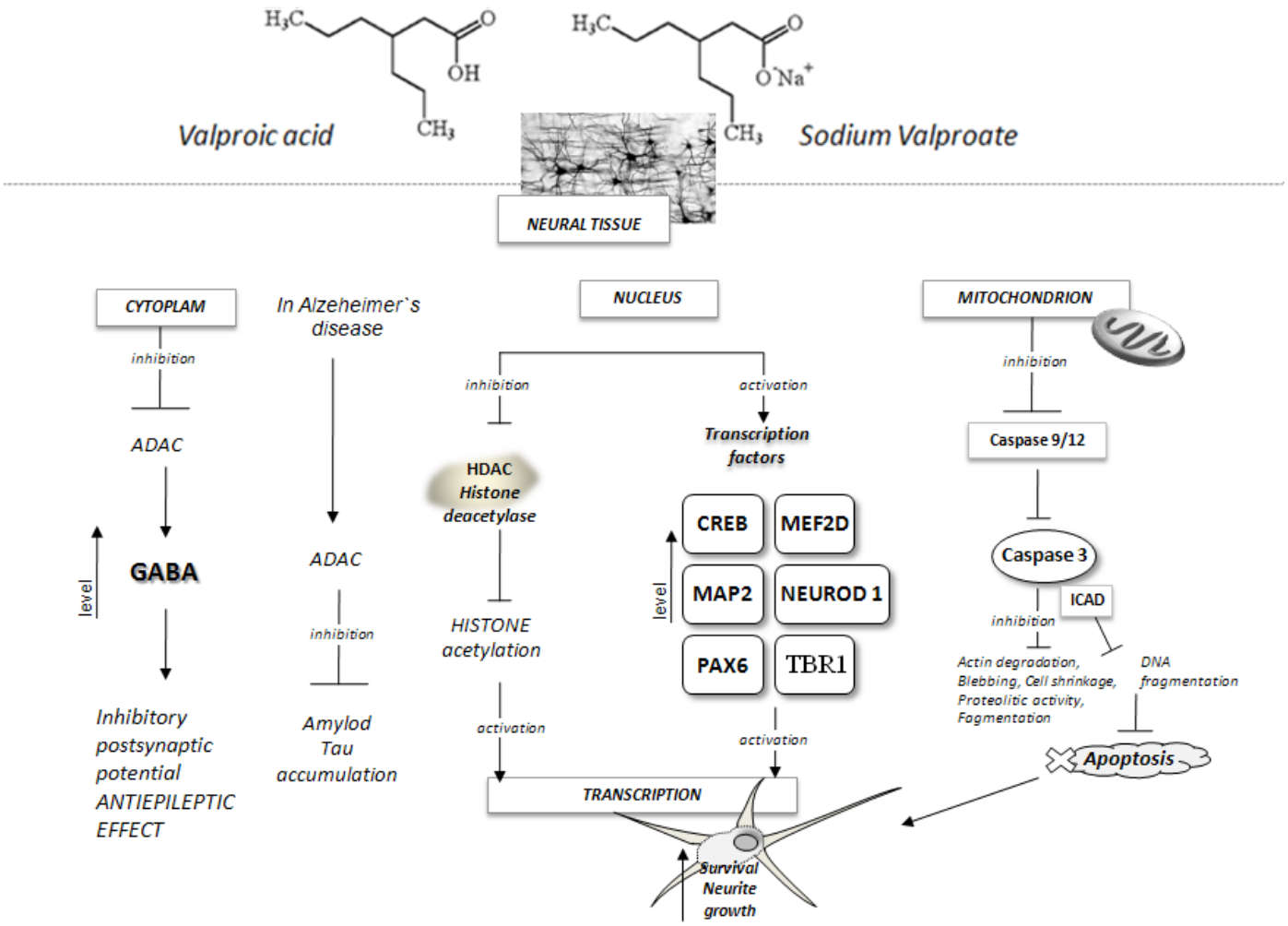
Pharmacokinetic and pharmacodynamic studies show that the absorption of short fatty acid VPA in the gastrointestinal system is close to 100 % (7,8). Because the molecular structure of VPA is similar to other short and long chain fatty acids, it follows the absorption pathways of all other triglycerides, cholesterol, and fatty acids.Nearly 90% of VPA is bound to the serum proteins. Only the unbound portion of the compound is active.The biological halftime of elimination in patients is 6-16 hours (1).The time of elimination is extended in children younger than 18 months of age. In the organism, there are at least 50

***Figure 1**. An overview of the physiological and adverse drug reaction effects caused by Valproic acid and Sodium Valproate treatment.*



known metabolites. The VPA molecule is partially metabolized in the cytoplasm, and partially in the mitochondrion where it is transported by the help of carnitine. Within the cytoplasm, it is oxidized in Phase I reactions by cytochrome P-450(CYP450)-catalyzed oxidation with the major enzymes being CYP2A6, CYP2B6 and CYP2C9, which form major toxic metabolites 4-eneVPA and 2-ene VPA. Cytoplasmic VPA can be immediately conjugated to UDP-glucuronic acid by UDP-glycotransferases (UGT1A3, -1A4, -1A6, -1A7, -1A8, -1A9, -1A10 and -1A15). The 4-ene VPA and VPA molecules enter the mitochondrion conjugated to CoA (4-eneVPA-CoA and VPA-CoA) where they undergo mitochondrion-mediated β-oxidation and cytochrome P-450(CYP450)-catalyzed oxidation. The major metabolites of CYP450-catalyzed oxidation are the 2-propyl-4-pentenoic acid (4-ene-VPA), and the β-oxidation metabolite 2-propyl-2, 4-pentadienoic acid (2, 4-diene-VPA). These metabolites are considered to be the main hepatotoxic metabolites of VPA. The metabolite 2-propyl-2-pentenoic acid (2-ene-VPA), which forms through the mitochondrial β-oxidation, is also converted to the 2, 4-diene-VPA *in vivo* (1;7;9). In Phase II of the biotransformation reactions, the majority of the formed metabolites are subjected to further glucuronidation, which is considered the major route for VPA and metabolite elimination. To a lesser extent, the adverse metabolite (2,4-diene-VPA-S-CoA) can be conjugated with glutathione (GSH) to form thiol conjugates, but more importantly it causes a decrease in mitochondrial glutathione levels, as described in rat hepatocyte cell cultures (9). The VPA glucuronides and their glucuronidated metabolites are excreted primarily by the urinary route and only in a small amount through the bile and into the intestines (1;7; 10).

Figure 2. *An overview of the physiological effects caused by Valproic acid and Sodium valproate in neural tissue*.



Mechanism of therapeutic action

It is thought that the major physiologic mechanism of VPA's therapeutic properties in epilepsy is the inhibition of 4-aminobutyrate aminotransferase (ABAT), a transaminase in the gamma aminobutyric acid (GABA) pathway. Therefore, VPA increases GABA release. It may also act by altering the properties of voltage-dependent sodium channels. In this way, it stops a seizure attack during epilepsy. VPA is also a histone deacetylase inhibitor. With this function, it promotes more transcriptionally active chromatin structures, which is a likely epigenetic mechanism for the regulation of many of the neuroprotective effects attributed to valproic acid. Intermediate molecules mediating these effects include the Vascular endothelial growth factor (VEGF), the Brain-derived neurotrophic factor (BDNF) and the Glial cell line-derived neurotrophic factor (GDNF) (1, 10, 11).

Adverse drug reactions

Liver adverse effects

In the liver VPA causes an accumulation of lipids, cholesterol, and fatty acids with the subsequent development of hepatic steatosis and nonalcoholic fatty liver disease (NAFLD) in patients as well as in *in vitro and in vivo* models.The hepatic adverse drug reactions caused by steatosis are probably due to the abnormalities in lipid transport, but the exact mechanisms of liver adverse drug reactions are not clear. Neither are the mechanisms by which VPA induces nonalcoholic fatty liver disease (NAFLD). Experimentally on human HepG2 cell lines, in vivo, VPA treatment causes leakages of

ALT, AST, and LDH in a dose dependent manner suggesting injury to hepatocytes (12).

The 4-ene-VPA and 2, 4-diene-VPA metabolites and enzymes CYP1A1, CYP2A6, CYP2C9, ABCG1, and CPT1A are associated with hepatic adverse drug reactions in humans and in certain hepatic cell lines (8;12). However, only the CYP2A6 polymorphism was found to be associated with higher concentrations of 4-ene-VPA and 2, 4-diene-VPA. Potential important risk factors include mutated genotypes of CYP2C9 and CYP2A6, and higher concentrations of VPA (8).

Exposure to sodium VPA has been shown to induce a down regulation of several transcripts in cultured hepatocytes. Their low levels resulted in time dependent fluctuations of cellular ATP, which can lead to cell death (13).

Hepatic adverse drug reactions caused by VPAhave also been associated with mitochondrial dysfunctions, with the inhibition of enzymes in the beta-oxidation pathwayand with oxidative stress.As aforementioned, VPA can form thiol conjugates particularly with glutathione (9). Such decreases of intracellular glutathione levels are known to change the redox status of the cell and lead to the adverse drug reactions of oxidative stress effects. For example, VPA incubation of rat hepatocytes in vitro caused glutathione depletion which suggests a possible cellular oxidative stress after VPA exposure (9). For VPA, the role as a potential oxidant is underexplored, and there are scarce literature sources on the subject. Experiments on human HepG2 cell lines showed that exposure to VPA for more than 72h increased levels of mitochondrial reactive oxygen species production (ROS), but decreased protein levels of mitochondrial superoxide dismutase SOD2, suggesting oxidative stress caused by impaired elimination of mitochondrial ROS (13).

Pourahmad et al. (14) and Jafarian et al. (15), both, in vitro, detected increases in ROS formation along with a decrease in mitochondrial membrane potential upon the treatment of rat liver mitochondria with VPA; all of these were events before cell death signaling began. Specifically, Jafarian et al. (15) showed that ROS is associated with increased lipid peroxidation, mitochondrial membrane collapse; mitochondrial swelling and finally the release of cytochrome c. release began apoptotic cell death signaling. Similarly, Pourahmadet al. (14) in their experiment demonstrated that the cytotoxic action of VPA manifests itself as lysosomal membrane leakiness in conjunction with ROS formation and a decline in membrane potential.Again, all were events before cell lysis started.

Chang et al. (16) investigated the genetic polymorphisms in the G protein beta three subunit (GNB3) and associations with the metabolic phenotypes of VPA treated human patients.Their study confirmed that patients, who are T allele carriers of GNB3 C825T polymorphism, have a lower risk of VPA induced metabolic abnormalities.They warrant further studies investigating mechanisms of VPA induced metabolic abnormalities and G protein.

An article by Stewart et al. (17) describes that the risk of hepatic adverse drug reactions induced by VPA is increased in patients with mitochondrial diseases, especially those with POLG1 gene mutations. POLG genes code for the mitochondrial DNA polymerase gamma and their mutations can cause Alpers-Huttenlocher syndrome, a neurometabolic disorder linked to an increased risk of developing fatal hepatic adverse drug reactions upon exposure to VPA. Stewart et al. (17) also described an association of VPA induced hepatic adverse drug reactions with a genetic variation in the POLG gene. In their case, it was a heterozygous genetic variation in the POLG gene which was primarily due to the p.Q1236H substitution, and it was strongly associated with VPA induced liver toxicity. In their experiment on primary human cell lines, therapeutic doses of VPA stopped human cellular proliferation and high doses of VPA induced non-apoptotic cell death, not mediated by mitochondrial DNA depletion, a defect infatty acid metabolism or mutation.Therefore, another mechanism of liver injury by VPA is impaired cellular liver regeneration.One of the ways of preventing it is by prospective genetic testing for POLG which could identify individuals at high

risk for potentially fatal consequences of VPA treatment (17).

Hepatic lipid metabolism is impaired during VPA treatment. The secretion of triacylglycerols and phospholipids at the sinusoidal pole of hepatocytes are reduced by an acute administration of VPA. This inhibition of secretion has been thought by Berllinger et al. (18) to be a factor in the development of steatogenic hepatic adverse drug reactions by VPA treatment (18) in rat hepatocytes, though so far shown only in an animal model.

Gene expression profiles in hepatocytes could be associated with the steatogenic hepatic adverse drug reactions of sodium VPA. Gene profiling data showed striking changes in the expression of genes associated with lipid, fatty acid, steroid metabolism, oncogenesis, and signal transduction and development in mice treated with VPA (19). Lee, et al. (19), found that 1156 genes were up and down regulated after exposure to sodium VPA. 60 genes were involved in lipid metabolism and were interconnected with the biological pathways for biosynthesis of triglycerides and cholesterol, catabolism of fatty acids and lipid transport (19).

Obesity, fatty acid, and cholesterol disorders in patients treated with VPA

Chronic treatment with VPA is commonly associated with weight gain, which potentially has important health implications, in particular increased central fat distribution. A positive correlation between chronic treatment with VPA and increased abdominal weight as well as increased blood pressure was recorded previously in human patients (20). An association was found between VPA induced weight gain and insulin resistance, hyperinsulinemia, hyperleptinemia and leptin resistance. Furthermore, the patients who had VPA induced weight gain were more likely to develop metabolic syndrome and dyslipidemia with associated long term vascular complications such as hypertension and atherosclerosis. In addition, they stated that long term VPA therapy carries a risk for atherosclerosis because of an accumulation of oxidative stress in combination with elevations of uric acid and homocysteine. Another experiment in human patients showed that VPA induced weight gain in the experimental group taking Valproate but noted weight gain was not due to decreased physical activity. In these participants of the study, there was a noted increased motivation to eat and decreased glucose levels in the experimental group compared to control group (21). Interestingly, quite opposite to these results in humans, Khan at al. (22) in their recent work in animal models discuss that the reason for the weight increase with VPA therapy might be increased appetite and irregular thirst, and consequent over-consumption of energy-rich alimentary. Also, they point out the dependence on the doses and duration of treatment, which changes the pharmacological signaling, differences in clinical and preclinical findings, as well as inter-species variability. The authors highlighted the role of histone deacetylases (HDACs) in insulin-resistance, gluconeogenesis and islet function, and formed a hypothesis that VPA treatment (in particular dose) might have a beneficial role in diabetic disorder. HDACs can modulate the expression of various genes, which directly or indirectly affect glucose metabolism. In their experimental work, Khan et al. (22), proposed and recorded the anti-diabetic role of VPA by the modulation of insulin signaling and forkhead box protein O1 (FOXO1)-mediated gluconeogenesis in type-2 diabetic Sprague-Dawley rats. In the proposed model diabetes was induced by the combination of a high-fat diet and low dose streptozotocin, and VPA was given at the doses of 150 and 300 mg/kg/day. VPA treatment significantly reduced the plasma glucose, HbA1c, insulin-resistance, and the fat deposition in the brown adipose tissue, white adipose tissue and liver, which is comparable to metformin effects—which the authors used as a positive control to create equal effects. The treatment also inhibited the gluconeogenesis and glucagon expression and recuperated the histopathological changes in the pancreas and liver. Detailed molecular mechanisms of the findings might elucidate the possible physiological mechanisms.

Adipokines

As expressed in the previous chapter, in recent years, research focused on adipokine signaling to elucidate the VPA effects in increased obesity. It seems that without doubt, VPA affects the neurohormonal regulation of appetite, as confirmed in both patients and animal models. Weight gain during VPA treatment may be related to increases in leptin, insulin and neuropeptide Y (NPY) levels. Tokgoz et al.(23), conducted a study on 20 prepubertal children which compared the BMI, leptin, insulin, NPY, adiponectin and gherlin levels with lipid profiles, and CAIMT(carotid artery intima media thickness), before and after a treatment period with VPA of six to twelve months. The study aimed to evaluate and to determine whether these parameters indicate the development of early atherosclerosis. The treatment did not affect plasma ghrelin, adiponectin levels, lipid profiles, and CAIMT after 6 and 12 months of treatment compared with pretreatment values. Although BMI and the appetite regulatory molecules were changed, early atherosclerotic changes were not triggered within a year of treatment (23). A similar study on VPA therapy, conducted earlier on 18 children (10.94 ± 3.78 years), showed, after 18 months of VPA exposure, an increase inthe serum leptin, insulin, and neuropeptide Y (NPY) levels, but also in the glucose, cortisol, galanin and ghrelin levels compared to the matching control. There was an increase of 2.3kg of body weight combined with an increase in named obesity signaling molecules in the treatment group compared to the control group (24).The findings of these two studies of decreased gherlin levels and increased weight gain are in stark contrast to the findings of the Gungor et al. (25) group which found increased levels of gherlin in proportion to an increase in weight of prepubertal patients. Meral et al. (26) included 44 children with idiopathic, generalized epilepsy treated with valproic acid (VPA) as an experimental group, and 40 children without therapy as the control group in their study (26). Neither the VPA treated group nor the control group showed any significant difference in terms of LDL cholesterol, total cholesterol and age. However, the VPA treated group subjects had significantly higher BMI-SDS as well as higher levels of visfatin, apelin, and triglycerides, but lower levels of adiponectin and HDL levels than the control subjects. The group concluded that visfatin, adiponectin and apelin can be considered as potential regulators of fat and glucose metabolism during valproic acid therapy (26).Grosso et al.(27) found that patients with VPA associated obesity had high concentrations of leptin in their blood. They did not find any differences in leptin concentration between patients who had VPA induced obesity and obese controls during their experiment. Li et al. (28) found that VPA can increase serum lipid levels in both juvenile and adult rats, but that higher levels of lipids could be found in juvenile rats (28).

Gherlin is affected by valproic acid treatment and there are potential effects of such interactions on weight gain.A study on 35 pediatric patients aged three to fifteen years were evaluated for gherlin, leptin, C-peptide, insulin, insulin like growth factor- 1, insulin like growth factor binding protein- 3 and glucose levels.Serum gherlin levels were increased significantly with a negative correlation with insulin like growth factor-1 and insulin like growth binding protein 3 in the prepubertal group at six months of treatment. Thus, the weight gain caused by valproic acid could be linked to the increased levels of gherlin levels in the early treatment period (25).

Polycystic ovarian syndrome (PCOS)

A higher occurrence of polycystic ovarian syndrome (PCOS) compared with other antiepileptic drugs is one of the major adverse drug reactions reported in women treated with VPA (29;30). Several molecular mechanisms could account for this epidemiological appearance. VPA treatment can be connected to hyperandrogenism, hyperandrogenemia, oligoovulaton, the appearance of PCO on anultra-sonogram, elevated levels of testosterone, and irregular menstrual cycles. The incidence of occurrence of PCOS in women taking VPA is 1.95 times higher than in women

being treated with other antiepileptics (31). It seems that VPA treatment during pregnancies possesses a significant risk of teratogenic effects based on a survey where a 15 out of 229 (6.6%) women prescribed valproate gave birth to a child with a major congenital malformation (29).

Since testosterone levels slightly increase, and progesterone and estradiol levels decrease over a longer treatment period with VPA, it is believed that such an imbalance induces polycystic ovarian changes and menstrual suspensions (amenorea) that later lead to polycystic ovarian syndrome (32).

Indeed, some authors, who also recorded increased levels of testosterone in women treated with VPA for bipolar disorder, proposed that a broad inhibitory action on glucuronidation systems and on cytochrome systems causes high concentrations of testosterone, dehydroepiandrosterone sulfate, and androstenedione. VPA treatment over a longer period of time is associated with increased levels of testosterone. It is also associated with a development of menstrual abnormalities. There is a significant correlation between VPA treatment and a reduction in the levels of mRNA encoding estrogen receptor alpha (ER alpha), which causes a lack of ER alpha protein in breast and ovarian cell lines. Beside the regulation of sex physiology and menstrual abnormalities, the weight gain and osteoporosis could be a result of the lack of estrogen signaling because of the clearance of ER alpha protein in cell lines (33). Furthermore, beside the VPA interference with the peripheral endocrine hormones, at the central regulatory level of the hypothalamus-hypophysis axis, VPA also causes decreased excretion of LH (luteinizing hormone), FSH (follicle stimulating hormone) and prolactin. Together, the steroid sex hormone imbalance, the hyperinsulinemia and obesity in humans, which occur due to VPA treatment, further contribute to an increase in sexual dysfunctions (34).

Impairment of synthesis of steroid hormones

Mechanisms of ovarian toxicity include a possible disruption of the pathways for the synthesis of sex hormones as a major cause in the development of PCOS. Brion et al. (35) expressed that VPA may increase mitochondrial cholesterol transport by a mechanism independent of the steroidogenic acute regulatory protein, as proven in an experimental model.

Some authors explain the hormonal imbalance as a direct consequence of differential activity onthe expression of CYP enzymes involved in hormone synthesis, which impairs the conversion of testosterone to estradiol (36, 37). A suppression of aromatase expression takes place in granulosa cells as an answer to treatment with VPA. For example, the follicular development of 14-day-old rats was suppressed, and testosterone, estradiol, androstenedione, and the combined levels of all steroid hormones tended to decrease over time with exposure to VPA (38). Gustavsen et al. (37), found in vitro, that the expression of genes coding for enzymes early in steroidogenesis was downregulated. Such changes did not occur with the use of other antiepileptics (37).

The suppression of aromatase expression means that the synthetic pathway from cholesterol to estradiol, including the de novo synthesis of cholesterol, is suppressed (38). Contrary to these in vivo results in isolated mitochondria, *in vitro*, VPA stimulates exogenous cholesterol conversion to progesterone (38). This indicates that the complex physiological mechanisms do not fulfill the whole picture of physiological interactions as *in vivo*.

The majority of the mechanistic studies on stereidogenesis of sex hormones and ovarian hyperandrogenism rely on *in vivo* and *in vitro* investigation of VPA exposure only, and measurements of CYP conversion in patients is scarce. There are inconsistencies in the literature regarding the effects of VPA on the female reproductive steroidogenesis, as pointed out by Glister et al. (39) in their review of literature on the subject. In vivo in rodent models, Glister et al. (39) mention both the increase in the number of

follicular "cysts" and total ovarian weight with decreased plasma testosterone level, then list experiments with no effect on serum androgen levels. However, reduced serum estrogen levels affect tthe androgen/estrogen ratio, where androgen hormones become relatively higher. Interestingly, Glister (39) mentions the experiment on primates where VPA treatment in a Rhesus monkey with normal cycling had no effect on androgen levels or ovarian morphology. The authors (39) further list similar inconsistencies in the in vitro studies with some experiments: (i) recordings of increased ovarian androgen synthesis and an increased transcription of steroidogenic genes, (ii) an inhibitory effect of VPA on hCG-induced androgen secretion, (iii) increased basal and LH-stimulated androgen secretion or decreased LH-stimulated androgen secretion and reduced basal and FSH-induced estradiol secretion. In their own experiment (39) in primary bovine theca (TC) and granulosa (GC) cells (a model closely relating human cycling), they exposed TC to VPA (7.8–500 μg/ml) with/without LH and GC with/without FSH or IGF analogue. In Theca cells, VPA reduced basal androstenedione secretion by 70% and in VPA/LH-induced theca cells by 93%. CYP17A1 mRNA was reduced by more than 99% and LHR, STAR, CYP11A1 and HSD3B1 mRNA was also lower. VPA only reduced theca cells progesterone secretion induced by the highest (luteinizing) LH dose. At higher concentrations (125–500 μg/ml) VPA inhibited basal, FSH- and IGF-stimulated estradiol secretion in granulose cells without affecting progesterone secretion. VPA reversed FSH-induced upregulation of CYP19A1 and HSD17B1 mRNA levels. Vice versa, VPA inhibits both LH-dependent androgen production and FSH/IGF-dependent estradiol production. The authors proposed that the named changes are consequences of the HDAC inhibitory properties and conclude that the VPA has a direct stimulatory action on theca cell androgen production (39).

Alzheimer's disease and VPA, new perspectives

In recent work, evidence was gathered that besides GABA promoting effects, VPA treatment has antiapoptotic and protective effects in neural tissue (Figure2), though as with steroidogenesis, mostly in model experiments. The deinhibition of histone acetylation caused by histone deacetylases (HDAC s) inhibitors could contribute to the recovery of learning and memory in rats. Histone hypoacetylaton of lysine residues contributes to cognitive impairments in Alzheimer's disease (AD).VPA can significantly elevate histone acetylation trough HDAC activity inhibition. Experiments showed that VPA treatment can boost the long-term recognition memory and spatial learning memory in AD transgenic mice. As such VPA could significantly improve cognitive function in AD (40, 41).

Experimentally, VPA ameliorates spatial memory impairment and amyloid beta deposition in transgenic mice (42). VPA treatment caused a decrease in senile plaque formation, and Amyloid beta 40 and Amyloid beta 42 accumulations. Several studies, including those by Longet al. (43) in 2016, proposed that gender differences play a role in determining how well VPA affects the treatment of Alzheimer's disease. It appears that gender differences play a role in the VPA effects on AD, since these effects were more notable in the male than in the female AD mice (43).

As a histone deacetylase inhibitor, VPA is able to upregulate neprilysin (NEP) expression and activity in human neuroblastoma SH-SY5Y cell lines, which usually express very little NEP protein. Upregulation of expression and activity of NEP in the rat hippocampus was, also, observed following i.p. injections of VAP to rats. NEP is an amyoloid degrading enzyme which in the healthy brain maintains Amyloid beta levels at physiologically low concentrations. The activity and expression of these enzymes decreases with age and, with some other pathological conditions, predisposes to late onset of AD (40).

The synapse damage caused by amyloid beta protein was reduced by pretreatment with physiologically relevant concentrations of VPA

(10 μM). VPA also decreased synaptic damage caused by other neurodegenerative associated proteins such as alpha synuclein, which is linked to Lewy Body dementia and Parkinson's disease (44).

VPA treatment inhibited the activity of GSK-3beta, decreasing hyper phosphorylated Tau by lower phosphorylation, as seen in the transgenic mice (45).

One of the main reasons of neuronal loss in AD is apoptosis of neurons. It seems that in neural tissue, VPA promotes the reduction of apoptosis by blocking and decreasing apoptotic signals and increasing survival (46). Evidence for reduction of apoptotic signals includes a significant reduction in the expression of interleukin 1-beta (IL-1ß) and the tumor necrosis factor alpha (TNF-α), as well as the micro and astrogliosis in the hippocampus and cortex of APP/PS1 transgenic mice (43). Besides the reduction of antiapoptotic signals, these results imply that VPA has anti-inflammatory properties in the brain that are opposite of the effects it has in the liver.

Further evidence of a decrease of apoptotic signaling in the mouse AD model showed that VPA acted via the suppression of both the mitochondrial and the endoplasmic reticulum pathways of apoptosis, by downregulating the expression of Caspase 3, Caspase 9, Caspase 12, and by reducing the level of cytochrome C and Bax. Antiapoptotic protein BCL 2 expression was increased, intracellular levels of Ca^{2+} decreased, and the mitochondrial membrane potential was elevated (46).These are yet other effects of VPA which seem to be completely opposite of those that are occurring in the liver where apoptosis inducing effects were demonstrated, as described previously in the text.

Most interestingly, beside the antiapoptotic effects, the proliferative effects in neurons, increase in synaptogenesis and novel connectivity between neurons are also important novel physiological roles of VPA in neural tissue. Experimentally, VPA caused an increase of CREB and BDNF expression, causing accelerated neurite outgrowth, modification of synaptic structure and improvement of behavioral deficits in AD mouse models. Accumulating evidence supporting such research included reports where VPA was able to induce MAP 2 gene expression, which mediated the process of de novo re-arborisation and neurite outgrowth of neurons. These functions add to the process of successful neuronal re-wirings (47).VPA treatment includes the activation of regulatory pathways that enhance neurogenesis and suppress gliogenesis. Genes which encode the transcription factors (TFs) that specify neuronal cell fate, including MEF2D, MYT1L, NEUROD1, PAX6 and TBR1, and their target genes, are induced by VPA (48). Neural stem cells (NSCs) derived from Niemann-Pick Type C disease (NPC) mice, a neurodegenerative and lipid storage disorder for which no effective treatment is known, showed impaired self-renewal and differentiation. VPA was able to induce neuronal differentiation and restore impaired astrocytes in NSCs from NPC1 (-/-) mice. Increasing levels of cholesterol within NSC from NPC1 (-/-) mice could be reduced by VPA. Necessary neurotrophic genes (TrkB, BDNF, MnSOD, and NeruoD) were upregulated through the repression of the REST/NRSF and HDAC complex by VPA treatment. These upregulated neurotrophic genes were able to enhance neural differentiation and cholesterol homeostasis in neural stem cells from NPC1 (-/-) mice (49). Such protective and beneficial role in AD, tau and amyloid accumulation will be the future of VPA research.

Discussion and conclusion

VPA is a short, branched chain fatty acid with strong anticonvulsant properties. It has diverse effects in different tissues, with opposing mechanisms of action that seem to be dose and exposure-time dependent (Figure 1). The number of papers published so far (Table 1), including approximately several hundred papers each year in the last decade, in all areas of physiological action, on both models and patients (Table 2), prove that the research on VPA physiology still raises interest in basic physiological research. The literature cited in this work, divided by subject area of research and

Table 2. *Cited literature on Valproic acid and/or Valproate, divided into categories by subjects of proposed physiological mechanisms and scientific research methods in vitro, in vivo and epidemiological studies on patients..*

Physiological Parameter	**In vitro (Cell culture)**	***In vivo* (Animal models)**	**Human patients (case, clinical and epidemiological studies)**	**No. of cited papers (TOTAL)**
Doses and therapy			1;2;3;4;5;6;10	7
Biotransformation	7;9;11;14	38	7;8;11;16	9
Liver adverse effects	9;12;13;14;15;17;18	18;19	8;17	11
Gonads, hormones, sexual physiology adverse effects	29;33;35;36;37;38	38	30;31;32;34	11
Stereidogenesis adverse effects (biotrasformation enzymes in sex hormones biochemistry)	35;36;37;38;39			5
Overweight, BMI, adiposity			16;20;23;24;25;27	6
Triglicerides, fatty acids cholesterol (biochemical mechanisms, adverse effects)	18;49	18;19;28	16;26	7
Adipokines		28	16;20;21;23;24; 25;26	8
Glucose and insulin, energy metabolism	22	29	16;21;23;24;25	7
Oxidative stress	13;14;15			3
Neural tissue beneficial effects, synaptogenesis, neural growth and beneficial effects in Alzeheimer disease models	44;49	40;41;42;43;44; 45;46;47;48		11
No. of cited papers (TOTAL)	30	17	37	

type of research (models and patients) (Table 2), is mainly from the last few years of research with only a few articles extended to the previous decade. From the comparison of cited physiological mechanisms, several major questions arise that will shape further research.

Although a relationship between VPA therapeutic mechanisms and GABA action is generally accepted, the mechanisms of its adverse reactions in other tissues have not been fully elucidated. The major questions that will impact future research directions are whether there is a unique fundamental physiological mechanism that triggers different reactions in the tissues with opposing action, or whether the different tissue responses are a consequence of diverse target pathways induced by VPA. Answering this fundamental question will direct

and focus the research toward a mechanistic explanation of adverse effects and hypothetical novel properties, such as a model based beneficial mechanism in Alzheimer's disease. The beneficial effects in neural tissue is mainly gained hypothetically in the models (Table 2) and has yet to be proven in human subjects. The models based on a novel proposed beneficial mechanism in neurons and especially in the protective role in the forming of Alzheimer's disease should also investigate the potential of VPA to prevent the transmigration of accumulated Tau and Amyloid in already formed plaques within the neural tissue. Research of such therapeutic potential in patients with early signs of plaque formation would be more important than VPA preventive potential, since it is highly unlikely that the healthy patients could be subjected to therapeutic exposure to VPA as a measure of prevention, even if the beneficial effects in prevention of the first steps of accumulation, and hyperphosphorilation of these molecules are proven in humans.

The problem of weight gain and energy physiology caused by the treatment with VPA still remains unsolved and limits all beneficiary actions described in neural tissues. Although noted in patients and animal models, the adverse drug effects on adiposity and lipid accumulation has not been clearly explained. It is believed that the hepatocyte mitochondrion disturbance is a major cause of imbalance of lipid metabolism in the liver and one of the causes of weight gain observed in patients treated with VPA. However, even though some genetic predispositions (such as POLG mutation) have been detected, it seems from the cited references that the effects could be more epigenetic in nature, and future research should focus on designing experiments connecting targeted genetic predisposition (mutations) and epigenetics. Furthermore, the hepatocyte lipid imbalance should be further investigated physiologically from the point of major conversing enzymes along the biochemical pathways of triglyceride, fatty acid and cholesterol synthesis/biochemical redistribution and lipoprotein synthesis and redistribution (for example Acetyl-CoA carboxylases 1 and 2 (ACC1 and ACC2), fatty acid synthetase (FAS), Stearoyl-CoA desaturase-1 or carnitine/palmitoyl-transferase 1 etc.). References in this direction of research are missing. Besides the proposed hepatic adverse drug reactions that influence lipid metabolism and transport, recently, the research concentrated on the role of appetite regulation pathways involving adipokines and other metabolic regulating hormones. This subject is abundant in the literature with both evidence from the patients and animal models. The exact question that future experimental design has to focus on would be whether adipokine disturbance (increase) is directly unleashed by VPA action as a cause or whether it is a consequence of adipocyte (and GI) reaction to differential fatty acids. There is a probability that adipokine activation and appetite enhancements are merely a consequence of disturbed lipid physiology within the adipocytes or hepatocytes. Besides lipid physiology, due to the abnormal effects on energy, fatty acid, and cholesterol metabolism, VPA treatment is a risk factor for the development of other metabolic diseases with its associated complications such as atherosclerosis, and metabolic syndrome, while in the CNS it has many beneficial effects. Research should also focus on the physiological pathways of cholesterol transport and metabolism not only in experimental models (Table 2), but in the liver, reproductive organs (stereidogenesis) and in neural cells of patients as well.

It seems that in the last decade the research field of oxidative stress has been abandoned or reduced in vivo (Table 2). In addition to the biochemical pathways of lipid metabolism, the cellular redox status of biochemical balance might also be important in contributing to the differential activation of physiological pathways of lipid metabolism and epigenetic changes that may enhance pathophysiological changes. Thus, future studies on VPA physiology should neither neglect this area of research. Within all prospect studies, special attention should be given to the doses of exposure.

In conclusion, although prescribed as an effective anticonvulsant, most of the pathogenic pathways of VPA's unwanted and beneficial molecular effects are not fully understood and further studies and experiments are warranted.

Disclosures

Competing interests: None to declare**.**

Acknowledgements and funding

This work is a part of the project of the Croatian Academy of Science and Arts "Cholesterol metabolism and oxidative stress"-MethCholOX2015", led by the corresponding author and the project grant no. IP-2014-09-9730ALZTAUPROTECT -"Tau protein hyperphosphorylation, aggregation and trans-synaptic transfer in Alzheimer's disease: cerebrospinal fluid analysis and assessment of potential neuroprotective compounds" of the Croatian Science Foundation, where the corresponding author participates as a collaborator.

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A Cross-Talk between the Renin-Angiotensin and Adrenergic Systems in Cardiovascular Health and Disease

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Abstract

It is well accepted that a number of cardiovascular (CV) and renal diseases are characterized by the long-term activation of both the renin-angiotensin system (RAS) and the sympathetic nervous system (SNS), which also contribute to the pathophysiology of structural and functional CV abnormalities as well as to the final clinical outcome. Moreover, there is a growing body of conclusive evidence that these systems do not operate independently, but interact at different levels throughout the CV system. The mediation of renin release from juxtaglomerular epithelioid (JGE) cells in kidney by SNS is well established and accepted. On the other hand, in recent years it became evident that RAS, by its main effect or angiotensin II (Ang II), induces SNS activity in various organs and tissues. Thus, there is a growing effort to clarify pathophysiological mechanisms of interaction and a more evident mutual potentiation of these two systems in different pathological states. Since it became evident that a high salt (HS) intake, which is a major risk factor for hypertension development, has a deleterious impact on vascular and endothelial functions (even in the absence of blood pressure changes), it became necessary to investigate and clarify the effect of HS loading on major regulating systems—RAS and SNS—precisely in healthy individuals. The present review aimed to summarize the interactions between the RAS and SNS in health and diseases (e.g. cardiovascular, renal), with a special focus on these two systems' interaction during HS intake in a healthy normotensive population.

(Stupin A, Drenjancevic I, Rasic L, Cosic A, Stupin M. A Cross-Talk between the Renin-Angiotensin and Adrenergic Systems in Cardiovascular Health and Disease. SEEMEDJ 2017;1(1);90-107)

Introduction

This review aimed to present the interactions between the renin-angiotensin system (RAS) and the sympathetic nervous system (SNS) in health and diseases (e.g. cardiovascular, renal), with a special focus on these two systems interaction during HS intake in healthy individuals. The mediation of renin release from JGE cells in kidney by β1-adrenergic receptors

Received: April 19, 2017; revised version accepted: May 8, 2017; published: May 9. 2017

KEYWORDS: renin-angiotensin system, sympathetic nervous system, cardiovascular function, chronic heart failure, chronic kidney disease, salt intake

activation is well established and accepted (1). However, there is a growing interest to understand how RAS affects SNS activity, and to evaluate whether these two systems potentiate each other's effects. It became evident that RAS, by its main effector, angiotenin II (Ang II), increases SNS activity in various organs and tissues (e.g. the central nervous system, the adrenal medulla, the sympathetic ganglia and the sympathetic nerve endings), and that this interaction is mediated mostly by the Ang II receptors type1 (AT1 receptors) located at the sympathetic nerve endings (2). The suggested physiological feedback loop in RAS-SNS interrelation is summarized in Figure 1. Most of these findings were brought by studies investigating the activity of these two systems in hypertension, chronic heart failure and/or chronic kidney disease (3, 4). Even though HS intake unequivocally suppresses RAS activity, the effect of HS intake on SNS activity that is independent of blood pressure changes is still not completely understood.

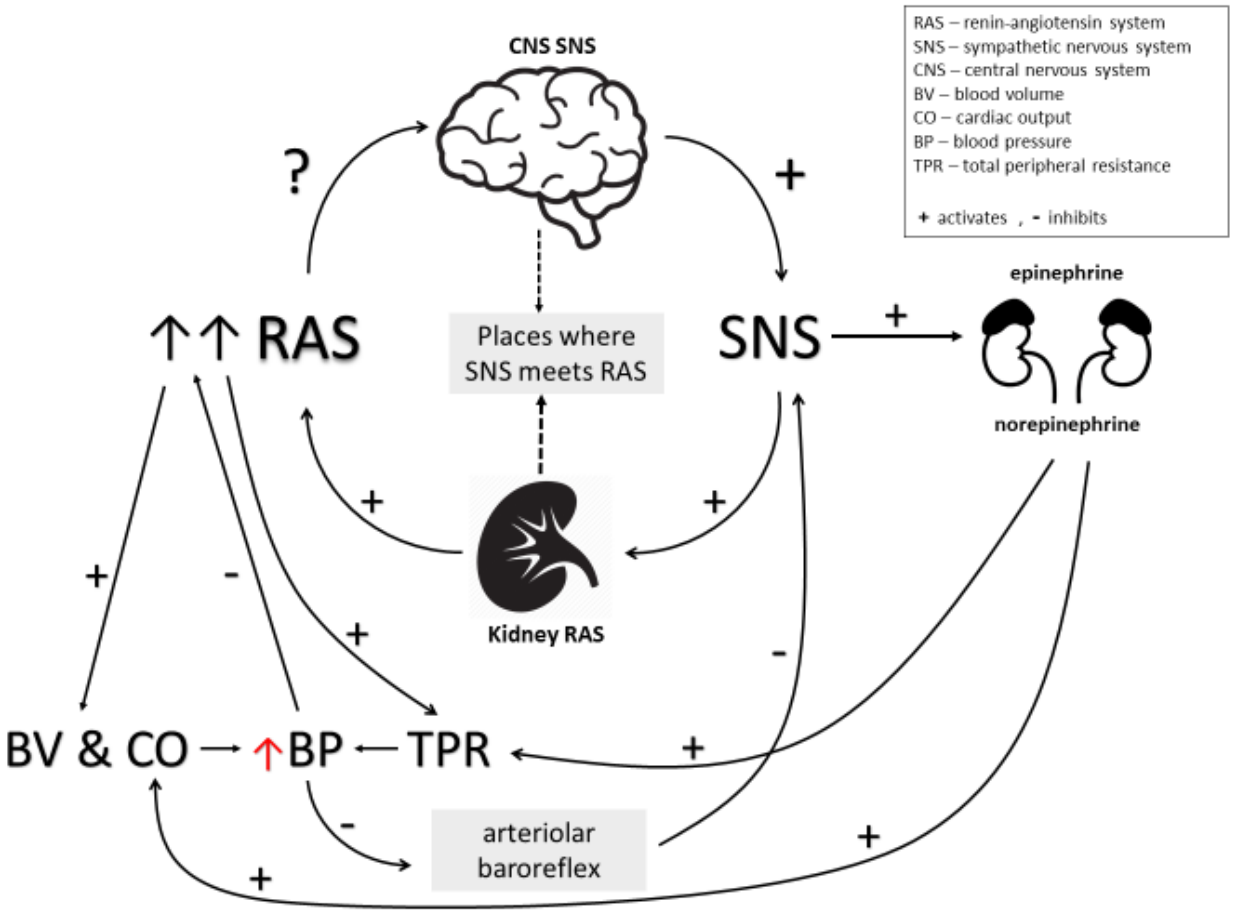
The role of the renin-angiotensin system in the cardiovascular physiological control system

The RAS is one of the most important hormonal systems which plays a key role in the arterial blood pressure, tissue perfusion, and extracellular volume homeostatic regulation, as well as in the regulation of neuronal and endocrine functions related to CV control (5, 6). RAS consists of a cascade of functional proteins and exhibits its effects through the effector molecule Ang II. In this cascade, the first is a release of an aspartyl protease called renin, which is synthesized and released from renal JGE cells located in the afferent and efferent arterioles of the renal glomerulus (1). Renin release is stimulated by various stimuli including decreased renal perfusion pressure (1), increased renal sympathetic nerves activity and decreased NaCl delivery to the macula densa of the juxtaglomerular apparatus (5, 6). Renin cleaves angiotensinogen, which is synthesized by hepatocytes, to form the inactive decapeptide angiotensin I (Ang I) (7, 8). Ang I is converted to the active octapeptide Ang II by angiotensin-converting enzyme (ACE) and non-ACE pathways (9, 10). Non-ACE pathways include Ang II production viae.g. chymase, which can be manifested in hypertensive patients treated with ACE inhibitors who have increased Ang II levels despite their therapy, a phenomenon called 'angiotensin escape' (11). To carry out its biological functions, Ang II binds to two specific and ubiquitous G-protein coupled receptors, Ang receptor type I (AT1) and type II (AT2) (12, 13).AT1 receptors mediate most of the established physiological effects of ANG II including actions on CV system (vasoconstriction, increased blood pressure, increased cardiac contractility, vascular and cardiac hypertrophy), kidneys (sodium reabsorption in renal tubule, inhibition of renin release), adrenal cortex (aldosterone synthesis) and SNS (1, 5, 6). AT2 receptors are generally assumed to counteract the vasoconstrictor and growth-stimulatory actions of AT1 receptors (14). There are several biologically active angiotensin metabolites, including angiotensin III, angiotensin IV and angiotensin-(1–7), which stimulate the AT2 receptors (but with low affinity), and/or newly discovered putative receptors (14, 15). The physiological relevance of these metabolites in various tissues is still under investigation. Aldosterone is another effector molecule of the RAS, whose synthesis and secretion is stimulated by the Ang II mediated activation of AT1 receptors in the adrenal zona glomerulosa cells. Aldosterone promotes sodium reabsorption, water retention, and potassium and magnesium loss and modulates blood pressure (16).

Circulating and local (tissue) RAS

Generally, RAS can be divided to the circulating and local (tissue) RAS (17). However, it is very hard to differentiate these two systems because of their extensive overlap (18). The circulating RAS implies renin produced in kidneys, which cleaves liver-derived angiotensinogen to generate Ang I that is converted by ACE into Ang II. On the other hand, a key feature of local RAS is the local synthesis of RAS components (e.g.

Figure 1. *Physiological feedback loop in renin-angiotensin system – sympathetic nervous system (RAS-SNS) interrelation.*



angiotensinogen, Ang peptides) independently of the circulating RAS. AT1 and AT2 receptors are invariably locally synthesized and are components of both circulating and local RAS systems (19). In recent years, it has been demonstrated that the local RAS includes many other potential components that may contribute to tissue-specific mechanisms of Ang peptide formation, and subsequently may participate in the pathophysiology of various diseases or contribute to the mechanisms that protect from tissue injury. These include the (pro)renin receptor(20), Ang generation from Ang-(1-12) that is independent of renin (21), intracellular (or intracrine) RAS that may contribute to CV disease (22, 23), and AT2 receptors and the ACE2/Ang-(1-7)/Mas receptor pathway that may mediate therapeutic benefit in CV disease (22, 23). Furthermore, several novel angiotensin peptides have been described, including angiotensin IV, angiotensin A, alamandine, and angioprotectin (22, 23), which are suggested to have the potential to contribute to disease or to protective mechanisms. The challenge of future research regarding the RAS is to investigate to what extent these two components of the RAS system are independent of each other, and what is the clinical relevance of the local RAS in CV disease development and progression.

Abnormal renin-angiotensin system activity in development of cardiovascular diseases

The RAS also has a central role in mediating and regulating vascular adaptive processes (24, 25) and is a major regulatory system involved in the regulation of a myriad of CV processes. Abnormal activity of the RAS leads to the development of different CV diseases and events (24), and results in vasoconstriction, muscular (vascular and cardiac) hypertrophy and fibrosis (25). Changed RAS activity is involved in secondary hypertension, including renin-secreting neoplasms, renovascular

hypertension, malignant hypertension, pheochromocytoma and primary hyperaldosteronism, and by primary hypertension, the plasma renin activity (PRA) depending on the particular case, can be high, normal or low (26). Furthermore, numerous signaling pathways, including cell proliferation, hypertrophy and apoptosis, in response to Ang II are mediated by reactive oxygen species, and oxidative stress is deeply associated with the progression of CV disease (27). The RAS, through its physiological effectors, plays a key role in promoting and maintaining inflammation, and has proinflammatory and profibrotic effects at cellular and molecular levels. Inflammation is an important mechanism in the development and progression of CV diseases such as hypertension and atherosclerosis. A dysfunctional endothelium is leaky and facilitates migration of inflammatory cells into the vascular wall and stimulates smooth muscle cells to proliferation (28). Ang II upregulates NF-κB and related inflammatory genes and activates endothelial and endocardial NADPH oxidase, which plays a central role in the generation of reactive oxygen species (ROS) in CV disorders (29). Interestingly, in almost all of the above-mentioned states, along with the RAS hyperactivity there is increased SNS activity as well (30), which sets the potential interaction of these two systems in the pathogenesis of CV and renal diseases in the focus of recent studies in this field.

The role of the sympathetic nervous system in the cardiovascular physiological control system

Activation of the SNS has long been recognized as a manifestation of various CV diseases including hypertension and the clinical syndrome of heart failure (30). Abnormal increase in the circulating plasma catecholamines level and increased muscle sympathetic nerve activity (MSNA) were some of the first documented evidences of increased SNS activity in CV disease patients (30). Still, even though increased SNS activity was generally (systemically) manifested in those patients, recent evidences implicate that SNS hyperactivity is not uniformly distributed through the body, but rather has regional distribution differences, with hyperactivity in some areas and modest or even absent activity in others. Thus, it seems that this generalized effect of SNS on the CV system was overestimated, and that a special focus of future studies should be set on its effect on individual organs and organ systems (31).

Sympathetic nervous system in central nervous system

The regulation of SNS activity in the CNS may have a crucial role in the pathogenesis of different CV diseases. The main sympathetic activity-regulating nuclei in the CNS are the paraventricular nucleus in hypothalamus (PVN), rostral ventrolateral medulla (RVLM) and nucleus tractus solitarius (NTS) (32, 33). NTS receives signals from the cardiopulmonary afferents, including baroreceptors and chemoreceptors, and has an indirect effect on of the neuronal activity of the RVLM (32, 33). In addition, when activated, NTS leads to the PNV activation as well, which is a major integrative nucleus that can influence SNS activity and extracellular fluid volume by producing antidiuretic hormone (ADH), which also has its repercussion on the activity of the CV system (34). PVN sends signals to RVLM which contains sympathetic premotor neurons for the CV system, and participates in SNS regulation via communication with the intermediolateral column of spinal cord (IML) (33, 35). These central SNS pathways are a focus of recent studies, since it has become more evident that the abnormal sympatho-excitation of the central SNS deteriorates renal and CV function in various diseases (e.g. chronic kidney disease, hypertension, heart failure) and contributes to disease progression (30).

Peripheral sympathetic nervous system

Peripheral effects of the SNS on the CV system, such as short-term—as much as long-term—control of blood pressure, are quite well known. The terminals of sympathetic nerves release norepinephrine, which binds to α-adrenergic receptors on vascular smooth muscle causing

vasoconstriction (36, 37). Many functional studies have confirmed that cutaneous blood flow is regulated by the SNS (38, 39). Sympathetic activity increases cardiac output by releasing epinephrine and norepinephrine (40) and also has effect on heart rate, which is mediated by the release of norepinephrine from postganglionic fibers that innervates the whole heart. This effect is mediated via β-adrenergic receptors on the heart muscle cells (40). Thus, a β-adrenergic blockade is an effective treatment for elevated blood pressure. The SNS regulates short-term transitions in blood pressure by the arterial barorefex (41), but also has a significant role in long-term blood pressure regulation. Studies have shown that the pharmacological blockade of sympathetic ganglions decreases blood pressure in both hypertensive and normotensive subjects (42). Chronic carotid baroreceptor stimulation evokes long-term reduction in the SNS and blood pressure in hypertensive patients (43).

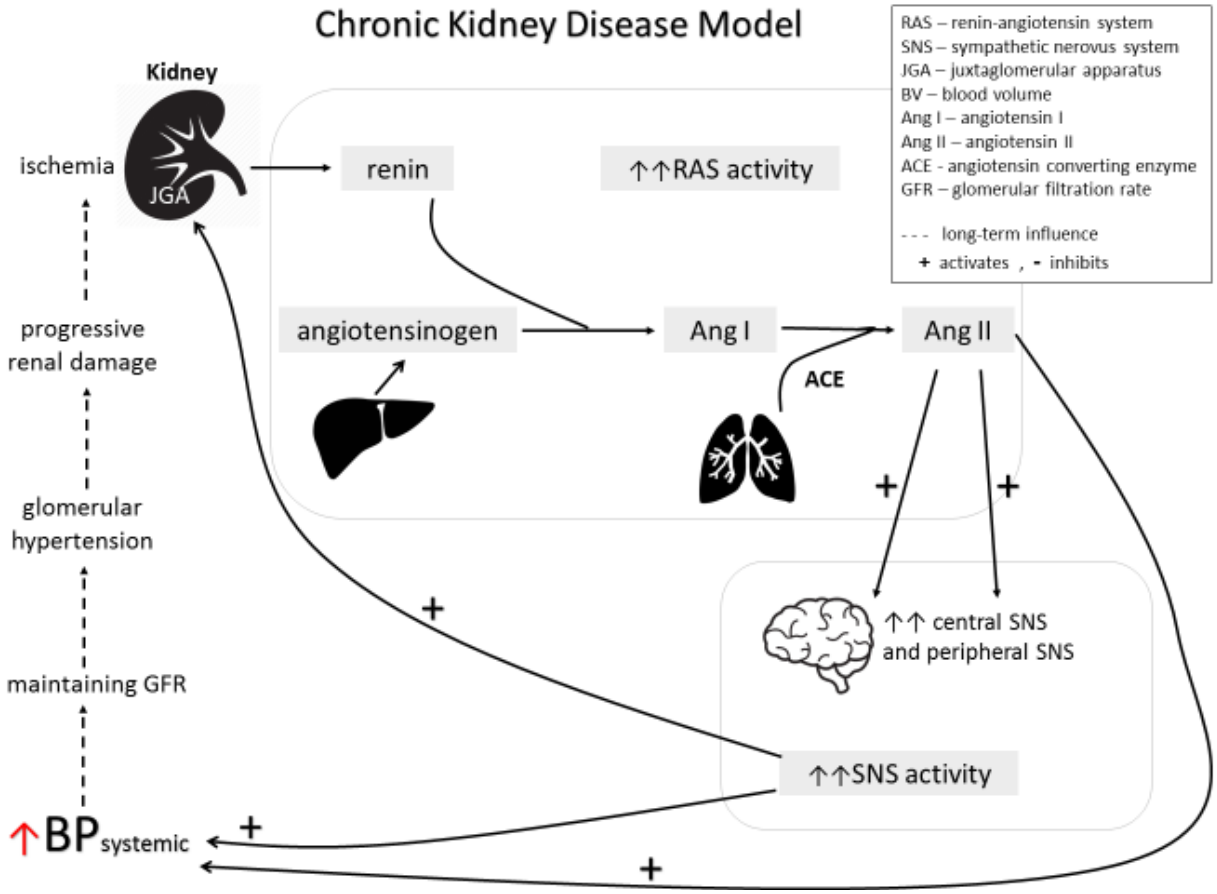
Another important part of the SNS is a muscle SNS. Muscle SNS positively correlated with total peripheral resistance, but negatively with cardiac output (44). Further, constriction of peripheral resistance vessels in the response to norepinephrine was lower in subjects with higher SNS activity, indicating that healthy people with higher muscle SNS had a second protective factor to prevent elevation in blood pressure (45). These findings may suggest that, in regulating the CV system to keep its function optimal, SNS activity affects not only well known and established pathways, but also many others not yet fully comprehensible.

The kidneys are important targets of sympathetic tone modulation in general, since the SNS is one of several factors that influences the efficiency of the renal regulation of blood pressure. Anatomical and physiological evidence has shown that the SNS innervates JGA cells, renal tubules and vasculature (46, 47).Thus, changes in renal SNS activity frequency mediate increases in urinary sodium and water excretion by regulating the renal tubular water and sodium reabsorption throughout the nephron, changes in renal blood flow and the glomerular filtration rate by regulating the constriction of renal vasculature, and changes in the activity of the RAS by regulating the renin release from JGA cells (46, 47). Increased renal SNS activity decreases renal blood flow and the glomerular filtration rate. JGA cells in the kidney have β-adrenergic receptors, which make the kidneys important targets of renal SNS activity and a place where it is generally accepted that the "SNS meets the RAS" (Figure 1). SNS activity in kidney increases renin release by activating JGA β-adrenergic receptor, along with an increase in tubular sodium reabsorption, and decreases in renal blood flow and the glomerular filtration rate (48). Furthermore, an additional effect on renal circulation is present via response to exogenous epinephrine and norepinephrine, with afferent arterioles showing higher sensitivity to the vasoconstrictive effect of circulating catecholamines than efferent arterioles (48). This reveals the importance and relevance of SNS activity on the kidney's compensatory mechanisms and management of volume expansion and high salt intake, which precedes pathological conditions such as hypertension.

The SNS also affects the immune system by contributing to leukocyte activation and extravasation, inflammation, oxidative stress and the production of chemokines and cytokines (49).

Thus, it is evident that understanding of the SNS modulatory effects on CV function implies its general (systemic), central and peripheral (individual organ or tissue) effects, and its interactions as well. While these individual effects of SNS are mainly well understood, there are many intertwined pathways between different SNS components in both health and disease, which make the effect of the SNS on the CV system more complex to understand and thus a focus of most recent studies on this issue.

Figure 2A. *Enhancement of renin-angiotensin system activity by sympathetic nervous system and vice versa in cardiovascular and renal diseases (chronic kidney disease)*



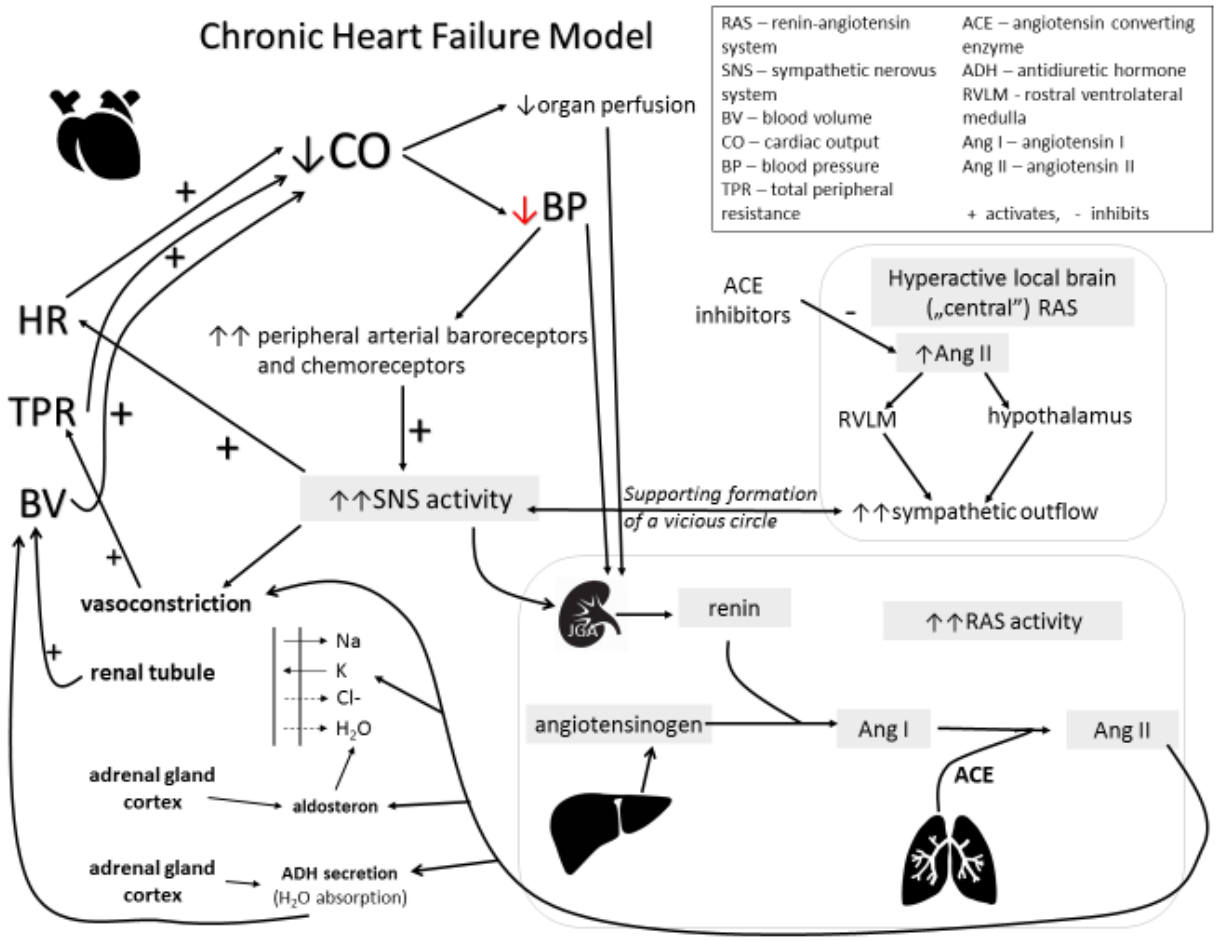
Interaction between the renin-angiotensin system and the sympathetic nervous system in the pathogenesis of cardiovascular and renal diseases

There are several CV and renal diseases characterized by both RAS and SNS activation in which these systems, beside their effect on blood pressure regulation, also contribute to the pathophysiology of both structural and functional CV abnormalities and contribute significantly to clinical outcome (4). It became evident that these systems do not operate independently, but interact at different levels throughout the CV system. Thus, there is a growing effort to clarify the pathophysiological mechanisms of interaction and more evident mutual potentiation of these two systems in different pathological states, including chronic kidney disease, essential hypertension, heart failure, obesity, metabolic syndrome, etc.

The renin-angiotensin system and sympathetic nervous system interaction in chronic kidney disease

Chronic kidney disease (CKD) is often characterized by enhanced activity of the RAS and SNS (4), which is summarized in Figure 2A. It is considered that kidney ischemia represents a central stimulation for renin secretion and RAS activation that subsequently increases SNS activity. Intravenous infusion of Ang II stimulates MSNA in humans, and even a small locus of injury in one kidney leads to hypertension associated with increased central sympathetic activity. Ang II can interact with the SNS on different sites, in the kidney, in the CNS and on peripheral sites, enhancing norepinephrine release from sympathetic nerve activity (50). When taken into account that increased SNS

Figure 2B. *Enhancement of renin-angiotensin system activity by sympathetic nervous system and vice versa in cardiovascular and renal diseases (chronic heart failure)*



activity enhances RAS activation by releasing renin from JGE, it is evident that there may be a reciprocal potentiation between these two systems in the development and progression of CKD. Thus, kidney injury, which is generally characterized by an increased RAS activity, can lead to high SNS activity, hypertension and finally end organ damage (50). Moreover, there is vast experimental evidence showing that increased SNS activity contributes at several levels to the development of CV organ damage (4, 51, 52). Thus, with these pathophysiological mechanisms in mind, it seems logical to hypothesize that RAS inhibition would reduce SNS activity in CKD. Moreover, recent studies reported that a SNS blockade in addition to RAS inhibitor treatment might be beneficial in a selected patients group (51-53).

The renin-angiotensin system and the sympathetic nervous system interaction in chronic heart failure

Chronic heart failure (CHF) is a multi-factorial disease that presents the end result of various insults to the myocardium (e.g. ischemic heart disease) (3). The most significant hallmark of CHF is the continuous interaction between the underlying myocardial dysfunction (e.g. decrease of cardiac output) and activated compensatory mechanisms in order to maintain blood pressure and organ perfusion. Activation of the RAS along with the SNS plays a crucial role in the pathophysiology of CHF, which is summarized in Figure 2B. Activation of the SNS is considered a major compensatory mechanism in the development of CHF, which may be due to the changes in peripheral baroreceptors and chemoreceptors reflexes, chemical mediators

that control sympathetic outflow and central integrative sites (33) (Figure 2B). In recent years Ang II, NO and/or pro-inflammatory cytokines were described as crucial mediators controlling sympathetic outflow. On the other hand, an increase in renin release in CHF state is caused by at least two pathways including renal sympatho-excitation as well as a decrease in renal perfusion pressure (3, 33). All of these compensatory mechanisms are initially beneficial; however, they became counterproductive if sustained for a prolonged time (Figure 2B).

Areas in RVLM and in hypothalamus mediate and increase sympathetic outflow in response to a microinjection of Ang II, which was inhibited after a central infusion of AT1 blocker losartan (54-56). Interestingly, this increment in central RAS activation could be associated with an increased oxidative stress level in CHF state (57, 58). The role of the central RAS in the supporting formation of a vicious circle in the development and progression of CHF is not limited to the CNS. Francis et al. reported that a central blockade of ACE decreased renal SNS activity, improved blunted baroreflex sensitivity, and normalized sodium consumption, urine sodium and urine volume in rats with CHF (59). Furthermore, in a myocardial infarction (induced by acute coronary artery ligation) rat model with transgenic deletion of angiotensinogen (rats which express an antisense RNA against angiotensinogen), deterioration of CHF was not as progressive as in control CHF rats (60). Taken together, it became evident that a hyperactive central RAS is a contributor to global physiological changes as well as the CV dysfunction seen in CHF. On the trail of these findings there is a growing trend to consider the use of both pharmacological and non-pharmacological therapy targeting the central RAS in the treatment of CHF (3). Clearly, the described cross talk between the central RAS and increased SNS activity is only one potential mechanism explaining the regulation of SNS activity in CHF (Figure 2B).

Changes in the renin-angiotensin system and sympathetic nervous system during high-salt loading

It is generally accepted that increased dietary salt intake is associated with an increase in arterial pressure, resulting in hypertension, which makes dietary salt a leading cause for CV, cerebrovascular, and renal morbidity and mortality (61). Various mechanisms were suggested to contribute to the development and progression of salt sensitive hypertension, including increased activation of the RAS and elevated SNS activity, well established hallmarks of arterial hypertension (62). However, in recent years it became more and more evident that HS dietary intake affects vascular and particularly endothelial functions even in the absence of changes in blood pressure (BP) (63-65).Thus, it should be taken into account that the concept of salt-sensitivity is not limited only to the effect of dietary salt modulation on BP, but to its effect on vascular function and the CV system in general as well (63). There is an overall consensus that HS intake, high levels of Ang II and increased sympathetic activity are all injurious to the CV system and play a role in a multitude of CV diseases (66). However, much less is known about the role of elevated salt intake on CV functions that are independent of arterial BP, especially in healthy humans. In clarifying the pathogenic sequence in which an HS diet is on one side and impaired CV function (e.g. endothelial dysfunction, hypertension) on the other, it is necessary to investigate the effect of salt loading on major regulating systems (e.g. RAS, SNS) in healthy individuals.

High salt feedback on the renin-angiotensin system

It has been demonstrated unequivocally in both humans and experimental animals that salt intake is inversely related to RAS activation: low salt intake stimulates RAS activity, and HS intake suppresses it (67). Dietary salt intake modulation affects RAS activity via at least four different pathways including: 1) the macula densa mechanism, which regulates renin release in response to changes in the renal tubular salt

concentration; 2) salt-dependent changes in arterial BP; 3) circulating salt-dependent hormones, particularly atrial natriuretic peptide (ANP); and 4) the SNS (67). Still, signal pathways that adjust renin synthesis and RAS activity to changes in salt intake are still not completely understood. Recent data suggest that macula densa mechanism is involved in adjustment of renin release in response to acute changes in salt loading (68, 69). Still, its effect on RAS activation during long-term changes in salt loading is less clear: it does not appear to have a function in this situation, but rather to modulate the general responsiveness of renin release (67, 70). Furthermore, numerous studies have reported that salt-dependent regulation of RAS can occur even in the absence of changes in arterial BP, especially in healthy individuals (64, 65, 71). Thus, BP is not a general controller of salt-dependent regulation of the RAS under normal conditions, but it can modulate renin synthesis when salt modulation provokes significant changes in BP levels. It has been shown that an increase in extracellular volume, induced by oral salt intake or intravenous saline infusion, is associated with elevated plasma levels of atrial natriuretic peptide (ANP), which consequently induce natriuresis and vasorelaxation, raise glomerular filtration and have capacity to directly suppress renin release from JGE cells (72). However, whether this mechanism is relevant in physiological condition in healthy humans or animals is still unclear. Studies have reported that modest acute salt loading in both healthy humans and animals did not elevate ANP levels and did not suppress plasma renin activity, indicating that RAS inhibition is not dependent on ANP (73, 74). Thus, ANP seems inessential for RAS suppression during acute salt loading, and its role in long-term dietary salt modulation should be addressed in further investigations. A number of studies investigated the correlation between salt intake and local renal sympathetic activity, indicating an inverse relation between the salt intake and renal nerve activity. Since renin-producing JGE cells have β1-adrenoreceptors whose activation results in renin release, one of the possible mechanisms mediating RAS inhibition by HS loading could be inhibition of local renal SNS activity (75, 76). Still, evidence indicating that renal nerves have a role as a mediator of the salt-dependent regulation of renin release and synthesis are insufficient.

This indisputable connection between salt intake and RAS activity indicates that the central role in mediating salt homeostasis within the body and its effect on CV, cerebrovascular or renal function belongs particularly to the RAS. In recent years, it became more evident that, besides its crucial role in body fluid volume, electrolyte balance and blood pressure regulation, the normal function of the RAS is critical for maintaining arteriolar structure, vascular reactivity and cardiovascular health in general (5). HS-induced increased oxidative stress (5) and impairment of vascular function (that is independent of BP changes) are related to low levels of Ang II and a normally functioning RAS has a protective effect in the maintenance of vascular function (77).

In contrast to numerous animal studies reporting that RAS inhibition provoked an increased oxidative stress level and endothelial dysfunction, the effect of RAS inhibition on the vascular function in a healthy normotensive human population was set in a focus of a very few recent studies. Most of these studies have found that HS intake (RAS inhibition) impairs the flow mediated dilation of the brachial artery in the absence of BP changes (65), which was likely associated with reduced vascular NO bioactivity (78-80). Furthermore, same deleterious effect of acute salt loading was observed in skin microcirculation as well (64, 65, 79). Cavka et al. reported that one week of a low-salt diet with oral losartan (a selective AT1 receptor inhibitor) administration led to a significant increase in plasma levels of the cyclooxygenase dependent vasoconstrictor thromboxane (TXA_2) without causing any changes in BP and/or skin microvascular blood flow responsiveness in young healthy women, suggesting that an AT1 receptor blockade may play an important role in the regulation of a cyclooxygenase-dependent pathway of metabolism of arachidonic acid (81).

High salt feedback onsympathetic nervous activity

Sodium retention is link between CV and renovascular diseases together with elevated SNS activity (3, 4). Interestingly, it still remains to be investigated how salt intake affects SNS activity in healthy individuals in the absence of BP changes (does it inhibit or potentiate SNS activity), and whether this effect is uniform for both local and systemic SNS responses (Figure 1). There is a paucity of studies investigating the effect of HS loading on systemic SNS activity in physiological conditions, both in animal and human models. So far, studies in animal models (rabbits) have reported that HS alone had no effect on baseline BP, water intake or SNS activity, but in combination with low-dose Ang II infusion HS provoked sympatho-excitation (82). HS intake induces the central sensitization of sympathetic circuits to result in exaggerated CV reflexes and an increase in BP variability in normotensive salt-resistant animals (83). Furthermore, a 6-day HS diet increased the mean systolic BP, decreased heart rate, and increased vagal activity in healthy, normotensive women (age 40–70) (84). These very few studies suggest that changes in autonomic nervous system balance should be taken into account during RAS modulation by salt intake, even in young healthy normotensive individuals.

Regarding potential mechanisms that mediate interaction between salt intake and SNS activity, it is well known that acute increase of plasma osmolality of sodium concentration in both plasma and cerebrospinal fluid can cause an increase of SNS activity (85, 86), which is presumably mediated by osmosensory neurons in the organum vasculosum laminae terminalis (OVLT) in the brain (86). The most recent study by Kinsman et al. has demonstrated that OVLT neurons are activated by modest rises in plasma or cerebrospinal NaCl, and their activation elevates BP and triggers a pattern of SNS activity that presumably facilitates renal sodium excretion in Sprague Dawley rats (87). Furthermore, many other agents, such as endogenous ouabain are involved in blood pressure (BP) elevation. Elevated sodium concentrations in body fluids can induce the secretion of the ouabain by both the hypothalamus and the adrenals, suggesting that ouabain could have an important role in linking together central and peripheral hypertension occurrences (88). Ouabain acts centrally in the brain to increase the sympathetic drive (89) and in the periphery causing vasoconstriction via myocytes and endothelium (90). Still, when taken into account that in most studies (both animal and human) in which moderate HS loading provoked vascular dysfunction independently of BP changes, there was no significant increase in sodium concentration in plasma. The above suggested effects of the described conditions are disputable and remain in need of clarification.

Another intriguing relation is that between the SNS and RAS during HS intake. An inverse relation of HS intake and renal SNS activity was observed (91, 92), showing that HS intake can suppress renal SNS activity. Renal SNS activity also affects RAS, whereas renal SNS activity stimulates renin release via the activation of β1 adrenoceptors placed in renin-producing JGE cells (75, 93), as already described. To examine this axis, studies using β1/β2 double knockout mice fed with a HS diet were performed (94). In this study β1/β2 double knockout mice were fed with a HS diet, and surprisingly they maintained the salt-dependent regulation of renin plasma concentration, since low salt intake stimulated renin release, and HS intake suppressed it (94). Similarly, dogs were fed with a HS diet and the changes in SNS activity effects were investigated via modulating baroreceptor activity (95). The suppression of central SNS activity by electrical stimulation of baroreceptors lowered BP without affecting the RAS and without impairing the normal salt-dependent secretion of renin. This may suggest that neither renal nor central SNS activity is crucial in the salt-dependent regulation of the RAS, but surely acts as a moderator in the RAS response to changes in salt load (67). Since most organs possess a local RAS that is regulated independently and is somewhat compartmentalized from the circulation, it has been suggested that the brain RAS may play an important role in CV regulation through its ability

to modulate SNS activity (96) (Figure 1). Studies in animal models have shown that increased levels of brain Ang II and the activation of brain AT1 receptors in the RVLM mediate the sympatho-excitatory actions (96). To date, the limited data indicate that elevated dietary salt intake enhances both sympatho-excitatory and sympatho-inhibitory responses evoked by a number of neurotransmitters exogenously applied to the RVLM (97-99), but these enhanced responses could not be attributed to changes in downstream sympathetic pathways or vascular reactivity (98-100). On the other hand, results obtained in studies on experimental animals have demonstrated that the brain Ang II activates posterior hypothalamic nuclei that increase efferent renal nerve activity and BP, suggesting the presence of renal/cerebral interaction in which the brain RAS can regulate the peripheral sympathetic activity and renal RAS (101).

Taken together, the relation between SNS activity and salt intake and its role in the development and progression of hypertension is yet unclear.

Conclusions

Many separated parts and mechanisms of the RAS and SNS affecting the CV system are discovered and explained, but the interplay of these separated mechanisms seems to have a pivotal role in explaining and understanding this highly interactive network. Studies conducted so far may suggest that some parts of the RAS and/or SNS effect on the CV system are overestimated, while others are underestimated. In a situation such as HS intake, both RAS and SNS activity may have a modulatory, compensatory role, and this role can increase as the CV and renovascular diseases progress.

metabolic syndrome and a series of cardiovascular problems (35), it is very important that the values were within healthy limits. Some researches show that, similar to BMI, values for PBF and WHR rise as people age (36).

The body composition (presented in Table 1) of the subjects shows that they have appropriate mass of fat and muscles, and their body composition is in accordance to weight status based on BMI value. In other words, the prevalence of obesity based on BMI distribution would probably be similar if they were distributed according to the BF% or SLM. Results reported by Grygiel-Gorniak et al. (37) show similar values of BMI, WHR and BF% with the results of the present study, as well as the similar differences between sexes. Our findings regarding differences in BMI and BF% between men and women showed that men had higher BMI, but lower BF% compared to women. This could be ascribed to the greater muscle mass in men. However, the correlation between BF% and BMI showed a statistically significant high positive value, indicating a strong connection between those two variables. Correlation was slightly lower in men than in women. There was also high correlation between BF% and WHR in both sexes. Collins et al., in their study of association of BMI and BF% among BMI-defined non-obese middle-aged individuals, found that the BMI category was not concordant with the %BF classification for 30% of the population. The greatest discordance between %BF and BMI was observed among %BF-defined overweight/obese women (38). A strong correlation of BMI and BF% in young women was reported in the study of Bakir et al. (39). They obtained correlation coefficients between BMI and BF% of 0.74 for women aged 18-30 years.

Proportions of weight categories were not significantly different over the years in which measurements were made. Students that choose to attend the College of Applied Sciences in Vukovar are similar in weight status throughout the years. This result is different compared to predictions of increase in obesity prevalence, and shows a steady state in the weight status of first-year students during the years examined, without any increase of obesity. It is possible, however, that it might have been too short a period for potential trends to reveal themselves.

Based on the presented results, the conclusion could be made that most of the freshman students at the College of Applied Sciences fall in the category of normal weight, with an

overweight prevalence of around 19-20%, including around 5% obese persons among them. There are also 6.5% of those who are underweight. There is a higher tendency toward the prevalence of overweight persons among men, while among women there is a higher tendency for underweight prevalence. The prevalence of obesity and the overall distribution across the weight categories, as well as the body composition of the first-year students have not changed during the period from 2008 to 2016.

Funding

This review article was supported by the Croatian Science Foundation under the project #IP-2016-06-8744 Interaction of Renin-Angiotensin and Adrenergic System in Oxidative Stress Induced Endothelial Activation (RAS-AdrenOX) and the Croatian Science Foundation grant #IP-09-2014-6380 Impaired Vasorelaxation and Endothelial Leukocyte Interaction (ELI) in Development of Atherosclerotic Lesions (V-ELI Athero).

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Pharmacogenomics: sex differences and application in pediatrics

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Abstract

Pharmacogenomics is a promising field which increasingly influences medicine and biomedical research in many areas. The aim of this article is to review recent advancements in the understanding of genetic polymorphisms and their influence on interindividual variability in drug response. Also, the main variabilities in drug response according to sex differences will be discussed. The translation of pharmacogenomics into the clinical routine as well as the challenges of achieving the goal of personalized medicine are also discussed. The role of pharmacogenetic tests in pediatrics has not been well defined yet, but it is clear that those tests could help in resolving some issues regarding the administration of drugs to children. At the conclusion, the foremost ethical, social and regulatory issues regarding the translation of pharmacogenomics into clinical practice and future perspectives in the field will be discussed.

(Bozic I, Omanovic T, Kuna L, Kizivat T, Smolic R, Vcev A, Smolic M. Pharmacogenomics: sex differences and application in pediatrics. SEEMEDJ 2017;1(1);108-120)

Introduction

Inter-patient variations in drug responses are a notable obstacle in everyday clinical practice. While a particular drug can be efficient and safe to administer in the majority of treated patients, in some individuals it might be ineffective and/or cause adverse drug reactions (ADRs), which may also sometimes be life-threatening (1). Both genetic and environmental factors influence the drug response of an individual patient (2). Since inter-individual variabilities in drug response are often genetically determined, the field of pharmacogenetics evolved for the purpose of assessing the influence of specific genetic biomarkers on the efficacy and safety of drugs. Pharmacogenomics, which emerged from pharmacogenetics due to the appearance of genome-wide association studies (GWAS), analyses the entire genome to find multigenetic factors related to an individual's drug response, representing another step towards personalized

Received: March 7, 2017; revised version accepted: April 26, 2017; published: May 9. 2017

KEYWORDS: pharmacogenomics, medicine, clinical, pediatrics

medicine (2-4). Furthermore, pharmacogenomics represents a promising area for the aims of maximizing the benefits of pharmacotherapeutic regimens and minimizing the risks of developing ADRs, which are significant causes of morbidity and mortality in patients worldwide (5-7). Most genetic biomarkers that have been found to affect the drug response in pharmacogenetic and pharmacogenomic studies are variations in the DNA sequences of genes encoding both enzymes and transporters included in the absorption, distribution, metabolism and excretion of drugs (8).

Pharmacogenetic and pharmacogenomic trials are usually designed by three different methodologies: candidate gene studies, genome-wide association studies, as well as whole-exome and whole-genome sequencing (9). In candidate gene studies, a hypothesis-driven approach is used, where a single targeted gene, encoding a protein included in the metabolism of the drug, is investigated (9, 10). However, in GWAS, two groups of patients with a different drug response profile are compared, and potential associations with many known genetic variants are investigated (9). The third and most comprehensive methodology is whole-exome and whole-genome sequencing, where all human genetic material is analyzed for variants related to drug efficacy and safety. However, the analysis of data collected in such studies remains difficult (9).

Genetic polymorphisms of drug-metabolizing enzymes

So far, data obtained from previous pharmacogenomic studies has yielded extensive reports about the genetic influence on treatment outcomes and side effect appearance (11).

Polymorphisms in genes encoding drug-metabolizing enzymes (DMEs), or transporters in phases I and II of the drug metabolism, often influence the drug response and determine the risk for the development of ADRs (12).

Single nucleotide polymorphisms (SNPs), minor insertions or deletions, as well as the amplification and deletion of gene copies, are genetic polymorphisms of DMEs (12).

More than 90% of human genes contain at least one SNP. Consequently, so far over 14 million SNPs have been discovered in the human genome (13). Factors that cause variations in drug response are complex and involve fundamental aspects of human biology (1).

However, several variants of DMEs have been discovered since the completion of the Human Genome project (1). As a reward for this laborious work, genetic polymorphism studies show clinically significant applications (1).

Altogether 57 cytochrome (CYP) genes encode CYP enzymes, which are subdivided into families, mainly involved in the metabolism of exogenous substances (14). Since CYP450 takes part in the metabolism of 65-70% of clinically used drugs, those enzymes play an especially significant role in the field of pharmacogenomics. In fact, CYP450 enzymes are responsible for 75-80% of all reactions of the phase I metabolism (15,16).

For instance, 20-25% of all marketed drugs are metabolized by CYP2D6, which is why its polymorphisms are very frequently investigated in pharmacogenomic trials (17,18). For example, it is the rate-limiting enzyme in the catalyzing process of tamoxifen's conversion intoactive metabolites, 4-hydroxytamoxifen and endoxifen. Therefore, CYP2D6 has a significant impact on the individual's response to tamoxifen therapy; as well it does for many other drugs (19).

A further example of the numerous CYP450 enzymes under pharmacogenomic investigation is CYP2C19, which catalyzes reactions in the metabolism of clinically important drugs, such as tolbutamide, glipizide, phenytoin, warfarin and flurbiprofen. More than 20 polymorphisms of genes encoding this enzyme have been studied, whereas the two most common variant alleles, CYP2C19*2 and CYP2C19*3, are null alleles, exhibiting reduced enzymatic activities (1, 18).

In addition, GWAS data confirmed previous discoveries, indicating the importance of DME variants in drug response (for instance, clopidogrel and CYP2C19), thus offering evidence for different levels of correspondence (12).

Also, more than 50% of the drugs used in clinical practice are metabolized by CYP3A4 in the human liver. So far, more than 20 variants of CYP3A4 are known. The frequency of individual CYP3A4 variants varies greatly across different ethnic groups and many of those variants exhibit altered enzyme activities, which means that polymorphisms of the CYP3A4 gene could be responsible for the difference in drug response between ethnic groups (1, 18, 20).

Recent studies have also emphasized the critical role of CYP2C19 polymorphisms for the therapeutic effects of clopidogrel. Furthermore, the importance of CYP2D6 polymorphisms for tamoxifen treatment appears to be relevant (12).

Importantly, non-CYP450 DMEs also play important roles in the metabolism of various drugs. Therefore, polymorphisms of genes encoding those DMEs also influence the therapeutic effect and could lead to potential adverse reactions after drug intake (1).

Pharmacokinetics and pharmacodynamics: sex difference

Even though the mechanisms are still not well understood, it seems that sex also plays a crucial role in the pharmacological response (21)(22). Generally, it is known that pharmacokinetic differences between men and women are more extensive compared with variations in pharmacodynamics (21). Some drugs frequently prescribed for the treatment of cardiovascular diseases, such as verapamil and amlodipine, show sexual dimorphism in their pharmacokinetic profile (14). Bioavailability of amlodipine slightly differed among sexes, with women showing higher bioavailability. However, after the adjustment of the obtained data to body weight, no significant SGD in the bioavailability of amlodipine remained (23). Some recent findings suggest that sex differences appear also in the field of pharmacogenomics (21).

Studies about sex-based differences in the response to pharmacotherapy show that women experience ADRs in a more frequent manner, and that those side effects may also be more severe compared with the ADRs in men (thiazolidinedione-induced bone fractures, iatrogenic long QT syndrome and iatrogenic systemic lupus erythematosus, etc.) (23). For instance, inhibitors of angiotensin converting enzymes (ACEIs) and antagonists of angiotensin receptors 1 (ARBs) are important compounds of therapeutic regimens in the treatment of cardiovascular diseases (14). Investigations have reported that during treatment with ACEIs, cough and angioedema are more frequent in women than in men. Furthermore, it has been shown that the XPNPEP2 C-2399A genotype is associated with an increased frequency of ACEI-associated angioedema in black men, but not in white men and women (21, 22). Because of this circumstantial evidence for the unfavorable safety profile in women, further studies should be conducted to clarify sex-gender related differences (23). Importantly, sex differences in drug response could have various reasons, and they are not all caused by the varieties of the DNA sequence of pharmacogenetically-relevant genes (24).

Due to the fact that not many genes that are known to be relevant for pharmagenomic studies are located on sex chromosomes, sex-gender differences in drug response are possibly caused by differences in autosomes, transcriptional gene regulation due to sex-specific epigenetic modifications, posttranscriptional modifications or by the effect of sex hormones (24, 25). Although animal studies suggest that sex-specific expression of CYP isoforms is a common case in rodents, this mechanism is subtler in humans (20). However, sex dimorphic metabolism does occur also in humans, for instance in the case of CYP3A4, as a very important isoform of CYP, which is expressed more extensively in females than in males (20).

Furthermore, evidence for sex-gender dependent gene expression exists, and this sexually dimorphic expression is seen in liver, muscle, fat and brain tissue (26, 27). Also, frequencies of specific allelic polymorphisms are unequally distributed among men and women, which also contributes to differences in the drug response and safety profile (24). Furthermore, psychological, physiological and lifestyle factors influence sex-gender differences, in particular in the case of therapy outcomes for drugs targeting the central nervous system (28,29). It is still unknown whether pathological conditions influence pharmacokinetic and pharmacodynamic parameters in a sex-gender dependent way (28, 29).

Until now, sex differences have been undervalued, and the study design in clinical trials during the drug approval process did not have a proper sex-based approach, all of which has led to a deficient understanding of drug response and side effect disposition among women, a deficiency that is a missing link in the path towards personalized medicine (21, 25). It is of great importance to carry out further studies with a more sex-based approach, so that it would become possible in the future to better adjust pharmacotherapeutic regimens and individual drug doses according to the sex of the patient (21).

Translating pharmacogenomic knowledge into clinical practice

The main long-term goal of pharmacogenomics is to translate observations regarding the genetic basis of drug responses into a more effective and less toxic treatment for individual patients in the everyday clinical practice (30). Until now, due to detailed pharmacogenomic studies for some drugs, the clinical application of this branch of personalized medicine has already become possible (9). Examples of successful clinical application of pharmacogenomics are listed in Table 1 (9).

Pharmacogenetic testing, in order to determine the suitability of a drug for the individual patient, is gradually moving from specialty medications, for instance drugs prescribed for patients with cancer, to more broadly prescribed medications, as are statins, codeine or warfarin (31).

Heretofore, the drug labels of 137 medications include pharmacogenetic-related information (32). It has been estimated that 16 percent of medications prescribed in primary care are pharmacogenetically impacted (33). The delivery modes of pharmacogenomic testing are yet unclear; therefore, the main goal is to write clinical practice guidelines with decision-making algorithms informed by controlled-clinical pharmacogenomics trials (34-37). These guidelines may increase precision, accuracy and the relevance of recommendations and subsequent applicability (38). Although pharmacogenomics offers significant potential to improve the clinical outcome of individual patients, the translation of pharmacogenomic knowledge and principles into clinical practice has been slow in most settings (30). One of the reasons could be the fact that in many cases, the variability of drug response involves many different factors other than pharmacogenomics (1). In fact, the understanding of the pathophysiological processes of the disease, the identification of important genes, as well as the recognition of the roles the genetic polymorphisms of receptors, transporters or DMEs play in the pharmacological outcomes, are all required for the complex process of achieving individualized medicine (1). For the reason that many factors that are not reflected in genomic information influence drug toxicity and efficacy, it is questionable whether personalized drug treatment will ever become attainable by pharmacogenetic testing alone (1). In addition to scientific difficulties, economic, ethical, social and regulatory issues are also very challenging (1).

Pharmacogenomic applications in pediatrics

During the past decade, much effort has been devoted to improve the safety and efficacy of medical products used for the treatment of the pediatric age spectrum, from premature newborns to adolescents, with a special accent

Table 1. *Examples of clinically relevant pharmacogenomic testing.*

Drug	Genetic variants	Influence	Notes
Warfarin *anticoagulant drug*	CYP2C9 gene and VKORC1 (78)	Genetic polymorphisms impact dose requirements of warfarin therapy (9, 79)	Only 59% of US patients have INR 2-3 (because of the narrow therapeutic range of warfarin many patients still receive an incorrect dose) (80)
Simvastatin lipid-altering agent used to treat hypercholesterolemia	SL-CO1B1*5 (81)	Increased risk of statin-induced myopathy patients suffering from cardiovascular disease (82)	Simvastatin is a widely used drug: SL-CO1B1 testing could reduce the incidence of statin-induced myopathies or rhabdomyolysis(83)
Codeine opioid analgesic drug	CYP2D6 (84)	Genetic polymorphisms affects metabolizing phenotype of patients (84)	Metabolizing phenotypes: ultra rapid (high risk for ADRs) and extensive metabolizers to intermediate and poor metabolizers (inadequate analgesia) (84)
Geftinib EGFR tyrosine kinase inhibitor (TKI)	EGFR (85)	EGFR mutation is a biomarker of geftinib efficacy (86, 87)	BRCA1 as well as compounds of the NF-κB pathway also affect the response of EGFR mutated patients to geftinib (88)
Irinotecan chemotherapeutic drug used for colorectal cancer	UGT1A1*28 (89)	UGT1A1*28 polymorphism leads to lower protein expression and irinotecan-induced toxicity (hematological and digestive ADRs) (89, 90)	Irinotecan is a prodrug of SN-38 that is conjugated via UGT1A1 (90)

on the progress in dosing strategies. Since the medicine registration protocols of the European Union, before the legislation called Paediatric Regulation in 2006, did not obligate pharmaceutical companies to assure in advance a Pediatric Investigation Plan (PIP), as reported by the European Commission, between 50% and 90% of the drugs used in pediatric medical care were administered off-label, without being adequately tested nor authorized with a tolerable risk benefit profile in children (39-41). There are two main issues regarding the administration of drugs to children under these circumstances. On the one hand, doses for children are usually obtained empirically in the absence of evidence by adapting adult doses to body weight in a trial-and-error pattern (42). Since absorption, disposition, metabolism and drug elimination are subject to developmental processes due to the ontogeny of DMEs, pharmacokinetic responses are not equivalent to those during adulthood (40, 43-45). Thus, dosing protocols for children should be independently obtained in pediatric studies.

On the other hand, along with changes in enzymatic activity due to the changes of gene expression during the development process, which is specific for the pediatric population, the influence of SNPs of genes for some receptors, enzymes and transporters involved in drug response and metabolism, also plays a fundamental role in the accurate prediction of treatment response in children (44, 46).

Consequently, additional pharmacogenomic trials should be performed in children if relevant biomarkers are available, since only about 70% of pharmacogenomic data obtained from studies on adult populations can be applied to children (45, 47-50). The role of pharmacogenomic tests in pediatrics has not been well defined yet and there is a lack of genotype-guided dosing strategies for children (51-53). Compared to adults, children have an increased level of complexness due to physiological maturation processes, as well as the ontogeny of gene expression, which contributes to the influence of specific genetic variants investigated in pharmacogenomic trials (54). As a result, the approach to results from pharmacogenomic trials on children should differ substantially from those yielded in studies on adult patients (54). Even though the DNA sequence persists throughout life, the pattern of gene expression is dynamic, and changes in protein synthesis occur. Indeed, some of the most important enzymes involved in drug metabolism, like CYP450 and UDP glucuronyl transferase, display gene expression depending on developmental changes (55). For instance, CYP3A7 gene expression is already observable in the fetal liver during the first trimester of pregnancy, but the level of CYP3A7 enzyme production decreases after the immediate postnatal period. As the CYP3A7 level of gene expression decreases, CYP3A4 and CYP3A5 expression boost after the end of the first postnatal week, until they reach 30% of the adult enzyme activity levels by the end of neonatal period (55, 56). Although the overall level of CYP3A protein expression remains constant, differences in specificities to substrates, as well as metabolic and catalytic capacities exist, depending on whether the prevailingly expressed genetic variant is CYP3A7 or CYP3A4 (55). Thus, drug metabolism via the CYP3A subfamily varies due to developmentally regulated gene expression and, depending on the moment of drug administration during the neonatal period, genetic variants in different members of the CYP3A subfamily are important. For instance, this might play an important role in altering the pharmacokinetics of tacrolimus, a calcineurin inhibitor and immunosuppressant drug used in children and adults after solid organ transplantation (57, 58). Since tacrolimus has a very narrow therapeutic index, it is still challenging to adjust the dosing regimen in children so that further pharmacogenomic trials and its implementation into clinical practice could potentially be crucial in this process (43, 59). Due to the breakthrough of pharmacogenomics in the field of personalized medicine, a number of pharmacogenomic studies are being conducted, mainly with adults as subjects in those trials. However, even though pharmacogenomic research on children lags behind, results published so far in this field accentuate the difference between children and adults in the framework of pharmacogenomics (54, 60). Therefore, it would be vague to directly extrapolate data from adult pharmacogenetic trials without putting them into the variable pharmacokinetic context of a developing child (54, 61). Indeed, there are some well-known clinical utilizations of pharmacogenetic tests in adult medical care, but there is still a lack of knowledge to translate those results to the pediatric patient spectrum (60). The most significant achievements in this study field have been made in pediatric hematology and oncology, but some trials were also conducted in the areas of rheumatology, endocrinology, neurology, gastroenterology, pulmology and organ transplantation in children (46, 54). For example, one of the most frequent chronic diseases in children is juvenile idiopathic arthritis (JIA), which can lead to persistent disabilities in adulthood (65). Methotrexate (MTX) is a disease-modifying antirheumatic drug (DMARD) important in the treatment of JIA that unfortunately exhibits heterogeneity in the clinical response (64). Pharmacogenomic studies suggest that polymorphisms in many genes encoding products included in the disposition and biochemical pathways of MTX, for instance, CACNA1I, ZMIZ1, TGIF1 or CFTR, can affect the clinical outcome and therefore have the potential to make pharmacogenetic testing of individual patients an integral diagnostic component before the application of MTX therapy (62-64).

Moreover, since pharmacogenomics seems to be one of the emerging tools used to improve drug safety by the avoidance of giving specific drugs to susceptible individuals who are likely to develop adverse drug reactions (ADRs), it is important to consider that some ADRs are specific to, or more frequent in, children. In fact, it is in some cases unreasonable to assume the genetic influence on the occurrence of some ADRs through adult pharmacogenomics study results, if these ADRs are unique to the child population (54).

Ethical issues concerning pharmacogenomics

Apart from the recognized benefits of pharmacogenomics in the future perspective of patient medical care, considerable ethical and legal questions, which could eventually overwhelm the unprepared legal framework, are arising (66). First of all, pharmacogenetic testing should not be administered without a signed informed consent, containing appropriate information about the benefits and risks of the procedure (67). Due to a child's inability to fully understand, or understand at all, the purpose and possible aftereffects of pharmacogenetic testing, parents or legal guardians must sign the consents. The ethical issue addressed here is that a person who signs the informed consent is not the person who receives the genetic testing, which could have unforeseeable consequences (68). As well as in other genetic tests, the DNA sample primarily used for one purpose, in this case, obtaining data about the individual efficiency and safety of a patient's treatment could unintentionally yield additional secondary information. Secondary information could be information about a predisposition to several diseases, a prognosis of current illness, or pharmacogenetic information about drugs used for other conditions, which could lead to both a patient's psychological and economical discomfort. For example, if the patient with a disease would be classified as a nonresponder to drugs currently used for the treatment of his condition, this could have an impact on the patient's insurance payments or even lead to discrimination in their search for employment because of the additional health care costs for employers (68, 69). Consequently, this example also raises the question of confidentiality and who should have access to data obtained by these tests. Also, if the patient, for instance, undergoes testing for the apolipoprotein E genotype, which is the most frequently investigated pharmacogenetic biomarker for statins, he might also receive unwanted information about his risk of developing Alzheimer's disease in his elderly years (70, 71). Also, due to the possible high costs of pharmacogenetic testing, it is likely that the economic status, whether of an individual patient or of a whole country, will influence and limit the accessibility of the method. Correspondingly, this could enhance the unethical socioeconomic divisions and inequalities in the health care system (72, 73).The pathway towards personalized medicine entails that the drug development by pharmaceutical companies should be genetically guided. Due to the further costs involved in such drug design, pharmaceutical companies might first want to evaluate the frequencies of alleles of interest. In the case of limited profits from the restricted drug market due to rare alleles and genotypes of nonresponders to available drugs, pharmaceutical companies might not be intrinsically stimulated to develop new drugs for those individuals. Just like in the case of orphan drugs for rare diseases, drug development for rare, orphan genotypes should be economically stimulated (72, 74, 75). In addition to the problem of orphan genotypes, it could be the case that developing drugs even for very frequent genotypes would not be of interest to major companies if the genotypes were geographically located in socioeconomically poorer areas (72).

Despite the ethical and legal questions, it seems that pharmacogenetic testing is one of the most promising steps towards personalized medicine. However, effort should be put into establishing legal parameters that can cope with the emerging needs of the evolving field of pharmacogenomics.

Future perspectives

Further trials with even more participants are likely to yield results in the near future that could extend the number of clinical implementations and make another step towards personalized medicine (76). Since the field of pediatric pharmacogenetics still falls behind the research on adults, advances in the research field are still expected so that the complex compound of genetic influence and ontogenic dynamics in children could be understood. A better and more profound explanation of those processes would certainly facilitate the clinical implementation of the future collected knowledge in the field of children's pharmacogenomics (51).

Furthermore, it is of great importance to educate clinicians about data interpretation of pharmacogenetic test results so that they can gain the required knowledge to accurately stratify patients into high-risk or low-risk groups regarding drug toxicity and consequently improve the therapeutic outcome without putting susceptible patients at risk of predictable life threatening ADRs. Therefore, new user-friendly and up-to-date guidelines should be provided to clinicians in order to help the future implementation of pharmacogenomic study results into the clinical daily routine (43, 46, 77).

It is likely that the further use of next-generation sequencing will lead to new advances in pharmacogenetics (43). Also, although still expensive, high-throughput screening methods could become more affordable in the future and help progress in the scientific field (77). Hence, these and further technological improvements could upgrade the current knowledge in pharmacogenomics to an advanced level, which could lead to more clinical aims, consequently increasing the safety of drugs used for the treatment of many diseases. However, since there are also emerging ethical concerns, an adequate legal framework should be established.

Conclusion

Pharmacogenomics is a rapidly emerging and promising scientific field in which an increasing amount of studies are being conducted. Although there are still challenges, it is promising that they could vanish with the improvement of study designs and the formation of international cooperation that would validate pharmacogenomic study results and promote the clinical use of pharmacogenetic tests (76). Sex-related differences have been reported in pharmacogenomics trials. Since more severe and more frequent drug adverse reactions have been found in women, whose pharmacological status is less studied, emphasis should be put on pharmacogenomic investigation in women (34). There are still no satisfactory data present regarding pediatric pharmacogenomic studies (54). However, legislations, both in the EU and USA, accentuate the need for clinical trials on pediatric patients so that an admissible level of safety in drug administration could be reached (40). The process of achieving individualized medicine for many diseases is complex, especially considering that many nongenetic factors influence drug toxicity and drug efficacy. In addition to scientific difficulties, economic, ethical, social and regulatory issues are also very challenging (1).

Acknowledgements

The support of the J. J. Strossmayer University of Osijek research grants (to MS and to AV) are gratefully acknowledged.

Disclosures

Competing interests: None to declare.

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Intrinsic Control and Environmental Factors in Food Consumption Related to Obesity

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Abstract

Obesity results from a complex interaction of genetic, hormonal, physiological, anatomical, psychological, behavioral and environmental factors causing an imbalance between energy intake and energy expenditure. According to the World Health Organization, the estimated number of obese people around the world has doubled from 1980, meaning that more than 600 million people worldwide are obese.

Obesity is associated with low-level chronic inflammation and represents a major risk factor for cardiovascular and metabolic diseases, but also some cancers. Centers that regulate food intake and energy balance are placed in the hypothalamus. Chemical signals are transmitted between hypothalamic neurons, and those neurons also affect the secretion of different hormones that are important for maintaining energy balance and metabolism. Moreover, genetic predisposition is also a risk factor for obesity development. Key neuronal populations for maintaining energy balance are the orexigenic agouti related peptide (AgRP)/neuropeptide Y (NPY) neurons and the anorexigenic proopiomelanocortin (POMC) neurons. This review attempts to present the prevalence and the major pathways regulating energy balance that may be affected by many environmental and social factors, such as emotions and human behavior, and can lead to obesity.

(Jukić I, Kibel A, Kibel D, Drenjančević, I. Intrinsic Control and Environmental Factors in Food Consumption Related to Obesity. SEEMEDJ 2017;1(1);121-129)

Epidemiology of obesity

Increased accumulation of fat tissue leads to obesity. The most common ways to evaluate obesity are by the calculation of the body mass index (BMI), the measurement of waist circumference (WC) and the calculation of the ratio of the circumference of waist and hip (WHR). A BMI of 20-25 kg/m2 is considered

Received: April 21, 2017; revised version accepted: May 10, 2017; published: May 25. 2017

KEYWORDS: obesity, POMC, melanocortins, chronic stress, social behavior

normal; 25-30 kg/m2 is overweight; 30-40 kg/m2 is defined as obesity and >40 kg/m2 is defined as extreme obesity. For children and adolescents, a BMI >95 percentile is considered overweight/obesity (1). A waist circumference (WC) of at least 88 cm for women and 102 cm for men is defined as obesity (2). A WHR above 1.0 in male subjects and above 0.6 in women is considered as an obesity pattern (3). The prevalence of obesity in the general population is increasing worldwide. Based on the Nutrition Examination Survey (NHANES) 1999–2000 in the USA, the prevalence of overweight in adults was 64.5% and the prevalence of obesity was 30.5% (4). The most comprehensive overview of the prevalence of obesity is provided by the World Health Organization (WHO) Global Status Report on noncommunicable diseases 2014 (5), which showed that the age-standardized prevalence of obesity in adults aged 18 years and over (cut-off point set to BMI ≥30 kg/m2) in the USA was 33.7%. Canada had a prevalence of obesity of 28%, Mexico 28.1%, Argentina 26.3% and Brazil 20%. The same publication reported that in Europe, the prevalence of obesity among the general population is as follows: UK 28.1%, Slovenia 25.1%, Spain 23.7%, Croatia 23.3%, Italy 21%, Germany 20.1% Turkey 29.5, France 23.9% and Bosnia and Herzegovina 17.9%. Interestingly, in Asia the prevalence of obesity was significantly lower: the prevalence of obesity was in China 6.9%, India 4.9% and Japan 3.3%. Australia has a prevalence of obesity in the general population of 28.6%. In Africa, there is a wide range of obesity prevalence, from 28.9% in Egypt to 10.5% in Zimbabwe, 11% in Congo and 11.4% in Cameroon (5). Based on WHO data, in 2014 more than 1.9 billion adults aged 18 years and older (39% [38% of men and 40% of women]) were overweight. Of these over 600 million adults were obese—about 13% of the world's adult population (11% of men and 15% of women). The worldwide prevalence of obesity more than doubled between 1980 and 2014 (5).

The prevalence of obesity is linked to the prevalence of various cardiometabolic diseases, such as diabetes mellitus. Recently, in 44 observational or clinical studies that were evaluated in a systematic literature review by Colosia et al, the obesity prevalence in T2DM subjects was determined based on BMI or WC (2). Based on BMI, the prevalence of obesity in that population group was 30-85.5% in Asia, 32.7-64.1% in Europe, 26.8-64.3% in North America, 32.5-59% in South America and 32-42.5% in Africa. The prevalence of obesity in T2DM patients based on waist circumference was 56-67% in Europe, 54.6% in Africa and 20-81% in Asia. However, there were no studies taking into account obesity based on WC in that publication, and not all countries were covered by the examined studies (2). Due to its effects on hormonal balance, autonomic system function and various organ functions, obesity represents a significant risk factor for many other chronic diseases such as hypertension, atherosclerosis, diabetes mellitus and chronic kidney disease..

The pathophysiological mechanism of obesity

The development of obesity is a consequence of several pathophysiological factors. First of all, it is an imbalance of food intake and energy expenditure. The central control of energy balance by the hypothalamus and the feedback loop of peripheral metabolic factors from the gastrointestinal and endocrine system, including adipose tissue, are very important factors, particularly in obesity related to chronic stress and other cardiometabolic diseases. Genetic predisposition accounts for approximately 30% of the risk for obesity development (6). The most recognized mutations are those of receptors or hormones related to the regulation of feeding behavior, and subsequently energy balance: e.g. mutations of the leptin gene and the leptin receptor gene, the mutation of the proopiomelanocortin gene (POMC) and mutations of genes for melanocortin receptors (e.g. MCR-4) (7). These conditions lead to pathological obesity with other metabolic and hormonal imbalances.

Central control of dietary intake (energy balance)

An individual's energy intake and expenditure must be balanced over time to ensure adequate body composition and function (8). The quantity

of food intake is regulated on a short-term basis, which controls the intake of food at each meal, and long-term, which is mainly concerned with the maintenance of normal quantities of body energy stores (9). The main homeostatic system involved in the regulation of energy balance is located in the hypothalamus, and the nucleus arcuatus has a central role in these processes. It functions by integrating neural and nutrient signals with hormonal signals that arise in the small intestine, liver, pancreas, adipose tissue and brainstem (10). The total daily energy intake is a function of the size, frequency, and qualitative composition of meals, and the perception of hunger and the decision to initiate a meal are determined by complex interactions between genetic, social, learned, environmental, circadian, and humoral factors (11).

Centers for the control of hunger and satiety are located in the hypothalamus, Stimulation of the lateral nuclei causes hyperphagia (their destruction has the opposite effect), while stimulation of the ventromedial nuclei causes satiety and aphagia (8). Other areas add to the complexity: Paraventricular nuclei destruction leads to excessive eating, dorsomedial nuclei destruction leads to depressed eating, and arcuate nuclei are centers where several hormonal signals from the adipose tissue and gastrointestinal tract are integrated to regulate food intake (9). In addition to these main structures of the homeostatic system, an important role is played by the brain's reward system — located in proximity to the hypothalamus with its main nodes centered on the ventral striatum. It includes the nucleus accumbens, the ventral pallidum, and the ventral tegmental area (10).

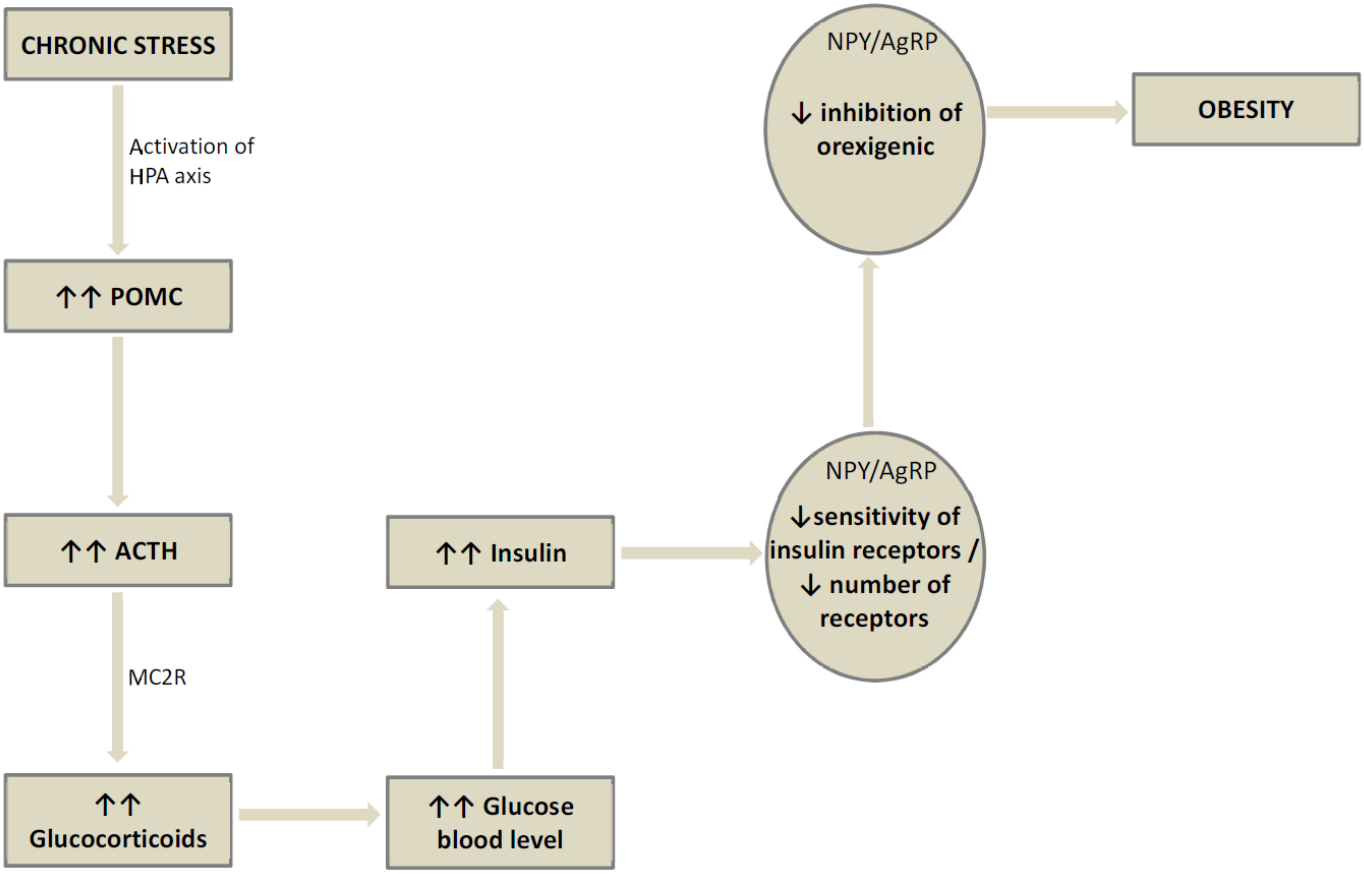
Evolutionary development led to the emergence of protective central mechanisms that are focused on the resistance of fat loss and on the maintenance of body weight, crucial features that helped enable the survival of the human species throughout time (12). Unfortunately, such a system predisposes humans to obesity in times of abundance, with all the known adverse effects. Obesity has become one of the leading medical challenges of the 21st century (11). "Hunger hormones," like orexin and ghrelin, as well as high-calorie food, incite individuals to eat, while "satiety hormones" like leptin, insulin and other so called "brain-gut peptides," suppress feeding behavior (12). Weight increase and obesity can result from long-term imbalance between the hunger and satiety signals (12).

There exist two neuronal populations that are crucial in energy balance homeostasis and are located in the arcuate nucleus of the hypothalamus: the orexigenic agouti related peptide (AGRP)/neuropeptide Y (NPY) neurons and the anorexigenic proopimelanocortin (POMC) neurons (10). Activation of the POMC neurons decreases food intake and increases energy expenditure. On the other hand, activation of the NPY-AGRP neurons exerts opposite effects, increasing food intake and reducing energy expenditure, with both of these neuronal groups having significant interaction (8). These neuronal groups are the main targets of hormones that regulate appetite, including leptin, ghrelin, insulin, cholecystokinin and others. POMC neurons secrete the alpha-melanocyte-stimulating hormone (MSH), which acts via several types of melanocortine receptors (MCR) and through them leads to a reduction in food intake and an increase in energy expenditure (9), as discussed below. AGRP are antagonists of MCR, increasing food intake. NPY increases appetite and is produced when energy reserves in the body are low (9).

Chronic stress and obesity

Stress is an incentive that occurs in response to certain experiences, representing the state of disrupted hemostasis. The organism reacts to stress via the central and peripheral segments of the autonomic nervous system and via the hypothalamic-pituitary-adrenal (HPA) pathway. Response to stress can be acute condition, and is necessary for the maintenance of homeostasis, or chronic stress, which is prolonged and may cause some disease states. Acutely, within a few hours, glucocorticoids act to inhibit the secretion of CRH and ACTH. That physiological effect is noticeably altered in

Figure 1. *Chronic stress and central mechanisms of weight gain*
Due to constant activation of the HPA axis during chronic stress, there is a marked increase in secretion of POMC, ACTH and glucocorticoids, which leads to enhanced blood glucose levels and increased insulin secretion. Persistent elevated insulin levels decreases sensitivity or number of insulin receptor in the NPY/AgRP region and reduce inhibition of the orexigenic neurons that leads to obesity.



chronic stress, where the effect of glucocorticoids on the brain is excitatory (13-15).

Many recent studies have shown that chronic stress may contribute to an increased risk for the development of obesity and other metabolic diseases. One of the elementary responses to stress is mediated by the activation of the para-ventricular nucleus of the hypothalamus that secretes a corticotrophin-releasing hormone (CRH), which stimulates the secretion of adrenocorticotrophin (ACTH) from the anterior pituitary gland. ACTH binds to its MC2R receptors on the adrenal cortex and thus stimulates the secretion of cortisol (13-15). Cortisol causes the elevation of blood glucose and insulin concentration, whose long-term effect may cause insulin resistance and diabetes mellitus type 2.

The long-term effect of chronic stress on the HPA axis leads to increased accumulation of visceral fat, but it also has been shown that obesity represents a state of chronic systemic low-grade inflammation that may cause an impaired function of the HPA axis. In the state of chronic stress, glucose metabolism is disrupted. The increased secretion of ACTH and hence a high level of glucocorticoids, primarily cortisol, is associated with weight gain and the high production of proinflammatory hormones and adipokines by adipose tissue depots (15-17).

The main central targets of insulin (and leptin) action are the POMC and AgRP neurons of the arcuate nucleus of the hypothalamus. Some studies suggest a major physiological importance of insulin receptors in the brain in the long-term modulation of energy balance, whereas the disturbance of the insulin receptor

gene may cause increased body fat and high plasma levels of insulin and leptin (18). Chronic stress also increases the activation of the sympathetic nervous system, and thus contributes to impaired glucose tolerance and to an increased risk for developing of cardiovascular events (19). Thus, chronic stress that activates the HPA axis, as well as the sympathetic nervous system, may progressively contribute to the occurrence of obesity and metabolic syndrome (Figure 1).

The role of MSH receptors

It is well accepted that the central melanocortin signaling pathway has a critical role in the maintenance of energy balance, and it is known that peptides generated from POMC have a key role in controlling food intake and weight gain (20, 21). Melanocortin receptors present a distinct family of G-protein-coupled receptors; they are coupled to adenylate cyclase and exert their effects primarily by activating a cAMP-dependent signaling pathway (20). Melanocortin receptors are differently distributed in various kinds of tissue and differ from each other in their affinity for binding various melanocortins and/or their antagonists, agouti signaling protein (ASP) and agouti-related protein (AgRP) (20).

Prohormone convertase 1 (PC1) is expressed in pituitary corticotrophs and produces ACTH, while the expression of PC1 and prohormone convertase 2 (PC2) within the hypothalamus leads to the production of α-, β- and γ-MSH (7). The actions of ACTH and melanocortins are mediated by specific interactions with five melanocortin receptors (MC1R to MC5R), two of which, MC3R and MC4R, are predominantly expressed within the central nervous system, and in the context of human energy balance and body weight regulation, they— especially MC4R— have the most important role (7,22). MC1R is expressed in pigment-producing cells of the skin and hair, and when α-MSH binds to it the production of pigment melanin is stimulated. Also MC1R is expressed on cells of the immune system, wherefore α-MSH has anti-inflammatory effects (7). MC2R is an ACTH receptor expressed in the adrenal cortex, while MC3R is expressed in the brain (mainly in the hypothalamus, cortex, thalamus and hippocampus), and over it γ-MSH plays a role in cardiovascular functions and sodium and energy homeostasis (7). The β-MSH peptide performs its action in weight regulation by binding to MC4R, helping to maintain energy balance (7), and in this context has the most important role. MC5Rs are expressed at low levels in numerous tissues, including the sebaceous gland, where it has a role in sebaceous secretion (7).

It is thought that the anorectic effect of POMC neurons is mediated via α- and β- MSH. Human and animal studies have shown that a lack of MC4R leads to obesity, hyperphagia and insulin resistance (23, 24), and in obese persons β- MSH mutations rather than α-MSH (25). As mentioned above, melanocortin receptors are distinguished by their ability to bind ligand, and under normal physiological conditions, ASP doesn`t have a role in the regulation of food intake since it antagonizes MC1R, but when ASP is synthesized from dermal papilla cells in very high concentrations, it acts as an antagonist of MC4R (20). It is thought that AGRP binds to neural MC3R and MC4R, and in genetically obese mice AGRP is overexpressed, indicating that AGRP has a physiological role in feeding behavior (20). No effect was observed on MC1R and MC2R. MC4R is specific since it binds both antagonists, ASP and AGRP, while MC3R binds AGRP, and MC1R binds only ASP (20).

Food availability – food quality (salt/sugar)

As never before, the food-based dietary diversity strategy has been put into the focus of worldwide political attention and has social, cultural, economic and environmental benefits (26). Although it is well known that dietary habits and health effects depend on ethnicity, gender, region and city (27), there is a widespread intention to improve world population health and individual health through diet (28). In the past, the concept of food quality was quite different compared to the present day, where an important role is given to modern marketing and

sophisticated industrial production methods of processed food rich in added sugar and salt (29). In the everyday diet, the sugar content has tripled during the last 50 years, and there is critical concern about dietary fructose, which has various negative effects on body metabolism (30). It is even popular to compare its effects with those that result from alcohol intake. Both metabolic processes are predominantly based on glycolysis, generate reactive oxygen species and result in a similar disease range (31).

Kitchen salt, sodium chloride, has a very important role in the food industry. The consumption of salt, even much more than is necessary for normal homeostatic process in the human organism, attenuates bitter taste and enhances taste in general. Bakery products, a widely available and popular food category for every age group, are considered to be a major hidden salt source.

In general, even though the word "addiction" has a negative connotation, much research suggests that consuming salty, sweet and fatty foods, for example fast food meals, can produce similar neurophysiological effects as in certain addictive drugs, leading to human obesity in the general population (32). That kind of processed food induces effects that are overwhelming to our brain-reward circuitry in a way natural food does not (30). Innovative solutions need to be implemented by the food industry to replace sodium with more acceptable substances without customer dissatisfaction (33).

The World Health Organization has taken aim at a 30% reduction in global salt intake by 2025 (34). Croatia is an example of a country with an organized national program for the reduction of excessive salt intake, with the goal of helping to develop consciousness about the negative impacts of salt on hypertension (35).

Social interactions as extrinsic factor (mirror neurons)

When discussing the physiological mechanisms related to food intake, we cannot avoid discussing what food means to human beings in addition to being simply "body fuel". How does one distinguish between food as an elementary human need and food as part of social and emotional interaction? The external influence on individual food consumption is very complex; it depends on a variety of factors, and therefore some studies were overlap in the search for proper answers (36). Human beings have a tendency to imitate the behavior of others including behavior related to food intake, a phenomenon that is known to exist in the animal world as well (37). Through many psychological studies, it has been argued that the act of perceiving another person's behavior creates a tendency to behave similarly oneself, which is called the "chameleon effect" (perception-behavior link) (38). In recent history, this and many similar correlations were explained by the discovery of "mirror" neurons —a distinct and intriguing group of neurons whose activities have been located in many different cortical areas, but were most extensively investigated in the ventral premotor region F5. They can transform specific sensory information into a motor format. Moreover, we can say in a way that "motor" or "sensory" neurons have an impact on either execution or observation, and the "mirror" neurons have both (39). They have a huge role in learning during the first developmental periods of life (40). The described functions of mirror neurons can be used to explain the adoption of the first dietary habits from our parents and other close persons, because imitation is a fundamental method through which children learn from their parents, including choices of food and food quantity. According to that concept, obese children display an altered conscious perception of their own weight (41). In that sense, the mirror neurons may represent independent neurophysiological pathways to stimulate overeating and in that way increase calorie intake (37). Furthermore, the perceived trend which includes a lack of physical activity combined with high calorie meals leads to a high BMI, and together they have been referred to as the "big two" contributors to obesity (41,42). As well as having a negative effect (43), peer relationships can also have positive influences on developing everyday habits (44). For instance, peer group physical activity is the strongest

predictor for individual physical activity (40). It is not well explained, but stigmas also have a huge effect on food intake, whereas with some exceptions, negative characteristics are assigned to those people who consume large amounts of food (45). By applying a multidisciplinary approach and using sophisticated neuroimaging methods, more useful information could be obtained not merely about the relationship between mirror neurons and food intake habits, but also about human behavior in general.

In conclusion, the pathophysiology of obesity includes the complicated interaction of the autonomic nervous system and hormonal system with the neuropeptide feedback loops from the gastrointestinal system to the hypothalamic centers that regulate energy homeostasis. Moreover, it is significantly affected by human behavior, emotions and food intake. Thus, obesity is a preventable risk factor for other cardiometabolic diseases.

Disclosures

Funding: No specific funding was received for this study.

Competing interests: None to declare.

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Interventions of Health Visitors in Making a Decision About Breastfeeding

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Abstract

Aim: The aim of this paper was to establish a link between the promotion of breastfeeding and the interventions of the visiting nurse.

Methods: The study was conducted in 2012 in the city of Đakovo and the surrounding area. The questionnaire was filled out by mothers after leaving the maternity ward, during the first visit of the community health nurse within the first seven days after the labor.

Results: From a total of 154 parturient women who filled in a survey after they had left the hospital, the decision about breastfeeding before the pregnancy was made by 58.4% of examinees. Primiparas from the city were older than primiparas from the surrounding rural area ($p<0.001$). Mothers received the majority of information about the proper placement of the child on the chest after birth from nurses in the maternity hospital (56.3% of the examinees). The study showed that 57.9% of the examinees had the support of their husband during lactation. The visiting nurses' first visit was within seven days after delivery at the home of 121 (78.6%) examinees, showing good awareness and collaboration between secondary and primary health care.

Conclusion: This research has confirmed that breastfeeding promotion and nursing interventions have a major impact on breastfeeding.

(Mikšić Š, Konjarik Z, Kralj-Vasilj M, Juranić, B, Pavlović D. Interventions of Health Visitors in Making a Decision About Breastfeeding. SEEMEDJ 2017;1(1);130-135)

Introduction

Preparing a pregnant woman and family members to accept positive attitudes about breastfeeding begins with educating and sensitizing the local community. Breastfeeding is good for the family, and society as a whole. The World Health Organization (WHO) and UNICEF are focused on the application of natural nutrition to ensure the health of the child and

Received: March 22, 2017; revised version accepted: May 25, 2017; published: May 26. 2017

KEYWORDS: promotion of breastfeeding, visiting nurse

mother (1). There is a need for intervention by gynecologists, maternity clinics, pediatricians, patrons, family physicians, nurses working in ambulances and pharmacists. Breastfeeding should be a choice, not a behavior that is imposed on the mother (2). It implies feeding the baby on the mother's chest, because breast milk is the best baby food for newborns (3). The length of breastfeeding is an indicator of the interest, time and energy that a mother intends to invest in her child's development. Rooming-in is a 24-hour stay of the mother and the baby with the goal of starting breastfeeding as early as possible. During breastfeeding, a mother transfers to her child feelings of warmth, safety and protection (4). Research has shown that early skin contact, besides stimulating breastfeeding, also affects the rhythm of sleep and sleepiness, and stimulates the motor and neurological behaviors of newborns after delivery. These positive effects were also observed in premature babies and term children (5). Mothers who breastfeed for a longer time spend more time with their child and achieve the interaction that favors their child's cognitive and intellectual development (6). According to the estimation of the health nurse, at least one or several patron intervention visits are arranged for the protection and care of the pregnant woman. During child monitoring, the mother should be instructed that she should be available to the child for unlimited breastfeeding (7). This process creates the proper bonding of mother and child and affects a different pattern of the mother's behavior. It is a special psychophysical stimulus for a mother and a child that improves the cognitive development of the child. Mother's milk, with its composition, fully meets the needs of the baby for food and fluids during the first six months of the baby's life, and as a dairy meal can be delivered even after the first year. The effects of the optimal composition of the mother's milk and the influence of certain ingredients stimulate the development of the baby's brain, and are not produced by other milk types. Establishing the importance of breastfeeding encourages an environment in which the mother has the support of family members, and the promotion of breastfeeding is carried out by health care professionals. The most important supportive role to the mother in breastfeeding belongs to the father of the child, who must be familiar with the benefits of breastfeeding and have a positive attitude towards it (8). The aim of this study was to establish a link between the promotion of breastfeeding and the interventions of visiting nurses.

Materials and methods

The research was conducted in the town of Đakovo and its surroundings, during the first house call made by health visitors after the mother had been discharged from the hospital. From a total of 202 babies born during the survey, there were 154 mothers who were breastfeeding their babies and were included in the research. The research was carried out by means of an anonymous survey. Other issues addressed concern the relationship between breastfeeding and the way of birth in these pregnancies, the duration of pregnancy, education on breastfeeding and support during breastfeeding. Afterwards, questions were asked related to previous births and the length of breastfeeding of children.

Statistical analysis

The frequency distributions for investigated variables were described by descriptive statistical methods. The Kolmogorov–Smirnov test was used to analyze the normal distribution of the variables. The mean values for the continuous variables have been expressed as the arithmetic mean and as the standard deviation for normally distributed variables, and for variables that do not have a normal distribution, as median and range. The nominal data have been expressed through the frequency distribution both in groups and its content. χ^2-test and Fisher exact test have been used to determine the differences between the two independent samples. The significance level $\alpha = 0.05$ was selected to evaluate the importance of the obtained results.

Figure 1. *Division of examinees (%) according to the person who taught them how to breastfeed*

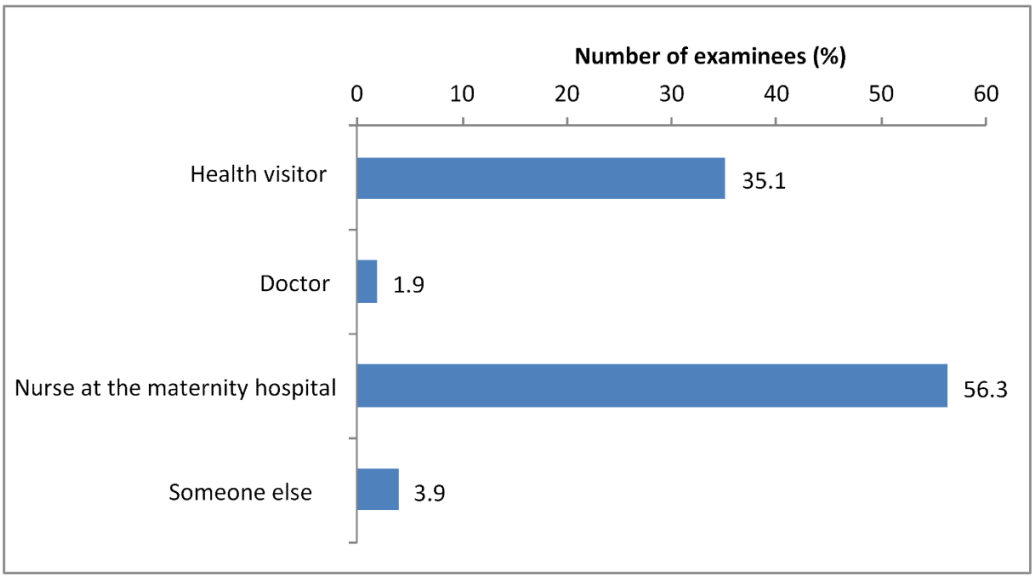

Table 1. *Breastfeeding signs regarding the time when the decision on breastfeeding was decided*

Breastfeeding signs	Time when the decision was made				Total N (%)	p*
	Before the pregnancy N (%)	During the pregnancy N (%)	After the delivery N (%)	Does not want to breastfeed N (%)		
BMI before pregnancy	21.68	3.18	1.20	24.42	5.58	
Mouth wide open, round cheeks, baby is close to the mother's body, baby is calm and relaxed, the baby holds the breast with its fingers by itself and lets go of the breast by itself after it has been fed	64 (71.1)	37 (72.5)	3 (27.3)	2 (100)	104 (67.5)	
Baby's mouth is not wide open, cheeks are sunken, breasts are painful and sore, there is only some contact between the mother and the baby	14 (15.6)	7 (13.7)	3 (27.3)	-	24 (15.6)	0.001
Baby's mouth is not wide open, cheeks are sunken, breasts are painful and sore – there are rhagades, there is no contact between the mother and the baby	3 (3.3)	3 (5.9)	-	-	6 (3.9)	
The mother is tense and in an awkward position, the baby is restless and cries, the baby can't take the breast by itself, breastfeeding is painful and short	-	2 (3.9)	1 (9.1)	-	3 (1.9)	
The baby does not suckle	9 (10)	2 (3.9)	4 (36.4)	-	17 (11)	
Total	90 (100)	51 (100)	11 (100)	2 (100)	154 (100)	

*Fisher exact test

Results

There were 154 women who took part in the research, of which 79 (51.3%) live in rural areas, and 75 (48.7%) in the city. The median age in which mothers had their first baby is 26 for the women living in town, and 23 for those living in rural areas (Mann Whitney U test, $p<0.001$). Mothers who live in the town have breastfed their first baby longer, whereas mothers who live in rural areas have breastfed their second baby longer. Most of the examinees received the information on breastfeeding from the nurses in the maternity hospital and from the health visitor (Figure 1).

During breastfeeding, 88 (57.9%) of the examinees were supported by their husbands, 56 (36.8%) by their extended family and 8 (5.3%) and by other services (i.e. health care service, BSG - Breastfeeding Support Group). Evaluation of examinees' breastfeeding has shown much better results in the cases of those who made the decision to breastfeed before or during their pregnancy. The lowest incidence of breastfeeding is in the group of examinees who made the decision to breastfeed after delivery (Table 1).

It was found that a group of women who gave birth to one, two and three children statistically differ significantly in the number of months of breastfeeding ($F(2.133) = 12.14$, $p < 0.01$, $\eta 2 = 0.154$). In a post-hoc comparison (Bonferroni), a statistically significant difference was found between women who had a first child ($M = 1.66$, $sd = 4.37$) and women who had a second child ($M = 7.34$, $sd = 8.87$).

In order to determine whether there was a difference between women who had a second or third child, a t-test was carried out. It was found that a group of women who gave birth to two and three children statistically differ significantly in the number of months breastfeeding another child ($t = 6.45$, $df = 65$, $p < 0.01$). There was a statistically significant difference between women who had a second child ($M = 1.02$, $sd = 0.15$) and women who had a third child ($M = 6.25$, $sd = 5.34$).

Discussion

By educating pregnant women and family members, the adoption of breastfeeding as the only way to properly feed the baby is accomplished. Interventions of visiting nurses are conducted through individual and group work (9). Mothers are more likely to make a decision about breastfeeding if they are well informed about the benefits of breastfeeding and if they have family, social, and especially their husbands' support. While 57.9% of examinees had the support of the husband during breastfeeding, only 5.3% had the support of outside groups, suggesting that breastfeeding support groups should be expanded because the mother is the best educator. Studies have shown that a woman whose husband does not support breastfeeding stops earlier or doesn't even start breastfeeding (8). The first visit of the nurses within 7 days after the birth occurred in 78.6% of the cases, indicating good communication and cooperation between the hospital and the patrons' service. It is very important for a mother to know where and how she can get all the information needed after birth. The visiting nurse must be familiar with the physiology of lactation to promptly detect potential problems with breastfeeding and to arrange, together with the new mothers, a health care and intervention plan for solving any identified problems. A significantly better estimation of breastfeeding was observed among those who decided to breastfeed before pregnancy (Table 1). Mothers with a clear plan for the duration of breastfeeding usually follow through with it, while hesitant mothers generally breastfeed for a shorter amount of time (10). Mother and child should master the breastfeeding technique, and the mother should be instructed to inflate the baby, which means that the frequency and length of breastfeeding depend on the child's needs and signs. Signs that breastfeeding is progressing or not progressing depend on the emplacement of mothers, breastfeeding and signs of the transfer of the milk (11). Our research has shown that previous experience is an important factor because the respondents' second child nursed longer than the third child.

Respondents of the study have consistently demonstrated that women who gave birth to more than one child have a higher breastfeeding efficacy than women who gave birth to one child (12, 13, 14). A positive experience of breastfeeding can improve the mother's confidence in breastfeeding, and a negative experience of breastfeeding can reduce it. Other studies have shown that 30-57% of mothers have problems with breastfeeding in the early postpartum age, where insufficient milk is reported as the most common problem and the primary cause of early termination of breastfeeding (15, 16). A well-educated mother is more prepared for problems that may occur during lactation, and she has a different pattern of behavior. Studies have shown that the mother who spends more time with her child is aware of the benefits of her milk for the growth and development of the baby, and also that in a short period of time the child developed a good grip on the breasts (17,18) (Table 1). Children have both the need for food as well as the need for touch, gentleness and love that only the mother can provide in the first days of life. Pinard's aphorism says "There is no replacement for mother's milk and her heart" (19). WHO, UNICEF and the American Pediatric Academy (AAP) plead for breastfeeding only up to 6 months of age and then a continuation of breastfeeding along with the right meal for frozen foods for at least one year (20) or two years (21), and even longer if so wanted by both mother and child.

Conclusion

This research has shown that the most important breastfeeding factors are the mother's decision before delivery and an early visit by a nurse after coming home from the maternity ward. The visiting nurse is the first health care worker to start training pregnant women during pregnancy about breastfeeding and continues after the mother's return from the maternity ward. Also, the visiting nurse's approach to the mother and newborn is of great importance, and their imparted knowledge is the basis for the development of appropriate skills (22) and attitudes so that habits and behavior changes will be adopted in order to preserve and improve the health of pregnant women, new mothers and children in the wider community.

Acknowledgments

The authors gratefully acknowledge the health visitors who made possible the conduct of the research and all the examinees who participated in the research.

This paper was presented as a Master Thesis at the Professional study in nursing, Faculty of Medicine Osijek, Josip Juraj Strossmayer University of Osijek, June 12, 2012

Disclosures

Funding: No specific funding was received for this study.

Competing interests: None to declare.

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Vocal Cord Paralysis and Parathyroid Cyst

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Abstract

Aim: Parathyroid gland lesions are an extremely rare clinical entity, mostly manifesting as adenoma and hyperplasia, rarely as parathyroid cysts, and most infrequently as carcinoma. Parathyroid cysts are frequently asymptomatic lesions of the neck and the superior mediastinum with only 300 cases reported in the literature. Symptomatic parathyroid cysts are very rare. Vocal cord paralysis due to recurrent laryngeal nerve dysfunction may herald the presence of neck and mediastinal disease including a variety of neoplastic, inflammatory and vascular conditions. The aim of this report is to describe their clinical presentation, diagnostic procedures, and therapeutic approaches. The objective of the study was to assess the presence of vocal cord paralysis and recurrent laryngeal nerve dysfunction, as well as their association in patients with recurrent laryngeal nerve.
Methods. We performed a 12-year departmental review of parathyroid lesions and parathyroid cysts. Retrospective analysis of clinical data on 20630 unselected patients submitted to thyroid gland and neck ultrasonography yielded 149 (0.007%) patients with parathyroid lesions, six (0.0003%) of them with parathyroid cysts. A comprehensive review of the literature revealed 18 patients with vocal cord paralysis and parathyroid cysts.
Results: Parathyroid adenoma were present in 97/149 (0.65%) and hyperplasia in 46/149 (0.30%) patients with parathyroid lesions. Parathyroid cysts were present only in six of 149 (0.04%) patients with parathyroid lesions. Five patients had asymptomatic nonfunctional parathyroid cysts, with vocal cord paralysis diagnosed in one female patient. In the 19 patients with parathyroid cysts and vocal cord paralysis reported in the literature (including the patient reported here), nine and ten patients had parathyroid cysts localized in the neck and mediastinum, respectively. Mediastinal cysts were twofold greater than those localized on the neck, while left recurrent laryngeal nerve dysfunction was recorded in 80% of cases. Cyst removal resulted in recurrent laryngeal nerve functional recovery in two-thirds of patients.

Received: April 27, 2017; revised version accepted: May 25, 2017; published: May 26. 2017

KEYWORDS: Parathyroid lesion; Cyst; Vocal cord; Paralysis; Cytology

Conclusion: Parathyroid cysts are rare, mostly asymptomatic pathologic lesions of parathyroid glands that should be taken into consideration in patients with hyperparathyroidism or vocal cord paresis.

(Đanić D, Fuštar Preradović Lj., Jović R, Đanić Hadžibegović A. Vocal Cord Paralysis and Parathyroid Cyst, SEEMEDJ 2017;1(1);136-143)

Introduction

Parathyroid glands and their lesions are present in four different histopathologic and clinical entities, mostly as adenoma and hyperplasia, and rarely as cysts or carcinoma. Parathyroid cysts (PTCs) are an uncommon cause of VCP. PTCs are benign neck tumors and that account for 0.8%-3.41% of all parathyroid lesions (1, 2). The true prevalence of PTC remains uncertain, and more than 300 patients with cystic lesions of parathyroid glands have been reported in the literature. About 200 of these lesions were localized in the neck, and the rest occurred in the mediastinum (2, 3). PTCs mostly occur at the age of 40-50 with a female predominance (female to male ratio, 2.5:1) and a few cases in pediatric patients. Generally, the left side of the neck and inferior parathyroid glands are involved. True PTCs, found in 80% of patients, are nonfunctional and asymptomatic. Occasionally, depending on their size and localization, PTCs may cause dysphagia, pain, and tracheal compression or RLN lesion (2, 3). Functional PTCs that develop by parathyroid adenoma cystic degeneration are found in 20% of patients (3). These PTCs usually occur as solitary lesions, whereas multiple functional PTCs are extremely rare and are associated with hyperparathyroidism (2, 3, 4, 5). Only one case of malignant PTC transformation has been described in the literature (6).

The etiology of vocal cord paralysis (VCP) is very heterogeneous and the true incidence of VCP in the general population is hard to estimate. According to Shafkat et al., 42 cases per 10 000 new patients can be expected at ENT clinic (7). Most frequently, VCP develops as a sequel of recurrent laryngeal nerve (RLN) dysfunction caused by a malignant tumor, various inflammatory processes or vascular malformations. A malignant tumor localized in the neck or mediastinum leads to RLN dysfunction by its aggressive growth, pressure or traction, and most frequently by nerve infiltration. Damage to RLN is considerably less commonly induced by benign tumors of the neck. The incidence of rare and idiopathic VCP varies from 16.3% to 31.1% (8, 9).

Current diagnostic tools including ultrasonography, multi-slice computed tomography (MSCT), magnetic resonance imaging (MRI) and technetium-99m sestamibi scanning can often differentiate solid tumors from cystic lesions. However, differentiating a thyroid cyst from PTC is challenging. Ultrasound-guided fine needle aspiration cytology (US-FNAC) is a useful test to confirm the diagnosis in case of clinically suspected parathyroid glands and parathyroid lesions, but also to detect a parathyroid gland of an unexpected localization, e.g., in the thyroid bed or within the thyroid gland. A parathyroid lesion may present as thyroid incidentaloma, a lesion incidentally found within or adjacent to the thyroid gland (10) (Fig. 1). The aim of the present study was to evaluate the clinical characteristics, diagnostic difficulty and our experience in therapy of PTCs.

Material and Methods

Study design: A retrospective analysis was performed on a cohort of 20 630 patients who submitted to ultrasonography examination of the thyroid gland and neck. Thyroid gland and neck ultrasonography reports were reviewed for diameter and the location of the suspect

Table 5. *Clinical and laboratory characteristics of parathyroid cyst patients (N=6)*

Patient No.	Initials, age (yrs)/gender	Symptoms	Localization	Cyst size	Serum PTH pg/mL	Cyst PTH pg/mL	Therapy: FNA/Surgery
1	51/F	Osteodynia	Left inferior	21 mm	35.43	240.1	FNA
2	76/F	Hoarseness Left vocal cord paresis	Left inferior	22 mm	30.31	389.5	Surgery
3	54/F	Dry cough Nonspecific breathlessness at exertion	Left inferior	24 mm	29.22	498.0	Surgery
4	44/F	Left-sided hemithyroidectomy	Right inferior	17 mm	64.3	21 770	FNA
5	50/M	Nephrolithiasis	Left superior	45 mm	77.61	103.1	FNA
6	79/M	Nonspecific swallowing disturbance	Right inferior	18 mm	110.90	337.0	Surgery

PTH = parathyroid hormone; FNA = fine needle aspiration

parathyroid lesions. Ultrasound-guided fine needle aspiration cytology (US-FNAC) of the parathyroid gland was performed under continuous, ultrasound visualization with use of an ACUSION X 300 device using 8.9 MHz and 11 MHz probes. The medical records were reviewed for clinical symptoms, biochemical measurements, ultrasonography reports, cytology data, surgical treatments and pathology reports.

Statistical Analysis

Proportions were expressed as percentages, whereas all continuous variables were calculated as median and range. There were no inter- or intra-group comparisons needing further statistical analyses.

Results

The analysis yielded 149 patients with parathyroid lesions out of 20630 examinations (0.007%), including 97 (65.1%) patients with parathyroid adenoma, 46 (30.8%) patients with hyperplasia and six (4.0%) patients with PTCs. There were four female and two male PTC patients with a median age of 52.5 (minimum 44, maximum 79, range 35) years and a median cyst size 21.5 mm (minimum 17, maximum 45, range 28 mm) (Table 1).

These six patients with PTCs underwent US-FNAC, and the presence of parathyroid hormone (PTH) was confirmed by aspirate biochemistry on a Cobas e411 analyzer (Roche, Mannheim, Germany). Nonfunctional PTCs were present in five patients and VCP in one patient. One patient had a functional PTC, with a parathyroid hormone (PTH) level of 77.61 pg/mL and normal serum and ionized calcium levels. The surgical extirpation of the cysts and histopathologic analysis were performed in three patients, whereas the other three patients underwent US-FNAC (Fig. 2 and 3). The study protocol was approved by the Hospital Ethics Committee and by the Zagreb School of Medicine Ethics Committee.

A comprehensive review of the literature from 1953 to the present showed 19 patients with PTC and VCP, including our patient reported herewith (Table 2). There were 53% males, with a median age of 54 (minimum 29, maximum 83, range 54) years, and a median cyst size of 48 mm (minimum 12, maximum 123, range 111 mm). Ten PTCs were localized in the mediastinum and nine in the neck; there were 16 nonfunctional PTCs. Functional PTCs were present in three

Table 6. *Reported 19 cases of VCP associated with benign parathyroid cysts*

Reference	Public. date	First author	Age/ Gender	Local.	Size mm	Functional cyst status	Neck side	Treatment	Postop. status
11.	1953.	Crile G. Jr	69/F	M	60	NF	R	S	N
8.	1966.	Hayashi S.	48/M	M	83	NF	R	S	N
12.	1978.	Clark O.H.	29/F	M	63	NF	L	S	N
13.	1983.	Marco V.	42/M	M	12	NF	L	S	N
14.	1988.	Takahashi E.	68/F	M	73	F	R	S	N
15.	1990.	Delauny T.	71/F	M	40	F	L	S	N
16.	1991.	Coates G.	60/M	N	80	NF	L	A	P
8.	1992.	Narita Y.	54/M	M	123	NF	L	S	N
17.	1993.	Grey A.B.	38/F	N	20	NF	L	A	N
18.	1997.	Landau O.	77/M	M	88	NF	R	S	P
19.	2000.	Sen P.	37/F	N	25	NF	L	A	N
20.	2006.	Coelho D.H.	49/M	N	48	NF	R	S	N
21.	2006.	Sihoe A.D.	51/M	M	95	NF	L	S	N
22.	2008.	Woo E.K.	83/M	N	39	NF	L	S	P
23.	2011.	Ghervian C.	50/F	N	42	NF	L	A	N
24.	2012.	Khan A.	58/F	N	32	F	R	S	P
25.	2013.	Menezes VC	56/M	M	72	NF	L	S	N
26.	2015.	Arduc A.	30/M	N	30	NF	L	S	N
	2016.	Danic D.	76/F	N	22	NF	L	S	P

N- neck; M-mediastinum; F- functional; NF- nonfunctional, S- surgery; A- aspiration; P- paralysis RLN; N- normal functional status

female patients, accompanied by elevated serum PTH and calcium levels. Left-sided VCPs were found in 15 patients. In 80% of patients, PTCs were surgically removed in toto, whereas 20% of patients underwent transcutaneous aspiration of the cyst content. In two-thirds of patients, PTC removal resulted in functional RLN recovery.

Discussion

Parathyroid lesions are rare clinical entities, and parathyroid cysts are uncommon forms of parathyroid lesions, possibly even referred to as 'a forgotten diagnosis' (27). Microcysts of the parathyroid gland are a common occurrence in the normal population and are found primarily in aging glands along with increased fat content. Macroscopic cysts of the parathyroid glands that are larger than 10 mm in diameter are referred to as parathyroid cysts and necessitate investigation. The clinical presentation is very different; functional parathyroid cysts are relatively easier to detect than nonfunctional ones. PTCs are diagnosed by clinical symptoms and biochemical parameters of hyperparathyroidism with elevated serum calcium levels, and postoperatively by histopathology demonstrating parathyroid gland tissue in the cyst wall and the postoperative correction of the PTH level and hypercalcemia (3). Generally nonfunctional parathyroid cysts are asymptomatic and incidentally diagnosed during clinical work-up

for thyroid disorders or other neck and chest soft tissue tumors (10, 27).

Figure 5. *Thyroid ultrasound showing cystic mass adjacent to the left lobe of the thyroid gland*

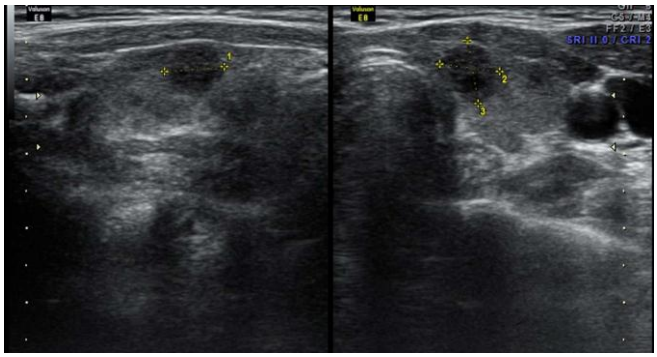


Figure 2. *Thyroid preparation with adjacent cystic of a parathyroid gland (own source)*

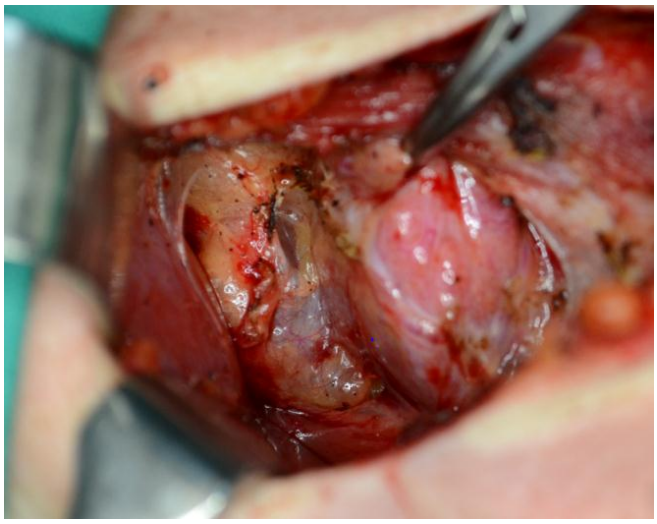
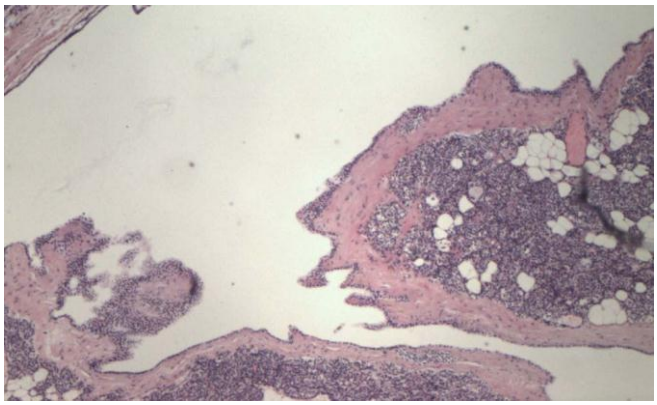


Figure 3*. Parathyroid gland histology (own source)*



Histopathology: inner cyst surface lined with a layer of flat epithelial cells; parathyroid gland tissue along the cyst connective wall (hemalaun-eozin stain)

Vocal cord paresis can be caused by recurrent laryngeal nerve lesions of various etiologies. Surgical traumas and tumors are most commonly involved, tough the cause remains unknown in one-third of lesions. The rare causes of VCP include cardiovascular syndromes, cervical spine osteophytes, tracheal diverticula, subclavian artery aneurysm, jugular vein thrombosis, and meningomyelocele (9). PTC has been described in several studies as a rare cause of VCP. Ho and McMullen report on 13 patients with various parathyroid gland lesions considered to have caused VCP. Functional PTCs were present in six patients, four of them with the clinical picture of hyperparathyroidism and a histopathology report indicating adenoma, and two patients with secondary hyperparathyroidism and a histopathology report indicating hyperplasia. Three patients had both PTCs and parathyroid gland nonfunctional hyperplasia, whereas five patients had true nonfunctional PTCs, two of them suffering from VCP and clinically present tumor mass on the neck, and one patient complaining of pain in the neck (27). PTCs belong to a group of extremely rare causes of VCP. In their study that included 93 patients with mediastinal PTCs, Shields and Immerman recorded nine (9.5%) patients with VCP. True nonfunctional PTCs were found in seven patients and functional PTCs in two female patients (5). In our unselected cohort of 20 630 patients that underwent neck and thyroid gland US, parathyroid lesions were recorded in 147 (0.007%) patients and PTCs in only six (0.0003%) patients, with VCP found in only one female patient (Table 1).

That patient was a 76-year-old female presented for clinical ENT examination due to hoarseness persisting for months. Fiberoscopy, laryngostroboscopy and electromyography studies revealed left-sided VCP. Standard biochemistry parameters showed no pathologic findings. The patient underwent neck and thyroid US examination (Figure 1). Based on these findings, the diagnosis of nonfunctional PTC was made. She underwent a minimally invasive surgical procedure. The cyst was surgically extirpated in toto, while saving RLN carefully (Figure 2). Follow up biochemistry parameters obtained 24 h postoperatively were within reference range. Histologic examination

confirmed the presence of PTC (Figure 3). At 12 months after the surgery, the patient had left-sided VCP and dysphonia, without breathing and swallowing difficulties. Serum levels of PTH, calcium and phosphorus were within the reference range.

About 300 patients with PTCs have been reported in the literature to date, 200 of these localized in the neck and 100 in the mediastinum (1, 5, 27). Analysis of the available literature yielded 19 patients with PTC and VCP, including our patient reported herewith (Table 2). The number of patients with neck and mediastinal PTCs was almost equal, i.e., nine and ten, respectively. A review of the present literature shows the prevalence of VCP was twofold greater (10 %) in patients with the PTC localized in the mediastinum than in those with the PTC in the neck (4.5 %). This finding may be due to the twofold greater median cyst diameter (75.5 mm) and probably a higher compression of the cyst wall upon RLN in patients with mediastinal PTCs as compared with the median neck cyst size of 37.5 mm. In our patients, the median PTC size was significantly lower, i.e., 24.6 mm. In 80% of patients, PTCs were localized on the left side of the neck or mediastinum with left-sided VCP. Left RLN is known to be more vulnerable than right RLN because of its length and localization. In two-thirds of patients, PTC removal resulted in the functional recovery of RLN. A review of the present 19 patients according to the functional cyst status shows that 16 patients had asymptomatic PTCs and three female patients had functional PTCs.

Loss of RLN function can be caused by a variety of mechanisms. In some patients, loss of RLN function can be explained by the known and verified pathologic mechanisms. A large cystic growth exerts nerve compression and/or traction, while local inflammation or edema can lead to neurapraxia and eventually to axonotmesis with a loss of function. In patients with long-standing renal insufficiency, the loss of RLN function can occur as a sequel of a hemorrhage into the PTC, frequently associated with inflammation (27). Major intracystic hemorrhages and RLN lesions were found in patients with hypercalcemic episodes and functional PTCs (24). In the majority of these patients, nerve and VCP recovery ensued after PTC removal. In malignant tumors, of the thyroid gland in particular, nerve lesions can be induced by direct invasion of malignant cells or by nerve compression by nodular hyperplasia in the case of a benign growth. However, in some cases, the cause of the loss of RLN function remains obscure, as in our patient. Accordingly, in some PTC patients, the loss of RLN function and VCP appear to occur due to some other unidentified cause, or VCP and PTC are present concurrently but without mutual interaction.

The modality and extent of PTC treatment is determined by its size, localization, functional status, and cyst-related complications. Minimally, invasive surgery for PTC removal is the gold standard in the treatment of functional PTCs. In patients with true nonfunctional PTCs and symptoms of adjacent organ compression, we believe that surgical removal of the cystic lesion is necessary, along with relieving the neck or mediastinum structure compression, with special attention paid to RLN. Percutaneous cyst aspiration is a satisfactory therapeutic procedure in small, asymptomatic, nonfunctional and uncomplicated cysts (27). In patients with permanent VCP, speech therapy, augmentation injections, or unilateral medialization laryngoplasty should be used. In our patient, three months of speech therapy resulted in satisfactory voice quality.

There were several limitations of our study. The most important ones were retrospective design, a single center experience, and a small number of cases that did not allow any statistical analyses.

In conclusion, our study has demonstrated that parathyroid cysts are rarely encountered in general neck and mediastinum pathology, but should not be forgotten as a possible cause of unilateral VCP. Functional and larger PTCs are easier to diagnose than small and asymptomatic ones. US-FNAC and aspirate biochemistry for PTH are the standard diagnostic procedures for this entity. Minimally invasive surgery with in toto cyst extirpation is the gold standard in the

treatment of all cystic lesions associated with complications.

Funding:
No specific was received for this study.

Competing interests:
None to declare.

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Etiology and the Genetic Basis of Intellectual Disability in the Pediatric Population

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Abstract

Intellectual disability/mental retardation (ID/MR) is defined as incomplete mental and cognitive development present before the age of 18. There are number of pre-natal and post-natal risk factors that can cause ID/MR but 25 %-50 % of all have genetic causes. In the general population, the prevalence of ID/MR is about 2 %-3 %. Use of standard cytogenetic methods analysis of chromosomes (GTG banding) and FISH (Fluorescent in Situ Hybridization) reveals only a small number of causes, but when using new molecular genetics techniques (like chromosomal microarray and next generation sequencing), the rate of causes of ID/MR is increased and new candidate genes for ID/MR have been discovered. Establishing a diagnosis of ID/MR is important for the patient and it provides genetic counseling for parents.

(Tomac V, Pušeljić S, Škrlec I, Anđelić M, Kos M, Wagner J. Etiology and the Genetic Basis of Intellectual Disability in the Pediatric Population. SEEMEDJ 2017;1(1);144-153)

Definition and prevalence of ID/MR

Intellectual disability/ mental retardation (ID/MR) is defined as a disability characterized by significant limitations in intellectual functioning and in adaptive behavior; condition covers everyday social and practical skills and begins before the age of 18. Intellectual functioning, also called intelligence, refers to general mental capacity, such as learning, reasoning, problem solving and so on. Adaptive behavior is the collection of conceptual (language and literacy), social (interpersonal skills, social responsibility) and practical skills (activities of daily living and personal care, occupational skills, healthcare) that are learned and performed by people in their everyday living (1).

Received: March 15, 2017; revised version accepted: May 25, 2017; published: June 2. 2017

KEYWORDS: mental retardation, intellectual disability, etiology, submicroscopic chromosome aberrations, genes

Table 1. *Environmental and genetic causes of intellectual disability*

Prenatal factors	**Perinatal factors**	**Postnatal factors**
Genetic: chromosomal abnormalities cryptic chromosome abnormalities deletions/duplications contiguous gene syndromes monogenic diseases	prematurity low birth weight asphyxia	sepsis/meningitis, encephalitis (HSV 1/2) various multifactorial causes (poverty and cultural factors)
Environmental: infections (toxoplasmosis, syphilis, rubella, cytomegalovirus and HIV infections) mother disease (diabetes) teratogenic factors (drugs and radiation)		
Metabolic: neonatal hypothyroidism		

The intelligence quotient test (IQ test) is a major tool in measuring intellectual functioning, which is the mental capacity for learning, reasoning, problem solving and so on. A test score below or around 70 or as high as 75 indicates a limitation in intellectual functioning. IQ testing became the way to define groups and classify people within them (1). According to IQ testing, ID/MR is categorized as: mild (IQ 50 - 55 to 70), moderate (IQ 35 - 49 to 50 - 55), severe retardation (IQ 25 - 20 to 35-40), or profound retardation (IQ below 20).

The prevalence of ID/MR varies considerably due to the different criteria and methods used in the diagnosis. This problem is present in 2 % to 3 % of the children's population, especially because 5 % to 10 % of children have motor impairment, isolated speech and language delay, severe primary sensorial deficits and pervasive disabilities. ID/MR is more frequent in countries of lower socioeconomic status due to increased incidence of anoxia, birth trauma and newborn brain infections (2). The prevalence of mental retardation in developed countries is thought to be 2% to 3%. The prevalence of mild ID/MR more often depends on external environmental factors (level of maternal education, access to education, opportunity and access to healthcare), while the prevalence of severe ID/MR is relatively stable (3).

Diagnosis is highly dependent on a comprehensive personal and family medical history, a complete physical examination and a careful developmental assessment of the child. When diagnosing ID/MR, it is very important to know how it is defined and classified.

Etiology and epidemiology of ID/MR

The etiology of ID/MR has heterogeneous environmental and genetic causes, summarized in Table 1 (4, 5). Prenatal factors are environmental (mother infection in pregnancy such as rubella infections, syphilis, toxoplasmosis, cytomegalovirus and HIV infections), teratogenic (the use of drugs such as thalidomide, phenytoin and warfarin sodium in early pregnancy, radiation), chromosomal abnormalities (e.g. trisomy 21), cryptic chromosomal abnormalities (deletions or duplications) and genetic mutations. Perinatal factors are prematurity and asphyxia, while postnatal factors are sepsis, meningitis, encephalitis (commonly caused by HSV 1/2) and various multifactorial causes (poverty and cultural factors).

Genetic factors are thought to cause ID/MR in about 25% to 50% of cases (6). Specifically, genetic factors are estimated to be the cause of moderate and severe ID/MR (IQ<50) in 0.3 % to 0.5 % of cases, of mild ID/MR (IQ ranging from 50 to 70) in 1 % to 3 % of cases and of severe ID/MR in 25 % to 50 % of cases (7).

Based on the symptoms' presentation, ID/MR is divided into two groups: syndromic and non-syndromic ID/MR. In non-syndromic ID/MR, the only pathological manifestation is cognitive deficit, and there are no changes in phenotype and no associated anomalies of organ systems. It can be inherited in three ways: autosomal recessive, autosomal dominant or X-linked mode. Syndromic ID/MR is related to phenotypic dysmorphy (craniofacial, skeletal), growth changes, neuromuscular changes and metabolic diseases (8).

Chromosomal abnormalities

Aberrations in the autosomal chromosome number in live-born babies are restricted to aneuploidies. These abnormalities represent about 10 % of the ID/MR that can be detected with conventional cytogenetic methods (9). The majority of cases involve trisomy 21 with a prevalence of 1 to 700, which is clinically expressed as Down syndrome (10). Other rare chromosomopathies include trisomy 13 (Patau syndrome) with a prevalence between 1 in 5,000 and 1 in 29,000 live births (11), trisomy 18 (Edwards syndrome) with a prevalence of 1 to 3600 and 1 to 8500 (12), and they are usually lethal in the first week of life. Monosomy of any autosomal chromosome is lethal in the earliest stage of embryonic life. There are autosomal structural abnormalities such as Wolf-Hirschhorn syndrome (microdeletion 4p) with a prevalence of 1 to 50,000 (13), Cri du Chat syndrome (microdeletion 5p) with a prevalence of 1 to 50 000 (14) and sex chromosomal aneuploidies such as Klinefelter syndrome (47,XXY) with a prevalence of 1 in 500 to 1,000 newborn males (15).

Contiguous gene syndromes

Contiguous gene syndromes are disorders caused by chromosomal abnormalities, such as deletions and duplications, which result in an alteration of normal gene dosage. For most autosomal loci, deletion causes a reduction of gene dosage to structural and functional monosomy. Haploinsufficiency for specific genes in the critical interval is implicated for del(7)(q11.23q11.23) in Williams syndrome, for del(8)(q24.1q24.1) in Langer-Giedion syndrome, del(17)(p13.3) in Miller-Dieker syndrome, and for del(22)(q11.2q11.2) in DiGeorge syndrome and velocardiofacial syndrome (16).

Genomic imprinting

Genomic imprinting is a situation in which there is gene expression from only one of the two alleles inherited from each parent, and it is based on epigenetic modifications of specific allele, such as histone acetylation/methylation and DNA methylation (17).

The deletion of a chromosome segment containing the active allele of an imprinted gene results in structural monosomy but functional nullisomy (e.g., paternal del(15)(q11.2q13) in Prader-Willi syndrome and maternal del(15)(q11.2q13) in Angelman syndrome). Uniparental disomy for the homologue containing the inactive allele results in structural disomy but functional nullisomy (e.g., maternal disomy 15 in Prader-Willi syndrome and paternal disomy 15 in Angelman syndrome).

Idiopathic ID/MR

Current research has been directed to clarify the genetic base of what was accepted as "'idiopathic ID/MR". The most prevalent structural variations in the human genome are copy number variations (CNVs), which appear predominantly in the subtelomeric regions. Genomic variations are a frequent cause of miscarriage, congenital anomalies (CA) and intellectual disability (ID) (18).

Pathogenic CNVs have been detected in 10 % to 15 % of patients with idiopathic ID/MR, especially with use of microarray technology. Most of CNVs are de novo mutations, but there are also rare inherited mutations with unknown significance (19).

Over the last few years, cryptic chromosomal anomalies, particularly subtelomeric and interstitial rearrangements (including microdeletions as well as balanced translocations and other chromosomal

aberrations) less than 3–5 Mb, have emerged as a significant cause of "idiopathic ID/MR" (20-22).

About half of all segmental aneusomies are found on subtelomeric and terminal regions of chromosomes that are gene-rich, and they are responsible for 5% to 7% of all cases of ID/MR (23, 24).

Monogenic causes

X-linked mental retardation (XLMR) is a common cause of monogenic intellectual disability, because most of genes causing ID/MR are found on the X chromosome. X-linked forms of mental retardation are estimated to cause 10-20% of all inherited cases of ID/MR. There is a higher prevalence of ID/MR among males relative to females (1.8 in 1000 males; carrier females 2.4 in 1000). However, female carriers may manifest mild symptoms, due to a skewed X-inactivation (25).

Based on symptoms' presentation, XLMR can be divided into three groups: 1) syndromes - characterized by multiple congenital anomalies (phenotypic dysmorphy, organ anomalies); 2) neuromuscular disorders - epilepsy, dystonia, spasticity, muscle weakness and so on without malformations and 3) nonspecific conditions (MRX) – isolated ID/MR is the only clinical manifestation. There are 215 XLMR conditions divided according to their clinical presentation: 149 with specific clinical findings, including 98 syndromes and 51 neuromuscular conditions, and 66 nonspecific forms (26).

Fragile X syndrome (FRAXA, OMIM 309550) is the most common form of syndromic XLMR (20 % of all XLMR cases), with a prevalence of approximately 1:5000 males, and causes intellectual disability in about 1 in 8000 females (27). Affected individuals have a folate-sensitive fragile site in the region Xq27.3, associated with an expansion of a trinucleotide repeat (CGG) in the 5'-noncoding region of a gene that encodes an RNA binding protein termed FMR1. Individuals with fragile X syndrome have a loss-of-function variant of FMR1 caused by an increased number of CGG trinucleotide repeats (typically >200) accompanied by aberrant CpG methylation of FMR1 (28).

Another common gene is MECP2 (methyl CpG binding protein 2 (OMIM 300005) on chromosome Xq28, which causes Rett syndrome, affecting approximately 1 in every 10,000–15,000 females worldwide (29). But it is also identified in the clinical spectrum seen in males with severe neonatal-onset encephalopathy or with X-linked intellectual disability associated with psychosis, pyramidal signs, parkinsonian features and macro-orchidism (PPM-X syndrome; OMIM 300055) (30).

Evaluation and Testing

The clinical geneticist has an important role in the evaluation of patients with intellectual disability and in making decisions about further genetic testing. Evaluation includes physical examination and the collection of family history information. The physical exam should focus on dysmorphological and neurological evaluation, congenital malformations, somatometric measurements and behavioral evaluations. In all patients with neurological symptoms, such as epilepsy and macro/microcephaly, neuroimaging studies - MRI (magnetic resonance imaging) should be performed for evaluation of brain malformations. If there are signs of metabolic disease, metabolic tests should be done (organic acid in urine, amino acids in serum, lactate, pyruvate) (31).

When investigating a patient with ID/MR, with or without dysmorphic features, the initial analysis several years ago usually began with cytogenetic testing (GTG-banding).

GTG banding (G-banding with Trypsin/Giemsa) is used for the detection of aneuploidy (abnormal number of chromosomes) and the identification of structural aberrations: deletions and translocations in chromosomal rearrangements only larger than 5–10 Mb. The overall yield of routine cytogenetic testing is 3.7 % (32).

Fluorescent in situ hybridization (FISH), using location specific probes, detects

submicroscopic alterations less than 5 Mb that cannot be observed using standard cytogenetic tests (GTG-banding). Today this method is used when a specific syndrome is suspected with high frequency in the general population (e.g., DiGeorge/velocardiofacial syndrome, Williams Beuren syndrome). The yield of FISH screening on patients with moderate to severe ID/MR is 6.8 % (33).

Candidates for subtelomere screening are patients with ID/MR and two or more dysmorphic features (mostly facial), congenital organ abnormalities, skeletal abnormalities, positive family history and pre/postnatal poor growth/overgrowth (34).

Several assays are currently available to detect subtelomeric rearrangements, but subtelomeric FISH and subtelomeric MLPA have been the most frequently used. MLPA results needed to be confirmed using other more accurate techniques such as FISH or aCGH (35).

With the introduction of comparative genomic hybridization on microarrays it is possible to screen the entire genome for evaluation of deletions and duplications of specific DNA sequences. Comparative genomic hybridization on microarrays (Array Comparative Genomic Hybridization - aCGH) and the technical basis of the method was first published in 1997 (36). Detection of subtle submicroscopic changes in a number of copies of DNA less than 1Mb is possible using different platforms. With the application of aCGH in patients with ID/MR it is possible to determine etiology in 20% of patients with normal karyotype and subtelomere screening with MLPA (36).

Copy number variations (CNVs) are the most prevalent structural variations in the human genome, which appear largely in the subtelomeric regions and can be detected by aCGH. ID/MR is associated with variable sizes of CNVs (18).

A disadvantage of the aCGH is that the identification of de novo CNVs of uncertain significance and unreported CNVs can be challenging to interpret. CNVs should be listed as benign or pathogenic, or reported as variants of unknown clinical significance (37). Pathogenic variants are detected in 15 % to 20 % of ID/MR patients (37, 38). Not all CNVs are fully penetrated or cause a spectrum of phenotypes, including intellectual disability, autism, schizophrenia, and dimorphisms. Such CNVs can pose challenges to genetic counseling. More variants of uncertain significance are found with higher density arrays (38). Sometimes, variants of unknown significance can be resolved by trio testing (mother, father and proband). Interpretation of those variants is very comprehensive and challenging, and demands bioinformatics and clinical knowledge.

Next-generation sequencing (NGS) is DNA sequencing technology that sequences all genes in one genetic test. Exome sequencing analyzes all exons of protein coding genes in the genome known as a cause of the diseases (clinical exome sequencing) or all of the genes in the genome (whole genome sequencing). NGS in a clinical setting opens up possibilities for discovering the genetic contribution for a large percentage of ID/MR individuals at the first onset of symptoms and the possible opening up of pathways for therapeutic interventions (37). NGS is progressively being set up in clinical laboratories for the diagnosis of ID/MR because of a higher diagnostic yield and devaluation in costs. Many studies revealed the usefulness of using an integrative approach to examine genotype-phenotype variability (37, 39, 40).

Whole exome sequencing (WES) is an impressive tool for identifying clinically undefined forms of ID/MR, especially when aCGH identified a de novo CNV of uncertain significance (37).

The vast majority of benign variants are single base pair substitutions. With better coverage depth, WES is adequate for the detection for close to all (99.7 %) pathogenic variants (41). CNV analysis is still an active area of research in NGS variant analysis and has long been important in ID research. CGH microarrays can only detect unbalanced structural variants, while apparently balanced chromosomal rearrangements occur in 1.54 % of live births and contribute to 6 % of abnormal phenotypes including ID (42). Whole

Table 2. *Advantages and disadvantages of genetic methods for diagnosing intellectual disability*

Method	Advantages	Disadvantages
GTG (G banding with Trypsin and Giemsa)	Whole genome analysis Detection of unbalanced and apparently balanced chromosomal rearrangements	Time consuming Small resolution (5 to 10 Mb)
FISH (fluorescent in situ hybridization)	Detection of unbalanced and apparently balanced chromosomal rearrangements and mosaicism Detection of small deletions and duplications	Time consuming Small resolution (depend on the size of FISH probe, 30 to 100 kb)
MLPA (multiplex ligation probe amplification)	High-throughput Simultaneously analyses of several samples Multiplex technique (study of several regions of the human genome in a single reaction) Low cost	Not whole genome analysis Sensitive to PCR inhibitors
aCGH (microarray comparative genomic hybridization)	Whole genome analysis High resolution (up to 40 kb)	Impossibility of detection of apparently balanced chromosomal rearrangements and mosaicism CNVs of unknown significance in clinic
NGS (next generation sequencing)	Whole genome analysis High resolution (covering all coding variation) Single strand sequencing	CNVs of unknown significance in clinic Expensive

genome sequencing (WGS) has the potential to uncover all forms of genetic variation in one test, and offers a higher diagnostic yield (43). In the study of Harripaul et al. of patients with severe ID, a diagnostic yield of 42 % was observed (42) which is a significant improvement over the diagnostic yield obtained by microarray, gene panels or WES (44). A summary of genetic methods used in diagnosing ID/MR is presented in Table 2.

Discussion

Defining the cause of intellectual disability/mental retardation (ID/MR) presents a diagnostic challenge. Mental retardation is present in about 1 % to 3 % of individuals in the general population, but there are many cases that cannot be explained despite novel technology and clinical investigations (24). Genetic factors are involved in many of the idiopathic cases of ID/MR. This conclusion is based on the fact that these patients often show signs such as dysmorphic features, growth retardation and malformations, or have a family history of mental retardation (6,7).

The genetic heterogeneity of intellectual disability requires genome wide approaches, including the detection of chromosomal aberrations by chromosomal microarrays and whole exome sequencing adequate for discovering single gene mutations (45).

For individuals with idiopathic ID/MR, autism spectrum disorders, or multiple congenital anomalies, chromosomal microarray analysis (CMA) is recommended as the first-line diagnostic test since it offers a much higher diagnostic yield (15 % to 20 %) compared with G-banded karyotype analysis (3 %) (38,42).

Despite those modern technologies, the genetic etiology of 80 % to 85 % of patients still remains unknown. NGS-based testing (targeted

multigene panels, whole exome sequencing or whole genome sequencing) for these cases, has a great potential to obtain diagnosis (44).

The vast majority of individuals with ID/MR currently receive no molecular diagnosis, which is a shortcoming that significantly impacts health and life span. There is also a strongly negative correlation of survival with the severity of ID (46). It is important to emphasize that knowing which genes carry mutations that cause ID/MR can have huge benefits for diagnosis in clinics, and can lead to better understanding of each patient's health issues, more appropriate care and treatment, improved overall health and life span, and appropriate counseling and planning for families.

Abbreviations

ID/MR - intellectual disability/mental retardation; GTG- G-banding with Trypsin/Giemsa; FISH - Fluorescent in situ hybridization; MLPA-Multiplex Ligation dependant Probe Amplification; aCGH - Array Comparative Genomic Hybridization; NGS – Next generation sequencing; CNVs - copy number variations; XLMR – X linked mental retardation; XLID – X linked intellectual disability; IQ- intelligence quotient test; FRAXA-fragile X syndrome; MRI - Magnetic resonance imaging; CA - congenital anomalies.

Funding

This work was supported by the scientific project VIF2015-MEFOS-11 'Etiology of mental retardation in pediatric population' Faculty of Medicine, J. J. Strossmayer University in Osijek

Transparency declaration

Competing interests: None to declare

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