Thyroid Autoimmunity and Infertility

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Abstract

Autoimmune thyroid disease (AITD) is one of the most common endocrinopathies and is more prevalent in women. The circulating thyroid antibodies and the hypothyroidism that often follows AITD have effects on many tissues. The endometrium and ovaries are not spared, and therefore this common morbidity might have an impact on fertility. Despite challenging data interpretation and contradictory results, a general takeaway from published studies is that there is a higher incidence of elevated levels of thyroid-stimulating hormone (TSH) and the presence of thyroid antibodies among infertile women. While a single specific and direct pathophysiological mechanism through which autoimmune thyroid disease causes infertility has not been identified, there are multiple gynecological comorbidities that might perpetuate infertility (endometriosis, premature ovarian failure, polycystic ovaries) and defective immunological functions (a shift to a proinflammatory Th1 response, increased levels of natural killer cells, cross-reactivity of antigens, etc.) that are affecting fertility. There is insufficient evidence suggesting that levothyroxine (LT4) treatment can help women suffering from AITD conceive and carry out a pregnancy.

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Introduction

The thyroid hormone plays an essential role in the growth, differentiation and development of virtually all tissues. It also impacts numerous metabolic pathways, making it a significant factor in maintaining metabolic homeostasis. The two forms of thyroid hormone, triiodothyronine (T3), which is more potent, and thyroxine (T4), which is more abundant, can have effects on many different cellular components (1). However, they mostly regulate the transcription of target genes by binding to their specific intranuclear receptors, found in several different isoforms (1, 2). Thyroid hormones also affect the female reproductive system, which is why dysfunction of the thyroid gland can subsequently lead to disturbances of the menstrual cycle and fertility issues (2).

Thyroid dysfunction, which leads to changes in circulating thyroid hormone levels, manifests itself as either hyperthyroidism or hypothyroidism. Hypothyroidism is the more prevalent variant and is linked to thyroid autoimmunity. Autoimmune thyroid disease (AITD) is the most prevalent cause of hypothyroidism in women of reproductive age (3, 4). Elevated levels of thyroid autoantibodies, such as thyroid peroxidase autoantibodies (TPO-

Ab) and thyroglobulin autoantibodies (TG-Ab) induce chronic inflammation in the thyroid gland, which leads to the loss of functional tissue. Hypothyroidism can manifest itself with typical signs and symptoms such as fatigue, constipation, depression, thinning hair, cold intolerance, bradycardia, hoarseness, etc. It can also be subclinical (SCH), which is classically defined as an increase in thyroid-stimulating hormone (TSH) concentrations over the upper limit of normal range (4.5 – 5.0 mIU/L) with normal fT4 levels (5).

Infertility is defined as an inability to conceive after at least 12 months of continuous intercourse without the use of contraception. Its current prevalence among couples ranges between 10 – 15% and has not changed significantly over the past few decades (6 - 8). Because of the effects thyroid hormone has on the ovaries (e.g., maturation of oocytes) and endometrial tissue, it is believed that AITD might play a role in the pathogenesis of infertility. Therefore, this review aims to interpret data collected on this subject thus far and summarize published studies to assess the association between AITD and infertility.

Pathophysiological links between autoimmune thyroid disease and infertility

Since AITD is the most common endocrine disorder in women of reproductive age, its effects on fertility have been investigated extensively and in detail. Nevertheless, a clear pathophysiological link connecting the two morbidities has not been identified and available data suggesting the prevalence of women with both AITD and infertility varies from study to study. Furthermore, interpretation of data from published studies on this subject matter is challenging due to several reasons, mostly relating to study designs and their flaws — retrospective studies providing incomplete information, small sample sizes, measurements of different thyroid antibodies, different populations investigated (heterogeneity of ethnicities and geographical locations), etc. Variations in SCH definitions and TSH ranges additionally contribute to the complexity of data interpretation.

The role of thyroid hormones

There is a clear connection between the thyroid gland and its hormones and the female reproductive system. Thyroid hormone receptors are expressed in the ovary and the endometrium. Furthermore, following egg fertilization, the thyroid hormone plays a role in the process of implantation and placentation (9). Generally, the proposed pathophysiological mechanisms contributing to infertility in women with AITD are either thyroid-dependent or thyroid-independent. Multiple studies have been conducted that aimed to establish the incidence of AITD in infertile women, as well as to connect AITD with certain morbidities affecting the female reproductive system. When observing the data collected and conclusions reached in each of those studies, it can be said that there is a significantly increased incidence of AITD in infertile women compared to controls (10 – 14). In a Danish study (10), conducted on 11,254 women, the conclusion reached was that higher TSH levels and higher TPO-Ab levels affect fertility. A study by Poppe et al. (11) demonstrated significantly higher levels of TSH in infertile subjects compared to controls (1.3 vs 1.1 mIU/L), however, TSH levels above or below the normal range were not more prevalent in infertile subjects (with fT4 levels within the normal range), indicating that women suffering from AITD might be affected by fertility issues even though they might seemingly be in a euthyroid state. In addition, women with AITD who are able to conceive are not without risk during pregnancy — a recent study among 10,990 patients, part of the FASTER (First and Second Trimester Evaluation of Risk) trial, shined a light on the connection between thyroid autoimmunity and pregnancy risks. It showed that there was an increased risk for preterm premature rupture of membranes when both TPO-Abs and TG-Abs were present in both the first and second trimester (15). On the other hand, there have been studies with different results and outcomes. A study by Abalovich et al. (16) observed a higher prevalence of SCH, but not

AITD, in 244 subjects consulting on infertility, providing an example of contradictory data making the interpretation difficult, as mentioned earlier. Another example is a study by Plowden et al. (17), part of the EAGeR (Effects of Aspirin in Gestation and Reproduction) trial. They found that there was no difference in the delay of pregnancy in women with TSH of at least 2.5 mIU/L or women with thyroid autoantibodies, compared with those with TSH under 2.5 mIU/L or without autoantibodies.

Couples undergoing ART (assisted reproductive technologies) treatments are a group of interest in this context. In a systematic review and meta-analysis of articles describing ART outcomes in the context of AITD, Busnelli et al. (18) concluded that AITD does not impact the outcome of ART treatments (IVF, ICSI) in terms of the number of oocytes retrieved and the likelihood of fertilization, implantation and clinical pregnancy. However, a wider implication similar to the one in the FASTER trial was made, that the presence of thyroid autoantibodies might have harmful effects on the course of the pregnancy, with an increased risk of miscarriage and decreased chance of live birth.

Poppe et al. also showed that elevated levels of TPO-Abs were higher in infertile subjects, while the highest prevalence of positive antibodies among all subgroups of infertility was observed with endometriosis (11). It is a benign condition also linked to altered immune conditions, where endometrial tissue appears outside of the uterus, most commonly in the pelvis, inducing a chronic inflammation (19). Endometriosis in the context of AITD has been investigated, but the results have been contradictory. While the study by Kim et al. and two other studies (16, 20) reported a higher prevalence of endometriosis in women with AITD versus controls, a study by Petta et al. (21) showed that the prevalence of AITD was similar in a group of subjects with endometriosis and in a control group. A more recent study identified anti-laminin-111 autoantibodies as having a potentially major role in the pathogenesis of endometriosis-associated infertility (22). Outside of the endometrium, research has been conducted that showed that the source of fertility issues among women with AITD might be the ovary. Monteleone et al. (23) hypothesized that the critical source of female infertility due to AITD was in the ovarian follicle itself. Their study showed that TG-Abs and TPO-Abs were measurable in all samples of follicular fluid and serum drawn from subjects with AITD, while they were absent in controls. Furthermore, all subjects with AITD were in a euthyroid state, which suggested that the absence of progression of thyroid hormone status towards hypothyroidism due to AITD does not exclude issues with fertility. Expanding on that theory, Kelkar, et al. (24) demonstrated that human anti-zona pellucida antigens reacted to murine thyroid tissue, therefore suggesting their similarity in antigens, which suggests, in turn, that zona pellucida may be affected by thyroid autoantibodies. A very common ovarian disorder that may be connected to AITD is polycystic ovary syndrome (PCOS). Thought to be one of the most common endocrinopathies in women (6.5 – 8 %), it is a complex disease that is multifactorial in its etiology (genetics, obesity, sedentary lifestyle, intrauterine androgen exposure, etc.) It is characterized by hyperandrogenism, increased LH-to-FSH ratio, poor glucose tolerance and hyperinsulinemia and over long-term, it can cause infertility and carries an increased risk for cardiovascular diseases, malignancies, type 2 diabetes mellitus and psychiatric disorders (25). A threefold higher prevalence of AITD in subjects with PCOS was demonstrated in a study on 175 subjects, compared to 168 controls, which was, according to the authors of the study, partly correlated with an increased estrogen-to-progesterone ratio (26). Further showing that the ovary is a key organ when investigating infertility in women with AITD, in a group of 244 subjects consulting on infertility, AITD prevalence reached statistical significance only in those with premature ovarian failure, which the authors believed to be the result of a shared autoimmune etiology between the two morbidities (16).

Thyroid hormone - independent immunological mechanisms

Independently of the thyroid, multiple immunological mechanisms have been described as potential contributors to impaired fertility and fecundity in individuals with AITD. A dominant Th1 immune response promotes inflammation evoked through cell-mediated mechanisms, which is harmful for pregnancy. Th2 cell subsets, on the other hand, regulate and control the inflammation and tissue injury implemented by Th1 reactions, as well as protect against autoimmune damage (27). The regulatory mechanisms and balance of Th1 and Th2 cells are impaired in women with fertility and fecundity issues, with multiple studies confirming a distinct Th1 bias (28, 29), even in the endometrial tissue itself (30). A study by Kwak-Kim et al. (30) showed significantly higher Th1/Th2 ratios of TNF-alfa/IL-4 and TNF-alfa/IL-10 in both women with recurrent pregnancy losses and women with multiple implantation failures, while IFN-gama/IL-4, IFN-gama/IL-10 were additionally higher in women with recurrent pregnancy losses. Links between impaired T-cell immunity and thyroid autoimmunity have recently been made. TNF-alfa/IL-10 T-cell ratios were significantly increased in women with AITD (12), thus showing that thyroid antibodies can serve as markers for abnormal immunity, which is an important factor in impaired fertility. When observing abnormal B-cell function and its potential contribution to infertility in women with AITD, a clear connection between the presence of certain non-organ specific antibodies (NOSAs) and infertile women with AITD has not been proven yet. Kim et al. (12) reported that women with AITD did not have a higher prevalence of antiphospholipid antibodies (APAs), but did have a higher prevalence of NOSAs than women with no thyroid antibodies. One study suggested that NOSAs, in addition to TPO-Abs and TG-Abs, may serve as independent risk markers for repeated pregnancy loss in women with AITD, but no such statement was made for unexplained infertility (31). Aggravation of infertility in women with concurrent thyroid autoimmunity and other systemic autoimmune diseases has also been described, specifically systemic lupus erythematosus (SLE) and Sjogren's disease (32, 33). In addition to effects T-cells and B-cells might have on the reproductive system, hyperactive and overproduced natural killer (NK) cells can also infiltrate the endometrial tissue and potentially alter the body's immune response, thus affecting fertility (34). TSH has been shown to have a stimulatory effect on NK cells (35) and studies have described increased levels and cytotoxicity of NK cells in AITD (12, 36 - 38). Therefore, NK cells are an additional potential contributor in the pathogenesis of infertility in women with AITD.

Autoimmune thyroid disease, infertility and nutritional deficiencies

Yet another contributor linked to both AITD and infertility is vitamin D deficiency. As an omnipresent protein, vitamin D plays a key role in the calcium and phosphate metabolism. It has also been identified as a beneficial factor in many morbidities, including autoimmune diseases, cardiovascular diseases and malignancies (39). Vitamin D deficiency (< 10 ng/ml), on the other hand, is, among other things, linked to both AITD and infertility. Kivity et al. (40) found that vitamin D deficiency was significantly more prevalent in subjects with AITD than in controls. Furthermore, vitamin D deficiency correlated to the presence of thyroid antibodies and abnormal thyroid function. In the context of fertility, vitamin D has been identified as mandatory for reproductive function in the murine model (41). Vitamin D deficient rats demonstrate, among other behavioral patterns and physiological changes affecting their reproductive systems, a diminished fertility capacity (42, 43). In humans, the vitamin D receptor (VDR) is expressed in the ovary, the endometrium and even the placenta. Furthermore, vitamin D is involved in better IVF outcomes, it plays a role in PCOS (deficiency is linked to obesity and metabolic morbidities) and it also affects the regularity of menstrual cycles and influences the production of sex hormones (44). With all that in mind, it is clear how vitamin D deficiency can contribute to both AITD and infertility.

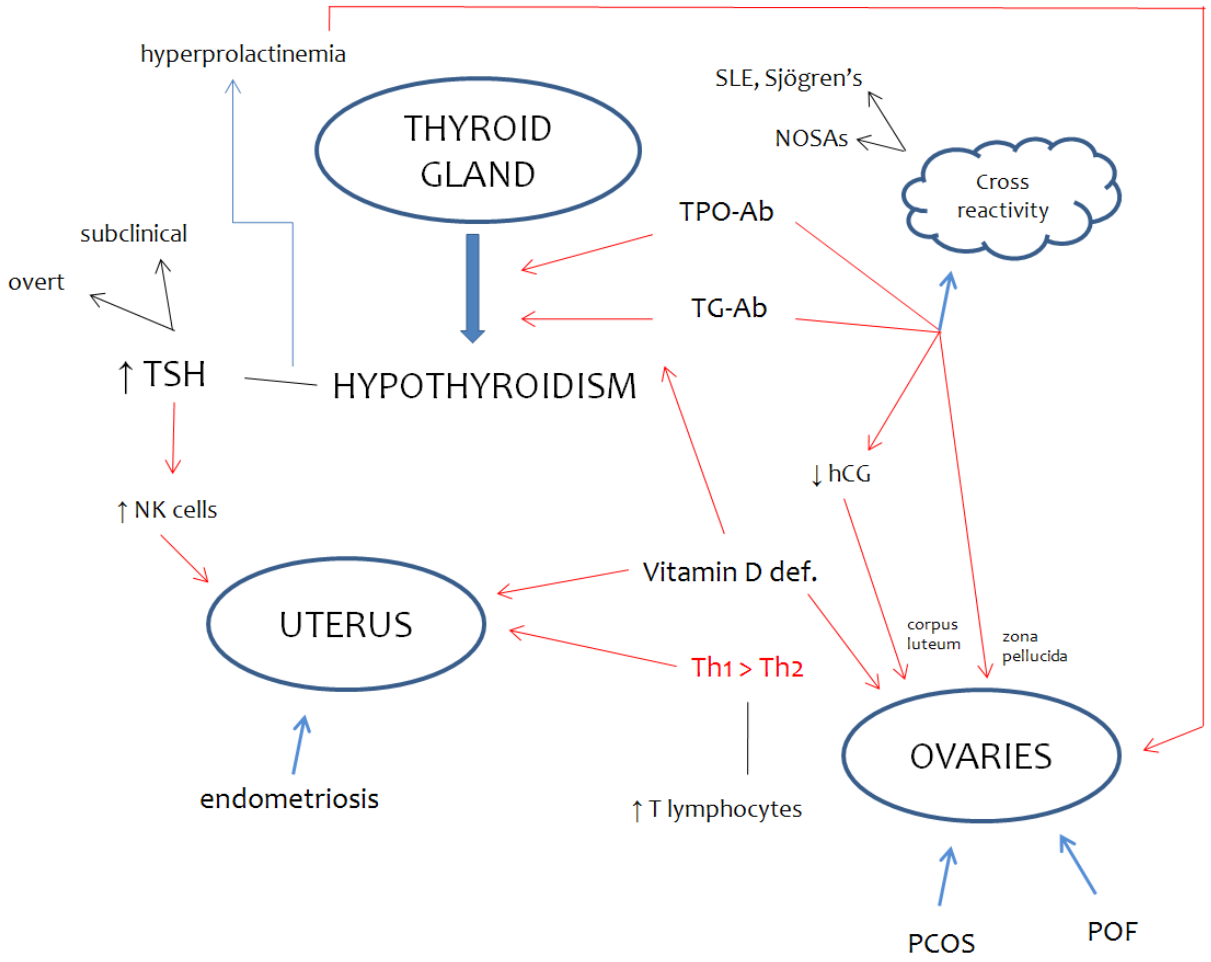
There are numerous other microelements and vitamins that play a role in the physiology of thyroid function, so their deficiency, overload or perhaps impaired functionality might also play a role in the development of thyroid autoimmunity and be linked to infertility. These effects haven't been extensively researched, but we know that microelements such as magnesium, iodine, selenium and zinc, as well as other molecules such as riboflavin, vitamin C and coenzyme Q10 have been associated with different forms of thyroid disease. Magnesium deficiency is the basis of mitochondrial dysfunction which can explain changes associated with thyroid dysfunction (45). An iodine overload increases the risk for an immune reaction, which additionally increases with selenium (Se) deficiency (46). Se is a necessary trace mineral because of its anti-inflammatory and antioxidant properties. While a study by Moncayo et al. (47) did not show a correlation between Se deficiency and thyroid autoantibodies, there have been several studies that showed lower Se levels in patients with Hashimoto thyroiditis (48, 49). Se levels were significantly decreased in patients with subacute or silent thyroiditis (47). However, Se supplementation during pregnancy and postpartum reduces inflammation in the thyroid and lowers the risk of hypothyroidism (50), while it decreases autoantibody levels and improves the ultrasound structure of the thyroid in patients with Hashimoto's thyroiditis (51). Antioxidant enzymes also depend on the availability of copper and zinc. A study by Stolinska et al. (52) did not confirm, however, that zinc supplementation in patients with normal zinc levels can affect thyroid metabolism. While riboflavin, vitamin C and coenzyme Q10 have been researched in the context of thyroid dysfunction, there is insufficient data to show a link between deficiency, overload or dysfunction of those antioxidants with AITD, especially in correlation with infertility (53, 54).

Hypothyroidism has also been associated with hyperprolactinemia - elevated levels of thyrotropin-releasing hormone (TRH) and TSH cause increased secretion of prolactin. Studies have shown that women of fertile age are most commonly affected by hyperprolactinemia as well as its presence in SCH, showing yet another consequence of this silent morbidity (55, 56). It can also impair fertility, since elevated prolactine levels and the pulsatile GnRH secretion may lead to a delayed LH response and affect corpus luteum function (56 – 58).

Finally, thyroid antibodies could also inhibit the activity of human chorionic gonadotropin (hCG) on the corpus luteum due to cross-reactivity that has been observed between hCG and TSH. The corpus luteum plays a key role in supporting and maintaining a pregnancy in the first trimester through progesterone and estrogen secretion and is largely dependable on hCG. This pathophysiological mechanism doesn't cause infertility per se, but it may impair fecundity, since TSH receptor blocking antibodies could also block luteinizing hormone (LH) and hCG receptors, causing a decrease in steroid hormone production, resulting in spontaneous miscarriages (59).

The American Thyroid Association published Guidelines for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum in 2017. They stated that evaluation of serum TSH concentration is recommended for all women seeking care for infertility. Additionally, they recommended levothyroxine (LT4) treatment for infertile women with overt hypothyroidism who desire pregnancy. However, no such recommendation was made for women suffering from AITD and euthyroid women due to insufficient evidence to determine whether or not LT4 therapy actually improves fertility in such cases. The only case where administration of low-dose LT4 was to be considered was in infertile women suffering from SCH, but were thyroid antibody-negative (60). There are several examples of conflicting data and evidence that is insufficiently strong to suggest LT4 therapy improves the chances of achieving pregnancy. Negro et al. (61) investigated the effects of LT4 treatment in TPO-Ab positive women undergoing assisted reproduction technologies (ART) and found that the pregnancy rate was not affected by treatment with LT4. In the study by Abalovich et

Figure 1. *Clinical entities and pathophysiological mechanisms linking female infertility with AITD. (TSH – thyroid-stimulating hormone; SLE – systemic lupus erythematosus; NOSAs – non-organ specific antibodies; hCG – human chorionic gonadotropin; NK – natural killer; PCOS – polycystic ovary syndrome; POF – premature ovarian failure)*



al (16), however, LT4 was prescribed to 34 subjects with SCH and after 6 months of follow-up, 44.1% achieved pregnancy.

Conclusion

AITD and infertility are two morbidities that have separately been widely investigated and detailed pathophysiological mechanisms for both entities have been described. However, there is a wide variety of overlapping factors that make it difficult to distinguish how and if AITD and infertility are interconnected. All the factors described in this review (abnormal lymphocyte production, a Th1 immune response bias, vitamin D deficiency, etc.) (Figure 1.) could be contributing to infertility as well as AITD to some extent, but the possibility remains that one or a few of them might have a more significant impact on the pathogenesis. Conflicting data and different study designs make shedding light on the interconnectedness of these morbidities difficult. There is even no consensus on the exact organ of origin of infertility caused by thyroid autoimmunity, with the uterus and the ovary both potentially playing roles and being targeted by the thyroid antibodies.

Based on the findings of the studies, there is still an insufficient amount of evidence that would suggest levothyroxine treatment is a viable treatment option for infertile women suffering from AITD. Recommendations from 2015 have only been made for infertile women suffering from SCH, but who are thyroid antibody-negative. Even in those cases, however, LT4 treatment should simply be "considered". That is

why it is crucial to continue researching these two entities to potentially increase the chances for women to conceive and carry out a healthy pregnancy.

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Coital urinary incontinence and female sexual function

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Abstract

Urinary incontinence (UI) is an everyday problem among a large proportion of adult women. The prevalence of UI ranges between 15% and 25% and the rate of incidence for each type of UI ranges between 10% and 58%. Because women are concerned about UI during sexual intercourse, it has a negative effect on female sexual health. The incidence of UI during sexual intercourse in incontinent women has been reported to range between 10% and 27%. The prevalence of female sexual dysfunction is estimated to be 43%. There are conservative and surgical methods of treating UI but the number of published scientific articles dealing with the assessment of the effects of these types of therapy on improving sexual health in women treated for UI is deficient.

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Introduction

The International Continence Society (ICS) terminology for lower urinary tract dysfunction (LUTD) from 2003, which can be divided into storage symptoms and voiding symptoms, defines urinary incontinence (UI) as a storage symptom and as the complaint of any involuntary loss of urine (1). UI can also be defined as the inability to control urination which is manifested in the range from temporary leakage of urine to complete involuntary voiding which can be seen as a social and hygienic problem (2, 3). The anatomical structures of the female reproductive and urinary system are closely related, which leads to the conclusion that urinary problems interfere with sexual health in females (4). According to the current World Health Organization definition, sexual health is a state of physical, emotional, mental and social well-being in relation to sexuality, it is not merely the absence of disease, dysfunction or infirmity (5). The normal male sexual response cycle can be divided into libido, erection, ejaculation, orgasm and detumescence (6). Sexual response cycle in women can be divided into libido, arousal, orgasm and satisfaction (7).

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Damage of the integrity of the sexual response cycle, which is essential to human sexual functioning, may result in sexual dysfunction (8). Female sexual dysfunction has been characterized as a persistent, recurrent problem with sexual response, sexual desire, arousal, orgasm or dyspareunia and vaginismus that distresses the affected or strains the relationship with their partner (9). As reported by the International Urogynecological Association and the International Continence Society in 2010, incontinence during sexual intercourse or coital urinary incontinence (CUI) is defined as the complaint of involuntary loss of urine during coitus (10). The objective of this study was to review the available evidence on incidence, prevalence, pathophysiology and treatment of UI and coital urinary incontinence (CUI) with the attempt to determine the impact of UI and its physical and psychological consequences on women's sexual health and function in order to advise in a timely manner, prevent and treat disorders associated with bladder control issues.

Urinary incontinence – definition, etiopathogenesis, epidemiology

Women seldom visit general practitioners regarding incontinence problems which occur during or independently of the sex act, being that they are not familiar with the possibilities of treatment and mainly because a lot of women are ashamed to admit their "condition" unless they are precisely inquired by specialists or asked to "fill out" related questionnaires (11, 12). UI has a negative impact on sexual health irrespective of whether or not unwanted urine leakage occurs during sexual intercourse (4). UI products (e.g. incontinence pads, diapers) keep women dry but wearing them all day can cause chronic skin changes of the genital region which leads to discomfort and pain especially throughout sexual intercourse (13). Furthermore, worrying about urinary leakage and odor during intercourse can cause feelings of anger, sadness, embarrassment, despair and low self-esteem. Consequently, women abstain from having intercourse, which can cause reduction in the frequency of sexual relations, reduction of sexual desire and ability to achieve an orgasm. What's more, men no longer find their partner sexually attractive and some men even experience erectile dysfunction (14, 15). The pathophysiology and frequency of CUI and impact of CUI on quality of life are still not conclusive because there is limited research data. UI is not a disease but a symptom that is caused by disorders of pelvic floor muscles, the urethral sphincter and bladder. UI is most commonly divided into 3 types: stress urinary incontinence (SUI) which is most prevalent (51%), urge urinary incontinence (UUI) (10%) and mixed urinary incontinence (MUI) (39%) (16). In women with SUI, involuntary urine leakage occurs during coughing, laughing, sneezing and other physical activities (e.g. exercise, lifting heavy objects, sexual intercourse). UUI is associated with an overwhelming urge to urinate that cannot be suppressed or delayed and is caused by detrusor overactivity. MUI is defined as the involuntary leakage of urine accompanied by a sense of urgency during physical exertion, exercise, sneezing or coughing. Urine loss during sexual activity may occur during arousal, penetration, orgasm, or resolution. Current research suggests that in women with SUI episodes of urine leakage are more likely to occur with penetration due to pressure on the bladder but in the case of UUI urine is more likely to leak during orgasm (17). In this fashion, we can determine two types of CUI: incontinence during penetration and incontinence at orgasm (17). Risk factors for the occurrence of UI are sex, age, cigarette smoking and high body mass index (BMI) (18). UI is three times more common in women than in men regardless of the type (19). The prevalence of UI increases with age and in women between 15 and 60, it ranges from 10 to 25% (20). In older age groups, the number of incontinent women is higher although the vast majority of older women have acceptable control over their urination. Among women aged 60 and over, prevalence of UI is 38% (21). The prevalence of female sexual dysfunction is estimated to be 43% and it increases with age (22). The incidence for each type of UI in females ranges between 10% and 58% depending on the observed population. It is generally accepted that the etiology of UI in women is associated with vaginal delivery, especially the first vaginal

birth, menopause and surgical procedures in the pelvis and abdomen (23). The particular obstetric event that causes incontinence has not been found but it is most likely associated with newborns with an excessive birth weight and difficult deliveries marked by prolonged pushing phases with or without instrumentation, which lead to nerve and muscle damage that provide a physiologic basis for this association (24). It is important to note that not all women who have had vaginal delivery experience symptoms of UI but they have more bothersome symptoms than women who have never had children. Hormone changes in menopause and surgical procedures can also affect muscle strength in the pelvic region. Cigarette smoking can induce chronic obstructive pulmonary disease with chronic cough that in time weakens the lower pelvic floor muscles and causes symptoms of SUI but on top of that, ingredients of cigarette smoke can cause urinary bladder mucous membrane irritation leading to urgency and UUI (25, 26). High BMI is a strong independent risk factor for UI and the mechanism of the onset of the disorder is that excessive body weight leads to an increase in intra-abdominal pressure which causes increased intravesical pressure, expanded mobility of the bladder neck and urethra, and also causes instability of the bladder detrusor, all leading to involuntary urine leakage (27). Menopause and partner status are important predictors for female sexual dysfunction (28).

Interrelation of urinary incontinence, emotional, mental and sexual health

UI has a negative impact on the quality of life of women, emotional health, physical and mental condition, impairs relationships, affects careers, and is also an additional financial burden (29, 30, 31). All incontinence types are associated with low self-esteem and a higher probability of psychiatric disease (32). The studies show higher levels of anxiety and psychological stress in women with UI (33). Nowadays women lead active lives and are trying to maintain a healthy body condition and normal sex life regardless of age. UI has been recognized as one of the predictors of female sexual dysfunction considering excessive acetylcholine release on the bladder during intercourse, increased intra-abdominal pressure with alteration of the urethrovesical angle and elevation of the bladder neck during intercourse, because of the fact that squamous epithelium of the trigone and urethra becomes thin and blood flow decreases with reducing estrogen in older postmenopausal women and the function of the main pelvic floor muscles deteriorates with age (34, 35). Urine leakage during orgasm may be caused by involuntary detrusor contraction and relaxation of the urethra (36). Involuntary urine leakage during sex is mainly a female problem because when a man has an erection the internal sphincter at the base of his bladder closes so urine can't pass into urethra (37, 38). Between 25 and 50% of women with UI experience decreased libido and decreased frequency of sexual activity because of the shame and fear of incontinence (39, 40). The incidence of UI during sexual intercourse in incontinent women has been reported to range between 10% and 27% (41). Recent studies reported high prevalence of CUI with results between 60% and 67% (42). The Pelvic Organ Prolapse/Urinary Incontinence Sexual Questionnaire (PISQ) and its short form version PISQ-12 are validated condition-specific female sexual function questionnaires purposively developed to assess sexual function in women with UI and/or pelvic organ prolapse (43, 44). Patients with a MUI diagnosis had significantly lower mean PISQ-12 scores than the ones with SUI and UUI, and patients with SUI had significantly lower PISQ-12 scores than those with UUI (45). On the other hand, one study showed that among sexually active women with UI, sexual function as assessed by the PISQ-12 does not differ according to type of incontinence (46). The result of one study was similar to most other research and actually established that the rate of overall female sexual dysfunction in women with UUI was higher than in those with SUI, that CUI was more frequent in women with UUI than in those with SUI, but that feelings of pain and impressions of more involuntary urine leakage during sexual intercourse were more common in women with SUI than in those with UUI (40). Some researchers have found that CUI was present to a greater degree in young

women than in elderly women and more frequent in women younger than 60 years of age (40, 47). In one study, 80% of women had incontinence during penetration, 93% had incontinence at orgasm and 92% had incontinence in both cases, indicating CUI as a common symptom during sexual activity in women with SUI and suggesting urethral dysfunction as the possible explanation of CUI (11). Maximal urethral closure pressure < 30 cmH2O was associated with CUI pointing out that urethral function plays a vital role in maintaining continence during coitus (48). In another report, 46% of women had UUI which led to the termination of sexual activity without orgasm and the rate of female sexual dysfunction was detected to be higher in women with UUI compared to that in the general population (49). Gynecologists and urologists need to pay more attention to sexual dysfunction in women with UI in their clinical practice. Additionally, one important thing to take into account is detailed medical history and sexual history of the occurring disorders during intercourse.

Treatment options

Once inflammation or anatomical disorders of the urinary system, neurological disease or cancer are ruled out, the following conservative and surgical treatment methods are available, most of which are scientifically proven with a high level of evidence and explained in the 2017 European Association of Urology guidelines: open communication with one's sexual partner, counseling with a therapist who specializes in sexual and relationship problems, bladder training, urinating prior to sexual intercourse, deferring intercourse, interrupting intercourse prematurely, avoiding certain positions, hurrying through sex, avoiding orgasm, regular physical activity and weight loss, smoking cessation, exercises to strengthen pelvic muscles, drug therapy (anticholinergics and ß3-agonist), periurethral bulking agents, intravesical Onabotulinumtoxin A injections, midurethral synthetic sling insertion, colposuspension and pubovaginal slings (50, 51, 52, 53, 54). The success rate is around 51% to 91% depending on the method, definition of cure and follow-up of every patient (55). In most cases, women try to perform the exercises of the pelvic floor on their own, in the comfort of their home, but without much success because they're not doing the exercises correctly. The efficiency of exercises can be increased if women exercise together with a specialized pelvic floor therapist. Oral anticholinergics and ß3-agonists are the mainstay of pharmacological treatment, but they have side effects leading to a high discontinuation rate. It has been observed that in 60% of cases anticholinergic therapy leads to improvement of disorders during intercourse in women with UI (56, 57). Intradetrusor injection of 100 units of Onabotulinumtoxin A significantly decreased the daily frequency of UI with clinically relevant improvement of symptoms and health related quality of life in patients inadequately treated with anticholinergics (58, 59). The midurethral synthetic sling insertion has become the "gold standard" of surgical treatment of SUI (60). In women who do not wish to have surgery or in whom surgical options are restricted (e.g. after irradiation), bulking agents should be considered as an alternative strategy because they are a minimally invasive approach to treat SUI (61). Their use should not be proposed as first-line treatment in women seeking a permanent cure for both primary and recurrent SUI (61).

Conclusion

UI is associated with a profound sense of humiliation and it is subject to social stigma and prejudices (4). UI and incontinence during sexual intercourse is still a taboo subject and many women feel uncomfortable talking openly about it. According to some estimates only 20% of women with UI seek help because of the problems that arise during sexual intercourse. There are many methods women can use to reduce the possibility of uncontrolled leakage during sex while working on a durable solution. It isn't an easy topic to discuss with one's sexual partner but the talk is necessary if the outcome is returning to the previous pleasurable sexual life. The treatment of UI and incontinence during sexual intercourse requires multidisciplinary

teamwork and cooperation among specialists, sexual partners and society. Conservative and surgical treatment of UI, irrespective of the type, can improve quality of life and enhance sexuality, but for more distinct conclusion further studies are needed.

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AgNOR Counts in Differential Diagnosis of Parathyroid Adenoma and Hyperplasia in Preoperative Cytologic Smears

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Abstract

Objective. Minimally invasive surgery is the method of choice in the management of hyperparathyroidism caused by parathyroid adenoma, whereas in case of parathyroid hyperplasia a radical operative procedure is necessary to prevent recurrence of the disease. The aim of the study was to investigate morphological and cytochemical parameters differentiating parathyroid adenoma from parathyroid hyperplasia in cytologic smears in preoperative work-up of patients with hyperparathyroidism.

Methods. Fifty parathyroid cytologic smears, preoperatively obtained by ultrasound-guided aspiration biopsy, were analyzed. Fifty parathyroid cell nuclei per smear were analyzed, and the number of nucleolar organizer region (AgNOR) was determined using SFORM software (Vamstec, Zagreb). The results obtained were compared with histopathology findings.

Results. The values of nuclear size parameters obtained by morphometric measurement revealed cells with larger nuclei and greater nuclear size diversity to be found in parathyroid adenoma, thus enabling differentiation of parathyroid hyperplasia from parathyroid adenoma in many cases. However, due to overlapping of borderline values, an attempt was made to reduce the possibility of error by determining AgNOR count and structure. The results obtained showed that neither AgNOR count, nor AgNOR classification into individual AgNOR, AgNOR clusters and annular AgNOR, had any role in differentiating parathyroid hyperplasia from parathyroid adenoma.

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Conclusion. Study results showed that AgNOR count and structure cannot help in determining more clearly the border between parathyroid adenoma and hyperplasia in cytologic smears.

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Introduction

Parathyroid adenoma is mostly found as a solitary tumor, therefore minimally invasive surgery has been accepted as the method of choice in the management of hyperparathyroidism caused by parathyroid adenoma. In contrast, parathyroid hyperplasia usually develops as a secondary lesion characterized by diffuse or nodular hyperplasia of one or more parathyroid glands. Recurrence is known to occur in patients with parathyroid hyperplasia even after radical and properly performed surgery that required bilateral neck exploration, and such cases have been described in the literature, pointing to the importance of preoperative differentiation of parathyroid hyperplasia and parathyroid adenoma (1,2,3). Nucleolar organizer regions (AgNOR) are segments located on the short arm of acrocentric chromosomes in which the rRNA gene is located during the interphase and mitosis. These regions can be shown cytochemically by silver staining using the method of identifying non-histone, argyrophilic proteins connected with the sites of protein rRNA transcription (AgNOR). AgNORs play a significant role in nucleic acid transcription to proteins, thus their number may serve as an indicator of cell proliferation and tumor growth (4-7). The present study was focused on identification of new morphological parameters for differentiation of parathyroid hyperplasia from parathyroid adenoma in preoperative cytologic smears by determining the number and structure of AgNOR per nucleus.

Material and Methods

Fifty parathyroid gland smears obtained by ultrasound-guided fine-needle aspiration biopsy (US-FNAB) were included in the study. The histopathologic diagnosis was unknown. Study material was obtained by ultrasound-guided fine-needle aspiration biopsy (UG-FNA) performed by a clinical cytologist (interventional cytologist) (8), who also performed the cytomorphological analysis of the material obtained. Each patient signed an informed consent form, while the study protocol was approved by the Hospital Ethics Committee and by the Zagreb School of Medicine Ethics Committee. US examinations were performed on an ACUSON X300 (Siemens, Erlangen, Germany) US device with 8.9 MHz and 11 MHz superficial tissue probes. UG-FNA was carried out by the free-hand technique (9). All smears were air dried and stained by the standard May-Grünwald-Giemsa (MGG) method (10) and cytochemical method of selective silver staining of AgNOR. For the cytochemical method, slides previously stained by the standard MGG method were used for allowing selection of appropriate slides with an adequate number of preserved cells. The selected slides were immersed in a solution containing 1 part of 2% gelatin solution in 1% formic acid and two parts of 50% aqueous silver nitrate solution, and then left to stay in a dark chamber at room temperature for 45 minutes. Then the slides were washed with distilled water and air dried (12). Upon silver impregnation, the AgNOR are seen as dark-brown spots in the nucleus (Figure. 1). Each slide was examined under Olympus BX 50 microscope connected to a computer image analyzer, initially at low magnification to select a representative area; then the selected area was systematically explored under immersion objective (magnification X1000) (11,14). In the present study, AgNOR analysis was performed by dividing them into three types: 1) homogeneous type (where dilutions were not even visible at magnification); 2) inhomogeneous

Figure 1. *PG Cells – AgNOR (Silver Nitrate, x1000)*

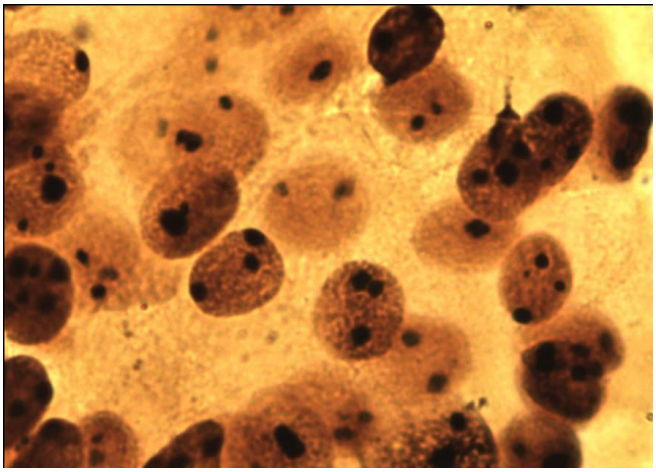
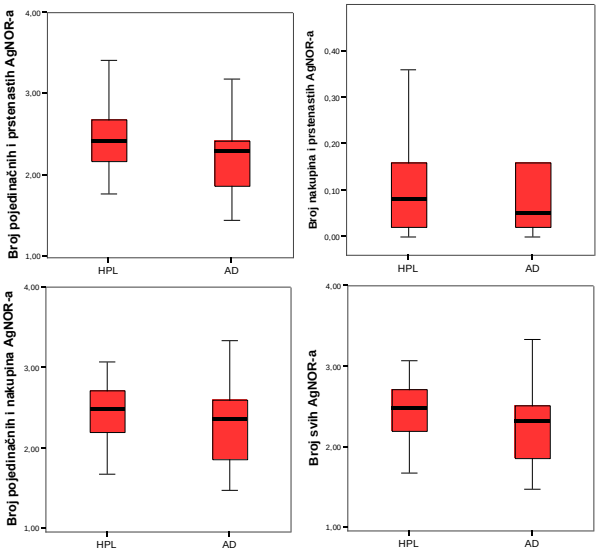


Figure 2. *Mean value and confidence interval for AgNOR numbers*



type (irregular AgNOR dilutions visible at X1000 and higher computer magnifications); and 3) annular AgNOR (annular configuration visible at standard magnification) (11,12). Intranuclear AgNOR number was determined for each AgNOR type. All patients were eventually treated surgically. Upon completion of testing, patients were divided into two groups based on histopathologic findings: parathyroid hyperplasia and parathyroid adenoma. The group of parathyroid hyperplasia included 36 (72%) patients and the group of parathyroid adenoma included 14 (28%) patients.

The basic descriptive parameters (minimal/maximal value and arithmetic mean) and variability measures (coefficient of variation and standard deviation) were calculated for each continuous variable. The pattern of distribution was assessed for each continuous variable. Determination was made of the mean value as a measure of central tendency and a central value based on the value size, as well as standard deviation (SD) as a measure of deviation of arithmetic means of samples from one population from arithmetic means of the other population. The correlation of continuous variables in the two groups was analyzed by the Mann-Whitney test as the most sensitive nonparametric test, along with the rank-sum calculation (13).

Results

Individual AgNOR count per nucleus was lower in the group of patients with parathyroid adenoma than in those with parathyroid hyperplasia, but the difference did not reach statistical significance (P=0.167) (Table 1). The mean number of AgNOR clusters was comparable in the two groups (P=0.763) (Table 2). The mean number of individual AgNOR and AgNOR clusters, the number of AgNOR clusters and annular AgNOR, the number of individual and annular AgNOR, and the number of all AgNOR, were greater in the group of patients with parathyroid hyperplasia than in those with parathyroid adenoma; however, the difference was not statistically significant (P=0.257, P=0.846, P=0.151, P=0.158) (Table 3) (Fig. 2).

Discussion

Preoperative morphological finding of parathyroid hyperplasia or parathyroid adenoma is fundamental for further treatment of patients with hyperparathyroidism. Optimal patient management requires the highest possible differentiation between patients with parathyroid hyperplasia and those with parathyroid adenoma because minimally invasive surgery has been accepted as the method of choice in the management of hyperparathyroidism due to parathyroid adenoma, whereas recurrence is quite frequent in patients with parathyroid hyperplasia, thus

Table 1. *Mean values of individual AgNOR parameters according to patient groups*

Parameter	Hyperplasia				Adenoma				P‡
	Mean	SD*	Min	Max	Mean	SD*	Min	Max	
Individual AgNOR count	2.48	0.63	1.12	4.90	2.25	0.51	1.44	3.18	0.167

*standard deviation; ‡Mann-Whitney test

Table 2. *Median of AgNOR cluster number according to patient groups*

Parameter	Hyperplasia				Adenoma				P‡
	Median	25%-75%*	Min	Max	Median	25%-75%*	Min	Max	
Number of AgNOR clusters	1.00	1 - 1	0.00	1.25	1.00	1 - 1	0.00	1.14	0.763

* interquartile range; ‡Mann-Whitney test

Table 3. *Median of AgNOR count according to patient groups*

Parameter	Hyperplasia				Adenoma				P‡
	Median	25%-75%*	Min	Max	Median	25%-75%*	Min	Max	
Number of individual AgNOR and AgNOR clusters	2.48	2.16-2.74	1.12	5.20	2.36	1.86-2.62	1.48	3.34	0.257
Number of AgNOR clusters and annular AgNOR	0.08	0.02-0.16	0.00	1.00	0.05	0.02-0.37	0.00	1.00	0.846
Number of individual and annular AgNOR	2.42	2.16-2.69	1.12	4.90	2.29	1.85-2.44	1.44	3.18	0.151
Number of all AgNOR	2.48	2.16-2.74	1.12	5.20	2.31	1.86-2.54	1.48	3.34	0.158

* interquartile range; ‡Mann-Whitney test

the other hand, subtotal resection of parathyroid gland or complete excision with partial parathyroid autotransplantation is performed on surgical removal of enlarged parathyroid glands in order to prevent consequential hypoparathyroidism; therefore, parathyroid tumor should be differentiated preoperatively from hyperplastic tissue. On morphometric measurements performed by the system of image analysis, the subjective investigator's error is reduced by use of quantitative parameters. In our earlier research, subsequent correlation with histopathologic findings revealed higher values of nuclear area, circumference, minimal radius, convexity and width in the group of patients with parathyroid hyperplasia compared with the group of patients with parathyroid adenoma (14). When extrapolated to cell morphology in cytologic smear, cells with greater nuclei were found in the group with hyperplasia in relation to those found in the group with adenoma. In the group with parathyroid hyperplasia, higher values of standard deviation were recorded for each of these parameters compared to parathyroid adenoma; applied to cell morphology in cytologic smear, it indicates greater heterogeneity and size diversity in hyperplasia as compared with adenoma (14). However, as the borderline values of the parameters analyzed showed an occasional overlap, we tried to more clearly determine the border between adenoma and hyperplasia in cytologic smears using additionally the cytochemical method and determination of nuclear AgNOR count. As the AgNOR count correlates with the level of DNA transcription and degree of cell proliferation, the features of nuclear organization provide a way to simply distinguish resting cells from those involved in the cell cycle (4-7,15,16). Black spots can be visualized in all cells of parathyroid epithelium by staining the nucleoli and other structures of nucleolar organization. AgNOR clusters were only detected in proliferating cells of various tissues, whereas individual spots were found in mature, nonproliferating cells (17-20). These findings point to physiological differences between individual AgNOR and AgNOR clusters, thus justifying their separate evaluation. There are literature reports on the studies where AgNOR count per nucleus was determined in postoperative parathyroid histopathologic smears and significant difference was found between parathyroid carcinoma and benign parathyroid lesions, while no such difference in AgNOR count per nucleus was recorded between parathyroid adenoma and parathyroid hyperplasia (21,22). Boquist reports on the comparable AgNOR count per nucleus in parathyroid adenoma and liver adenoma (2.6 and 2.3 AgNOR per nucleus, respectively), breast fibroadenoma and thyroid follicular adenoma (23). Mourad et al. have introduced the mean AgNOR (mAgNOR) count per nucleus, which correlates with ploidy (aneuploid cells have ≥2.4 mAgNOR per cell and diploid cells <2.4 mAgNOR per cell (24). In the present study, AgNOR analysis was performed by dividing them into three types: homogeneous, inhomogeneous and annular AgNOR (8). As AgNOR clusters are only found in proliferating cells, the number of AgNOR clusters is understandably low in adenoma and hyperplasia; instead, they are only found in individual cells. In our study, annular AgNOR were only detected in several individual nuclei. Irrespective of their number, all spots were enumerated and their role in differentiating parathyroid hyperplasia from parathyroid adenoma was assessed upon statistical data processing. The mean number of all AgNOR per nucleus was greater in parathyroid hyperplasia compared to parathyroid adenoma (2.48 vs. 2.31); the difference is not statistically significant (P=0.158) (Table 3) and is consistent with the values reported by Boquist in histopathologic material (23). Statistical data processing indicated the AgNOR classification according to their proliferation activity into individual AgNOR, AgNOR clusters and annular AgNOR to have no value in differentiating parathyroid hyperplasia and parathyroid adenoma. These cells are low-proliferating cells predominated by homogeneous AgNOR, while AgNOR clusters and annular AgNOR are few and found in comparable number in both parathyroid hyperplasia and parathyroid adenoma. Cytologic smears are considered more appropriate for AgNOR determination than histologic sections because the spots are more

easily enumerated and individual spots are better visualized within the nucleoli in cytologic smears. In the latter, whole cells are seen, thus the number of spots is expectedly higher than in histologic sections. Yet, even materials that are so different can be compared if the ratio of AgNOR count and nucleus area is determined. This is of great value because there are few reports on studies performed on preoperatively obtained cytologic FNAB smears, thus obviating possible errors caused by different sample processing (25-27). Our own observations and subjective assessment that differences in nuclear size are substantial features to differentiate parathyroid adenoma and parathyroid hyperplasia in cytologic smear, and that these features can help differentiate these two entities in preoperative cytologic US-FNAB smears, were quantitatively confirmed by use of objective morphometric measurement and statistical analysis of the data obtained. Our results still point to justifiable utilization of cytodiagnosis, along with morphological and morphometric methods in the preoperative identification of parathyroid tumors. Particular nuclear morphometric parameters may influence the selection of patients as potential candidates for minimally invasive or radical surgical procedure (14). However, the borderline values of the study parameters may occasionally overlap, and quantitative determination of AgNOR per nucleus in preoperative cytologic smears cannot help in differentiating parathyroid adenoma from parathyroid hyperplasia, thus it cannot be used as a diagnostic criterion, which is consistent with literature data obtained on postoperative histopathology material (23,28,29).

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Risky behavior and exposure to noise among adolescents

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Abstract

Aim: Adolescents are under greater risk of noise influence which affects their psychophysical health. The most common noise sources are too loud cinema halls, concerts, sports events, different outdoor events, street noise, and listening to music that is too loud on iPod and MP3 gadgets. The aim of this study was to investigate risk behaviors and attitudes among adolescents toward noise exposure; to explore how adolescents recognize symptoms and signs of noise influence to their own psychophysical health.

Methods: The study included 533 high school students aged 15-19 years. The data were collected using a questionnaire that contained a total of 32 questions/ attitudes/ statements. SPSS statistical software was used for data processing.

Results: Students in higher grades (third and fourth) show a better understanding of noise types that can damage hearing and they agree with the statement that listening to loud music can damage hearing, whereas students of first and second grades disagree. Younger students usually associate hearing loss with aging.

Conclusion: The study showed that such risky behavior of adolescents is in accordance with global trends. It can be and it has to be influenced by integrated programs within primary and secondary education. By continuous education of both children and parents when it comes to noise level in their living, working or entertainment environment, and when it comes to the ways in which they can decrease the noise level, damage to hearing can be prevented, or at least postponed.

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Introduction

Noise is an unwanted, i.e. unpleasant or unexpected sound; a mixture of sounds having various properties which can be permanent, abrupt and striking. The properties can vary in level, duration and distribution and can have multiple adverse effects on human health and

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hearing. Noise is an audible acoustic energy which can negatively affect the physiological and psychological state of humans. People are exposed to noise on a daily basis, including environmental, community, public and domestic noise, all of which represent one of the main issues of human environment, especially in city areas (1). Main noise sources in outdoor spaces are traffic, industry, construction and public activities, sports and leisure. Noise usually found in indoor spaces includes servicing equipment, music equipment and home appliances. Traffic is one of the main causes of noise. In cities, noise emitted by cars accounts for 80 % of noise in city areas. What is more, next to busy road junctions, the noise can reach up to 90 dB (2). About 80 million of Europeans live in areas where the level of noise exceeds recommended exposure limits. By reducing partial or total noise primarily in the public and work environment as well as the time of exposure, at least half of hearing damage cases could be prevented. Despite the fact that noise is an integral part of nature, factors such as industrial development, population growth and density and increased number of roads and vehicles, especially in urban settings, have led to an increase of noise exposure representing a threat to human health (Table 1). The impact of noise on human health can be direct, resulting in damage to the organ of hearing and balance. Also, it can be indirect and can affect the nervous, vascular, digestive and endocrine system. Direct noise results in partial and complete hearing loss, tinnitus, various speech disorders, problems related to communication and balance disorders such as unsteadiness while walking. Nowadays, noise is the main cause of hearing impairment. Prolonged exposure to noise, such as during the whole day, permanently damages our hearing. Noise induced by traffic, noise we are exposed to in school and at the work place, loud music, and most of all, neglecting to pay attention to our hearing, leads to noise-induced hearing loss (NIHL). Indirect effects on health include neurovegetative reactions such as hypertension, endocrine disorders and other metabolic disorders, as well as exhaustion, mental reactions (irritability) and low performance. Depending on their interests, upbringing, education and habits, adolescents are at higher risk of noise influencing their health and physical and mental development. Sources of noise are mostly extremely loud sound levels in cinemas, concerts, sports events, restaurants, open air events, shopping malls, street noise and loud listening to music on headphones. Music played on iPods, CDs and MP3 players is one of the least known, but one of the most dangerous killers of hair cells. The volume of sound emitted by these devices sends music via headphones/ earphones directly to our ear so that we no longer hear people around us or the noise of cars and trams, which means that the sound is too loud and potentially life-threatening. More than 78% to 90% of young people and approximately 50% of adults listen to music through headphones/earphones. About 50% of young people use personal music players from 1 to 3 hours per day and a significant number of young people even longer than that (3). It has been proven that exposure to noise of up to 85 dB during an 8-hour period causes hearing loss, while the level of sound volume emitted by iPods and MP3 players is much louder (usually more than 100 dB). Adolescents and young adults consciously expose themselves to very loud music, very often for several hours in succession. Loud music at concerts and in clubs, emitted by audio devices, represents a potentially dangerous source of noise. The noise level recorded at rock concerts ranges from 120 to 140 dB, while in restaurants, at weekends, the noise level exceeds 95 dB. According to a group of authors, professional exposure in nightclubs considerably exceeds the recommended noise level. Taking the weekly exposure to noise in nightclubs into account, a 5-hour stay in a typical club will expose the ear to noise equivalent to 98 dB (4). Extremely high and very dangerous noise levels can be found next to loudspeakers at big music events (e.g. open-air concerts). On the back of every event ticket, there is a sentence written in very small letters which warns the visitors that loud music can damage the hearing. Hence, the use of hearing protection, such as earplugs, which decrease the noise level but do not affect the quality of sound, is recommended to concert visitors. Two decades ago, a group of authors came to the conclusion that a temporary

threshold shift of more than 10 dB can be observed after 3 hours of listening to music on medium noise levels via portable audio devices (5).

Table 1. *Environmental sounds and the corresponding sound level (noise) in decibels*

Environmental sounds	Sound exposure level (dB)
Threshold of hearing	0 – 25
Whisper	20
Conversational speech	40
TV	55
Apartment on a busy street, noise in a large business office	60
Busy traffic, noise in a cafe,	70
Car, hair dryer	70
Metro, big crowd	80
Truck, busy crossroads	90
Train passing	95
Chainsaw, drilling machine	100
Air hammer	105
Loud music	110
Rock concert near the speaker, thunder	120
A singer whose volume has reached the maximum	130
Threshold of pain	130 –140
Takeoff and landing of a plane	140
Space shuttle platform	180

The aim of this study was to explore risk behavior of adolescents with respect to noise and their understanding of consequences of auditory overload on their psychophysical health.

Material and Methods

The study included 533 students, aged between 15 and 19, attending "Pakrac" high school during November and December in 2016. The data was collected using a survey questionnaire especially designed for the purposes of this research. The questionnaire was divided into four parts. The first part included general data, like gender, date of birth, field of study the students are enrolled in. Employing a Likert-type scale, other parts of the questionnaire referred to statements on attitude, risky behavior and symptoms. The questionnaire contained a total of 32 questions/attitudes/statements. The research was approved by the school ethics committee. Furthermore, the study was performed in accord with the ethical principles of the Declaration of Helsinki. In a meeting of all form teachers of "Pakrac" high school, the aim of the questionnaire and the instructions on how to fill it in, were briefly presented. The questionnaires were filled in during homeroom class. The whole process of conducting the survey questionnaire went on smoothly, with occasional questions regarding the meaning of particular questions/attitudes stated in the questionnaire.

Statistical analysis

The Kolmogorov-Smirnov test was performed to test the data against normal distribution. The mean values of the continuous data are expressed by the median and interquartile range, while nominal indicators are shown by absolute and relative numbers. The differences between categorical variables were tested using the $\chi 2$ test. In order to determine the difference between two independent samples, the Mann Whitney test was employed, while for three and more samples the Kruskal Wallis test was performed. Also, originally written database programs and the statistical software package SPPS (version 15.0) were used, using a significance level of $\alpha = 0.05$.

Results

The study involved 533 participants (students) aged between 15 to 19. The sample included 200 (37.5%) male and 333 (62.5%) female students. While analyzing the research results, the participants were divided into two categories, i.e. junior and senior participants. The term "junior" refers to first- and second-year high-school students, while the term "senior" involves participants in the third, fourth and fifth grade. According to the obtained results, a statistically significant difference can be observed in the following attitudes: senior high-school students

Table 2. *Participants distribution according to risk behavior*

Risk behavior	Number (%) of respondents					
	I do not agree at all	*I mostly disagree*	*I neither agree nor disagree*	*Mainly I agree*	*I agree*	*Total*
I listen to music every day for more than 1h through the headset	139 (26.1)	76 (14.3)	100 (18.8)	73 (13.7)	144 (27.1)	532 (100)
Family members complain that I listen to music or TV too loudly	209 (39.4)	108 (20.3)	59 (11.1)	72 (13.6)	83 (15.6)	531 (100)
Once a week (or more) I go to a nightclub or cafe bar	104 (19.6)	59 (11.1)	95 (17.9)	94 (17.7)	179 (33.7)	531 (100)
I adhere to warnings of my smartphone that the music is too loud	216 (40.7)	84 (15.8)	97 (18.3)	75 (14.1)	59 (11.1)	531 (100)
I often go to concerts and similar events	104 (19.6)	116 (21.8)	157 (29.6)	89 (16.8)	65 (12.2)	531 (100)
When I'm exposed to noise, I use protection in the form of earplugs or ear pads	416 (78.6)	61 (11.5)	24 (4.5)	16 (3)	12 (2.3)	529 (100)
I often participate in activities where I am exposed to noise	81 (15.2)	116 (21.8)	183 (34.4)	102 (19.2)	50 (9.4)	532 (100)
I live near some sources of noise	301 (56.6)	96 (18)	69 (13)	40 (7.5)	26 (4.9)	532 (100)

(third and fourth grade) show a statistically significant higher level of knowledge about different types of noise that can damage the hearing ($p<0,05$). Also, they agree on the statement that loud music damages hearing ($p<0,05$) unlike the first-year and second-year students. Furthermore, senior participants believe that noise causes the highest damage during leisure time ($p<0,001$), which can result in anxiety, depression and high blood pressure ($p<0,05$). In contrast to their senior counterparts, junior students often connect hearing loss with older people. The distribution of participants with respect to risky behavior is shown in Table 2. A statistically significant difference regarding risky behavior can also be observed in the senior population of high-school students, who stated to rarely listen to music using headphones/earphones for more than an hour a day ($p<0,05$). All other statements relative to risky behavior do not point to a statistical significance between the different age groups. The highest overlap can be observed in the statement that noise has a negative effect on the students' ability to learn, while the statements that the participants experienced ear pain as a result of listening to music using headphones/earphones and that the noise emitted by church bells and electronic devices in their living area (e.g. television, radio, air conditioner, refrigerator, washing machine, computer) made them feel uncomfortable, displayed the lowest level of agreement.

Discussion

A lot of research has been carried out so far on the influence of noise on human health, especially that of young people, due to increasing exposure to levels that can directly and indirectly affect the health and quality of life. However, studies show that exposure to daily noise levels still represents a great public health and social issue, which has resulted in increased preventative measures. Preventative measures first included the adult population exposed to professional noise, while, at the same time, the number of children and adults with hearing loss

is constantly on the increase. The results of this research point to the necessity to educate adolescents with the aim to reduce their risky behavior and to influence their attitude towards noise. Out of the total number of respondents, 80% disagree with the statement that listening to loud music through headphones/earphones is connected with hearing impairment, which represents a worrying fact about the perception of noise among young people. A study was carried out among 1547 students of 13-19 years of age in Swedish schools. A significant difference was identified between socioeconomic and age groups. In other words, the age group of 13-15 displayed a better attitude towards noise, if compared to the age group ranging from 16 to 19. Older adolescents stated to better take care of their hearing than younger adolescents (6). The results of the study in question are compatible with the results of this research, where older participants (3rd, 4th and 5th graders) are better acquainted with different noise types, risky behavior (listening to loud music) and the possible adverse effects. On the other hand, risky behavior of younger participants (1st and 2nd grade) is displayed in the amount of time spent listening to music using headphones/earphones, which is more than one hour. The same group of respondents (78.6%) do not use any kind of ear protection, be it earmuffs or earplugs. The cause of such an attitude could be found in the fact that younger participants connect hearing loss with older people. A six-year study carried out in the USA showed that 12% of children aged 6 to 19 suffer from noise-induced hearing-threshold shifts (7). Research of some authors shows that 1% of all school children suffer from some kind of hearing damage (8). Several years of exposure to loud music suffices to cause damage to the inner ear. It is estimated that after 10 years of exposure to loud music via audio devices in clubs and concerts, approximately 10% of people will suffer from irreversible bilateral hearing loss at a frequency of 3 kHz (9). During 2005, a group of researchers performed a study on hearing loss among young people caused by noise. Due to the fact that a large number of young people expose themselves to loud music in their free time, the researchers assumed that young people are unaware that exposure to loud music can result in hearing loss. In their research, they used a questionnaire which consisted of 28 questions. They presented the questionnaire on the public web site of a television network with the aim to find out the attitude of young people towards general health issues, including hearing loss. Only 8% of respondents recognized the problem of hearing loss as a great health issue. The majority of participants experienced tinnitus or temporary, reversible hearing loss after visiting concerts (61%) and clubs (43 %). Only 14% stated to have used earplugs. Still, it is encouraging that the majority of the respondents would have been willing to use ear protection if educated or warned by a doctor about possible permanent hearing impairment (10). Despite the evidence on the adverse effects of noise on young people, neither guidelines nor safety standards have been defined yet. Scientists warn that today's generations of young people are at higher risk of facing hearing damage at an earlier age than older generations. If music is listened to too often, doctors recommend keeping the volume at 60% of the maximum. As many as 22.4% of our participants sometimes experience buzzing in the ears; however, we do not possess any information as to whether this percentage increases with age. Today's studies indicate that the number of tinnitus cases will rise with age in children due to long-term exposure to noise. 45.1% of our participants indicated to have concentration problems due to environmental noise, while 57.3% answered to have problems with learning when noise is present. Furthermore, 66% of the students study the easiest and most effectively when in silent environments. The results confirm that the presence of noise makes young people feel uncomfortable and that it causes lack of concentration. Hearing loss can be prevented if children and parents are adequately educated about noise levels in the environment they live, work or entertain themselves in and about the methods they can use to reduce noise.

Conclusion

The research has shown that risky behavior in adolescents follows global trends. The trends

can be altered by using integrated programs during primary and secondary school education. Also, it is very important to educate the educators, who will then better convey their knowledge in a way that is more appealing to young people. Young people are more prone to change their habits, hence quality education can lead to easier changes in young people's attitude towards noise. Raising public awareness and promoting activities regarding the prevention of hearing loss will improve the health of the ear and hearing. Some of the preventative activities include restriction on exposure to loud noise, reduction of noise levels whenever possible or avoidance of noise sources. It is of utmost importance to perform further research on risky behavior and exposure to noise in youth in order to publish quality literature adapted to the current situation. This way, we can achieve better results and education aimed at the core of the problem.

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Dietary supplements and sport performance – A comprehensive review

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Abstract

Aim: Comprehensive articles on dietary supplements and their impact on sport performance, which would provide professional and recreational athletes with evidence-based information, are sparse.

Methods: Investigation involved eight different dietary supplements, commonly used among recreational and endurance athletes according to available literature obtained by searching the PubMed database, namely: *Antioxidants, beta-alanine (B-alanine), branched-chain amino acids (BCAAs), caffeine, carbohydrates, creatine, nitric oxide/nitrates, and proteins*. Their mechanisms of action have been briefly presented, along with their potential beneficial and harmful side effects and safety.

Results: i.) *Antioxidants*: A sufficient amount of antioxidants is available in a balanced diet ii.) *B-alanine*: Supplementation is likely to be beneficial in high-intensity exercises. iii.) *BCAAs*: No review articles in English were available iv.) *Caffeine*: Caffeine supplementation is beneficial in endurance exercises v.) *Carbohydrates*: Carbohydrate supplementation is probably beneficial in exercises lasting longer than one hour vi.) *Creatine*: Creatine supplementation is effective in high intensity, short-lasting exercise, while it does not seem to have any ergogenic effect in aerobic exercise. vii.) *Nitric oxide/nitrates*: Nitrate supplementation has a small but significant performance-enhancing effect, most apparently in situations of insufficient perfusion. viii.) *Proteins*: Protein supplementation, in combination with resistance exercise, most likely has beneficial effects on lean body mass and muscle strength.

Conclusion: Most of the analyzed dietary supplements, if used for intended exercise regime, provide a kind of sport performance enhancement. On the other hand, long-term studies about their safety are mostly lacking.

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KEYWORDS: dietary supplements, sports, endurance, performance-enhancing substances

Abbreviations

BCAAs – branched-chain amino acids

ROS - reactive oxygen species

cAMP - cyclic adenosine monophosphate

C-P - creatine phosphate

NO – nitric oxide

B-alanine – beta-alanine

B-carotene – beta-carotene

Introduction

Professional athletes and amateurs alike are in constant search for new means which would enable them to improve their sport results in shorter time. Among those means, a prominent place belongs to dietary supplements (1). The use of dietary supplements among athletes varies quite substantially (2–5), and can reach numbers as high as 98% (4), with one of the main reasons for their utilization being the improvement in sport performance (2–5).

There are a lot of studies, assessing different dietary supplements and their influence on athletic performance, as well as numerous review articles and meta-analyses evaluating individual supplements or family of supplements (whey proteins, branched-chain amino acids (BCAAs), antioxidants…) with respect to sport performance. Yet, a systematic review of literature that could give an athlete a more comprehensive overview of the vast and diverse field of dietary supplements is lacking.

Review articles and meta-analyses are tools used in science to evaluate evidence and also to make scientific conclusions, since they sum up and critically assess the knowledge about a particular topic that is usually dispersed in the form of original articles. The goal of this paper was to gather currently available and evidence-based information in the form of review articles and/or meta-analyses on most currently used sport performance-enhancing dietary supplements and present them to a reader in an understandable manner, as it has been shown that the acquisition of information about dietary supplements usually does not come from evidence-based source of information, but rather from family members, friends and coaches (6). Accordingly, we have identified the most commonly used performance-enhancing dietary supplements and studied recently published review articles/meta-analyses about those products, with an aim to write a comprehensive overview of dietary supplements that are used for sport performance enhancement.

Methods

PubMed was searched using the search string "(spor*[Title] OR athl*[Title] OR train*[Title]) AND performan*[Title]", to recognize papers that presented any facts connected to enhancing sport performance (different types of dietary supplements, different exercise procedures …) published in the last five years. Analyzing the titles and abstracts of those articles, we identified the most commonly used dietary supplements or groups of dietary supplements associated with enhancing sport performance, and then searched the PubMed library to find review articles/meta-analyses that evaluated each individual dietary supplement or a group of dietary supplements (i.e. string "(carbohy*[Title]) AND (sport*[Title] OR athl*[Title] OR exercis*[Title]) AND (revie*[Title] OR analys*[Title])" was used to find review articles/meta-analyses focusing on carbohydrate supplements used in sports). If a search string returned many review articles/meta-analyses, just the most recent ones were included in the analysis (and reported in the results section). The reference lists of included articles (articles writing about supplements used to enhance sport performance in general, not inside a specific context [i.e. just endurance sports]) were examined to find additional papers.

Results

Eight different dietary supplements or groups of dietary supplements were recognized: i.) antioxidants, ii.) beta-alanine (B-alanine), iii.)

branched-chain amino acids (BCAAs), iv.) caffeine, v.) carbohydrates, vi.) creatine, vii.) nitric oxide (NO)/nitrates and viii.) proteins (whey and other proteins).

3.1. Antioxidants: The review articles/meta-analyses for the effects of antioxidants were found using the search string "antiox*[Title] AND (sport*[Title] OR athl*[Title] OR exercis*[Title]) AND (revie*[Title] OR analys*[Title])" on PubMed. The search returned four articles, one of which was about exercise and pregnancy and was therefore excluded from the analysis. Out of the three that remained, the most recent one (7) was included in the analysis (it was published in 2015; the other two were written in 1999 and 1993, respectively). Another review article, describing vitamin C and its effect on performance, was found while searching through the reference list of the first article and was also included in the analysis.

3.1.1. Mechanism(s) of action: During exercise, the production of reactive oxygen species (ROS) in the skeletal muscle cells increases (8), and this can have damaging effects as ROS can alter cell structure and function, and cause fatigue (9). On the other hand, ROS are speculated to be involved in glycogen resynthesis (10) and also in adaptive responses induced by training (11–14). The use of the adequate amounts of antioxidants can therefore, in theory, optimize the balance between pro and anti-ergogenic effects of ROS and consequently improve performance.

3.1.2. Meta-analysis/revision conclusions: Braakhuis and Hopkins (2015), who analyzed 71 studies examining the impact of various antioxidants (vitamin E, quercetin, resveratrol, beetroot juice, other food derived polyphenols, spirulina and N-acetylcysteine) on performance, concluded that the only antioxidant exerting beneficial acute effects on performance was N-acetylcysteine when injected intravenously, but this route of administration was not recommended (7).

The review of Braakhuis (2012), which considered 11 articles that examined potential effects of vitamin C on sport performance, found that large doses (more than 1 g/day) of vitamin C appeared to reduce the training-induced adaptations by reducing mitochondrial biogenesis or possibly by altering vascular function (15). They also concluded that small doses of vitamin C (approximately 0.2 g/day), provided by five servings of fruit and vegetables per day, may be sufficient to reduce oxidative stress without reaching the threshold that would impair optimal training adaptations. Moreover, a short-term intake (during a period of one to two weeks) of doses greater than 2 g/day seems to be beneficial for athletes during times of increased stress (15).

3.2. B-alanine: The review articles/meta-analyses for the effects of B-alanine were found using the search string "*alanin*[Title] AND (sport*[Title] OR athl*[Title] OR exercis*[Title]) AND (revie*[Title] OR analys*[Title])" on PubMed. The search retrieved two papers. Of the two, the first was a review article written in 2010 (16), and the other one was a meta-analysis conducted in 2012 (17).

3.2.1. Mechanism(s) of action: The effects of B-alanine supplementation can mostly be attributable to an increase in concentration of carnosine (a dipeptide of the amino acids B-alanine and histidine concentrated in muscle and brain tissue) in the skeletal muscle cells, as concentration of B-alanine is thought to be the rate-limiting step in carnosine synthesis (B-alanine and L-histidine [carnosine synthase]) (18–20). Carnosine is speculated to exert buffering (20), antioxidant (21,22) and calcium (Ca^{2+}) regulatory (23,24) effects, that could potentially enhance performance.

3.2.2. Meta-analysis/revision conclusions: The meta-analysis was conducted on 15 papers. Authors concluded that B-alanine supplementation elicited a significant performance-enhancing effect on high-intensity exercise, particularly when exercise lasted between one and four minutes (17). As B-alanine improved predominantly short-lasting anaerobic exercise performance, the authors argue that those data provide further evidence that supplementation of B-alanine increases intramuscular carnosine, which has pH buffering capacity (17).

3.3. *BCAAs*: The review articles/meta-analyses for the effects of BCAAs were found using the search string "(BCAA*[Title] OR branch*[Title]) AND (sport*[Title] OR athl*[Title] OR exercis*[Title]) AND (revie*[Title] OR analys*[Title])" on PubMed. The search retrieved only one review writing about BCAAs and sport performance enhancement (of the nine retrieved by the search, seven were focused on exercise and heart bundle branch blocks, and one was focused on exercise and dissection of the celiac trunk and its branches), As it was written in Spanish, we only assume the proposed mechanisms of *BCAAs* actions.

3.3.1. Mechanism(s) of action: BCAAs are thought to improve performance by diminishing the exercise-induced increase in serotonin, which is thought to be at least partly responsible for the central feeling of fatigue during exercise (25). The exercise-induced increase in serotonin is thought to be induced by an increase of plasma free tryptophan. The concentration of free tryptophan rises during exercise because of increased levels of non-esterified fatty acids, the levels of which are also higher during exercise, and which compete with tryptophan for the same binding sites on albumin (26). The transport of tryptophan across the blood-brain barrier is considered to be the rate-limiting step in the synthesis of serotonin. BCAAs compete with tryptophan in that transport (27).

3.3.2. Meta-analysis/revision conclusions: The search string on PubMed returned only one article, which was in Spanish, so we have no results to report in this section.

3.4. Caffeine: The review articles/meta analyses for the effects of caffeine were found using a search string: "(caffei*[Title]) AND (sport*[Title] OR athl*[Title] OR exercis*[Title]) AND (revie*[Title] OR analysi*[Title])" on pubMed. The search returned seven articles. According to the titles and abstracts of those articles, none was appropriate (some of them were dealing with caffeine and sport performance in a specific setting [endurance performance, high intensity performance, …] others were about pharmacological aspects of caffeine, while another one dated as far back as to the year 1994), so we included a review (28) article in the "similar articles" section in the PubMed.

3.4.1. Mechanism(s) of action: Caffeine exerts various effects on many organ systems. It is an inhibitor of phosphodiesterase (increasing the intracellular cyclic adenosine monophosphate [cAMP]) and also an antagonist of adenosine receptors (its psychoactive effect) (29). Its actions on sport performance are thought to be mediated through its effects on the cardiovascular system (an increase in heart rate and blood pressure), the pulmonary system, the endocrine system and the central nervous system (28).

3.4.2. Meta-analysis/revision conclusions: Authors of the review (28) concluded that caffeine is beneficial in endurance exercise, as it has been shown to increase work output and the time to exhaustion. They also stated that it should be ingested one hour before prolonged endurance events and that, if an athlete decides to stop consuming caffeine before competition with an aim to increase its ergogenic effects during competition (since a person can get habituated), one should reduce caffeine consumption at least one week before the competition, to be completely free from its withdrawal effects. Furthermore, to avoid potential negative symptoms, the dose should gradually be reduced over three to four days, instead of quitting abruptly. Resuming caffeine on the day of competition can again provide the desired ergogenic effects, similarly as it would for a nonuser (28).

They have also concluded that because caffeine increases the plasma concentration of lactate and hence decreases pH, it may be contraindicated in athletes engaging in sprint events which last between 30 seconds and three minutes (28).

3.5. Carbohydrates: The review articles/meta analyses for the effects of carbohydrates were found using a search string "(carbohy*[Title]) AND (sport*[Title] OR athl*[Title] OR exercis*[Title]) AND (revie*[Title] OR analysi*[Title])" on PubMed. The search returned nine articles; yet, only one was appropriate for inclusion in the analysis (30), since others were

either about carbohydrates taken in combination with proteins, or about the effects of carbohydrates on lipid metabolism and about different ways of use of carbohydrates (mouth rinse).

3.5.1. Mechanism(s) of action: There are two proposed mechanisms when trying to explain how carbohydrate supplementation during exercise improves performance (30): i.) mental cognitive stimulation of the central nervous system by carbohydrate exposure during exercises of shorter duration (less than one hour - the glycogen stores are not a limiting factor) (31–33) and ii.) a direct exogenous carbohydrate contribution to carbohydrate oxidation during muscle glycogen limiting exercises (duration longer than two hours) (34–36).

3.5.2. *Meta-analysis/revision conclusions:* The revision included 61 different performance studies performed on 679 subjects. Eighty-two percent of the studies showed performance benefit, while 18 % showed no performance improvement compared to placebo (30). Based on the revision of the studies, authors recommend that for exercises of shorter duration (less than one hour) and high intensity, small amounts of liquid carbohydrate solutions provide performance benefit through stimulation of the pleasure and reward center in the brain (30). By increasing the duration of exercise (duration of one to two hours), moderate amounts of carbohydrate supplementation (30-60 g/h) consumed frequently throughout the exercise appear to maximize the performance advantage of carbohydrate supplementation (liquid carbohydrate sources are recommended (30)). With the duration of exercise extending beyond two hours (with concurrently reduced intensity), a greater variety of carbohydrate foods and fluids can be included to meet the high hourly carbohydrate intake recommendations of 40-110 g/h (30).

3.6. Creatine: The review articles/meta analyses for the effects of creatine were found by using a search string: "(creati*[Title]) AND (sport*[Title] OR athl*[Title] OR exercis*[Title]) AND (revie*[Title] OR analysi*[Title])" on PubMed. The search string retrieved seven articles, four of which were appropriate (they were about sport performance in general with regard to creatine use), with the most recent one dating to 2003 (37).

3.6.1. Mechanism of action: Creatine supplementation is thought to increase intramuscular storage of creatine phosphate (C-P), which is responsible for anaerobic ATP resynthesis during high-intensity, short-duration exercises (38,39). Based on the available data, positive correlations between muscle creatine uptake and exercise performance (40) were shown.

3.6.2. *Meta-analysis/revision conclusions:* Based on the available research data, authors concluded that creatine supplementation could increase the skeletal muscle C-P content, which may improve performance involving short periods of extreme exertion , especially during repeated bouts of exercise (37). On the other hand, creatine supplementation did not appear to increase the maximal isometric strength, the rate of maximal force production, or the aerobic exercise performance (37).

3.7. Nitrates: The review articles/meta-analyses for the effects of nitrates were found using a search string: "(nitr*[Title]) AND (sport*[Title] OR athl*[Title] OR exercis*[Title]) AND (revie*[Title] OR analysi*[Title])". The search retrieved five articles: only two (41,42) of them were appropriate (dealing with nitrate supplementation and exercise performance), while others were mainly focused on other issues, such as the effects of exercise and nitrates on vascular endothelial function, the effects of nitrates on exercise-induced bronchospasm, etc.), so the most recent one (42) was analyzed.

3.7.1. Mechanism of action: It is believed that inorganic nitrate can be converted to its antecedents (nitrite and NO), especially in the presence of hypoxia and acidosis (41). NO is thought to exert multiple effects in the body, including its effects on neurotransmission (43), on vascular tone control (44), mitochondrial respiration (45) and skeletal muscle contraction (46), thus its influence on exercise is complex.

3.7.2. *Meta-analysis/revision conclusions:* The authors state that nitrate supplementation has no or little effect on maximal oxygen consumption (V_{O2max}) but it can lower the oxygen deficit of submaximal exercise (that is, improve skeletal muscle efficiency or economy), at least in subjects who are not highly trained (42). This may lead to a small increase in the sustainable power output for a given metabolic rate. They also conclude that at least in situations where some fraction of the working muscle mass may be relatively under-perfused (in hypoxia, during high-intensity exercise in subjects with low aerobic fitness - when their muscles are relatively under-perfused with respect to the heart) (42), nitrate supplementation can speed up the oxygen kinetics and thus have an ergogenic effect. The optimally efficient nitrate dose is likely to vary between subjects but is hypothesized to be generally more pronounced in highly trained individuals (42).

3.8. Proteins (whey): The review articles/meta analyses for the effects of proteins were retrieved using the search string: "(protei*[Title]) AND (sport*[Title] OR athl*[Title] OR exercis*[Title]) AND (revie*[Title] OR analysi*[Title])" on PubMed. The search returned 27 articles, three of which were appropriate for analysis (dealing with protein supplementation and sport performance), and since the most recent one (47) was funded by a commercially orientated consortium, the second most recent one was chosen for analysis (48).

3.8.1. Mechanism(s) of action: Protein balance in the human body depends on protein synthesis and protein degradation. When the synthesis is higher than the breakdown, one will have a positive protein balance, otherwise the protein balance will be negative (49). Amino acid intake (50,51) and resistance exercise training (52–56) have separately been shown to have a positive effect on protein synthesis. Moreover, their combined effect (i.e. ingesting proteins after resistance training) was shown to be greater than just adding two separate effects together (57–59).

3.8.2. Meta-analysis/revision conclusions: The meta-analysis consisted of 22 randomized controlled trials that, in the aggregate, included 680 subjects (48). Authors concluded that dietary protein supplementation represented an effective dietary strategy to augment the adaptive response of skeletal muscle to prolonged resistance-type exercise training in healthy younger (younger than 50) and older (older than 50) adults. Furthermore, in younger adults, protein supplementation during prolonged (more than six weeks) resistance-type exercise training significantly augmented the gains in fat-free mass, type I and II muscle fiber cross-sectional area, and one-repetition maximum leg press strength compared to resistance-type exercise training without a dietary protein-based cointervention (48). The findings in the group of younger adults were evident, despite the fact that, before the intervention, all groups were already consuming a more than adequate dietary protein intake of 1.2 g/kg body weight/day (48). From a practical point of view, it is worth noting that subjects were supplemented with an average of 50 ± 32 g proteins / day (in excess of their normal diet) and, in most cases, the protein supplements were ingested before or immediately after each exercise session.

Discussion

In the era of highly competitive sports, more and more individuals are using performance-enhancing nutritional supplements to enhance not only their sport performance but also their visual appearance and self-confidence. Consumers of those supplements are overwhelmed by the manufacturers' claims of increased strength, weight loss, and improved body definition, but information about the efficacy is rarely acquired or presented in an evidence-based manner (6).

There is a large body of information in the form of original articles and/or review articles and meta-analyses about a particular nutritional supplement, but a more comprehensive overview of the vast and diverse field of dietary supplements is lacking. Keeping that in mind, a review article was written, identifying the common sport performance-enhancing dietary

supplements, finding the relevant and recent review articles/meta-analyses about those supplements, and summarizing their findings and conclusions.

Eight dietary supplements/groups of dietary supplements were identified and focused on: antioxidants, B-alanine, BCAAs, caffeine, carbohydrates, creatine, NO/nitrates and proteins (whey and other proteins).

Regarding antioxidants, of all the tested antioxidants (vitamin E, quercetin, resveratrol, beetroot juice, other food-derived polyphenols, spirulina and N-acetylcysteine), the authors found strong evidence for performance-enhancing effect only for N-acetylcysteine (but with the very impractical administration route, namely i.v.) (7). The authors also speculated that antioxidants could reduce the impairment of performance during tournaments or repeated efforts, presumably by reducing the severity of inflammation (7). Similar speculations were hypothesized in a review about the influence of vitamin C on performance (15), where the authors concluded that the amount obtained in five servings of fruit and vegetables would be sufficient to reduce oxidative stress, and that higher doses might be beneficial only during periods of intensified stress (15). Accordingly, based on the conclusions of those two reviews, the amount of antioxidants we get in a balanced diet is probably sufficient, even for athletes, and thus, the supplementation of antioxidants should be considered only in periods of increased stress (tournaments, world series). The safety of long term antioxidant use should also be taken into account, when considering antioxidant supplementation for enhancing sport performance, since a double-blinded, placebo-controlled study about the use of vitamin A and beta-carotene (B-carotene) and their effect on lung cancer, the Beta-Carotene and Retinol Efficacy Trial, showed, on 18314 participants, that there was a significantly higher relative risk for lung cancer and lung cancer associated mortality among the patients who were receiving a combination of B-carotene and vitamin A, compared to placebo (60). The results of this study were concordant with the results of the randomized, double-blinded, placebo-controlled trial conducted on 29133 Finnish smokers, which examined the effects of alpha-tocopherol and/or B-carotene, and found an eight percent higher overall mortality among patients receiving B-carotene (compared to the ones who did not receive it) (61). The results of a recently published Cochrane meta-analysis (approximately 300,000 participants) which compared the effects of antioxidant supplements (beta-carotene, vitamin A, vitamin C, vitamin E, and selenium) versus placebo or no intervention, were also worrying as they found that B-carotene and vitamin E seemed to increase mortality; a similar possibility was suggested for larger doses of vitamin A (62).

As for B-alanine supplementation, the authors of a meta-analysis conducted in 2012 (17) concluded that B-alanine supplementation had an ergogenic effect on high intensity exercises lasting between one and four minutes, possibly contributable to pH buffering capacity of intramuscular carnosine, the concentration of which rises with supplementation. The authors of a 2010 review (16) also noted similar observations, concluding that B-alanine carries potentially beneficial effects in high-intensity exercise including anaerobic sprints and resistance training. B-alanine supplementation is thus likely to be beneficial in high-intensity exercises. A systematic review from 2014 (63) found similar results regarding performance, indicating that B-alanine may increase power output and working capacity, decrease the feeling of fatigue and exhaustion, and have positive effects on body composition and carnosine content. In addition, this study concentrated on side effects of B-alanine supplementation, and found that they were mostly mild, consisting of paraesthesia and/or infrequent mild transient symptoms such as tingling in hands and fingers. The study also stressed the need for more studies dealing with long term effects of B-alanine supplementation.

The ergogenic effect of caffeine on endurance performance was demonstrated by multiple meta-analyses/review articles which also concluded that in habituated caffeine consumers, the abstinence from caffeine at least seven days before competition would give the

greatest chance of optimizing its ergogenic effect (28,64). However, the effect of caffeine on high-intensity performance is not well established, since caffeine increases plasma lactate concentration and hence decreases pH (28). On the other hand, a systematic review (65) of the effect of acute caffeine ingestion on short-term high-intensity exercise performance showed that 11 (of 17) studies revealed significant improvements in team sports exercise and power-based sports after caffeine ingestion, while further six (of 11) studies showed significant benefits for resistance training. Based on the results of the above studies, caffeine supplementation for endurance exercises seems to be beneficial, but when it comes to the effects of caffeine on high-intensity exercise, further investigation is required. Long-term ingestion of moderate doses of coffee (three to four coups [less than 400 mg/day]), is suggested to have mostly beneficial effects and has been shown to be inversely associated with the risk for various diseases (66). Epidemiological data support the view that habitual coffee consumption lowers the risks of Parkinson's and Alzheimer's disease, has a favorable effect on liver function, a possible role in weight loss (66), and also decreases the risk for developing certain types of cancer (endometrial, prostatic, colorectal, liver) (67,68). On the other hand, coffee intake has been associated with bone loss and adverse effects in pregnancy (66). Nevertheless, the growing body of evidence from epidemiological studies supports the notion that moderate coffee consumption exerts mostly beneficial effects on health and reduces mortality (67). Unfortunately, the association does not show causality, so large double-blinded, placebo-controlled studies are needed to clarify the effects of coffee consumption on health per se. Moreover, the effects of coffee consumption are not attributable solely to caffeine (since coffee has more than 1,000 different compounds (66)), while the supplements that are used to increase sport performance use mostly caffeine as an active compound, and lack other compounds found in a cup of coffee. Consequently, to determine the safety of caffeine-containing sport performance-enhancing supplements, additional studies on those supplements should be more thoroughly evaluated before giving adequate evidence-based statements about the safety of those supplements.

Several systematic reviews (30,31) confirmed the efficacy of carbohydrate mouth rinse on exercise performance with activities lasting less than one hour, potentially attributable to activation of pleasure and reward center in the brain (30).

As for ingestion of carbohydrates, a systematic review (69), which included only studies mimicking real life situations (subjects exercising in the postprandial state) and excluding studies that followed rigorous criteria like keeping athletes in a fasted state, concluded that carbohydrate supplementation during exercise bouts of less than 70 minutes is unlikely to be beneficial (69). On the other hand, a beneficial effect of carbohydrate supplementation during exercise lasting more than 70 minutes was confirmed in a systematic review that included all types of studies (those mimicking real life situations and also studies following rigorous inclusion criteria) (30) and also in the review (69) that included just studies mimicking real life situations. The most probable beneficial effect was attributed to the replacement of exhausted glycogen storage (30, 69). Taken together, carbohydrate supplementation is probably beneficial in exercises of longer duration (more than one hour), while in short-lasting exercise (less than one hour), more research is required to determine the efficacy in 'real-life situations mimicking' scenarios.

As for creatine, several review articles/meta-analyses (37,70) showed that it was effective in increasing performance in high-intensity, short-lasting (less than 30 seconds) exercises, and also that the effect of creatine diminished with increasing duration of exercise (70). It seems that creatine does not have any ergogenic effect on aerobic exercise (37). Short-term administration of creatine supplementation seems to be safe, since studies have not found clinically significant deviations from normal values in the renal, hepatic, cardiac, gastrointestinal or muscle function (71,72). In fact, most reports on its

potential side effects, such as muscle cramping, gastrointestinal symptoms, changes in renal and hepatic laboratory values, remain anecdotal (72). Studies which would evaluate the safety of long-term creatine supplementation are lacking. A few studies that examined the long-term effect of creatine supplementation on kidney function (73), or on various blood and urinary markers of health (metabolic markers, skeletal muscle and liver enzymes, electrolytes, lipid profile, hematological markers, and lymphocytes) (74), showed no differences between the measured outcomes in the creatine-taking group compared to the control group. Additional studies on larger number of participants are needed to give firm evidence-based conclusions regarding long-term safety of creatine supplements.

Regarding nitrate supplementation, the analyzed review articles/meta-analyses (41,42) have concluded that nitrate supplementation had a small but significant (41) performance-enhancing effect. The effect was most apparent in situations where skeletal muscles were insufficiently perfused, such as during hypoxia, or high-intensity exercise in subjects exhibiting low VO2max, when the contracting muscle is in a disadvantageous position relative to the heart and is relatively under-perfused (42).

A recent study has shown that short-term nitrate supplementation (up to 28 days) was safe, and all hematological safety markers remained in a normal range (75), but long-term studies regarding safety are needed.

The effects of protein supplementation (50 ± 32 g/day [above the usual diet]) were shown to be beneficial in augmenting the gains of fat-free muscle mass, type I and II muscle fiber cross-sectional area, and muscle strength, especially in combination with resistance training of longer duration (more than six weeks) (48). On the other hand, another review (76) showed that for untrained individuals, consuming supplemental proteins most likely had no impact on lean mass and muscle strength during the initial weeks of resistance training. However, as the duration, frequency, and volume of resistance training increase, protein supplementation may promote muscle hypertrophy and enhance gains in muscle strength in both untrained and trained individuals (76). Additionally, a third meta-analysis (47) showed similar beneficial effects of whey protein consumption on body composition parameters, which were most pronounced with resistance training exercise. We may conclude that protein supplementation, in combination with resistance exercise of longer duration (more than six weeks), most likely has beneficial effects on body composition (lean body mass) and possibly also on muscle strength.

Mounting evidence suggests that protein intake in excess of two to three times the recommended daily allowance may have harmful effects on the homeostasis of calcium and, possibly, bone mass, particularly for proteins from primarily animal sources and in situations where potential loss of bone calcium has not been minimized by additionally supplementing other dietary elements such as potassium or bicarbonate. Additional potential harmful effects of high-protein intake on kidneys, the cardiovascular system, and carcinogenesis have been suggested but available data remain inconclusive, and further research is necessary (77).

Conclusion

Most of the revised dietary supplements, if used for the intended exercise regime, provide some sort of sport performance enhancement. Sport performance enhancement can be a clear-cut one, or it can be observed only in strict laboratory conditions, it may involve unusual routes of administration or may only be successful in a subset of people. Nevertheless, at least in professional sport, where milliseconds decide between success and failure, the competitive edge that dietary supplements can bring, can give an athlete an advantage to succeed. On the other hand, the safety of long-term supplementation, being another and even more important aspect, should also be taken into account when considering whether or not performance-enhancing supplements should be encouraged and consumed. Unfortunately,

these kinds of studies, dealing with long-term safety of the supplements, are mostly lacking.

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Isotope studies of karst springs included in the water supply system of the City of Rijeka (Croatia)

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Abstract

Aim: To provide information about the results of isotopic analyses of karst springs, Rječina Spring and Zvir, which ensure potable water for more than 200,000 people in Rijeka County (Croatia)

Methods: Specific activities of selected radionuclides were determined by high resolution gamma spectrometric analysis and radiochemical separation method. Values of hydrogen and stable isotope contents were determined by water equilibration method on isotope ratio mass spectrometer in conjunction with dual inlet and equilibration peripheral unit.

Results: Anthropogenic radionuclides were detected in trace amounts. The results of the analysis show that the calculated yearly dose introduced to an adult human consuming 2 liters of water per day is approximately 20 μSv. Stable isotopes content of the spring waters indicates a dominant recharge of the analyzed hydrological system by winter precipitation. Different isotopic variations of spring water as a consequence of sudden precipitation inputs in summer and autumn indicate that water discharged at Rječina Spring mainly originates from a big water reservoir situated in the wide mountainous region in the hinterland of the spring.

Conclusion: The examined water is potable and radiologically safe. Stable isotope variations of the spring water show a fast reaction to sudden precipitation inputs, confirming the ecological vulnerability of karst springs. In light of recent heavy precipitation and flooding in different parts of Croatia, a more systematic research on isotopic water composition should be encouraged.

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Introduction

Rječina Spring (RJ) and Zvir (ZV) are the most important springs of the water supply system ensuring potable water for the City of Rijeka and its surroundings. These are springs with relatively similar annual average discharges but different flow dynamics. RJ springs at 325 m above sea level, has a mean annual output discharge of 7.38 m^3s^{-1} and regularly dries out for several months a year. Namely, RJ is a seasonal spring that functions as an overflow for high and medium groundwater discharges (1). It provides high quality water for the City of Rijeka and neighboring settlements through most of the year and its protection is of the highest priority. RJ average water supply rate measures 0.69 m^3s^{-1}. ZV springs at 3 m above sea level, has a mean annual distribution of 5.2 m^3s^{-1} and a nominal minimum discharge of 1.5 m^3s^{-1}. The pumping from ZV is activated, i.e. water from ZV is used for water supply, when the RJ discharge is reduced to 0.29 m^3s^{-1} or it dries out completely (2).

The two springs are under regular quality and health control checks by the Water Supply Company and the Teaching Institute of Public Health of Primorje & Gorski Kotar County (3), and have been subjects of many scientific investigations (1, 4). Nevertheless, isotopic studies of these spring waters are not as frequent. The need for better understanding of complex processes taking place in this ecologically and socially important karst area is often emphasized (5).

In order to expand the knowledge about RJ and ZV, we present the results of measurement for selected radionuclides in RJ and ZV water, as well as the results of monitoring hydrogen (^2H) and oxygen (^{18}O) stable isotope variations in RJ water as a reaction to precipitation input events.

To the best of the authors' knowledge, there have not been any reports about RJ and ZV from the radiological point of view. The first report about the results of a systematic analysis of a stable isotope composition of the springs was given by Mance et al. (6) and, before that, there were only sporadic reports (7, 8). The results presented here relate to short-term data collection, i.e. sampling during particular storm events, including observations related to rainfall inputs and stable isotope composition variations related to changes in RJ discharge. The results of this type of studies can contribute to identifying water storage processes and mechanisms of groundwater recharge and discharge in the karst aquifer (9). They are also important for public health as they reveal information about the reaction time of karst springs to potential pollutants.

Materials and Methods

Study area

RJ and ZV are the largest springs of the Rječina River (RR) basin, a typical Dinaric karst basin. The basin is located in the Dinarides of western Croatia and southern Slovenia (Figure 1A).

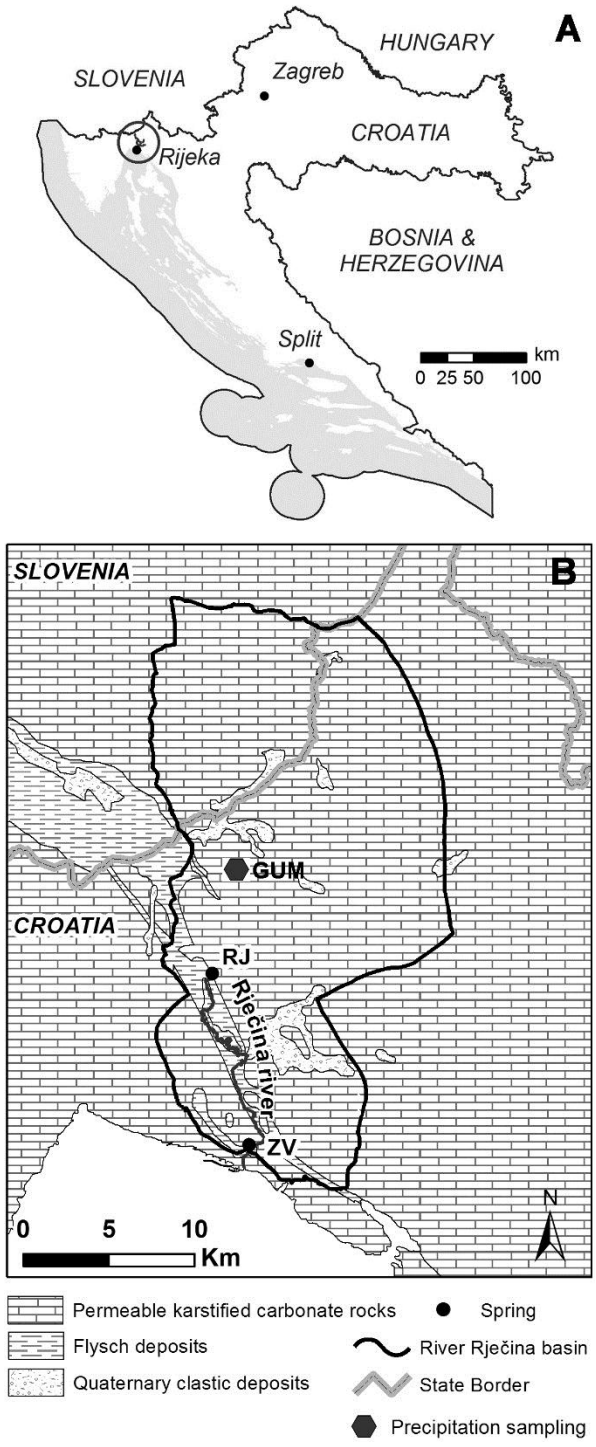
The RR basin covers approximately 500 km^2 and has its main recharge area in the mountains of Gorski Kotar (Croatia) and the Snežnik massif (Slovenia). The previous isotopic analysis found substantial evidence in favor of the conjecture that RJ and ZV are mainly recharged from the same average altitude and/or share the same groundwater reserves (6). The main lithological sedimentary units of the region are carbonate rocks, flysch and Quaternary clastic deposits (Figure 1B).

According to the Köppen-Geiger climate classification (10), the associated climate type for the study area changes from Cfa on the coast to Cfb in hinterland. These are temperate climates, with precipitation regimen without regular dry seasons, and with hot (Cfa) and warm summers (Cfb). The mountainous part of the study area has an annual rainfall greater than 3000 mm, making it one of the rainiest regions in Croatia (11).

Measurement methods and sampling

For the purpose of this study, spring water samples were collected at RJ and ZV, while

Figure 1. *A) Map of Croatia showing the study area (circle); B) sampling locations and distribution of the main lithological structures in the study area. RJ – Rječina Spring; ZV – Zvir; GUM – Gumance*



precipitation samples were collected at Gumance (GUM), the location that belongs to the recharge area of RJ and ZV basin. Precipitation sampling site can be seen on Figure 1B, while its coordinates and altitude are indicated in Table 1.

Table 2. *Coordinates and altitudes (in m above sea level) of precipitation sampling site*

Precipitation sampling site	Coordinates	Altitude (m a.s.l.)
Gumance (GUM)	N 45° 28' 4'' E 14° 24' 27''	688

Specific activity concentrations for natural (^{40}K, ^{210}Pb, ^{226}Ra, ^{228}Ra, ^{232}Th, ^{235}U, ^{238}U) and anthropogenic (^{134}Cs, ^{137}Cs, ^{241}Am) radionuclides have been determined for two samples collected in March 2012 at springs RJ and ZV. Activity concentrations were determined by high resolution gamma spectrometric analysis. Additionally, for the determination of ^{226}Ra activity concentration, the radiochemical separation method was also used (12). Measurements of activity concentrations were performed at the Institute for Medical Research and Occupational Health (Zagreb, Croatia). Effective dose calculation and results analysis were performed according to the regulation of Croatian Ministry of Health and recommendations of the World Health Organization (WHO) (13, 14). The annual effective dose *E* (mSv/year) resulting from water intake was estimated as (14):

$$E = q \cdot \sum_i C_i \cdot h_i$$

where:

q - annual ingested volume of drinking water, assumed to be 0.73 (m^3/year),

C_i - the activity concentration of radionuclide i (Bq/m^3),

h_i - dose coefficient for ingestion of radionuclide i by adults (mSv/Bq).

Drinking water is considered to be safe from the radiological point of view if the annual dose calculated based on the presence of radioactive isotopes in water, under the assumption of an average daily consumption of 2 liters, is below 0.1 mSv (14). Since there are no strict upper limits for activity of individual radionuclides in drinking

Table 2. *Results of gamma spectroscopic analysis of water samples*

Radionuclide	Activity concentration (Bq / m³)	
	Rječina Spring	Zvir
^{40}K	42.2 ± 2.5	44.7 ± 3.4
^{134}Cs	< 1.2 ± 0.2	4.4 ± 1.5
^{137}Cs	< 1.1 ± 0.2	< 1.4 ± 0.4
^{210}Pb	17.2 ± 4.2	32.5 ± 7.5
^{226}Ra (γγ)	< 25.6 ± 7.1	< 12.7 ± 4.2
^{226}Ra (αα)	21.1 ± 6.3	5.4 ± 2.8
^{228}Ra	3.4 ± 0.5	2.6 ± 0.7
^{232}Th	3.4 ± 0.5	2.6 ± 0.7
^{235}U	< 1.6 ± 0.8	< 2.0 ± 1.0
^{238}U	< 31.5 ± 5.3	30.2 ± 7.4
^{241}Am	< 3.5 ± 0.8	< 4.6 ± 1.1

water, screening for gross α and gross β radiation activity was also carried out (14).

In order to investigate the karst aquifer discharge response to sudden precipitation inputs, daily sampling during storm events at spring RJ was organized and precipitation samples were taken on GUM. Samples were taken during two storm events in late summer of 2013, followed by storm event sampling in the fall of 2013. On those occasions, both rain water (N = 10) and groundwater samples (N = 30) were collected. Stable isotope contents of collected samples were measured by the water equilibration technique in Stable Isotope Laboratory at the Physics Department of the Faculty of Medicine at University of Rijeka. For the measurement, an isotope ratio mass spectrometer (Delta^{plus} XP; Thermo Finnigan, Germany) coupled with equilibration unit and dual inlet system was used. Results were reported in δ-values (‰) on VSMOW scale (15). The measurement precision was < 1 ‰ for hydrogen (δ^2H), and < 0.1 ‰ for oxygen (δ^{18}O).

Globally, for natural waters that are not under the influence of evaporation, the linear relationship between δ^2H and δ^{18}O is represented by the Global Meteoric Water Line (GMWL): $\delta^2H = 8 \cdot \delta^{18}O + 10$ ‰ (16). There are local deviations from the GMWL, due to various different factors such as altitude, proximity to the sea, etc., which required the measurement of local meteoric water lines. One of these local meteoric water lines is the Western Mediterranean Meteoric Water Line (WMMWL): $\delta^2H = 8 \cdot \delta^{18}O + 13.7$ ‰ (17). Stable isotopes can be used as "fingerprints" to detect geographical origins of natural waters (18, 19). One of the ways to do this is by using d-excess, the hydrological parameter introduced by Dansgaard (20): d-excess = $\delta^2H - 8 \cdot \delta^{18}O$. Precipitation that originates from the Atlantic Ocean typically has d-excess values ≈ 10 ‰ and precipitation originating from the Mediterranean has d-excess values > 15 ‰ (21).

Results

Gamma spectrometric analysis proved the existence of natural as well as anthropogenic radionuclides in examined water samples. The latter were only found in trace amounts. Results of the analysis, i.e. activity concentration for radionuclides of interest, are presented in Table 2. Activity concentration of ^{226}Ra has been determined by using both gamma spectrometric method and radiochemical separation method, and results of these two methods do not differ significantly (Table 2).

Calculated gross α radiation activity for RJ is $2.831 \cdot 10^2$ Bq / m³, and for ZV it is $2.712 \cdot 10^2$ Bq / m³, while gross β radiation activity for RJ is $2.509 \cdot 10^2$ Bq / m³ and for ZV it is $2.443 \cdot 10^2$ Bq / m³. Estimated effective dose for an adult whose daily intake is 2 L of water, for RJ is 18 μSv / year and for ZV 23 μSv / year.

As it can be seen in Figure 2, δ^2H and δ^{18}O values of spring water correspond to WMMWL, while the majority of precipitation samples lie between GMWL and WMMWL. Figure 2 also shows spring water values corresponding to the lower part of value cluster of precipitation samples.

D-excess values of precipitation in our study range from a minimum of 6.98 ‰ to a maximum

Figure 2. *δ^2H versus $\delta^{18}O$ correlation diagram of Rječina Spring (RJ) samples and precipitation samples collected at Gumance (GUM). Global Meteoric Water Line (GMWL) and West Mediterranean Meteoric Water Line (WMMWL) are shown for comparison.*

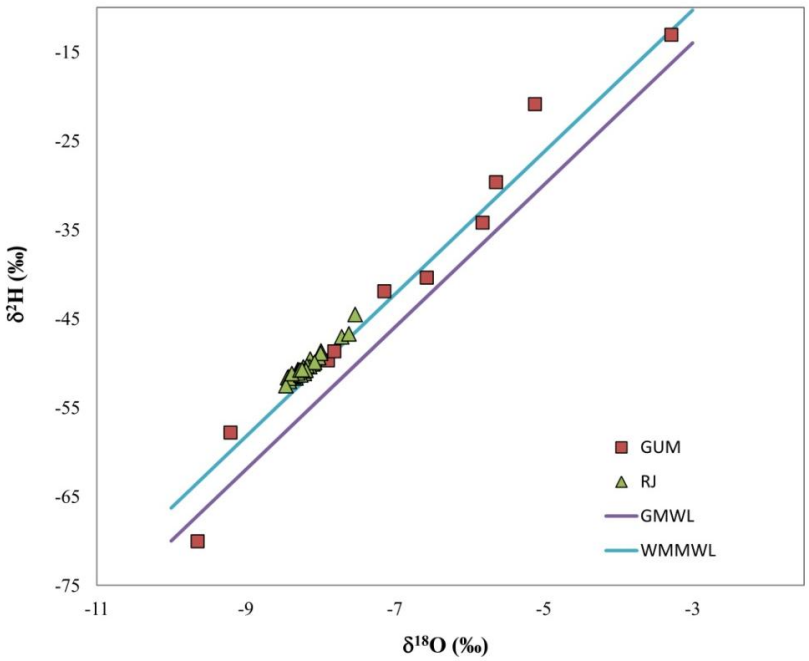


Figure 3. *Deuterium excess values of Rječina Spring (RJ) samples and precipitation samples collected at Gumance (GUM). The mean analytical uncertainty (± 1.04 ‰) was as indicated by the error bars.*

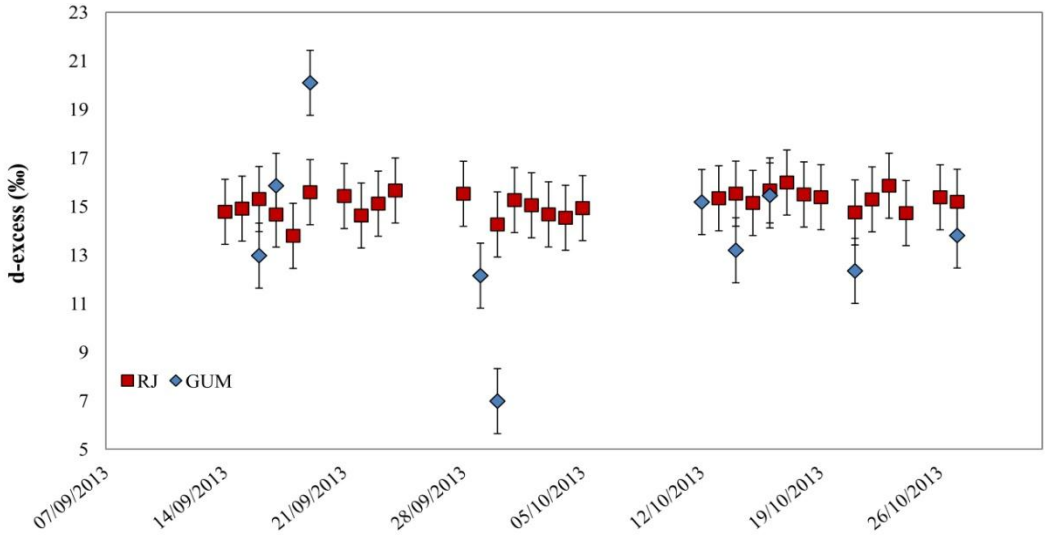
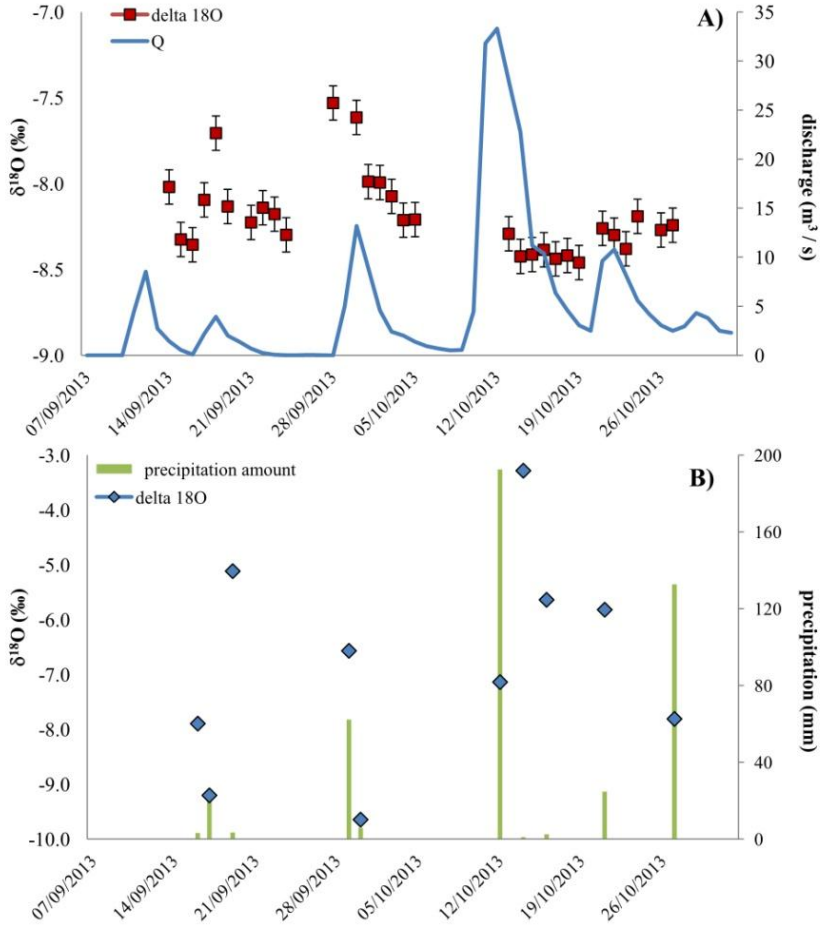


Figure 4. *A) Daily discharge and $\delta^{18}O$ groundwater series for samples collected at Rječina Spring; B) precipitation amounts and corresponding $\delta^{18}O$ values of samples collected at Gumance (GUM). The mean analytical uncertainty (± 0.1 ‰) is indicated by the error bars.*



deviation of 1.45 of 20.09 ‰ (Figure 3). Other precipitation d-excess values fluctuate about a mean value of 13.87 ‰, with standard deviation of 1.45 ‰. ~~‰~~. Unlike the precipitation samples, d-excess values of spring water are all within the measurement error with a mean value of 15.13 ‰ and a standard deviation of 0.49 ‰.

$\delta^{18}O$ time series of RJ spring water collected in September 2013 show shifts towards more positive values that coincide with an increase of RJ discharges (Figure 4). The first such shift occurred on 18th September (RJ, $\delta^{18}O$ = -7.71 ‰), although rain water collected on 17th of September had a lower value (GUM, $\delta^{18}O$ = -9.2 ‰) than values of RJ water sampled prior to that rain event (≈ -8.0 ‰ to -8.3 ‰).

Next significant rain event occurred between the 28th and 29th September 2013, with 62.3 mm of rain and $\delta^{18}O$ = -6.57 ‰. RJ sample collected on the 28th September had the value of -7.53 ‰, although the discharge was very low. The discharge reached the highest value on 30th September, with corresponding $\delta^{18}O$ = -7.61 ‰. During the following days, both discharge rates and $\delta^{18}O$ values continued to decrease (Figure 4A).

After the described events, RJ $\delta^{18}O$ values oscillations were not significant any more, although there were major precipitation events in October 2013. The two most important of those precipitation events took place on 12th and 27th October, with precipitation amounts of 192.6 mm and 132.7 mm, respectively. $\delta^{18}O$ value for the first event was -7.14 ‰, and for the second one -7.81 ‰ (Figure 4B), but a reaction to these events could not be detected in isotopic values of RJ (Figure 4A).

Discussion

Values of radionuclide activity in spring waters of RJ and ZV reported here are well below guidance levels for radionuclides in drinking water recommended by WHO (14). The calculated yearly effective doses, obtained by using the concentration values of radionuclide activity in spring waters of RJ and ZV, assuming that the water is introduced into the human body on a daily basis, are ≈ 20 μSv. This is significantly below the acceptable limit of a dose (13). The analyzed RJ and ZV samples are not radiologically contaminated and water may thus be considered radiologically safe and used for drinking.

Mance at al. (6) showed that RJ is under the influence of different hydrological conditions in comparison to other springs in the area. In order to provide some new, additional information about this complex system, samples of GUM precipitation and RJ groundwater were taken in late September 2013 and in the fall of 2013.

Both, an isotopic values correlation diagram (Figure 2) and d-excess values of the spring samples (Figure 3) indicate a Mediterranean origin of the analyzed water, confirming the dominant recharge of the system by winter precipitation (6).

An analysis of stable isotope dynamics shows a different composition of stable isotopes in groundwater during the three storm events. The first September storm event occurred after a relatively long period without significant precipitation. This event is difficult to comment given that a shift towards a more positive groundwater $\delta^{18}O$ value occurred one day prior to the rain event on GUM, and this rainfall had the second most negative $\delta^{18}O$ value of all rain samples collected during this study (Figure 4). A possible explanation is that a rain event that triggered the groundwater shift toward more positive $\delta^{18}O$ value had happened earlier than the one on GUM and that it had taken place deeper in the mountainous hinterland of the system, most probably on the Slovenian Snežnik massif.

The second September event had significantly less negative $\delta^{18}O$ precipitation values in comparison to RJ values collected previously during the month (with the exception of the value that corresponded to the highest RJ discharge of the previous event). As it already happened during the previous event, a shift towards less negative groundwater values occurred again a day prior to the event on GUM (Figure 4). Once more, this suggests that an earlier event, taking place further in the catchment area, had activated the system prior to the event on GUM. Also, this indicates a fast reaction of the hydrological system to sudden precipitation inputs and a short retention time of newly infiltrated water in the underground. This is very important from the environmental protection point of view as it indicates an area highly susceptible to contamination. The introduction of a pollutant to the system would probably cause an immediate pollution of drinking water.

Stable isotope composition of groundwater samples collected after the third storm event was without significant oscillations although the precipitation amount was considerable and its δ values were less negative than those for groundwater sampled prior to the storm. Bearing in mind that the system may be described by a dual porosity model (6), this situation might suggest that September storm events triggered the passage of groundwater through short and wide karst channels, while well mixed groundwater discharged in October originated from groundwater reservoirs. Accordingly, it can be concluded that there is a scant amount of groundwater in the vicinity of RJ, and the water discharged at RJ mainly originates from a large water reservoir located in the mountainous region in the hinterland of the spring.

The last precipitation event in this study occurred on 27th October, but the isotopic value of the collected precipitation was too close to the value of previously collected groundwater (Figure 4), so this event could not be used for further analysis.

Although we got new insights in the functioning of the system, such as the one indicating that precipitation from the hinterland has greater influence on RJ than precipitation occurring closer to it, there are still many unresolved questions. The reaction of the system to the precipitation inputs proved to be very fast, and daily sampling was probably not frequent enough to give a complete picture about the hydrological conditions. More frequent sampling, for example on an hourly basis, could give a better insight into the situation (9).

Conclusions

The authors present the results of radioactive and stable isotopes analyses for RJ and ZV, the two largest and most important water supply springs of the city of Rijeka, as well as precipitation samples collected at the assumed recharge areas of the two springs. The purpose of the paper is to provide a radiological analysis of their water quality and to expand the sources of available data for stable isotopes.

Measurements of radionuclides showed that the water is safe for human consumption. Stable isotope data indicates a rapid reaction of the system to sudden precipitation inputs, thus confirming vulnerability of karst springs to potential pollution. We can confirm that the main water reservoir of the system is situated in the mountainous region of the basin, and that in the vicinity of the Rječina Spring there are only scant amounts of groundwater.

The authors conclude that isotopic investigations of the spring water and precipitation collected on the assumed recharge area reveal some useful information on the hydrological system. Recently, there have been catastrophic floods in different parts of Croatia. We may treat the floods as indicators of a lack of detailed knowledge about the hydrological conditions in corresponding aquifers. Therefore, more systematic isotopic analyses are strongly encouraged.

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PROGINS mutation of progesterone receptors and its role in premature birth – an overview

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Abstract

Premature birth (prior to 37 weeks of gestation) is a big medical and socioeconomic problem. It accounts for 8-12% of the total number of births, and apart from causing increased mortality of newborns, it is also the cause of increased morbidity. Fifteen million babies per year are born preterm. Despite the frequency, consequences and costs of premature delivery, very little has been done for preventing it, especially for preventing extremely premature deliveries (before the 28th gestation week).

Etiology of premature labor is multifactorial, and includes pathophysiology, genetic and environmental factors. Recent scientific research shows that genetic factors, mostly present in the mother's genome, account for up to 40% of variation in the delivery time.

It is believed that premature birth exhibits the same cascade of events like a normal birth, only it starts sooner. This process is controlled by a series of hormonal effects between the fetus, the placenta and the mother. One of the key signaling pathways in this series is the progesterone signaling pathway.

PROGINS allele is a progesterone receptor gene modification. It is made of three variants: V660L, H770H and alu insertion. Progesterone receptors with PROGINS mutation are less susceptible to progesterone activity, and it seems that the withdrawal of progesterone causes the beginning of birth cascade. Mutation of +331 G/A progesterone receptor is a newly discovered mutation. It is believed that this mutation leads to a PR-A and PR-B receptor quantity disorder before the delivery term.

The aim of this review is to summarize all recent knowledge about PROGINS and +331 G/A mutation of progesterone receptors and to estimate whether this genetic mutation has a value in modulation of risk of preterm birth.

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Introduction

Premature or preterm birth is recognized as a worldwide problem. The percentage of preterm births has not been reduced despite existing research and therapy. It is defined as birth between 22nd and 37th week of gestation, and it is one of the major causes of prenatal mortality and morbidity. Prematurity accounts for 70% of neonatal mortality and 75% of neonatal morbidity (1).

According to gestational age, preterm birth is divided to extremely preterm birth (between 24th and 28th week of gestation), early preterm (between 28th and 34th week of gestation) and late preterm birth (between 34th and 37th week of gestation) (2). The percentage of preterm births varies from 5% of all deliveries in Europe to 18% in South African area (3). There are also variations of the percentage of preterm birth in diverse ethnical groups; for example, the percentage of preterm births is bigger in the population of African Americans than in the population of Caucasians (4,5). In 2010, there were around 15 million preterm births in the world (3,5). In the same period in Croatia, the percentage of preterm births was between 5.19 % and 7.88 % of all births, with a tendency of increasing since 2008 (6).

Etiology and risk factors of preterm delivery

Etiology of preterm birth is heterogenic and is connected to different metabolic pathways in the human body. For almost 50 % of preterm births, the cause is unknown. Preterm birth is divided in three subtypes (2):

- **spontaneous preterm birth** (spontaneous start of labor); 50% of all preterm births, occurring more frequently in the population without any risk factor for preterm birth
- preterm premature rupture of membranes (**PPROM**); 25% of all preterm births, occurring more often in the African-American population, in most cases as a result of infection
- **iatrogenic preterm birth** before 37 weeks of gestation, due to maternal or fetal medical reasons, or other non-medical reasons that could jeopardize the health of mother and/or fetus (e.g., preeclampsia, placenta previa, placental abruption, multiple gestation, grow restriction of fetus); 25% of all preterm births.

There are many risk factors that relate to preterm birth, as indicated in Table 1. In an ideal situation, risk factors for preterm birth should be identified prior to or during the first trimester of pregnancy and, if possible, that should lead to interventions which would result in term delivery. Some pathways of starting a preterm delivery and related risk factors are presented in Table 2.

Nowadays it is more common to use the term "preterm parturition syndrome" (7), or even "great obstetric syndrome", because of its multifactorial etiology.

There are four major factors leading to preterm labor (7,8,9,86): 1) Pathological uterine distension; 2) Maternal fetal stress (premature activation of the maternal or fetal hypothalamic-pituitary adrenal axis); 3) Abruption (decidual hemorrhage) and 4) Infection / exaggerated inflammatory response. All these processes could lead to cervical shortening and could start long before obvious signs of preterm birth are shown (10). Also, although they start at different ends of pathophysiology of preterm birth, they end in the same way, by activating choriodecidual reaction, uterine contractility and changes of the cervix. All these changes lead to preterm birth.

Table 1. *Etiological risk factors associated with clinical presentation of preterm birth (2).*

Medically induced preterm birth	**PPROM (Preterm premature rupture of membranes)**	**Spontaneous preterm birth**
Maternal factors	Infection	Previous preterm birth
Gestational hypertension and vascular disorder	Uterine distension	Low body mass, poor weight gain
Acute illness or chronic condition	Cervical anomalies	Strenuous physical workload, ergonomic factors
Obstetrical complication	African-American ethnicity	Uterine anomalies
Antepartum bleeding	Disadvantaged population	Psychosocial stress
Maternal age > 35 years		Lifestyle, smoking
Fetal factors		Drug abuse
Intrauterine growth restriction		Maternal age < 18 years
Unstable fetal condition		Unknown
Fetal anomaly		
Multiple pregnancies		

Table 2. *Causes and pathological pathways of preterm birth (7)*

• Uterine distension
• Ischemia
• Infection
• Cervical disease
• Abnormal allograft reaction
• Allergic phenomena
• Endocrine disorders

Pathological uterine distension

It is highly possible that uterine overdistension (caused by multifetal pregnancy, polyhydramnios or any other cause of uterine distension) can cause expression of contraction-associated proteins (CAPs) in the myometrium. Overdistension of the uterus also induces formation of gap junctions, upregulates oxytocin receptors and produces prostaglandins and inflammation cytokines (11). All this can initiate events that could change timing of uterine activation and lead to uterine contractions and cervical dilatation.

Maternal-fetal stress (premature activation of the maternal or fetal hypothalamic-pituitary adrenal axis)

In situations of stress, some circumstances disturb normal functions of a person. Maternal stress (infection, multiple pregnancies or psychological stress, such as depression or anxiety) can activate maternal HPA axis and cause preterm birth.

Premature fetal HPA activation, on the other hand, can be the result of stress of uteroplacental vasculopathy. It is more highly correlated with preterm birth than mother's stress (12). The main pathway of this cause seems to be a change in fetal adrenal-placental endocrine cascade, which leads to early rise of maternal CRH (corticotropin-releasing hormone) and estrogen levels. CRH plays a role both in term and preterm birth. Usually it is released by the hypothalamus, but during pregnancy it is released by trophoblast and decidual cells, too (13,14). Increased production of placental CRH stimulates production of ACTH, which further

stimulates production of cortisol. Cortisol inhibits hypothalamic CRH and ACTH, but on the other hand, stimulates CRH from the placenta. CRH also induces production of prostaglandins in the placenta (15). The increase of prostaglandins results in parturition through elevation of proteases in the genital tract (e.g., MMP) and higher myometrial contractility (16). Also, prostaglandins influence the PR-A: PR-B ratio and induce functional progesterone withdrawal (17).

Stress, maternal and/or fetal, can also stimulate steroid-induced immunophilin cochaperone FKBP51 in the decidua. It can also cause functional progesterone withdrawal through inhibition of progesterone receptors (18).

One more pathway of preterm birth caused by activation of fetal HPA axis is the estrogen pathway. Fetal ACTH induces synthesis of DHEA. DHEA in fetal liver is converted to 16-hydroxy-DHEA-S. The placenta converts these precursors to E, E2 and E3, which further activate the myometrium through increase of gap junction formations, oxytocin receptors, activity of prostaglandins and increasing of enzymes responsible for myometrium contractions (e.g., calmodulin) (19,20). All the pathways mentioned above cause contractions of the myometrium and the start of labor.

Infection / exaggerated inflammatory response

It is the only evidence-based and proven cause of preterm birth, which activates different pathways leading to preterm birth. Infection activates a cascade in the immunological response of the mother and leads to preterm birth. Inflammation is a coordinated process, and its role is basically to protect the host. In a normal situation, when the immune system is properly controlled, inflammation is protective. In other cases, it is harmful.

Preterm birth can be caused by both systemic and local genitourinary pathogens. In most cases, the cause can be symptomatic or asymptomatic bacteriuria (21), presence of genital infections (22), periodontal disease (23) and clinical and subclinical chorioamnionitis. The last-mentioned infection is the cause of as much as 50 percent of preterm births before 30 weeks of gestation (24).

Studies have proven that the actual cause of preterm birth is not the infection itself but rather a disorder of maternal immunity (21-24). Pathways of preterm birth in infection start by binding of bacterial ligands to toll-like receptors (TLRs) in placental, decidual, amniochorion and cervical cells. TLRs and local leucocytes activate NFkappaB, which in turn, starts the maternal and/or fetal inflammatory response. Whether TLRs will start the activation of NFkappaB or not, depends on the presence of some intracellular signaling adaptors (e.g., MyD88), coreceptor molecules (e.g., CD 14) and receptor modulators (soluble IL 6 receptor, soluble TNF receptor -1, etc.) (25-27).

Activation of NFkappaB leads to activation of neutrophils, macrophages and various proinflammatory mediators. The most important mediators of this response are TNF and IL 1 Beta. They induce COX-2 expression and production of prostaglandins. TNF also initiates expression of various MMPs in the amnion, chorion, decidua and cervix, and degrade the matrix of cervix and fetal membranes (28,29). TNF alfa can also induce apoptosis in amniotic epithelial cells, which leads to PPROM.

Not just the immune response, but some bacteria themselves (e.g., Pseudomonas, Staphylococcus, Streptococcus) can have a direct role in the pathogenesis of preterm birth. They can produce enzymes that can degrade fetal membranes, as well as phospholipase A2 and endotoxin, which stimulate uterine contractions (30).

Abruption / decidual hemorrhage

Vaginal bleeding caused by decidual hemorrhage is a risk factor for preterm birth and PPROM. According to one study, vaginal bleeding lasting more than one trimester increases the risk of PPROM seven times (31). PPROM develops after decidual hemorrhage, due to high concentration of decidual tissue factor. It combines with factor VIIa of hemostasis,

and in the end, thrombin is generated. Thrombin binds to decidual protease-activated receptors, which induce expression of proteases (e.g., MMP). Abruption can also be related to an inflammatory reaction without infection. It starts as a result of activation of the immune response by free hemoglobin chains and protease.

Pathological cervical change

Most cases of cervical changes prior to term pertain to cervical insufficiency. The changes that cause preterm birth may be the result of a congenital disorder, post-surgical trauma or damage caused by trauma. In most cases, though, cervical shortening is the result of inflammatory or hemorrhagic pathways.

As mentioned above, main changes in preterm birth happen on the placental level. They include effects on the level of prostaglandins (immunology and infection pathways) and the endocrine level (progesterone/estrogen pathway). Recently, new pathways have been identified and they seem to be connected with biological and psychosocial factors. These pathways include, as mentioned above, influence of genetic factors, factors of stress, factors that can be attributed to the mother or to the fetus, conditions which cause mechanical stimuli, and cases of inflammation and infection.

The role of genes in preterm birth

Investigations about genetic influence on preterm birth have been common during the last two decades. Studies have shown that the risk of preterm birth is higher in women born prematurely. Women who have had a previous preterm birth are at greater risk to have it again. After the first preterm delivery, the chance of another preterm delivery in the same mother goes up to 30-50% (32). Also, it has been shown that mothers whose sisters, mothers or female cousins have had a preterm birth bear greater risk of having a similar preterm birth themselves (33,34,35). An extensive study, conducted in Sweden in 2010, showed that both maternal and fetal genes are involved in preterm birth (36,37,38). Fetal genetic factors accounted for 13.1% of the variation in gestational age at delivery, while maternal genetic factors accounted for 20.6% (37). In a similar study, estimation of the percent of variation connected to fetal genetic factors ranges from 11% to 35%, while the range for the maternal genetic contribution is 13-20% (39). Another study, by Svensson et al., showed that 25% of variation in preterm birth was explained by maternal genetic effects, 5% by fetal genetic effect, 18% by the environment created by the couple and 52% by unshared environmental effect (40,41). This and other studies showed that paternal genetic influence on preterm birth is minimal (up to 5%) or there is no influence at all. That fact is in discrepancy with two Norwegian studies (42,43). In the same study by Svensson et al. it is shown that fetal genetic effect is higher if preterm birth is induced for medical reasons. Genes can influence different pathways in the body connected with preterm birth. Most of the studies involved gene contribution in immunology and inflammation pathways (32,44). Induction of proinflammatory mediators, especially TNF and its receptors, has been suggested to have a crucial role in activation of labor, both term and preterm (45). Pro-inflammatory and anti-inflammatory cytokines (interleukins) have also been investigated (46,47). There have also been investigations on genetic mutations leading to change of uterine contractility and change of cervical tissue, which leads to cervical shortening and spontaneous preterm birth. They involve genetic mutations in dopamine receptors, OST receptors, progesterone pathway, etc. Recently there have been studies about stress influence and genetic mutations in preterm birth (48).

A big genome-wide association study of a large cohort of women of European ancestry has shown that maternal variants at the EBF1, EEFSEC, AGTR2, WNT4, ADCY5, and RAP2C loci were associated with gestational duration, and that maternal variants at the EBF1, EEFSEC, and AGTR2 loci were associated with preterm birth (49).

In this review, the authors will try to see the influence of specific genetic polymorphisms connected to progesterone pathway that has an

important role in maintenance of pregnancy and changing contractility of the uterus.

The role of progesterone in onset of preterm birth

Progesterone (P4) is one of essential hormones in establishing and maintaining pregnancy. It is a 21-carbon steroid hormone which is mainly produced in the ovaries, placenta, brain and the adrenal glands (50). In early pregnancy, it is produced by corpus luteum whereas from 7th week of pregnancy onwards, its production occurs in the placenta. Progesterone is required for maintenance of pregnancy and one can see its influence on uterus contractility by inhibition of cervical ripening and decreasing of the production of chemokines. Progesterone also prevents apoptosis in fetal membranes in both basal and proinflammatory conditions, and it prevents PPROM and, consequently, preterm birth (51,52).

Progesterone produces its physiological effects through progesterone receptors (PGRs). PGRs are expressed in the central nervous system, ovaries, breasts, and the female reproductive tracts, including the vagina, cervix, fallopian tubes and uterine endometrium and myometrium. At term, depending on the species, either withdrawal of P4 by a decrease in hormone levels or alteration of PGR signaling relieves the suppression of inflammation and contraction, which allows the myometrium to start contractions and lead toward labor. Multiple mechanisms, including P4 metabolism, regulation of PGR gene expression, PGR post-translation modifications and PGR co-regulators, which mediate or regulate uterine P4/PGR signaling, have been identified and reviewed (53). One of the hypotheses about preterm birth is that the cascade of events in preterm birth is similar as the events at term birth, with the difference being only that it starts earlier.

Progesterone receptors are members of the group of steroid hormone receptors. They are made of central DNA-binding domain (DBD), N-terminal part with proximal activation function (AF1), distal AF3 in B upstream segment, and nuclear localization signal which is found upstream of LBD. AF1 is ligand-independent, while AF is not. AF1 has an influence on direction of transcription (54). There are two types of progesterone receptors: nuclear and membrane receptors (8). The nuclear PRs function as ligand-activated transcription factors and they influence gene expression. Membrane PRs are on the cell surface; they are related to G-protein coupled receptors and single transmembrane receptors, and they appear to mediate direct non-genomic actions of progesterone (8). Maintenance of pregnancy is mostly regulated through nuclear progesterone receptors, while membrane progesterone receptors are less sensitive to the influence of progesterone. By influencing PRs, progesterone activates a variety of pathways and induces expression of other genes, which leads to activation or deactivation of myometrium. Those pathways include activation of certain CAPs (contraction-associated genes) such as connexin, ion channels (e.g., calcium channels), uterotonin receptor and enzymes that influence synthesis of local prostaglandins (1,55).

Nuclear receptors are coded by the prostaglandin gene located on chromosome 11 (11q22-q23) (56). Two isoforms of nuclear progesterone receptors are most significant for progesterone influence; more specifically, PR-A and PR-B. Both receptors have the same DNA-binding domain, ligand-binding domain and hinge region. The only difference between these two is the fact that there are additional 165 amino acids present in the N-terminus of PR-B (18). There are some other forms of nuclear PRs known, such as PRC, PRM, PRS, PRT etc., but their significance in human birth is irrelevant (55).

PR–B have the function of transcriptional activators of genes involved in maintaining pregnancy, and PR–A repress the activity of PR-B. According to literature, most of the PRs in the myometrium during pregnancy are PR-B, and as the term of delivery comes nearer, the number of PR-A increases (36).

PR isoforms influence the expression of diverse downstream genes through a complex regulatory network which includes the NF-κB, ZEB-microRNAs and UPR pathways, as well as direct transcriptional regulation. They have a

combined influence on activities of downstream effectors. The overall P4/PGR signaling-dependent molecular profiles are modified by activities of PGR isoforms, co-regulators and the ligand availability. P4/PGR signaling mediates and utilizes these interconnected pathways to determine the state of the myometrium throughout pregnancy (53).

Different influences of PR-A and PR-B isoforms are reflected in their influence on expression of proinflammatory and anti-inflammatory genes. PR-A act proinflammatory, by increasing expression of proinflammatory genes for PTGS2, IL8, IL1A and PTX3. On the other hand, PR-B inhibit expression of proinflammatory genes (57).

In mammals, it has been proven that birth starts at the moment of progesterone withdrawal. However, in humans, measurements of the levels of serum progesterone in the blood at the time of birth showed that there was no declination of progesterone level.

At least five pathways of how withdrawals of progesterone influence the start of birth were examined. They include: 1) reduced bioavailability of progesterone, 2) increased cortisol concentration in late pregnancy, which leads to progesterone and cortisol competing for binding to glucocorticoid receptors (58), 3) conversion of progesterone to an inactive form, 4) changes in isoforms of PG receptors (59), 5) changes in progesterone co-regulators (54).

Some studies also include functional estrogen activation (60) and inflammation resulting in NFkappa mediated PR repression (61).

In this short review, the authors have reviewed literature data about changes in isoforms of PG receptors. There is a hypothesis that human parturition involves changes in expression of myometrial nPRs and that change of expression leads to functional progesterone withdrawal and start of birth.

The main theory is so-called IST (isoform switch theory) (36,62), according to which, as mentioned above, the ratio of PR-A and PR-B is changed in favor of PR-A. PR-A repress PR-B and reduce transcription of pregnancy promoting genes. Increasing level of PR-A at the end of pregnancy occurs because of a change in methylation of PR-A promoter region. That eventually leads to pre-term contractility of the uterus.

Chai et al. wanted to clarify epigenetic mechanisms that contribute to the control of PR isoform expressions in the pregnant human myometrium. They researched the change in methylation of the cPg island in promoter region of PRs and uncovered an epigenetic mechanism for elevated PR-A:PR-B expression ratio in term myometrium during progesterone withdrawal. PR-A promoter loses H3K4me3 selective demethylase JARIDIA, which leads to increased methylation of the PR-A promoter, change of its transcriptional activity and change of PR-A:PR-B ratio (63).

In further investigations, Nadeem et al. identified a mechanism by which P4 action of maintaining the pregnancy has been withdrawn even in the presence of elevated levels of this hormone in circulation. Unliganded PR-A localize to the nucleus, where they paradoxically activate transcription of Cx43 gene through interaction with AP 1 heterodimers (64). Namely, during pregnancy, under the influence of P4, PR-B create a complex with transcriptional repressors and inhibit transcription of Cx43. In labor, as a result of change of PR-A:PR-B ratio, PR-A become unliganded and encourage expression of Cx43.

A similar mechanism can be applicable on other labor-associated genes, such as PTGS2, OXTR, OXN, PTGDS and NFKB2 and some proinflammatory cytokines and matrix proteins. This study has also showed increased level of progesterone metabolized with 20 alpha HSD enzyme, which might be important for the usage of appropriate progesterone for therapy of preterm birth (59).

Beubaker et al. showed that the repressive activity of PR-A and their amount in the myometrium are increased by pro-inflammatory stimulation (65).

Genetic variants of PRs and their influence on preterm birth

Common variant in human PRs is the so-called PROGINS allele. It is present in some frequency in more than 20% of population. PROGINS mutation of progesterone receptors is extensively investigated. One of the research groups, Romano et al. (66), showed that it is characterized by a 320 bp PV/HS-1 Alu insertion in intron G and two-point mutations, V660L in exon 4 (rs1042838 SNP) and H770H (silent substitution) in exon 5 (rs1042839 SNP). The Alu element contains a half estrogen-response element/Sp1-binding site (Alu-ERE/Sp1), which acts as an in-cis intronic enhancer leading to increased transcription of the PROGINS allele in response to 17beta-estradiol. Moreover, Alu insertions in the human genome are frequently methylated. Some data indicate that the PROGINS-Alu does not affect gene transcription due to DNA methylation. However, the Alu element reduces the stability of the PROGINS transcript compared with the CP allele and does not generate splice variants. The amino acid substitution (V600L) in exon 4 leads to differences in PR phosphorylation and degradation in the two PR variants upon ligand binding, likely because of differences in the three-dimensional structures of the two PR variants. Consequently, the PR-L660 (PROGINS) variant displays decreased transactivation activity in a luciferase reporter system and is less efficient in opposing cell proliferation in hamster ovarian cells expressing human PR-A, when compared with the PR-V660 (most common variant).

PROGINS variant of PR is less responsive to progestin compared with the most common PR because of reduced amounts of gene transcript and decreased protein activity (66).

It has been proven that PROGINS allele has its influence, and represents a risk factor in some patients with breast cancer, endometrial cancer and endometriosis. In pregnancy, we see its influence either in decreased effectiveness of PGRs on P4 or through increased risk for conditions associated with preterm birth.

Another SNP that the authors wanted to emphasize is +331 G/A SNP of PR, which is a newly described mutation. Its significance lies in its influence on PR-A/PR-B isoform ratio, more specifically, its ability to change the ratio in favor of PR-A. The possible significance of these mutations could be decreased transcriptional regulation of progesterone target genes, which leads to change of pathways and start of preterm birth.

Several studies have tried to show whether there is an influence of these four SNPs on modulation of preterm birth. Results are biased. Some studies (Diaz Cueto et al., 2008; Guoyang et al., 2008; Kurtz et al., 2001; Oliveira et al., 2011) (67,68,69,70) did not find any connection between polymorphism of PGR and risk of preterm birth. On the other hand, some studies (Langmia et al., 2015; Ehn et al., 2007; Tiwari et al., 2014; Mann et al., 2013) (71,72,73,74) have found that mutations of PGRs have a significant influence on modulation of preterm birth. There is a possibility that either mutation in the mother's or in fetal PGR genes leads to a difference and, consequently, preterm birth. Ehn at al. found that mutation in both fetal and maternal PRGs contributes to greater possibility of preterm birth. In addition, it has been proven that these mutations of PGR receptors differently influence preterm birth depending on race. This influence is bigger in the African-American population. Other studies have only confirmed that women with these mutations have a greater risk of preterm birth.

Progesterone as therapy for prevention of preterm birth

Supplement of progesterone as prevention of preterm birth appears to be effective, but it has to be kept in mind that it is not an ideal medicine. Usage of progesterone as therapy for prevention of preterm birth depends on various factors. First of all, it is important to use the appropriate type of progesterone and to choose appropriate patients. It is also important to use a proper dose, route of delivery and plasma concentration (76,77). The pathway leading to preterm birth is also of importance (78,79).

Table 3. *Recommendations for progesterone supplementation to prevent preterm birth (85).*

Progesterone preparation	Dosage and route of administration	Indications
Hydroxyprogesterone caproate a 250 mg	250 mg intramuscularly once a week from between 16th and 20th week of gestation until the 36th week of gestation	Singleton pregnancy, prior spontaneous singleton preterm birth, normal cervix length. Singleton pregnancy, prior spontaneous preterm birth of twins, normal cervix length. Twins, prior preterm birth.
Natural progesterone	Vaginally	Singleton pregnancy, prior spontaneous singleton preterm birth, normal cervix length. Singleton pregnancy, prior spontaneous preterm birth of twins, normal cervix length. Twins, prior preterm birth. Twins, short cervix.
Micronized progesterone vaginal gel/vaginal tablet	90 mg /100 mg per day, vaginally	Singleton pregnancy,prior spontaneous preterm birth of twins, short cervix < 20 mm.
Progesterone suppository	90-200 mg per day, vaginally, from the moment of diagnosis until the 36th week of gestation	Singleton pregnancy, prior spontaneous preterm birth of twins, short cervix < 20 mm.

Studies have shown that clinical efficacy and safety of progesterone therapy can also be altered due to gene polymorphisms of PGRs (75). That fact is especially important in the African-American population, where genetic mutations of PGRs occur more often, and lead to resistance to progesterone therapy.

There are two major preparations of progesterone used in premature birth therapy/prophylaxis; hydroxyprogesterone caproate, a synthetic progesterone usually used in 250 mg dosage once a week, given as an intramuscular injection; and natural/micronized progesterone in a dosage of 100 mg per day, given vaginally.

There is also a rare application of progesterone as vaginal gel in a dosage of 90-200 mg.

Most common usage of progesterone preparations as prophylaxis of preterm birth starts from 16-20 weeks and lasts until the 36th week of gestation.

Several meta-analyses and studies have been made about the efficiency of progesterone treatment in preterm birth.

Dodd et al. conducted a meta-analysis of usage of progesterone for prevention of preterm birth in singleton pregnant women with high risk for preterm birth. Usage of progesterone made the risk for preterm birth in their current pregnancies lower. (80)

Meis et al. researched the usage of 17 hydroxyprogesterone caproate in women who had a documented preterm singleton birth in their obstetric anamnesis. Therapy started between 16th and 20th week of gestation and was used until the 36th week. Prophylaxis reduced the risk of preterm birth in their current pregnancy. (81)

Da Fonseca et al. analyzed the use of progesterone vaginal suppository between 24th and 34th week of gestation. Therapy was given to singleton pregnant women with risk factors in their anamneses. Risk of preterm birth in their current pregnancies was reduced with prophylaxis. (82)

Contrary to those findings, OPPTIMUM trial, led by Norman et al., showed that vaginal progesterone therapy did not reduce fetal and neonatal mortality and morbidity in preterm birth, or preterm birth itself. (83)

PROGRESS study, led by Crowther et al., also did not find any reduction in mortality or morbidity of fetuses or mothers related to preterm birth (84).

Conclusion

In conclusion, usage of progesterone in prophylaxis of preterm birth is still indicated and used worldwide. The most important thing is to choose the right candidates with the right risk factors for preterm birth. Recommendations for usage of progesterone for prevention of preterm birth are given in Table 3 (85).

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Nurses' Attitudes Toward Nursing Research

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Abstract

Aim: The aim of the study was to examine the attitudes of nurses towards research in nursing.

Methods: The study involved 202 respondents. The respondents were nurses employed at Osijek Clinical Hospital. The Boothe's Attitudes on Nursing Research Scale was used as an instrument of research – a modified version by Bostrom, A. C.

Prior to statistical data processing, respondents were divided into two groups, considering the level of education: vocational nurses and Bachelors of Science in Nursing.

By age, respondents were divided into three groups: aged 20 to 35, 36 to 50 and 51 to 65. The differences between the observed groups were tested by the t-test and analysis of variance.

Results: There is a statistically significant difference in attitudes towards research given the level of education of the respondents (p = 0.015). Bachelors of Science in Nursing have more positive attitudes towards research ($\bar{x}$=148.5) compared to vocational nurses ($\bar{x}$=141.1). A significant difference in attitudes towards nursing research was determined also with respect to the age of respondents (p = 0.002). Younger nurses have a more positive attitude towards research in nursing ($\bar{x}$ = 151.5) than middle-aged ($\bar{x}$=140.9) and senior-aged nurses ($\bar{x}$=140.1) do. There is also a significant difference in the level of motivation for continuing professional education given the respondents' level of education (p = 0.019).

Conclusion: Nurses show mildly positive attitudes towards nursing research.

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Introduction

"Nursing research is systematic inquiry designed to develop trustworthy evidence about issues of importance to the nursing profession" (1).

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Nursing research, in the narrow sense, refers to "a scientific process that validates and refines existing knowledge and generates new knowledge that directly and indirectly influences clinical nursing practice" (2).

Scientific research in nursing dates back to Florence Nightingale. Her data collection and analysis of factors affecting mortality and the illness of soldiers during the war resulted in changes in health care (3). More precisely, although F. Nightingale recommended conducting clinical nursing research back in the mid-19th century, it was almost 100 years later that most nurses accepted her counsel. Still, there is reason for optimism. The number and quality of research in nursing is growing today (4). With the development of nursing education in the Republic of Croatia, the number of research projects in the field of health care has increased. However, in comparison to Western world countries, there is still not enough research in nursing in our country. The availability of nursing journals through online databases provides nurses in Croatia with a detailed insight into what has been hitherto investigated in the area of health care, allowing a comparison of the research results with those conducted in the world and facilitating the provision of evidence-based health care. Evidence-based clinical nursing practice represents a connection between nurses' personal experience and patients' value system on the one side and evidence on nursing and medical literature on the other (5). Evidence-based practice has shown correlation with better patient outcomes, as patient care decisions are then backed up by scientific evidence (6).

Research is an important factor in establishing and maintaining high standards of health care (7). Some of the limitations of scientific research application, both in general and in healthcare, are: moral or ethical problems, problems of complexity of human functioning, problems related to measurement and control issues (8). Research shows that nurses find the following to represent main obstacles to research: lack of time, lack of interest and motivation, insufficient awareness of available research literature, insufficient authority to change the practice, lack of peer support, lack of support for the implementation of research findings in practice and insufficient understanding of the research process (6,9).

Some of the goals of nursing research are to promote evidence-based nursing practice, to secure the credibility of the nursing profession, to determine nursing practice responsibility, and to document the cost-effectiveness of nursing care (4).

Estabrooks et al. (10) have conducted an integrative review of literature and a meta-analysis of studies that deal with the impact of individual nursing personalities on research use. There were 20 studies involved in the meta-analysis, and 6 categories of potential individual characteristics were found, divided by the authors as follows: a) beliefs and attitudes, b) involvement in research activities, c) search for information, d) professional characteristics, e) education, and f) socio-economic factors. The authors concluded that all of these studies show methodological problems and it would be very difficult to suggest that any other individual characteristic, other than one's attitudes toward research, affects the use of research as such.

Attitude is an acquired, relatively durable and stable organization of positive or negative emotions, evaluations and responses to an object. Once formed, attitudes are resistant to change and are quite durable. Nevertheless, they can change under the influence of changed circumstances and new experiences. As the social behavior of a human is related to their attitudes, there is a great interest in examining attitudes in order to explain and predict behaviors or to influence behaviors by means of planned attitude-changing procedures (11).

Many studies have focused on the attitudes of nurses towards research, as well as on conditions that facilitate their implementation.

In 1989, Bostrom A. C. et al. conducted a survey on 720 Bachelors of Science in Nursing ("BSNs") and vocational nurses. The results of this study showed that both groups perceived that there

was support for research activities both among their peers and in the hospital in which they were employed. They expressed their personal interest in participating in research activities and they strongly believed that clinical nursing practice was a suitable source of research in nursing. Lack of time was identified as a leading factor for their personal lack of involvement in research, as well as a lack of cost-effectiveness in terms of rewards or job promotions (9).

The results of Marsh and Brown's research, conducted in 1992 on 144 nurses employed in a private hospital, showed that nurses had mildly positive attitudes towards research. The degree of education was the only respondents' characteristic that was shown to be related to their attitudes (12).

Bjorkstrom and Hamrin concluded in their research that nurses generally have positive attitudes towards research. They found some discrepancies between age groups (younger nurses have a more positive attitude towards research) and in terms of the level of education (nurses with higher levels of education have more positive attitudes towards research) (13).

In 2007, Hofmeister examined the attitudes of nurses towards research and found that they had slightly positive attitudes. She also stated that, as the level of nurses' education increases, so do their positive attitudes towards research. She concluded that time, support and education are the main factors that could lead to more positive attitudes towards research (6).

A study conducted in Sweden on a sample of 1054 nurses was based on the attitudes of nurses towards research as well as their awareness and use of research findings. The results of the survey show that nurses generally have positive attitudes towards research (14).

The main purpose of this study is to examine attitudes of nurses towards research in nursing. Specific goals are focused on the following issues: 1) to investigate whether there is a difference in attitudes towards research in nursing with respect to the level of respondents' education, 2) to examine whether there is a difference in attitudes towards research in nursing with regard to the respondents' age; 3) to find out whether there is a difference in the level of motivation for continuing professional education given the level of respondents' education, and 4) to examine whether there is a difference in the level of motivation for continuing professional education with regard to the respondents' age.

Materials and Methods

A cross-sectional study was conducted (15). The research was conducted in the period from May to August 2016 at Osijek Clinical Hospital. The study involved 202 respondents. The respondents were nurses employed at the Clinics, Institutes and Departments of Osijek Clinical Hospital. The research instrument used was Boothe's Attitudes on Nursing Research Scale, modified version by Bostrom A. C. (9), with prior permission to use. The original questionnaire has 46 questions. The question: "Nursing research is more essential in the medical setting than in the psychiatric setting" was eliminated from the questionnaire because of inappropriate application in our health system. The scale contains 3 subscales: *Interest and Environmental Support*, *Payoff and Benefits*, and *Barriers to Conducting Research*. Answers are given on the Likert 5-degree scale starting from 1 *(I completely disagree)* to 5 *(I completely agree)*. The questionnaire results range in total from 45 to 225. A larger number indicates more positive attitudes towards research, while a high score on negative particles reflects negative attitudes. The total score on the first subscale can range from 20 to 100, on the second subscale from 17 to 85, and on the third from 8 to 40. Reliability coefficients for the whole questionnaire and for all three subscales have been calculated. Chronbach alpha for all the particles (with five reversed points) is 0.904. For the first subscale it is 0.869, for the second subscale it is 0.839, and for the third subscale it is 0.439. The Hofmeister's study produced similar reliability coefficients for the three subscales (*Interest and Environmental Support* 0.879, *Payoff and Benefits* 0.844, and for the third subscale *Barriers to Conducting Research* it was 0.571) (6). The socio-

demographic characteristics of respondents were collected through 6 questions (gender, age, level of education, workplace, level of motivation for further professional education and the number of nursing journals commonly read by the respondents). Prior to statistical data processing, the respondents were divided into two groups, with regard to the level of education: BSNs and vocational nurses. According to age, respondents were divided into three groups: aged 20 to 35, 36 to 50 and 51 to 65.

Statistical analysis

Categorical data were represented by absolute and relative frequencies. Numerical data were described by arithmetic mean and standard deviation. The differences between the observed groups (level of education, age) were tested by t- test and analysis of variance. After the analysis of variance as a post hoc analysis, Scheffe test was used. The level of significance was set at $\alpha = 0.05$. The analysis of the data obtained was done by SPPS for Windows (version 15.0, SPSS Inc., Chicago, IL, USA) (16).

Ethical principles

Prior to the research, the written consent of the Commission for Ethical and Vocational Issues of Nurses at the Clinical Hospital Osijek was obtained on 21st April 2016, as well as the written consent of the principal nurses of the Clinics and the Institutes where the research was conducted. The research was conducted in accordance with ethical principles and human rights in research.

Results

The study involved 202 respondents, of whom 19 (9.4%) were male and 183 (90.6%) female. Out of the total number of respondents, 98 (48.5%) were BSNs, and 104 (51.5%) were vocational nurses. The largest number of respondents worked at hospital departments (wards), 134 (66.3%). The age of respondents ranged from 21 to 64 (mean age was 40.4; SD = 11.2).

Overall results on the Boothe's Attitudes on Nursing Research Scale range from 89 to 198, with arithmetic mean of 144.7 (SD = 21.7). The results on the first subscale (*Interest and Environmental Support*) in this study range from 34 to 90, with an arithmetic mean of 62.7 (SD = 11.8), and the possible range from 20 to 100. On the subscale *Payoff and Benefits*, the results range from 29 to 76, with an arithmetic mean of 55.1 (SD = 10.0), and the possible range of 17 to 85. The results on the third subscale (*Barriers to Conducting Research*) range from 18 to 37, with an arithmetic mean of 26.9 (SD = 4.0), and the possible range from 8 to 40 (Table 1).

Average scores of individual particles for the three subscales are shown in Tables 2, 3 and 4.

BSNs have achieved a significantly higher total score on the Boothe's Attitudes on Nursing Research Scale compared to vocational nurses (t-test, $p = 0.015$). A statistically significant difference between BSNs and vocational nurses has been found on the second subscale (*Payoff and Benefits*). In this subscale, vocational nurses score an average result of 53.1 (SD = 10.7), while the BSNs achieve the average score of 57.2 (SD = 8.7) (t-test, $p = 0.003$). No statistically significant differences have been found between the observed groups on the two remaining subscales.

In order to determine differences in attitudes towards research according to the age of respondents, the subjects were divided into 3 age groups: 20 to 35, 36 to 50 and 51 to 65 years of age.

The analysis of variance confirmed that there was a statistically significant difference between the three age groups of respondents on the Boothe's Attitudes on Nursing Research Scale. A post hoc analysis (Scheffe test) confirmed the statistically significant difference between the first and the second age groups ($p = 0.010$), and

Table 1. *The attitudes of nurses toward nursing research*

The Boothe's Attitudes on Nursing Research Scale	min	max	$\bar{x}$ (SD)*
Interest and Environmental Support	34	90	62.7 (11.8)
Payoff and Benefits	29	76	55.1 (10.0)
Barriers to Conducting Research	18	37	26.9 (4.0)
Total score	89	198	144.7 (21.7)

** arithmetic mean (standard deviation)*

Table 2. The average scores of individual particles for subscale **Interest and Environmental Support**

Item	Interest and Environmental Support	$\bar{x}$ (SD)*
1.	I would like to conduct research.	3.0 (1.3)
2.	I would like to put research high on my list of priorities.	2.8 (1.2)
4.	I believe my place of employment would provide me with ample assistance during the research process.	3.4 (1.1)
5.	I believe my place of employment would provide me with ample consultive assistance for conducting research.	3.5 (1.1)
6.	My supervisor would allow time in my daily assignment to conduct research.	3.3 (1.2)
8.	I know what is expected of me when submitting my research proposal to the hospital nursing research committee.	3.1 (1.0)
11.	I am familiar with selected statistical procedures used for the analysis of research findings.	2.9 (1.1)
12.	I believe my job provides the time necessary to conduct research.	2.4 (1.1)
13.	My colleagues (other professionals) would encourage me to conduct research.	3.3 (1.1)
14.	My peers in nursing would encourage conducting research.	3.5 (1.0)
15.	I believe my peers in nursing would assist in conducting research.	3.5 (1.0)
16.	My job provides ongoing educational programs in order to conduct research.	2.9 (1.1)
18.	I believe my working environment provides ample opportunity to conduct research.	3.3 (1.0)
19.	I believe my place of employment has ample secretarial assistance for anyone wishing to conduct research.	2.9 (1.1)
20.	I believe my place of employment has ample statistical assistance for anyone wishing to conduct research.	2.8 (1.1)
21.	I believe my place of employment has ample assistance for anyone for the analysis of results and findings of the research that is conducted.	2.8 (1.1)
27.	Nursing research requires more from me than I am willing to give to my job.	3.0 (0.9)
34.	Time spent giving patient care is more important than time spent conducting research.	4.1 (1.0)
35.	I am interested in conducting research.	2.9 (1.2)
44.	Nursing research should be initiated by nurse researchers.	3.4 (1.2)

Table 3. The average scores of individual particles for subscale **Payoff and Benefits**

Item	Payoff and Benefits	$\bar{x}$ (SD)*
3.	Nursing research is conducted because it allows nurses to be promoted.	3.5 (1.1)
9.	The informed consent necessary for employee participation in research prevents me from conducting research in my work areas.	3.3 (1.0)
22.	I would conduct research if I had the time.	3.1 (1.2)
23.	I would conduct research if I knew how to write the proposal, conduct and analyze the results and findings.	3.0 (1.2)
24.	Research findings that are advantageous to good patient care can be implemented in my working environment.	3.4 (1.1)
25.	Nursing research is the means whereby the theoretical basis for nursing practice is derived.	3.3 (1.2)
26.	Members of the treatment team other than nurses should conduct research relative to patient care.	3.3 (1.0)
29.	I would like to conduct a study of a problem in patient care.	3.1 (1.1)
30.	I would conduct research if patient assignments were lightened.	2.8 (1.1)
31.	Nursing research should be initiated by nurses in the clinical area.	3.4 (1.2)
33.	Nurses would conduct more research if more funds were available for them to use for this purpose.	3.7 (1.0)
36.	Nurses receive praise from their peers and colleagues when they conduct research.	2.8 (1.1)
37.	Nurses would conduct research if they were provided time for research.	3.7 (0.9)
38.	Nurses would conduct research if relief time were given to conduct research.	3.8 (1.0)
40.	I would do research if I knew more about it.	2.9 (1.2)
41.	Nurses are criticized too much by their peers when they conduct research.	2.9 (1.0)
43.	I believe that I would conduct research if someone more knowledgeable would help me in the process.	3.2 (1.2)

** arithmetic mean (standard deviation)*

The post hoc analysis did not find any difference between the second and third age groups (p = 0.977).

Regarding the level of motivation for continuing professional education in relation to the respondents' level of education, the results show that there is a statistically significant difference between the examined groups (p = 0.019). A higher level of motivation is present in BSN's. In the group of BSN's, a total of 8 (8.2%) respondents reported high motivation for continuing their education, whereas in the group of vocational nurses a total of 5 (4.8%) respondents reported high motivation. Also, in the group of BSNs, 7 of the respondents (7.1%) were not motivated to continue their professional education, while in the group of vocational nurses 19 of them (18.3%) were not motivated at all.

Results on the level of motivation for continuing professional education in relation to the respondents' age show that the three age

Table 4. The average scores of individual particles for subscale **Barriers to Conducting Research**

Item	Barriers to Conducting Research	$\bar{x}$ (SD)*
7.	The process of submission of the research proposal to the hospital nursing research committee is too detailed.	3.3 (0.8)
10.	The informed consent necessary for patient participation prevents me from conducting research in my work areas.	3.2 (1.0)
17.	I have the skills and knowledge necessary for me to conduct research.	3.3 (1.0)
28.	Nursing research should be conducted by nurses with a baccalaureate degree.	3.7 (0.9)
32.	Nursing research should be initiated by nurses in education.	3.4 (1.1)
39.	Nursing research should be conducted by nurses with a doctorate.	3.3 (1.3)
42.	Nursing research should be conducted by nurses with a master's degree.	3.3 (1.2)
45.	Patient participation in nursing research is difficult to obtain.	3.3 (1.0)

** arithmetic mean (standard deviation)*

groups differ to a statistically significant extent in terms of the level of motivation for further education ($p < 0.001$). Although in all three age groups most of the respondents are partially motivated to continue their professional education, the highest level of motivation is present in younger nurses (aged 20 to 35). Also, among the nurses of younger age, only 2 of them (2.6%) were not motivated to continue their education, while the number of unmotivated nurses aged 51 to 65 was 13 (25%).

The results also demonstrated that BSNs commonly read more nursing journals compared to vocational nurses. The difference between these two observed groups was statistically significant ($p = 0.001$). Most of the respondents in all three age groups usually read one nursing journal. However, 31 (40.8%) respondents in the youngest age group and only 7 (13.5%) of them in the age group of 51 to 65 read no nursing journals whatsoever.

Discussion

The results of our research have shown that nurses show mildly positive attitudes towards nursing research. Since the minimum possible score on the Boothe's Attitudes on Nursing Research Scale is 45, and the maximum is 225, the resulting mean of 144.7 (SD = 21.7) indicates slightly positive attitudes towards research. These results are in line with the findings of previous studies (6, 7, 12, 17, 18), which also confirm that nurses' attitudes towards research are positive. As Hofmeister points out, such a positive attitude of nurses towards research is the key indicator of evidence-based practice (6).

Analyzing the overall outcomes on some subscales of Boothe's Attitudes on Nursing Research Scale also confirms the positive attitudes of nurses towards research. The analysis of the results on the three subscales is consistent with the findings of Hofmeister (6) and Bostrom (9), who also found that nurses perceive that research support exists among their peers and at the hospital; they find that research is important and useful for nursing practice and express their awareness about obstacles to conducting research.

In further analysis of the nurses' attitudes towards research, an examination of subjects' responses in individual particles of each subscale was performed. The average scores in individual particles of *Interest and Environmental Support* subscale show that the respondents agreed with the statement that time spent in patient care was more important than the time taken to conduct research. This is also the particle for which the respondents showed the

highest agreement in the whole questionnaire. Also, respondents expressed a somewhat higher degree of agreement with statements saying that they believed they would get the help needed to conduct the research at their workplace and that their associates would help. The highest disagreement was expressed with regard to the statement that they had enough time at work to carry out the research. A somewhat lower score has also been achieved in the particle that says research is high on the list of their priorities. It can be concluded from such results that nurses, despite their generally positive attitudes towards research, nevertheless give greater priority to patient care, rather than conducting research. It is therefore questionable if they understand the importance of implementing research findings in their practice and if they see the resulting benefit to patients. Concerning the *Payoff and Benefits* subscale, it has been shown that respondents agree the most with claims that nurses would conduct research if they were given free time and if they had more money available for that purpose. They agreed the least with the statement that nurses get praise from their associates and colleagues when conducting research. Such results on the *Payoff and Benefits* as well as *Interest and Environmental Support* subscale support earlier research results (6, 9), which also demonstrated that the time available, as well as working environment support, are important factors associated with the nurses' decision whether they will implement and apply research findings or not.

Average scores on particular particles of the *Barriers to Conducting Research* subscale show the respondents' highest agreement with the statement that the research should be conducted by the BSNs, which means that nurses are aware, to a certain extent, of the importance of education for acquiring knowledge of research methods.

Further analysis of nurses' attitudes towards research is focused on examining differences in attitudes with regard to the level of education and the age of the respondents.

Our results show that BSNs achieve a significantly higher total score on the Boothe's Attitudes on Nursing Research Scale compared to vocational nurses. This finding is in line with the findings of earlier research (7, 12, 17, 18), which also showed that the higher the level of education among respondents, the more positive the attitudes towards research. Given that nurses who attain a higher level of education get more familiar with the research process during their formal education, such findings are not surprising. As Marsh and Brown (12) point out, nurses who attended research methodology courses, either participating in research or were researchers themselves, have more positive attitudes towards research. Bostrom (9) finds some additional differences between BSNs and vocational nurses, and points out that BSNs claimed that conducting research was a desirable part of their nursing role and considered themselves better prepared for conducting research, while vocational nurses believed that research is also important for patient care, but they were not willing to put research before patient care. Consequently, the level of education is undoubtedly linked to positive attitudes towards research. Education should be a fundamental element for motivating and strengthening positive attitudes towards research (18).

There was a statistically significant difference between the three age groups of respondents on the Boothe's Attitudes on Nursing Research Scale. Although the results of some studies (12, 17) have not confirmed the correlation between age and attitudes towards nursing research, the findings of this study show that younger respondents have more positive attitudes towards research, as opposed to middle-aged and senior respondents. Fugleberg (19) also found that younger nurses achieve higher results on scales that measure involvement in research and attitudes towards research. This finding is also in line with the results of our research and one may say that nurses of younger age have more positive attitudes than nurses of middle and senior age. Regarding the level of motivation for continuing professional education, relative to the respondents' level of

education, most respondents from both groups were only partially motivated to continue their professional education. Nevertheless, the results show that a higher level of motivation is present in BSNs. The results of the present study have also shown that the three age groups of respondents differ to a statistically significant extent in terms of the level of motivation for further education. Although most of them are partially motivated to continue education, in all three age groups, it can be concluded that the highest level of motivation is present in younger nurses.

Although our research has shown that nurses have slightly positive attitudes towards research in nursing, which is consistent with previous research on this topic, the results of this study cannot be generalized to the entire population of nurses. A topic which has not been covered by this research, and may certainly be useful to examine, is the link between motivation for continuing education in one's profession in general and their motivation toward nursing research specifically. Another interesting topic for future research could be the relationship between attitudes towards research and actual conducting of research. Namely, it is well-known that the attitude itself does not necessarily result in change of behavior. The fact that nurses have a positive attitude does not necessarily mean that they will also practice research. We may say that identifying the attitudes of nurses to research is only the first step that should link future studies to other significant variables in order to effectively assess the acceptance of changes in nursing practice and the conducting of evidence-based health care.

Conclusion

Based on the research conducted for the purposes of this paper, it can be concluded that nurses show mildly positive attitudes towards research in nursing. There is a significant difference in attitudes towards research with regard to the level of education and age of the respondents. BSNs have more positive attitudes towards research than vocational nurses do. Younger nurses have more positive attitudes towards nursing research than it is the case with middle-aged and senior-aged nurses. There is a significant difference in the level of motivation for continuing education in one's profession with regard to the level of education and age of the respondents. A higher level of motivation is present in the BSNs. The highest level of motivation is present in nurses aged between 20 and 35.

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The Quality of Life of a Patient With Colostomy

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Abstract

Aim: The aim of this research was to examine the quality of life of patients with temporary or permanent colostomy regarding age, gender, marital status and time spent with an ostomy.

Materials and Methods: The study involved 41 members of the Ostomy ILCO Clubs in Slavonski Brod and Osijek as respondents. The standard version of the "Quality of Life Questionnaire for a Patient with an Ostomy" was used as the research instrument.

Results: The mean value of the overall scale was 5.4. The respondents rated the physical well-being the highest, a median of 5.9 (interquartile range 4.6 to 7.2), and social well-being the lowest, median 5.0 (interquartile range 4.1 to 6). Women gave higher marks for physical, social and spiritual well-being, and overall for the scale median of 5.5 (interquartile range 4.9 to 6.1), but with no significant differences compared to men. Married respondents ranked psychological, social and spiritual well-being higher, with a median of 5.4 (interquartile range 4.7 to 6.0), but with no significant differences compared to those who live alone. Physical, psychological and spiritual well-being is somewhat lower in patients under the age of 65, and social well-being is lower in subjects aged 66-75, the median of 4.7 (interquartile range 3.6 to 5.6). Correlation between the subscales pertaining to quality of life of the respondents with regard to their age and with regard to living alone (single, divorced or widowed) is significant in terms of the physical (Spearman coefficient of correlation $\rho = 0.945$, $p < 0.001$), mental (Spearman coefficient of correlation $\rho = 0.943$, $p = 0.005$), social (Spearman's correlation coefficient $\rho = 0.829$, $p = 0.042$) and spiritual well-being (Spearman coefficient of correlation $\rho = 0.886$, $p = 0.019$).

Conclusion: Quality of life of patients with an ostomy is satisfactory. There are differences in quality of life regarding age, gender, marital status and time spent with an ostomy. There is also a connection between the subscales "quality of life regarding age" and "quality of life regarding marital status" of patients with colostomy.

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KEYWORDS: colostomy; quality of life

Introduction

Year in, year out, cancer incidence and mortality rate are increasing worldwide. Colorectal cancer accounts for more than 9% of the world's cancer incidence. It is the third most common cancer in the world and the fourth most common cause of death (1). Globally, colorectal cancer (CRC) is the third most common cancer diagnosed in men (after lung cancer and prostate cancer), and the second most commonly diagnosed in women (after breast cancer) (2).

Carcinoma is the second most significant cause of death in Croatia: every fourth resident dies from cancer. Compared to other European countries, Croatia is a country with a medium incidence but high mortality rate (3). Colorectal cancer is usually caused by changes on the colon mucous membrane or on a polyp protruding from a mucous membrane (4). Like all cancers, it is most curable if detected at an early stage of the disease.

In many cases a surgical operation is performed that ends with the creation of a colostomy on the lower left side of the abdominal wall, but it is also possible to create an ostomy on the right side of the abdominal wall. In most cases, the colostomy is temporary and is removed but sometimes remains a permanent solution in cases of poor operation outcome and metastases (5). The main purpose of creating an ostomy is to release the stool and gas, that is, to keep the continuity of the digestive tract (2).

Before surgery, the patient often feels anxiety, worry and fear, and this is where a nurse plays a major role. Health education always begins with preoperative psychological preparation of the patient, as it allows the nurse to assess the patient's level of knowledge of the illness and operation itself, as well as about their family support, work, hobbies, cultural and spiritual beliefs. The success of mental preparation depends largely on the patients themselves and their acceptance of the new situation. If a patient cannot care for him/herself, the education involves a spouse or a family member (6).

Hospital discharge planning requires team work of doctors, nurses, enterostomal therapists and dieticians. At the discharge, patients and caregivers are provided with special information on the proper way of treating the ostomy and the surrounding skin, changing ostomy bags and using ostomy aids. Discharge information also includes instructions about prevention of possible complications. Family members participate in the process of cleaning the wound and skin and in changing ostomy bags so they can do it themselves when the patient returns home. Patients also get clear directions about when they need to call a doctor (7). The quality of life of a patient with colostomy depends to a great extent on how well they and their family have adopted procedures important for ostomy care and accepted the new lifestyle (6).

Although a minor surgery from the medical point of view, the creation of temporary or permanent ostomy greatly reduces the quality of the patient's life (8). Colostomy has a significant effect on the life of the patient, regardless of the diagnosis of the disease. Cohen et al. emphasize that for patients with cancer, concern about the creation of the ostomy goes beyond all the other patients' concerns (9). The influence of colostomy on physical, psychological, social and spiritual well-being is not unexpected, but is insufficiently described in the literature.

Research shows that quality of life is increasingly recognized as an important measure of the outcomes in survivors of large surgical and medical treatments. In patients with colon cancer, various physical problems are present after surgical treatment, such as problems with the stool and urination, and problems with intimacy, which significantly affects the quality of life. Evaluating the quality of life is considered crucial for evaluating clinical outcomes after surgical treatment since it considers the patient's perspective in the decision-making process (10).

New studies have shown that the ostomy has, as expected, a great influence on the patient's quality of life and a great influence on the patient's daily life. The easiest way to find out how an individual perceives his or her quality of

life and how satisfied they are with their everyday life after the creation of an ostomy is to use an interview or a questionnaire that assesses the quality of life (11).

The quality of life is multi-dimensional, dynamic, and subjective. It is focused on the patient, involving physical, functional, emotional and social/family well-being. This is why quality of life is important for assessing the effects of disease on individuals, their families and their community (12).

Many studies have shown a reduction in the quality of life in patients with colostomy. Dissatisfaction with preoperative preparation and postoperative care, complications related to ostomy, psychiatric history of the patient, and negative thoughts and beliefs associated with ostomy contribute to the reduction of life quality (13). On the other hand, ostomy gives many people hope, prolongs their life, and enables their activity and realization of life plans.

Adaptation to a new life situation lasts from several months to two years and is conditional upon a number of factors that include one's general health but also the level of knowledge and skills that the person possesses. The support of patient's family and health care professionals, as well as joining support groups where they will be able to talk about difficulties and identify with people who have experienced the same situation, is particularly important. All this enables the patient to restore their everyday activities and social roles as soon as possible (14). The patient's attitude toward their own well-being and functional status is an important outcome and the priority of each research.

According to the guidelines on care quality provided by the Swedish National Health and Social Care Board, the patients should be treated respectfully; they must receive satisfactory information and have the opportunity to participate in the decision-making process. Quality of life is essential for patients with colostomy and the factors that affect it should be enabled. Assessing the quality of life of patients with an ostomy will lead to a better understanding of the patients and improve their life quality. Psychosocial needs as well as their impact on quality of life have been researched, but a small number of studies point to interventions which could solve problems and meet the needs of patients with a colostomy (1, 10).

The main purpose of this study was to examine the quality of life of patients with a temporary or permanent colostomy. Specific goals were focused on the following issues: a) to examine whether there is a difference in the quality of life of patients with temporary or permanent colostomy with regard to the respondents' age; b) to investigate whether there is a difference in the quality of life of patients with temporary or permanent colostomy with regard to the patients' gender; c) to find out whether there is a difference in the quality of life of patients with temporary or permanent colostomy given the respondents' marital status, d) to examine whether there is a difference in the quality of life of patients with temporary or permanent colostomy regarding the time spent with a colostomy and e) to find out whether there is a correlation between the "quality of life of patients with colostomy" subscales and their age, gender, marital status and time spent with a colostomy.

Materials and Methods

Our respondents, a total of 41, were members of Ostomy ILCO Club Slavonski Brod and Ostomy ILCO Club Osijek who have either a temporary or a permanent colostomy. Subjects were aged 44 to 74, and there were both male and female respondents. All respondents were regular at their monthly Ostomy Club meetings.

As an instrument of research, an anonymous questionnaire was used, consisting of general demographic data (age, gender, marital status, temporary / permanent colostomy, how long a person has had a colostomy). The questionnaire used to assess the quality of life of a person with a colostomy was the "Quality of Life Questionnaire for a Patient with an Ostomy", City of Hope and Beckman Research Institute (9, 15, 16). This questionnaire consists of 43 questions evaluating the quality of life using a scale from 0 to 10. The questionnaire is divided into four

subscales: "Physical well-being" (questions 1 to 11), "Psychological well-being" (questions 12 to 24), "Social well-being" (questions 25 to 36), and "Spiritual well-being" (questions 37 to 43). Answers are scored, with 0 being the worst possible result, and 10 the best possible one. However, a few answers are scored in the opposite direction, with 0 being the best and 10 the worst result. Particles 11-12, 15, 18-19, 22-30, 32-34, and 37 are scored reversely.

The results were obtained by adding points to each particle in the subscale, then dividing the sum by the number of particles in each subscale. The total QOL score was obtained by summing the results of all the particles and dividing by the total number.

Statistical analysis

Categorical data were represented by absolute and relative frequencies. Numerical data were described by the median and the limits of the interquartile range. The normality of the distribution of numeric variables was tested by the Shapiro-Wilk test. Differences in numeric variables by gender and marital status, due to deviations from normal distribution, were tested by the Mann-Whitney U test. The difference in numeric variables by age groups and duration of the ostomy, due to the deviation from the normal distribution, was tested by the Kruskal-Wallis test. All P values were two-sided. The level of significance was set at = 0.05. For statistical analysis, the statistical program MedCalc (version 16.2.0, MedCalc Software bvba, Ostend, Belgium) was used.

Ethical principles

Prior to the research, a written consent of the Commission for Ethical and Vocational Issues at the J. J. Strossmayer University of Osijek, Faculty of Medicine Osijek was obtained (Class: 602-04/16-08/15, No.: 2158-61-07-1683, 10th June 2016.). The approval for the use of the standard questionnaire "Quality of Life Questionnaire for a Patient with Ostomy" was obtained from the City of Hope National Medical Centre Principal, Marcie Grant.

The research was conducted in accordance with ethical principles and human rights in research.

Results

The study included 41 respondents aged 44 to 74 (mean age 66 years, interquartile range 61 to 74 years). Out of the total number of respondents, 26 (63%) were male and 15 (37%) were female patients. Most of the subjects were married, a total of 35 (85%). There were 6 (15%) patients with a temporary colostomy and 35 (85%) patients with a permanent colostomy. The patients' time spent with the colostomy was 1 to 360 months (mean 48 months, interquartile range 12 to 102 months).

The overall quality of life of the examinees with the colostomy includes physical, psychological, social and spiritual well-being. The full-scale reliability coefficient, Cronbach Alpha, was 0.957.

When it comes to physical well-being, 5 respondents (12.2%), had serious sleep problems, followed by problems with smells – 3 (7.3%) of them, and 2 (4.9%) of them reported problems with diarrhea. Problems with itching or pain, diarrhea, gases, leakage of the bag contents, and constipation / absence of stool, were the least apparent.

Regarding their psychological well-being, 6 (14.6%) respondents expressed fears that their illness would relapse. Also, it was difficult for them to adjust to the ostomy, and they reported a feeling of uselessness. Some respondents feel embarrassed by having a colostomy, some consider the ostomy hard to look at and take care of, some have memory problems and some of them do not feel happy with their lives as a consequence.

Regarding overall results pertaining to the social well-being components, privacy in the care of the ostomy is not a problem for most respondents. Nevertheless, for ten (24.4%) respondents, their condition is completely stressful for the family; six (14.6%) respondents state that their ostomy is a problem during travel, and five (12.2%) respondents report troubles with sports activities and recreation. As for the results of the participants with respect to

Table 1. *Rating of subscales and the overall scale of the quality of life with a colostomy by age of respondents*

	Median (interquartile range) according to age groups				**p***
	Up to 65 years (N=18)	66 – 75 years (N=16)	76 and over (N=7)	In total (N=41)	
Physical well-being	5.7 (4.6 – 6.8)	6.5 (4.5 – 7.1)	6.7 (4.5 – 8.2)	5.9 (4.6 – 7.2)	0.671
Psychological well-being	5.1 (4.1 – 5.8)	5.3 (4.8 – 5.8)	5.8 (5.2 – 6.4)	5.2 (4.8 – 5.8)	0.385
Social well-being	5.0 (4.4 – 6.1)	4.7 (3.6 – 5.6)	5.1 (4.3 - 7)	5.0 (4.1 – 6.0)	0.364
Spiritual well-being	4.9 (3.9 – 5.9)	5.5 (4.3 – 6.3)	5 (2.9 – 6.1)	5.3 (4.1 – 6.1)	0.444
Overall scale	5.3 (4.7 - 6)	5.3 (4.8 – 5.9)	5.6 (4.6 - 7)	5.4 (4.7 – 6.0)	0.727

** Kruskal Wallis test*

Table 2*. Rating of subscales and the overall scale of the quality of life by the length of time a person has spent living with a colostomy*

	Median (interquartile range) according to the length of time spent with a colostomy				**p***
	Up to 2 years (N=16)	From 3 to 10 years (N=19)	For 11 or more years (N=6)	In total (N=41)	
Physical well-being	6.1 (4.8 – 7.4)	5.4 (4.3 – 6.7)	7 (5.5 – 7.8)	5.9 (4.6 – 7.2)	0.255
Psychological well-being	5.2 (4.4 – 5.7)	5.2 (4.8 – 5.8)	5.5 (5.2 – 5.8)	5.2 (4.8 – 5.8)	0.413
Social well-being	5.1 (4.2 – 6.1)	4.8 (3.2 – 5.3)	5.9 (4.8 – 6.8)	5.0 (4.1 – 6.0)	0.110
Spiritual well-being	4.7 (4.1 - 6)	5.4 (4.3 – 6.1)	4.6 (3.6 – 7.3)	5.3 (4.1 – 6.1)	0.657
Overall scale	5.3 (4.7 – 6.2)	5.3 (4.4 – 5.6)	5.6 (5.3 – 6.2)	5.4 (4.7 – 6.0)	0.489

** Kruskal Wallis test*

the aspects of spiritual well-being, seven respondents (17.1%) find that they have a reason to live, whereas four (9.8%) respondents state that they are completely uncertain about their future. The same number of respondents find prayer and meditation sufficient to satisfy their spiritual needs.

In order to determine the differences in the quality of life with a colostomy in terms of age of the respondents, they were divided into three groups: the first group with respondents of up to 65 years of age, the second one with those between 66 and 75 years old, and the third group with respondents older than 76 years of age (Table 1).

Analysis of the impact of time spent with an ostomy on the quality of life with respect to physical well-being, mental well-being, social

Table 3. *Rating of subscales and the overall scale of the quality of life of a person with a colostomy*

	Median (interquartile range)	Minimum - maximum
Physical well-being	5.9 (4.6 – 7.2)	1.5 – 9.3
Psychological well-being	5.2 (4.8 – 5.8)	3.4 – 7.5
Social well-being	5.0 (4.1 – 6.0)	0.9 – 7.4
Spiritual well-being	5.3 (4.1 – 6.1)	1.9 – 8.6
Overall scale	5.4 (4.7 – 6.0)	3.0 – 7.4

Table 4*. Assessment of the correlation between the age of the subjects and the length of time spent living with a colostomy, according to marital status*

	Spearman coefficient of correlation (p-value)					
	Age of respondents	**Length of life with colostomy**	**Physical well-being**	**Psychological well-being**	**Social well- being**	**Spiritual well-being**
Married						
Physical well-being	0.082 (0.638)	-0.103 (0.554)	-			
Psychological well-being	-0.038 (0.829)	0.112 (0.520)	0.118 (0.500)	-		
Social well-being	-0.139 (0.426)	0.027 (0.879)	0.683 (<0.001)	0.350 (0.040)	-	
Spiritual well-being	-0.036 (0.838)	0.028 (0.874)	-0.211 (0.224)	0.137 (0.432)	-0.083 (0.637)	-
Overall scale	-0.140 (0.423)	-0.032 (0.856)	-	-	-	-
Living alone (single, divorced, widower)						
Physical well-being	0.945 (<0.001)	0.543 (0.266)	-			
Psychological well-being	0.943 (0.005)	0.371 (0.468)	0.943 (0.005)	-		
Social well-being	0.829 (0.042)	0.543 (0.266)	0.829 (0.042)	0.714 (0.111)	-	
Spiritual well-being	0.886 (0.019)	0.429 (0.397)	0.886 (0.019)	0.943 (0.005)	0.829 (0.042)	-
Overall scale	0.987 (<0.001)	0.543 (0.266)	-	-	-	-

well-being, and spiritual well-being, is shown in Table 2.

Respondents rated physical well-being the highest, with a median of 5.9 (interquartile range 4.6 to 7.2), ranging from 1.5 to 9.3, and gave the lowest scores for social well-being, a median of 5.0 (interquartile range 4.1 to 6), ranging from 0.9 to 7.4 (Table 3).

There is a significant correlation between the quality of life subscales according to the respondents' age for those living alone (single, divorced or widowed) in terms of their physical (Spearman coefficient of correlation $\rho = 0.945$, $p < 0.001$), mental (Spearman coefficient of correlation $\rho = 0.943$, $p = 0.005$), social (Spearman's correlation coefficient $\rho = 0.829$, $p = 0.042$) and spiritual well-being (Spearman coefficient of correlation $\rho = 0.886$, $p = 0.019$). The overall scale is related to the age of the respondents (Spearman's coefficient of correlation $\rho = 0.987$, $p < 0.001$).

Physical well-being is significantly associated with psychological well-being (Spearman coefficient of correlation $\rho = 0.943$, $p = 0.005$), social well-being (Spearman correlation coefficient $\rho = 0.829$, $p = 0.042$) and spiritual well-being (Spearman coefficient of correlation $\rho = 0.886$, $p = 0.019$). Spiritual well-being is significantly and strongly associated with psychological (Spearman's coefficient of correlation $\rho = 0.943$, $p = 0.005$) and social well-being (Spearman coefficient of correlation $\rho = 0.829$, $p = 0.042$) (Table 4).

Discussion

The overall quality of life of patients with a colostomy involves physical, psychological, social and spiritual well-being.

In the context of physical well-being, the results of this study show that respondents are most concerned with sleep problems, smells and diarrhea. They have least problems with itching or pain, gases, leakage of the bag contents, and constipation / absence of stool. In the research conducted by Grant et al., the subjects complained most of gas problems, smell, diarrhea and leakage of the bag contents (17). In the study of Krouse, Herrington et al., it was reported that subjects had the most problems with the skin around the ostomy, with unpleasant smells and leakage of the bag contents (18). In the research conducted by Dabirian et al., the respondents complained about the skin around the ostomy, sleeping problems, unpleasant smells and gases (19). It is evident that, in addition to all the physical problems mentioned, all respondents (both in the present study and in other ones), complained about the smell. Pittman et al. point out that these problems can lead to psychological and social problems (20).

The results of this research with regard to psychological well-being of persons with a colostomy are supported by the results of earlier studies. Thus, for example, Krouse, Grant et al., state that most of the subjects experience problems with anxiety, getting to know new people, and fearing the return of the disease (21). Krouse, Herrington et al. report that the survey respondents complained most about having problems when they wanted to travel somewhere because they lacked privacy in maintaining the ostomy and therefore felt anxious (18). Mitchell et al. find that subjects in their research felt anxious and depressed (22). Orbach and Tallent state that dissatisfaction with one's appearance may last for 5 to 10 years after surgery (12).

In the context of social well-being, the results of our research show that privacy in maintaining the ostomy is not a problem for most respondents. However, most patients reported that their condition was completely stressful for their family. Some of them stated that their ostomies disturbed their intimacy or posed problems in sports and recreation, which is consistent with the results of other studies (17, 18, 20, 21). In research results of Mitchell et al. we can find that respondents felt isolated from society (22).

The results of this research show that, in the context of spiritual well-being, most respondents feel that they do have a reason to live. Some of them are completely uncertain

about their future, while others find prayer and meditation to be enough to satisfy their own needs. Conversely, in the research by Grant et al., the number of respondents who stated that they have a reason to live was the smallest. Similar to the findings of our study, respondents in Grant et al. research are uncertain about their future, but have religious support, and inner peace (17).

In the research of Krouse, Grant et al., the greatest problems are related to inner peace, reasons for living, and religious support (21). Dabirian et al. stated in their findings that the most problematic issue for their respondents was the conduct of religious activities, i.e. going to worship (19). In the research of Mitchell et al., patients are least likely to find hope for the future (22).

The nurse, as one of the persons who play a role in health education, provides the patient with physical and psychological preparation. In this process, the most important thing is to include family members and spouses. Given that both before and after surgery the patients feel anxious, afraid and worried about the outcome, the nurse must have patience and be extremely sympathetic to their health problems, including those pertaining to their physical, psychological, social and spiritual well-being. The nurse also provides support after the setting up of the colostomy, provides patient and family education and psychological support, and helps with the patient's participation in an ostomy club, so that the patient understands that he is not alone.

Conclusion

Quality of life of patients with a colostomy is satisfactory. Physical, psychological and spiritual well-being is somewhat lower in subjects aged 65 and under, and social well-being is lower in subjects aged 66 to 75 but with no significant differences in other age groups. Women have given somewhat higher grades for their physical, social, and spiritual well-being, and for overall scale, but without any significant differences in relation to men. Married respondents have ranked their psychological, social and spiritual well-being as well as the overall scale higher, but without any significant differences in relation to those living alone (single, divorced, widowed). The assessment of physical and social well-being is the lowest in patients living with an ostomy for three to ten years. Spiritual well-being is graded the lowest in respondents living with an ostomy for up to two years, while assessments of psychological well-being are equal according to the time spent living with a colostomy. Although present, the differences are not statistically significant. There is also a connection between the subscales "quality of life regarding age" and "quality of life regarding marital status" of patients with an ostomy.

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