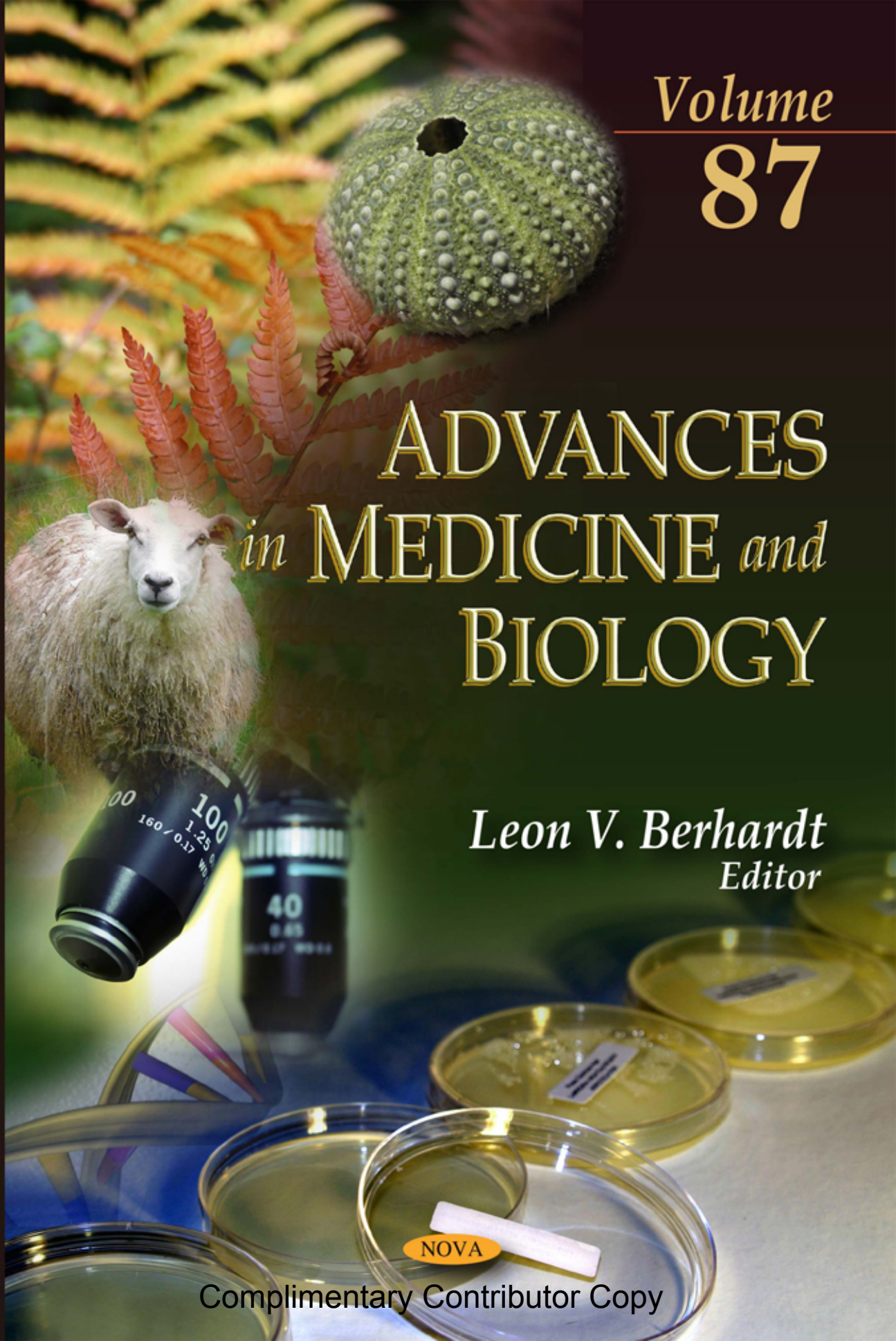

ADVANCES IN MEDICINE AND BIOLOGY

VOLUME 87

ADVANCES IN MEDICINE AND BIOLOGY

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ADVANCES IN MEDICINE AND BIOLOGY

VOLUME 87

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Contents

Preface

This book presents the latest developments in medicine and biology. Chapters include research on trends in the birth prevalence of boys with isolated hypospadias and undescended testis in Hungary during the last 50 years; alleviating premenstrual syndrome (PMS) symptoms using a natural factor; neutralization-enhancing RF antibodies; advances in the diagnosis, assessment, management and outcome of Takayasu's arteritis; macronutrients and premenstrual syndrome; pressurized intraperitoneal aerosol chemotherapy (PIPAC); the control of MAO expression; and what we know about iMAO.

Chapter 1 – Previously an increasing birth prevalence of hypospadias and undescended testis was recorded in some countries, including in the Hungarian Congenital Abnormality Registry, 1962-2011, i.e., during the last 50 years. However, the critical evaluation of cases with recorded isolated hypospadias (IHS) and isolated undescended testis (IUT), particularly to compare with the so-called reference birth prevalence of IHS (2.25 ± 0.30 per 1000 live-births) and IUT (6.86 ± 0.62 per 1000 live-births) determined in a Hungarian clinical-epidemiological study based on the physical examination of newborns with well-defined diagnostic criteria did not indicate a real increase in the birth prevalence of IHS and IUT. Thus, the increase of recorded cases with IHS and IUT can be explained by the more complete reporting of cases and a larger proportion of mild subgroup of IHS and the lack of differentiation between birth IUT (with many postnatal descents of testes) and true IUT (diagnosed at 3,5 months after birth). In conclusion, birth defect-registries are not appropriate for the estimation of real birth prevalence of IHS and IUT. In addition the socio-demographic variables of mothers were also analyzed without significant association of changes in birth prevalence of recorded IHS and IUT. Finally maternal risk factors were evaluated in the large population-based Hungarian Case-Control Surveillance of Congenital Abnormalities, 1980-1996, some of them showed association with the risk of IHS and IUT but without connection of the trend in the birth prevalence of cases with IHS and IUT.

Chapter 2 – There is hardly any other element of the physical environment which has caused so much confusion and controversies as negative air ions (NAI). Despite more than 100 years of study in the field, the knowledge on ion specter is still in an embryonic stage.

However, NAI have found to have good or excellent results, as therapeutic, as well as a prophylactic factor. The authors used a therapy of moderate doses of negative air ions emitted by a generator, for up to $14\text{-}20^{th}$ days to treat the symptoms of discomfort in Premenstrual Syndrome (PMS). On female students with PMS, the results were very significantly different from the witness group. The premenstrual intense pain decreased from 38% to 9.5%

($p < 0.001$), and generally pain completely disappeared in 45% of cases. Acne decreased from 76% to 43%, and irritability from 76% to 19%.

It is worth mentioning that the premenstrual irritability completely disappeared in 75% of students. Before the experiment 81% of the cases used pain killers, while after the experiment only 24% were still addicted to medication, especially as a preventive measure. Another effect was the regulation of the menstrual cycle, in 89% of the cases with irregular periods. All of these improvements lasted in half of cases up to one year after the treatment (no other known medicine has such a long effect). The items referring to the energetic basis of the neuro-psychic tonus have an ascending trend, except the well-being sensation and self confidence, which were situated from the very beginning at the higher levels of the scale. Negative air ions are not a universal panacea, but they could be considered a physiological factor, very cheap, with no side effects, not addictive, with very few contraindications, and which do not bring any foreign substance inside the organism.

In conclusion, considering the advantages, the artificially ionized air might be a potentially effective biologic factor, which, if properly harnessed, controlled, and utilized, may become a valuable adjunct to other forms of therapy.

Chapter 3 – Monomeric specific high-affinity IgG antibodies arising during infection process have low avidity to multimeric structures of the pathogen. Moreover, in the case of viruses such antibodies may sometimes enhance FcR-mediated infection of monocytes.

Multistage generation of antibody complexes with high affinity and avidity are important for efficient neutralization of the pathogen. Multimeric IgM or IgA rheumatoid factor (RF) antibodies may specifically bind Fc parts of IgG molecules complexed with pathogen and thus enhance the neutralization activity of pathogen-specific monomeric IgG. Such neutralization-enhancing RF antibodies amplify IgG response via increased aggregation and clearance of antibody-pathogen immune complexes.

Neutralization-enhancing RF antibodies (NeRFa) might be induced via prolonged repeated immunization with protein antigens. Induction of NeRFa would be an important task for creating effective therapeutic and prophylactic vaccines against HIV, malaria, Ebola and other pathogens. Single-administration vaccine technology with pulsatile release of protein antigens from biodegradable polymeric microspheres opens new possibilities to achieve such a goal.

The idea that specific induction of NeRFa can be a universal approach to eradicate any pathogen, including cancer-associated viruses, will be put forward in this review article.

Chapter 4 – Takayasu arteritis (TA) is a rare, chronic large-vessel vasculitis (LVV) characterized by granulomatous inflammation of the vessel wall with an unknown etiopathogenesis. TA predominantly affects young females during the 2^{nd} or 3^{rd} decades of life and mainly involves the aortic arch and its major branches, ascending aorta, thoracic descending aorta and abdominal aorta. A physical examination is the first step for disease assessment. Systemic inflammatory response does not always show a positive correlation with inflammatory activity in the vessel wall. Imaging modalities are very important for establishing the diagnosis of TA. Conventional angiography, the gold standard for initial diagnosis, seems to be replaced with the new imaging modalities such as magnetic resonance angiography, ultrasonography, computerized tomography and, 18F-fluorodeoxyglucose positron emission tomography in recent years. Prognosis is possibly getting better with lower mortality in recent years. The most commonly used agents include corticosteroids and conventional immunosuppressive agents such as methotrexate, azathioprine, micophenolate

mofetil and leflunomide. However, in resistant and/or intolerant patients, biologic drugs including anti-TNF agents (mostly infliximab), rituximab and tocilizumab seem to be promising. Antiplatelet treatment may also lower the frequency of ischaemic events in TA. Endovascular interventions (balloon angioplasty or stent graft replacement) or by-pass surgery may be useful for critical arterial occlusions. There is a clear need to develop a validated set of outcome measures in TA, such as measures of disease activity, health-related quality of life and disease-related damage. The OMERACT Vasculitis Working Group has taken on this task and aims to develop a core set of outcomes for LVV.

Chapter 5 – Premenstrual syndrome (PMS) has few established risk factors and an unknown etiology. Effective treatment options are limited and most have substantial limitations. It is important to identify modifiable risk factors for PMS including dietary aspects. A number of recommendations concerning macronutrient intake have been made regarding prevention or treatment of PMS. For example, the American Congress of Obstetricians and Gynecologists and the Association of Reproductive Health Professionals recommend consuming regular meals with adequate protein and complex carbohydrates, with reduced fat and sugar intakes to alleviate PMS. However, these recommendations are not evidence-based and largely unsubstantiated.

Potential physiological effects of fats on PMS include inflammation and altered hormonal milieu. Saturated fats act as pro-inflammatory factors and increase estradiol and luteinizing hormone levels, whereas polyunsaturated fats like omega-3s act as anti-inflammatory factors. To date, two cross-sectional studies have examined fat intake and PMS symptom severity, and five small clinical trials have tested whether supplements containing fatty acids are effective at relieving symptoms. Total, saturated, and monounsaturated fats have overall higher symptom scores, particularly pain but total fat intake is related to lower cravings and bloating. The treatment trials show that gamma-linoleic acid supplements reduce symptoms.

Potential effects of carbohydrates include increasing serotonin levels, regulation of blood glucose and insulin sensitivity, and reducing estrogen levels via whole grain fiber. Few studies have directly assessed how carbohydrates impact premenstrual symptoms. These studies have shown inconsistent relationships suggesting that higher carbohydrate intakes are associated with lower symptom experience, particularly for pain and negative affect. Women with PMS show increased intake of total carbohydrates primarily from simple sugars, which are associated with higher symptom severity, whereas whole grains and fiber are not associated, except for breast pain.

Dietary protein may potentially affect premenstrual symptoms through action of the renin-angiotensin-aldosterone system, lowered calcium levels, negation of serotonin increases from carbohydrates, and altered hormonal milieu. Soy protein is related to lower estradiol and progesterone levels, whereas red or processed meats have increased steroid hormones and sex hormone binding globulin. To the authors' knowledge, only three studies have examined the association of protein intake and premenstrual symptoms. It appears that while women with higher intakes of protein have fewer symptoms, there is no difference in severity. Low-fat vegetarian diets show reductions in symptoms and severity, though not related to soy intake.

Several major limitations exist among available literature. All studies have assessed macronutrients as a potential treatment rather than a causal factor of PMS. Among the observational studies, it is unknown whether observed associations are due to reverse causation; specifically, it is unclear whether macronutrient intake contributes to PMS or whether women with PMS have altered their diet to control their symptoms. Lastly, many

studies have looked at each macronutrient separately and have not assessed the effects relative to each other, to kilocalorie intake, or to confounding factors. Future research should use prospective designs to determine the temporal relationship between macronutrient intake and PMS controlling for other macronutrients and confounders. This research has the potential to help women prevent PMS via modifiable risk factors or treat symptoms effectively and safely.

Chapter 6 – Pressurized IntraPeritoneal Aerosol Chemotherapy (PIPAC) is an innovative drug delivery system applying chemotherapy as a pressurized therapeutic aerosol into the abdominal cavity. PIPAC has superior pharmacological properties and first clinical results concerning efficacy and safety are promising. However, applying chemotherapeutic substances as a toxic aerosol is a challenge for occupational health and safety management. A risk assessment of PIPAC was performed to meet legal requirements. It was not possible to replace chemotherapeutic drugs with other substances since platin-based drugs and/or anthracyclines are the drugs of choice in many peritoneal cancers. However the dose of toxic substances could be reduced by 90%. Exposure of health workers to the chemotherapeutic drugs can be cutaneous (liquids) or respiratory (aerosol). Wearing chemotherapy gloves, protective clothing and glasses was effective for preventing cutaneous or ocular exposure, since the drugs applied are not absorbed through the skin. A three-level confinement system was designed to prevent any inhalative exposure of health workers. The first level is the closed abdomen itself, which tightness is controlled before chemotherapy application. The second level is the volume of the operating room together with air exchange through high-flow ventilation. The third confinement is the physical operating room, since the PIPAC procedure is remote-controlled and the team leaves the room during application. At the end of the procedure, the toxic aerosol is exhausted through a Closed Aerosol Waste System (CAWS) over a special line into the external environment, using the same system used for eliminating narcosis gases. Repeated environmental measurements in two different hospitals according to NIOSH protocols at the potential working places of the surgeon and of the anesthesiologist detected no traces of platin in the air. Biological monitoring in the blood of 5 surgeons after 500 procedures showed no traces of platin or doxorubicin. Mathematic modelling of the worst case scenario (immediate release of the toxic aerosol into the working environment) showed a potential respiratory uptake lower than 1/100’000 of a usual systemic chemotherapy dose. Over 650 procedures, to minor incidents related to disconnection in the tubing system were reported in the Critical Incident Reporting System (CIRS). No severe incident, in particular no leakage of the toxic aerosol, was recorded. No pressure- or technology-linked patient complication was noted. Work place measurements remained below the tolerance margin. The safety measures and conditions as defined above are sufficient. PIPAC can be used safely in the clinical setting if the conditions specified above are met. For the drugs tested, PIPAC is in compliance with European Community working safety law and regulations.

Chapter 7 – Mitochondrial monoamine oxidases (isoforms A and B; MAO-A and MAO-B respectively) are ubiquitous enzymes located at the outer mitochondria membrane facing toward cytoplasm. This localization results strategic allowing products of enzyme catalysis distributing in cytoplasm as well as into organelles. In this respect, several studies indicate the deleterious role of the hydrogen peroxide produced by MAO catalysis in inducing oxidative damage of structural or mitochondrial proteins (Kaludercic et al., 2010), triggering tissue loss of function and senescence.

Interesting enough, experimental evidence indicate that MAO-A isoform over-activation occurs in degenerative pathologies including heart and kidney failure. These evidence suggested that MAO-A overactivation might be considered as a novel source of free radicals participating to unbalancing the cell redox state, a ground condition for the onset of several diseases.

Accordingly, inhibition of enzyme activity has been proposed as a novel antioxidant strategy to reduce tissue oxidative status. This approach has however, intrinsic limitations including the increase of the the sympathergic tone.

Bach et al., (1988) cloned MAO-A and MAO-B describing interesting differences and similarities between the two isoforms. In this respect they clearly indicated that the promoters of the two isoforms are controlled, as expected, by different regulatory factors. In particular, in addition to sex hormones, they reported that angiotensin-II (AT-II) selectively activates MAO-A promoter activity. This evidence, which can have clinical implications, has been neglected largely by pharmacologists.

Drugs targeting AT-II and its intracellular cascade are considered gold standard treatments for cardiovascular diseases (CVD). The beneficial effects of such drugs may include the control of MAO-A over-activation, thus working indeed as "indirect antioxidants." This aspect may over-come the use of MAO inhibitors, which *di per se* are endowed of scavenging activity in respect of oxidants, and be considered an added value for drugs rescuing against the cardiovascular risk.

Chapter 8 – MAO inhibitors (MonoAminoOxidase) - iMAO, are a group of antidepressant agents acting through the blocking of the enzymatic pathway of the disintegration of the serotonin - of MAO enzyme decomposing serotonin. One distinguishes two isoenzymes: MAO-A and MAO-B differing in the location and action. Isoenzyme MAO-A is found in a nervous tissue and its action is based on controlling the level of affection, whereas MAO-B is located in non-nervous tissues and with its action it regulates the appropriate blood pressure. Also numerous side effects, which may appear in the course of therapy with MAO, are based on these relations, especially on nonselective ones.

iMAO action was revealed by chance, as these medicines were used in treating tuberculosis, and their influence on the mood was quickly connected with action on neurotransmitters. The entire group of MAO inhibitors are not only drugs used in the treatment of depression, and these are divided in three subgroups. The first, oldest group includes drugs restraining MAO permanently and not selectively. Isoniazid, applied up till today in curing tuberculosis is in this group. The following second group, entails medicines blocking MAO irremediably, but selectively, e.g., Selegiline (MAO-B) - used in therapy of Parkinson's disease. Last, most contemporary group, are selective and reversible medicines, e.g., moklobemid, (MAO-A) that is the most important antidepressant agent being a representative of iMAO entire group.

iMAO group is used universally worldwide, which does not mean that these medicines are deprived of side effects. Above all, an amount of interactions they can enter with other medicines, elevating the serotonin level is quite large. Effects of such combinations are very serious, including serotonin syndrome and death. A wrong diet, containing lots of the tyramine, also decomposed by MAO, has a similar effect. Other adverse effects characteristic of MAO inhibitors are a hepatotoxicity and hypertensive action.

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Chapter 1

Trends in the Birth Prevalence of Boys with Isolated Hypospadias and Undescended Testis in Hungary during the Last 50 Years: A Population-Based Study

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Abstract

Previously an increasing birth prevalence of hypospadias and undescended testis was recorded in some countries, including in the Hungarian Congenital Abnormality Registry, 1962-2011, i.e., during the last 50 years. However, the critical evaluation of cases with recorded isolated hypospadias (IHS) and isolated undescended testis (IUT), particularly to compare with the so-called reference birth prevalence of IHS (2.25 $\pm$ 0.30 per 1000 live-births) and IUT (6.86 $\pm$ 0.62 per 1000 live-births) determined in a Hungarian clinical-epidemiological study based on the physical examination of newborns with well-defined diagnostic criteria did not indicate a real increase in the birth prevalence of IHS and IUT. Thus, the increase of recorded cases with IHS and IUT can be explained by the more complete reporting of cases and a larger proportion of mild subgroup of IHS and the lack of differentiation between birth IUT (with many postnatal descents of testes) and true IUT (diagnosed at 3,5 months after birth). In conclusion, birth defect-registries are not appropriate for the estimation of real birth prevalence of IHS and IUT. In addition the socio-demographic variables of mothers were also analyzed without significant

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association of changes in birth prevalence of recorded IHS and IUT. Finally maternal risk factors were evaluated in the large population-based Hungarian Case-Control Surveillance of Congenital Abnormalities, 1980-1996, some of them showed association with the risk of IHS and IUT but without connection of the trend in the birth prevalence of cases with IHS and IUT.

Keywords*:* Hypospadias, undescended testis, increased birth prevalence, birth defect registries, population based study, socio-demographic variables of mothers, birth outcomes, risk factors

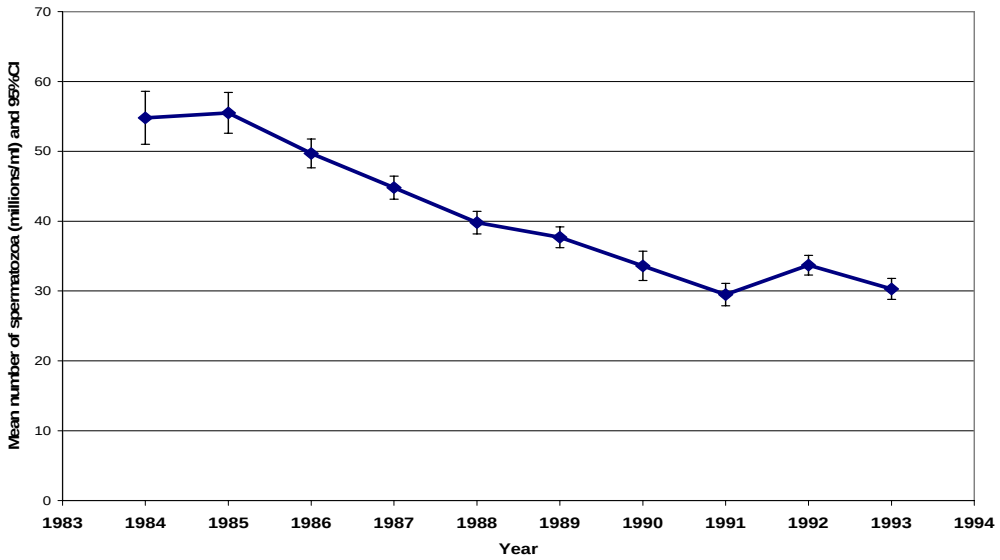
Introduction

Several studies indicated the disturbance of reproductive function in males during the last decades.

Carlsen et al., (1992) reported that sperm density had been progressively declining worldwide during much of the 20th century. Later, Swan et al., (2000) re-examined the issues and reported that there were declines of 1-3% per year in sperm density, with secular declines that were strongest in Europe, weaker in North America and either small or nonexistent in other continents. Similar trend in the quality of sperm was observed in Hungary as well. The Hungarian Periconception Service (HPS) was established in 1984 [Czeizel, 1999] and one of important issues in the protocol is the sperm analysis of male participants. After the collection of data regarding the history of previous conceptions and genitourinary infections, qualified nurses suggested a voluntary semen analysis in all males after obtained the sperm at home by withdrawal method after 3 days of sexual abstinence and provided for laboratory analysis within 1-3 hours. About 76% of male participants produced sperm for analysis during the study period, though this rate was 89% in some years [Czeizel, 2012].

The data of sperm analysis were unexpected (Figure 1). In the mid 1960s the sperm density (million/ml) was 74 millions, and this figure was accepted as referent. At the start of the HPS, i.e., in 1984 and 1985, this figure was 57 million but after 1987 it decreased fewer than 40 millions and since 1990 the mean value has been 31 million without further decline. Thus 23% of male participants had less than 20 millions/ml of spermatozoa. The mobility of spermatozoa did not show significant change while the proportion of teratoid spermatozoa increased during the last decades (Table I) [Czeizel et al., 2013]. Thus about 35% (!) of males were referred to the andrologist for final diagnosis and specific treatment in the HPS. The success of treatment was checked in the second visit 3 months later. This decline in sperm density has been accompanied by an increasing incidence rate of testicular cancer [Coleman et al., 1993; Forman and Moller, 1994; Adami et al., 1994] including in Hungary [Klujber, 1992]. The above threatening phenomena have been accompanied by an increasing prevalence at birth of hypospadias [Källen and Winberg, 1982; Czeizel, 1985; Czeizel et al., 1986] and undescended testes (Chilvers et al., 1984; Matlai and Beral, 1985; John Radcliffe Hospital Study Group, 1986]. These topics will be discussed in this chapter. The dangerous trend in the indicators of male reproduction malfunctions such as drastic decrease of sperm density and a robust increase of testis cancer in young age, in addition to the increased birth prevalence of hypospadias [Aschim et al., 2004b; Toppari et al., 2010] and undescended testis [Aschim et al., 2004b] resulted in the delineation of testicular dysgenesis syndrome

[Skakkebaeck et al., 2001]. There are some hypotheses for the explanation of testicular dysgenesis syndrome.



Year	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993
No.	360	423	825	734	600	815	410	428	446	738
Mean(S.D.)	54.8(36.6)	55.5(30.6)	49.7(30.1)	44.8(22.9)	39.8(20.2)	37.7(21.8)	33.6(21.5)	29.5(17.1)	33.7(14.9)	30.3(20.6)
95 % CI	51.0 - 58.6	52.6 - 58.4	47.6 - 51.8	43.1 - 6.5	38.2 - 1.4	36.2 - 39.2	31.5 - 35.7	27.9 - 31.1	32.3 - 35.1	28.8 - 1.8

The drastic drop of sperm density was found in 5,779 male participants in the HPS between 1984 and 1993 compared to the Hungarian baseline figure of healthy early adult males determined in 1965/66 (74 millions/ml); later further decrease was not observed. These findings explain that 23% of male participants had less than 20 millions/ml of spermatozoa. There was a significant increase in the proportion of teratoid spermatozoa from 11% to 31% in the above period, it was not increased later. The mean proportion of spermatozoa with good motility was between 67-71% between 1984 and 2010 without significant change. In addition 16% of males were screened out with pyosperm. Thus about 35% (!) of males were referred to the andrologist for final diagnosis and specific treatment.

Figure 1. Changes in mean number of spermatozoa (millions/ml) from 1984 to 1993.

Table I. The mean proportion (%) of spermatozoa with good mobility and teratoid spermatozoa density in the Hungarian healthy males in the years of 1965-1966 as reference and later in the male participants in the HPS

Years	Number of males	Mean proportion (%) of spermatozoa with good mobility	Mean proportion (%) of teratoid spermatozoa
1965-1966	50	71	11
1984/1985	783	68	16
1986-1989	2,974	67	21
1990-1994	2,022	70	28
1995-2010	9,901	68	31

There was no significant change in the proportion of spermatozoa with good monbilty while the proportion of teratoid spermatozoa increased.

One hypothesis that has been advanced to explain this decline in male reproductive health is that some environmental exposures act as “endocrine disruptors” [Sharpe and Skakkebaeck, 1993; Jensen et al., 1995; Toppari et al., 1996].

These endocrine-disrupting chemicals are supported to exert antiandrogenic effects on developing male fetuses and children. Among these chemicals the contraceptive hormonal pills are also mentioned.

Although we have no evidence to offer against the above hypothesis, we suggested an alternative hypothesis for a decline in male reproductive health and it is relaxed reproductive selection [Czeizel and Rothman, 2002].

Our hypothesis is based on the proposition that until recent times there has been strong natural selection against poor reproductive health, but on the past decades this elective selective pressure has been relaxed. For example in Hungary the average number of offspring per fertile couples was 11 at the end of the 18th century, but this number declines to 7 in 1920s, and subsequently dropped substantially to about 1.3 in the past decades [Demographic Yearbook, 2014]. Similar demographic changes have occurred in other European countries [Toulemon, 1988].

In addition at the start of this period of changing demographics, the proportion of childless was about 15% [Czeizel and Tóth, 1990], and fertile couples did not have effective birth control. By the second part of the 20th century, fertile couples had access to widely available and efficient birth control, while infertile couples had access to fertility treatments that enabled many subfertile couples, who might have previously been childless or had fewer children than they whished, to produce more offspring. Whereas the number of offspring from fertile couples used to be many times that from subfertile couples, these numbers have converged.

As a result of these changes, the proportion of children in the general population born to subfertile couples increased from an extremely low proportion of 1-4% at the start of the 20th century to about 14-20% by the end of the 20th century [Czeizel and Tóth, 1990]. Because of this trend, the proportion of men in the population with an inherited tendency toward subfertility has increased substantially during the period that sperm density has been reported to have declines. Subfertility is also related to both hypospadias [Fritz and Czeizel, 1996] and testicular cancer [Harland, 2000], and may help to explain the increasing trend of these structural birth defects, i.e., congenital abnormalities (CAs) of male genital organs.

In conclusion, we hypothesize that the relaxation of natural selection against subfertility may account for much of the decline in male reproductive health.

The first and major aim of this chapter is the critical evaluation of increasing trend of recorded birth prevalence of hypospadias and undesecended testis in Hungary based on our previous studies [Mavrogenis and Czeizel, 2013] and undescended testis [Mavrogenis et al., 2015]. The second aim of this analysis is the evaluation of maternal socio-demographic changes as the possible factors in the in the trend of hypospadias [Mavrogenis et al., 2014a] and undescended testiss [Mavrogenis et al., 2014c]. Finally, as the third aim of our project, the maternal factors were evaluated in the origin of hypospadias [Mavrogenis et al., 2014a; Mavrogenis et al., 2014d; Mavrogenis et al., 2014e] and undescended testis [Mavrogenis et al., 2014b].

Materials and Methods

Hungarian Congenital Abnormality Registry (HCAR)

The HCAR was established 1962 based on the mandatory reporting of cases with CA from the birth until the end of first postnatal year by medical doctors [Czeizel, 1997; Czeizel et al., 2014]. As far as we know it was the first national population-based CA-registry in the world. The reporting of cases with CA is mandatory for physicians to the HCAR, and most are reported by obstetricians (in Hungary practically all deliveries occur in inpatient obstetric clinics and birth attendants are obstetricians) and pediatricians (who are working in the neonatal units of inpatient obstetric clinics and various general and specialized, e.g., urologic inpatient and outpatient pediatric clinics). Autopsy was mandatory for all infant deaths during the study period and pathologists sent a copy of the autopsy report to the HCAR if defects were identified in infant deaths. Since 1984 prenatal diagnostic centers were also asked to report malformed fetuses diagnosed prenatally with or without elective termination of pregnancy to the HCAR.

Cases with CA are differentiated into isolated and multiple or syndromic categories in the HCAR. The multimalformed cases had 2 or more CAs in different organs with or without minor anomalies [Czeizel et al., 1988]. Thus, the unit of recording was the person and not the CA in the HCAR. Reported isolated minor anomalies/morphologic variants (i.e., unusual morphologic features that are of no serious medical or cosmetic consequence to the children) were recorded but not evaluated at the calculation of CA rates in the data set of the HCAR. In addition, CAs were classified into 3 groups from clinical aspect: lethal, severe (together major) and mild (CAs that require medical intervention but life expectancy is good) on the basis of their severity [Czeizel et al., 1993].

The recorded total (birth + fetal) prevalence of cases with CA was 35 per 1000 informative offspring (live-born infants, stillborn fetuses and electively terminated malformed fetuses) between 1980 and 1996 [Czeizel, 1997] and about 90% of major CAs were recorded in the HCAR [Czeizel et al., 1993].

According to their request, parents of cases with different CAs, among them hypospadias, were invited to attend annual meetings in the institute of the HCAR between 1980 and 1996. The staff of the HCAR provided information for parents regarding the possible causes of CAs, and initially medical geneticist of the HCAR examined children, and advised parents, if they needed, on further examinations, treatments, and prevention of recurrence risk. One of the major benefits of these parental meetings was that the physical examination of cases improved the quality of CA-diagnoses including cases with hypospadias and helped to differentiate subgroups.

Hungarian Case-Control Surveillance of Congenital Abnormalities (HCCSCA)

The HCCSCA was established in 1980 [Czeizel et al., 2001]. However, the method of data collection was changed in 1997 (after the retirement of the last author of this paper). Since all case and control mothers are visited and questioned at home by regional nurses, but

these data have not been validated at the time of this analysis. This fact explains that here only the 17 years' dataset of the HCCSCA, 1980-1996 are evaluated.

Patients, i.e., cases affected with CA including hypospadias and undescended testis were selected from the HCAR for the HCCSCA. Only those cases were selected from the HCAR into the HCCSCA who were reported after the first 3 months of birth (thus 23% of all cases, mainly with mild CA were excluded). Cases with CA-syndromes caused by gene mutations or chromosomal aberrations with preconception origin were also excluded.

Controls were defined as newborn infants without CA. Controls were selected from the National Birth Registry of the Central Statistical Office for the HCCSCA by the case lists for each quarter of the years from the staff of the HCAR. In general two controls were matched to every case according to sex, birth week in the year when the case was born and district of parents' residence. If controls were twin, only one of these twin-pairs was selected randomly for the HCCSCA [Czeizel et al., 2014].

There were 3 sources of exposure and confounder data [Czeizel et al., 2001]:

1. Prospective medically recorded data. An explanatory letter and a printed informed consent were mailed continuously to the address of mothers of cases and controls immediately after their selection for the HCCSCA. Mothers were requested to send us the prenatal maternity logbook, discharge summary of their delivery and every medical record concerning their diseases during the study pregnancy and their child's CA. These recent data helped us to modify the diagnosis of CAs if it was necessary. (These medical documents were sent back within 4 weeks.) Prenatal care was mandatory for pregnant women, thus nearly 100% of pregnant women visited prenatal care, on average 7 times. The task of obstetricians in prenatal care was to record all maternal diseases and medicinal products used by women during the study pregnancy in the logbook.
2. Retrospective maternal self-reported information. A structured questionnaire with a list of medicines and diseases were also mailed to the mothers of cases and controls. This questionnaire comprised of questions regarding socio-demographic data, pregnancy complications, acute and maternal diseases and related drug treatments, folic acid and multivitamin uses of mothers and birth outcomes of cases and controls. Mothers were asked to read the enclosed list as memory aid before they filled-in the questionnaire.

The mean ± S.D. time elapsed between the end of pregnancy and the return of the "information package" (including logbook, discharge summary, questionnaire and informed consent) in our prepaid envelope was 3.5 ± 2.1 and 5.2 ± 2.9 months in cases and controls, respectively.

3. Supplementary data collection. Regional district nurses were asked to visit all non-respondent case mothers and to help them to fill-in the same questionnaire used in the HCCSCA and to evaluate the available medical documents. District nurses could visit only 200 non-respondent [Czeizel et al., 2003a] and 600 respondent control mothers [Czeizel and Vargha, 2004] in two validation studies because the ethics

committee considered this follow-up to be disturbing for the parents of all healthy children [Czeizel et al., 2001].

The necessary data were available for 96.3% of cases (84.4% from replies and 11.9% from visits) and 83.0% of controls (81.3% from replies and 1.7% from visits). The signed informed consent was sent back by 98% of mothers, the name and address were deleted in 2% of subjects without signed informed consent.

Unclassified multiple CAs including hypospadias of undescended testis as component CA were excluded from the study.

Hypospadias

Introduction

Hypospadias is structural birth defect, i.e., congenital abnormality (CA) of male genital organ with displacement of the urethral opening along ventral surface, in general associated with hypoplasia of penis, dorsal hooded foreskin and chordee.

The birth prevalence rate of hypospadias covers a wide range from 4 to 43 patients per 10,000 births in different countries/studies [Kurahashi et al., 2004; Nassar et al., 2007]. In addition, an increasing birth prevalence of hypospadias was reported in Sweden [Källen and Winberg, 1982], in England and Wales [Matlai and Beral, 1985] and Hungary [Czeizel, 1985; Czeizel et al., 1986] in the 1960-1970s. Later increasing prevalence of hypospadias was observed in the USA [Paulozzi et al., 1997], Australia [Nassar et al., 2007], China [Sun et al., 2009], and Denmark [Lund et al., 2009] as well. However, an increasing birth prevalence of hypospadias was not found in Finland [Aho et al., 2000], Scotland [Ahmed et al., 2004], northern England [Abdullah et al., 2007], in the USA: New York State [Fisch et al., 2009], California [Carmichel et al., 2003], Washington State [Porter et al., 2005] and in Japan [Kurahashi et al., 2004]. Thus there was a debate about whether or not the birth prevalence of hypospadias is increasing, and the question is whether the previously found increasing trends were connected with different diagnostic criteria, the more complete ascertainment, and/or reporting of cases or exposure to new etiological factors (e.g., endocrine disruptors) in the origin of hypospadias [Czeizel, 2009].

First we have to consider diagnostic criteria. First the so-called isolated (i.e., hypospadias without other CA) and syndromic/multiple (hypospadias associates with other CA) should be separated in patients because of their different severity and origin. In our project, only cases with isolated hypospadias (IHS) were evaluated.

In the second step the different subgroups of hypospadias: anterior (glandular, coronal), middle (distal penile, midshaft, proximal penile) and posterior (penoscrotal, scrotal, perineoscrotal) are differentiated [Van der Zanden et al., 2012]. We use the following definitions of these subgroups:

Glandular: the opening is distal to the sulcus coronarius.

Coronal: the opening is within the sulcus coronarius.

Penile: the opening is proximal to sulcus coronarius with distal, midschaft, proximal location.

Penoscrotal: the opening is in the immediate vicinity of penoscrotal junction.

Scrotal: the opening is in the scrotal region.

Perineoscrotal: the opening is behind the cleft scrotum.

In fact glandular IHS is a minor anomaly without any need for surgical/medical treatment, thus practically we did not want to evaluate these cases among CAs in the HCAR. However, if IHS was not specified, we included these cases to the group of IHS. Coronal IHS induces a dilemma because this subgroup in general also does not need surgical intervention but in general associate with the dysfunction of penis, therefore this subgroup is included in the data set of cases with CA.

Results

The first aim was the critical evaluation of recorded cases with IHS in the population-based HCAR during last 50 years, 1962-2011, because we determined previously the true birth prevalence of cases HCAR [Czeizel and Tusnády, 1984]. Thus we were able to compare the recorded and true annual rates of cases with IHS in our project, particularly regarding the previously found increasing recorded trend of IHS [Czeizel et al., 1986]. Of course, at the evaluation of cases with IHS, CA-syndromes due to major mutant genes, chromosomal aberrations and unidentified multiple CAs including hypospadias as component CA were excluded in this study. The dataset of the HCAR, 1962-2011, included 11,498 cases with recorded IHS in live-born cases. IHS was not diagnosed in stillborn fetuses and malformed fetuses due to prenatal diagnosis. The annual number of live-births in Hungary, recorded annual numbers and birth prevalence of cases with CA, in addition to recorded annual numbers and birth prevalence of cases with IHS, are shown in Table II.

The trend of annual birth prevalence rates of CAs between 1962 and 2011 can be separated into 4 stages: (i) Cases with CA during the preliminary period between 1962 and 1969. (ii) After 1970 (i.e., from the nomination of the last author of this chapter for the directorship of the HCAR) there was an obvious increase to the maximum rate of cases with CA: 47.84 per 1000 births in 1983. (iii) There was a continuous decrease in the recorded rate of cases with CA until 1990 followed by a drastic drop after the change in the directorship of the HCAR from 1995. (iv) The new co-workers of the HCAR were able to reorganize the reporting system with the help of regional public health officers from 2000 and it resulted in a significant increase in the annual rate of CAs with a maximum (57.49/1000) in 2006.

The recorded annual birth prevalence of IHS in the HCAR is shown in Figure 2. There was a significant increase in the rate of IHS from 1962 until 1982 (2.41 per 1000) followed by a decline but not so robust than in CAs and finally there was a new increase from 1999 with a peak between 2005 and 2010, the maximum of 3.42 per 1000 was recorded in 2010.

The recorded live-birth prevalence of IHS was 0.48 per 1000 in the HCAR between 1962 and 1969 based on the data of one pediatric urological inpatient clinic; other similar institutions were not available in Hungary during this period. The prevalence rate of IHS was 1.59, 2.24, 2.07, 2.53 per 1000 live-births in 1970-1979, 1980-1989, 1990-1999 and 2000-2009, respectively, with the total rate of 2.04 per 1000 between 1962 and 2011. The rate of IHS was 3.12, 4.39, 4.05 and 4.96 per 1000 male live-births in 1970-1979, 1980-1989, 1990-1999 and 2000-2009, respectively, with a total figure of 4.00 per 1000.

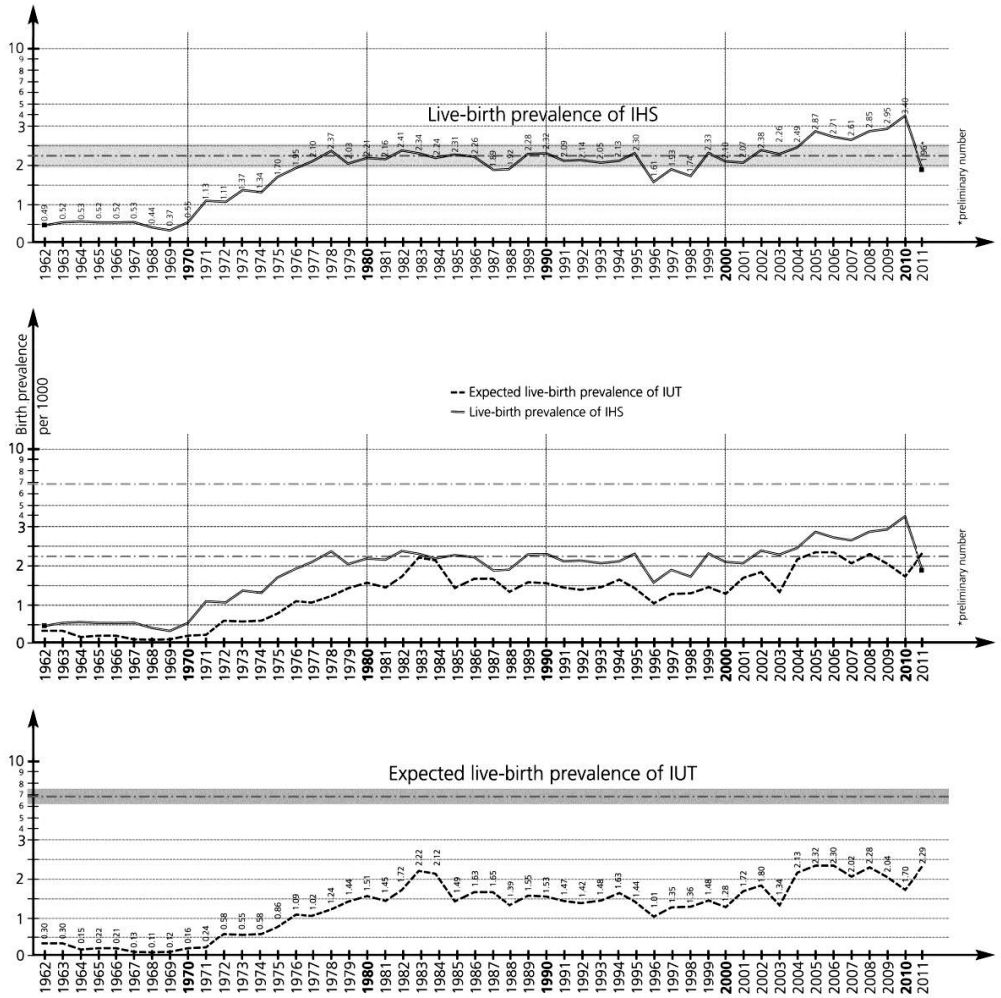


Figure 2. Annual live-birth prevalence rates of cases with total CA and isolated hypospdias (IHS) and isolated undescended testis (IUT) between 1962 and 2011 in the HCAR.

However, recorded birth prevalence of cases with IHS depends on the completeness of the reported cases. Thus it was necessary to compare the recorded and true birth prevalence of cases with IHS. The so-called true birth prevalence of IHS was determined in a previous clinical-epidemiological study when newborns were physically examined according to the previously defined protocol of diagnostic criteria by the same experts [Pazonyi et al., 1975; Czeizel and Tusnády, 1984]. In this study 10,203 newborns, among them 5,211 boys, born in the first half of 1973 were examined during the first day of life by obstetricians and later by neonatologists until the 6th postnatal day to diagnose IHS in 7 leading Hungarian obstetric hospitals. The rate of IHS was 2.25 $\pm$ 0.30 per live-births, and this figure was accepted as the referent. The confidence interval of this true baseline rate was 1.95-2.55 per 1000. This rate was 4.41 per 1000 male live-births.

Table II. Annual number of live-births in Hungary and recorded number and birth prevalence per 1000 of cases with total congenital abnormalities (CAs), isolated hypospadias (IHS) and isolated undescended testis (IUT) in the HCAR, 1962-2011

Years	No. of live-births	No. of cases with CA	Birth prevalence of total CAs	No. of cases with IHS	Live-birth prevalence of IHS	No. of cases with IUT	Live-birth prevalence of IUT
1962	130,053	1,951	15.00	64	0.49	39	0.30
1963	132,335	2,770	20.93	69	0.52	40	0.30
1964	133,690	2,888	21.60	72	0.53	21	0.15
1965	134,525	2,911	21.63	70	0.52	30	0.22
1966	140,004	3,001	21.43	73	0.52	30	0.21
1967	150,465	3,112	20.68	80	0.53	20	0.13
1968	155,966	2,999	19.22	69	0.44	18	0.11
1969	155,848	2,801	17.97	58	0.37	19	0.12
1970	153,339	3,711	24.20	85	0.55	26	0.16
1971	152,159	4,843	31.82	172	1.13	38	0.24
1972	154,688	4,802	31.04	173	1.11	90	0.58
1973	157,623	4,780	30.32	217	1.37	88	0.55
1974	187,957	6,301	33.52	252	1.34	110	0.58
1975	195,847	6,909	35.27	334	1.70	169	0.86
1976	186,916	7,582	40.56	365	1.95	204	1.09
1977	179,152	7,219	40.29	377	2.10	183	1.02
1978	169,152	7,277	43.02	402	2.37	210	1.24
1979	161,677	6,905	42.70	329	2.03	233	1.44
1980	149,673	7,011	46.84	331	2.21	227	151
1981	142,890	6,186	43.29	310	2.16	208	1.45
1982	133,559	6,197	46.39	322	2.41	230	1.72
1983	127,258	6,089	47.84	298	2.34	283	2.22
1984	129,359	5,428	41.96	290	2.24	275	2.12
1985	130,200	4,999	38.39	301	2.31	194	1.49
1986	128,204	4,961	38.69	290	2.26	210	1.63
1987	125,840	4,151	32.98	239	1.89	208	1.65
1988	124,348	4,319	34.73	285	1.92	173	1.39
1989	123,304	4,287	34.76	282	2.28	192	1.55
1990	125,679	4,295	34.17	292	2.32	193	1.53
1991	127,724	3,786	29.64	268	2.09	189	1.47
1992	121,724	3,227	26.51	261	2.14	174	1.42
1993	117,033	3,157	26.97	241	2.05	174	1.48
1994	115,598	2,897	25.06	247	2.13	189	1.63
1995	112,054	2,634	23.50	258	2.30	162	1.44
1996	105,272	2,206	20.95	170	1.61	107	1.01
1997	100,830	1,783	17.68	195	1.93	104	1.03
1998	97,857	2,627	26.84	171	1.74	133	1.35
1999	95,116	2,794	29.37	222	2.33	141	1.48
2000	98,135	2,864	29.18	207	2.10	126	1.28
2001	97,597	3,236	33.15	203	2.07	168	1.72
2002	97,327	3,628	37.27	232	2.38	176	1.80
2003	95,177	3,375	35.46	216	2.26	128	1.34
2004	95,613	4,950	51.77	239	2.49	204	2.13
2005	98,002	5,310	54.18	282	2.87	228	2.32
2006	100,360	5,770	57.49	272	2.71	231	2.30

Years	No. of live-births	No. of cases with CA	Birth prevalence of total CAs	No. of cases with IHS	Live-birth prevalence of IHS	No. of cases with IUT	Live-birth prevalence of IUT
2007	98,098	5,185	52.85	257	2.61	199	2-02
2008	99.580	4,579	45.98	284	2.85	228	2.28
2009	96,961	4,561	47.03	287	2.95	198	2.04
2010	90,722	4,683	51.61	311	3.42	155	1.70
2011	88,441	4,482	50.67	174*	1.96*	203	2.29

The comparison of the annual recorded birth prevalence rates of cases with IHS in the HCAR with the true birth prevalence of IHS showed that in general the recorded birth prevalence of recorded IHS did not reach the level of true birth prevalence of IHS. The exception period was between 2005 and 2010 in the HCAR, when the increase of IHS rates exceeded the upper confidence limit of true birth prevalence (2.55 per 1000) with the highest rate of 3.42 per 1000 in 2010.

At the evaluation of IHS is worth evaluating different subgroups, but unfortunately specified subgroups were reported rarely in the HCAR. These subgroups could be evaluated only in 52.0% of cases with IHS (1,580/3,038) between 1980 and 1996 in the HCAR due to physical examination in the parental meetings and reported subgroups in the HCAR completed by the recent medical documents in the HCCSCA 3.5 $\pm$ 2.1 months later (Table III).

Thus, the dataset of the HCCSCA provided a more detailed analysis of cases with IHS on the basis of available recent medical document on 3.5 $\pm$ 2.1 months after birth. There were 4,385 live-born cases with IHS in the HCAR, 1980-1996, but we focus this analysis for 3,038 live-born cases with IHS in the dataset of the HCCSCA, 1980-1996. The difference, i.e., 1,347 cases is explained by late notification, i.e., after the third postnatal month (1,246 cases), non-respondents and unknown new addresses (77 cases), misdiagnosis (18 cases mainly due to syndromic hypospadias) and 6 families refused the collaboration.

We differentiated two study periods after the new coworkers of the HCAR. There were 1,685 cases with IHS between 1997 and 2004, the subgroups were reported only in 605 cases (35.9%) and the coronal type was recorded in 383 cases (40.0%). However, the proportion of reported coronal subgroup was much higher in the HCAR between 2005 and 2010. Of 1,693 reported cases with IHS, only 533 (31.5%) had specified subgroup, but 288 (54.0%) were classified as coronal subgroup.

In addition we compared the percentage rate of IHS subgroups in the cases in our study material with the IHS subgroups in previous clinical study (Pazonyi et al., 1975) and the previous Hungarian epidemiological study (Czeizel et al., 1986) (Table III). The subgroups of penile and more severe cases did not show significant differences among these datasets, but the proportion of coronal subgroup was higher in the material of HCAR 1962-2011, explained mainly in the period between 2005 and 2010. Thus the increase of cases with IHS between 2005 and 2010 may be connected with larger proportion of reported mild coronal subgroup.

In the next step we compare the birth outcomes of cases and controls in the HCCSCA, 1980-1996. There were 2,146,574 total births in Hungary during the study period, but all IHS occurred in live-born babies, thus it is worth calculating with 2,134,151 live-births. Thus 38,151 population controls represented 1.8% of all Hungarian births, but only 24,814 male newborns were evaluated in the study. The group of 3,084 cases with IHS had 4,981 matched

controls. However, the variables of matched controls and population controls did not show significant differences therefore only the data of populations controls are shown in this chapter.

Table III. Distribution of different subgroups of IHS in three Hungarian studies

Subgroups of hypospadias	HCAR 1962-2011		Pazonyi et al 1973 (1975)*		Czeizel et al 1975 (1986)*		Present study
	No.	%	No.	%	No.	%	%
Glandular: the opening is distal to the sulcus coronarius	372	23.5	69	23.5	85	29.7	23.6
Coronal: the opening is within the sulcus coronarius	658	41.6	80	27.2	103	36.0	41.7
Penile: the opening is proximal to sulcus coronarius	490	31.0	134	45.6	84	29.4	31.0
Penoscrotal: the opening is in the immediate vicinity of penoscrotal junction	41	2.6	0	0.0	8	2.8	2.6
Scrotal: the opening is in the scrotal region	11	0.7	0	0.0	2	0.7	0.7
Perineoscrotal: the opening is behind the cleft scrotum	8	0.5	11	3.7	4	1.4	0.4
Total	1,580	99.9	294	100.0	286	100.0	100.1
Subtotal without glandular hypospadias	1,208	76.5	225	76.5	98	34.3	34.6
Subtotal without glandular and coronal hypospadias, i.e., severe cases	550	34.8	145	49.3	201	70.3	76.4

*publication year.

The seasonality of births of cases with IHS based on the monthly figures is shown in Table IV. There was a higher rate of cases with IHS born between January and April with exception of February.

Table IV. Number of monthly live-births in Hungary and cases with IHS in the HCCSCA, 1980-1996 and their rates

Months	Live-births, 1980-1996		Cases with IHS in HCCSCA, 1980-1996	
	No.	%	No.	per 1000
I: January	181,101	8.5	306	1.68
II: February	167,757	7.8	236	1.40
III: March	183,738	8.6	284	1.54
IV: April	172,816	8.1	278	1.60
V: May	179,771	8.4	256	1.42
VI. June	181,682	8.5	258	1.42
VII. July	192,823	9.0	275	1.42
VIII. August	186,592	8.7	272	1.45
IX: September	181,644	8.5	225	1.23
X: October	173,315	8.1	217	1.25
XI: November	166,005	7.8	205	1.23
XII: December	170,907	8.0	226	1.32
Total	2,138,151	100.0	3,038	1.42

Table V. Live-birth outcomes of male cases with IHS and male controls

Live-birth outcomes	Cases (N = 3,038)		Controls (N = 24,814)	Comparison
Quantitative	Mean	S.D.	Mean	S.D. t = p =
Gestational age (wk)*	39.2	2.2	39.4	2.0 5.14 p<0.0001
Birth weight (g)**	3,127	604	3,323	514 19.44 p<0.0001
Categorical	No.	%	No.	% OR 95% CI
Twins	50	1.6	263	1.1 1.56 1.15-2.12
Preterm birth*	304	10.0	2,073	8.4 1.22 1.07-1.38
Postterm birth*	35	1.3	458	1.8 0.73 0.51-1.09
Low birthweight**	396	13.0	1,239	5.0 2.85 2.53-3.22
Large birthweight**	15	0.5	188	0.8 0.65 0.38-1.10

*Adjusted for age, parity (birth order) and employment status of mothers.

** Adjusted for age, parity (birth order), employment status of mothers and gestational age of newborns.

Table VI. Main socio-demographic variables of mothers who delivered cases and population controls

Variables	Case mothers (N = 3,038)		Population control mothers (N = 24,814)	
Quantitative	No.	%	No.	% Comparison
Maternal age, yr				
- 19	347	11.4	2,141	8.6
20 – 29	2,107	69.4	17,991	72.5 χ^2 = 27.5 p<0.001
30 -	584	19.2	4,682	18.9
Mean, S.D.	25.2	5.2	25.4	4.9 t = 2.11 p = 0.035
Birth order				
1	1,533	50.5	11,880	47.9
2	1,014	33.4	9,318	37.6 χ^2 = 21.1 p<0.001
3 or more	491	16.2	3,616	14.6
Mean, S.D.	1.7	1.1	1.8	0.9 t = 5.63 p<0.001
Pregnancy order				
1	1,389	45.7	10,645	42.9
2	948	31.2	8,806	35.5 χ^2 = 21.8 p<0.001
3 or more	701	23.1	5,363	21.6
Mean, S.D.	2.0	1.3	1.9	1.1 t = 4.63 p<0.001
Categorical	No.	%	No.	%
Unmarried	168	5.5	944	3.8 χ^2 = 21.0 p<0.001
Employment status				
Professional	266	8.8	2,902	11.7
Managerial	699	23.0	6,736	27.1
Skilled worker	892	29.4	7,701	31.0 χ^2 = 141.5 p<0.001
Semiskilled worker	543	17.9	3,943	15.9
Unskilled worker	223	7.3	1,455	5.9
Housewife	305	10.0	1,515	6.1
Others	110	3.6	562	2.3

The birth outcomes of cases and population controls are shown in Table V. Of 3,084 cases with IHS, 50 (1.6%) were twins, this rate is somewhat higher that the twin rate of population controls. The mean gestational age at delivery was 0.2 wk shorter in the mothers of cases than in the mothers of population controls while the mean birth weight of cases was smaller with 196 grams compared to population controls. These findings were in agreement with the rate of preterm births and particularly low birthweight newborns. The rate of preterm

births was 1.2 fold higher in the group of cases than in the group of population controls while the rate of low birthweight was 2.6 fold higher in cases than in population controls.

Thus, the higher rate of low birthweight is characteristic for the cases with IHS, and the latter indicates intrauterine growth restriction of fetuses.

The second aim of our project was to analyze maternal variables in general, in addition to the detailed analysis of time trends because if maternal variables associate with the birth prevalence of cases with IHS, the time trend of these cases may be connected with the changing maternal variables. Maternal variables of cases with IHS and population controls are shown in Table VI.

Table VII. Main variables of mothers who delivered cases in 1980, 1990 and 1996

Variables	1980 (N = 197)		1990 (N=188)		1996 (N=125)	
Quantitative	No.	%	No.	%	No.	%
Maternal age, yr						
- 19	24	12.2	21	11.2	8	6.4
20 – 29	130	66.0	136	72.3	96	76.8
30 -	43	21.8	31	16.5	21	16.8
Mean, S.D.	25.2	5.1	25.1	5.4	25.6	4.8
Birth order						
1	94	47.7	92	48.9	62	49.6
2	76	38.6	63	33.5	29	23.2
3 or more	27	13.7	33	17.6	34	27.2
Mean, S.D.	1.8	1.3	1.7	0.9	2.1	1.4
Pregnancy order						
1	88	44.7	87	46.3	58	46.4
2	63	32.0	56	29.8	26	20.8
3 or more	46	23.4	45	23.9	41	32.8
Mean, S.D.	2.0	1.5	1.9	1.2	2.3	1.6
Categorical						
Unmarried	6	3.0	17	9.0	10	8.0
Employment status						
Professional	25	12.7	12	6.4	11	8.8
Managerial	35	17.8	47	25.0	22	17.6
Skilled worker	67	34.0	64	34.0	47	37.6
Semiskilled worker	33	16.8	24	12.8	16	12.8
Unskilled worker	10	5.1	14	7.4	12	9.6
Housewife	25	12.7	27	14.4	14	11.2
Others	2	1.0	0	0.0	3	2.4

The mean maternal age was lower in cases with IHS because of the larger proportion of case mothers under 19 years than that of population control mothers. However, the mean birth order (parity) was somewhat higher in the mothers of cases than in the mothers of population controls explained mainly by larger proportion of primiparous mothers of cases. The interval between mean birth order and pregnancy order (birth + miscarriages) was similar in the mothers of cases and population controls, and it is against a higher rate of fetal death in previous pregnancies.

The rate of unmarried mothers was somewhat higher in the group of cases than in the group of population controls. The distribution of maternal employment status showed a significant difference among the study groups and it indicated the lower socioeconomic status of case mothers: the proportion of professional and managerial mothers was lower (31.8%) in

the mothers of cases than in the mothers of population (38.8%). Thus, vice versa, the proportion of semi- and unskilled workers and housewives was larger in case mothers (35.3%) than in population control mothers (27.9%). Mainly the proportion of housewives was higher in case mothers than in population control mothers, most of these women belonged to the poor women in Hungary.

The time trend of maternal variables was also analyzed compared to the data in 1980, 1990 and 1996. The distribution of maternal age showed difference because the proportion of youngest (19 yr or less) and oldest (30 yr or more) women decreased and the proportion of 3 or more birth order increased. The employment status of mothers did not know significant changes though the proportion of professionals showed a mild decrease while the proportion of unmarried and unskilled workers increased. However, these trends did not associate with the trend of birth prevalence of cases with IHS.

Thus the third aim of our project was to evaluate the possible environmental factors which have a role in the triggering of polygenic predisposition for IHS therefore pregnancy complications, acute and chronic diseases with related drug treatments and pregnancy supplements were analyzed in the population-based HCCSCA, 1980-1996. The critical period of IHS is estimated between 5th and 14th postconception weeks, i.e., 7th and 16th gestational weeks calculated from the first day of the last menstrual period, thus exposures were evaluated between the second and fourth gestational months [Czeizel, 2009; Czeizel et al., 2008].

The incidence of pregnancy complications recorded prospectively in the prenatal maternity logbooks is presented in Table VIII. Preeclampsia/eclampsia and gestational diabetes occurred more frequently among the mothers of cases than in the mothers of matched and population controls. The diagnosis of preeclampsia was based on the increased blood pressure accompanied by proteinuria measured in prenatal clinics after the 20th gestational week. Of 176 cases in the group of preeclampsia/eclampsia, 6 had mothers with eclampsia. The important role of maternal preeclampsia in the origin of low birthweight is indicated by the comparison of low birthweight rate in mothers of cases with preeclampsia (23.3%; 41/176) and without preeclampsia (12.7%; 363/2,862). Gestational diabetes is defined as glucose intolerance of any degree (mean blood glucose exceeded 6.1 mmol/L) that begins or is first recognized during pregnancy. On the other hand, the incidence severe (treated) nausea and vomiting pregnancy was lower in the mothers of cases with IHS than in the mothers of controls.

The incidence of acute maternal disease groups (e.g., influenza) did not show any association with the risk of IHS.

The evaluation of chronic maternal diseases was based on medical records in prenatal maternity logbook. Two chronic diseases associated with a higher risk of IHS in the mothers of cases compared to the mothers of population controls. Of 13 epileptic case mothers, 3 were not treated with antiepileptic drugs during the study pregnancy, while 7 had monotherapy (valproate 4, phenytoin 2, diazepam 1). Of 3 women with polytherapy, 2 had valproate + diazepam, one phenytoin + diazepam. If 6 pregnant women with valproate treatment were removed from 13 epileptic women, the previously found association disappeared.

The rate of medically recorded cervical erosion was also higher in the mothers of cases, than in the mothers of population controls. The cervical erosion is cervical ectopy because the cells at the os of the cervix change from squamous cells normally found in this region to columnar cells and this pathological condition gives a red and eroded appearance. Only

pregnant women with cervical erosion with or without cervicitis, but without pelvic inflammatory diseases and vulvovaginitis/bacterial vaginosis were included into this group. Of 3,038 cases with IHS, 9 (0.3%) had mothers with cervical erosion while this figure was 0.1% in population controls (18/34,814) (OR with 95% CI: 4.09, 1.84-9.12). (The number of matched controls was too low for this analysis.) The usual drug treatment in these pregnant women was topical and oral antimycotic and antiparasitic drugs, mainly clotrimazole, metronidazole and metrodinazole + miconazole.

Table VIII. Incidence of medically recorded pregnancy complications in the mothers of cases, matched controls and population controls

Pregnancy complications	Mothers of cases (N = 3,038)		Mothers of matched controls (N = 4,981)		Comparison	Mothers of population controls (N = 24,814)		Comparison
	No.	%	No.	%	OR, 95% CI	No.	%	OR, 95% CI
Threatened abortion	478	15.7	840	16.9	0.92 0.81-1.04	4,290	17.3	0.88, 0.79-1.09
Nausea/vomiting, severe	223	7.3	430	8.6	0.84 0.71-0.99	2,328	9.4	0.77, 0.66-0.88
Gestational hypertension	89	2.9	152	3.1	0.96 0.74-1.25	720	2.9	1.01, 0.81-1.26
Preeclampsia/eclampsia	176	5.8	153	3.1	1.94 1.55-2.42	770	3.1	1.92, 1.62-2.27
Pregnancy related renal disease	47	1.5	76	1.5	1.01 0.70-1.46	310	1.2	1.24, 0.91-1.69
Oedema with excessive weight gain	60	2.0	121	2.4	0.81 0.59-1.11	595	2.4	0.82, 0.63-1.07
Gestational diabetes	35	1.2	34	0.7	1.70 1.06-2.72	148	0.6	1.94, 1.34-2.81
Anemia in pregnancy	482	15.9	805	16.2	0.98 0.86-1.11	4,070	16.4	0.94, 0.76-1.19
Placental disorders**	35	1.2	86	1.7	0.66 0.45-1.09	391	1.6	0.73, 0.51-1.03
Polyhydramios	8	0.3	20	0.4	0.65 0.29-1.49	126	0.5	0.52, 0.25-1.06
Oligohydramnios	2	0.1	2	0.0	1.64 0.23-11.65	9	0.0	1.82, 0.39-8.41
Threatened preterm delivery***	355	11.7	691	13.9	0.84 0.72-1.04	3,520	14.2	0.88, 0.78-1.09
Prolonged pregnancy	24	0.8	62	1.2	0.63 0.39-1.01	320	1.3	0.66, 0.40-1.09
Others	22	0.7	26	0.5	1.41,0.76-3.32	120	0.5	1.50, 0.96-2.37

There were 59 drugs used at least by 15 mothers of cases. (The previously mentioned valproate was used only in 6 epileptic case mothers.) Two drugs were used more frequently during the critical period of IHS by the mothers of cases compared to the mothers of population controls (Table IX). The use of progestin derivative allylestrenol (Gestaton^{R}, Organon; Turinal^{R}, Richter) tablets contained 5 mg and their indication was the treatment/prevention of threatened and habitual/repeated abortion. However, if only medically recorded allylestrenol treatments in the prenatal maternity logbook were evaluated, this association disappeared.

Nystatin (Nystatin^{R}, Chinoin) was available as tablet including 500,000 IU and the usual dose of oral nystatin treatment was daily 1.5-3.0 million IU for 3-6 days. Of 3,038 cases with IHS, 22 (0.7%) had mothers with nystatin treatment while this figure was 0.4% (93/24,814) mothers of population controls (OR with 95% CI: 1.94, 1.22-3.09). If only medically recorded nystatin treatments in the critical period of IHS were evaluated, this association was confirmed (OR with 95% CI: 3.17, 1.63-6.18).

Another progestin derivative hydroxyprogesterone was not used more frequently by the mothers of case than by the mothers of population controls (Table IX).

All other sex hormones and three related drugs (clomifene, bromocriptine and oxytocin) were also evaluated independently on the number of case mothers (Table IX).

Table IX. The use of female sex hormones and related drugs during the study pregnancy of case and population control mothers

Medicinal products	Case mothers (N = 3,038)		Population control mothers (N = 24,814) Comparison				
	No.	%	No.	%	OR	95% CI*	
Allylestrenol	462	15.20	3,450	13.90	1.11	0.91	1.23
Hydroxyprogesterone	39	1.28	270	1.09	1.17	0.89	1.99
Oestradiol (Akrofollin^{R})	1	0.03	4	0.01	2.04	0.23	18.28
Ethinylestradiol (Mikrofollin^{R})	5	0.16	11	0.04	**3.72**	**1.29**	**10.71**
Ethinylestradiol + ethisterone (Limovan^{R})	9	0.29	21	0.08	**3.51**	**1.61**	**7.67**
Oestradiol + progesterone (Limovanil^{R})	5	0.16	11	0.04	**3.72**	**1.29**	**10.71**
Oestrone (Hogival^{R})	2	0.06	15	0.06	1.09	0.25	4.76
Diethylstilbestrol (Syntestrin^{R})	1	0.03	7	0.02	1.17	0.14	9.49
Lynestrenol (Orgametril^{R})	13	0.42	4	0.01	**26.66**	**8.69**	**81.80**
Norethisterone (Norcolut^{R})	4	0.13	35	0.14	0.93	0.33	2.63
Progesterone (Glanducorpin^{R})	3	0.09	13	0.05	1.89	0.54	6.62
Dihydrogesterone (Duphaston^{R})	3	0.09	22	0.08	1.11	0.33	3.72
Chorionic gonadotrophin (Chorion^{R})	12	0.39	73	0.29	1.34	0.73	2.48
Clomifene (Clostilbegyt^{R})	10	0.32	69	0.27	1.18	0.61	2.30
Bromocriptine (Bromocritin^{R})	2	0.06	6	0.02	2.72	0.55	13.50
Oxytocin (Oxytocin^{R})	1	0.03	36	0.14	0.23	0.03	1.65
Ethinylestradiol + ethynodiol (Bisecurin^{R})**	2	0.09	11	0.04	1.49	0.33	6.70
Ethinylestradiol + levonorgestrel (Rigevidon^{R})**	1	0.03	6	0.02	1.36	0.16	11.31
Ethinylestradiol + levonorgestrel (Ovidon^{R})**	3	0.09	15	0.06	1.63	0.47	5.65
Ethinylestradiol + levonorgestrel (Tri-Regol^{R})**	4	0.13	3	0.01	**0.90**	**2.44**	**48.74**
Ethynodial diacetate (Continuin^{R})**	3	0.09	26	0.10	0.94	0.29	3.12
Contraceptive pills together	13	0.42	61	0.24	1.74	0.96	3.18
Grand total	68	2.23	388	1.56	**1.44**	**1.11**	**1.87**

Thus the number of these treatments during pregnancy was limited. These drugs were used during the first or second gestational months, the exception was oxytocin.

Lynestrenol (Orgametril^{R}) showed a strong association with the risk of IHS because it was used more frequently by the mothers of cases than by the mothers of population controls. Lynestrenol is a derivative of progestins with strong progesterone effect for endometrium. Orgametril^{R} contains 5 mg, in general the daily dose was one tablet, its use was stopping after

the recognition of unplanned pregnancy in the second month, thus it was not used during the third and fourth months. Of 13 pregnant women, 12 had medically recorded Orgametril^{R} use during pregnancy; therefore this association was confirmed based on medically recoded data as well.

In addition oral tablets of ethynilestradiol (Mikrofollin^{R}, Richter containing 0.05 mg) and its combination with ethisterone (Limovan^{R}, Richter: ethynilestradiol 0.01 mg + esthiterone 10 mg) and oil injection Limovanil^{R} (Richter: oestradiol 2.5 mg + progesterone 12.5 mg) was used more frequently by the mother of cases than by the mothers of population controls. Limovanil^{R} contains large doses, frequently used for abortion induction. If we combined the number of these three drugs, the mothers of 19 cases (0.63%) and 43 population controls (0.17%) had these treatments and this rate was highly significantly higher in the groups of cases with IHS. Of 19 pregnant women with these treatments, 17 were medically recorded in the prenatal maternity logbook.

Among contraceptive pills, only ethynilestradiol (30/40/30 micrograms) + levonorgestrel (50/70/125 micrograms) (Tri-Regol^{R}, Richter) showed an association with the higher risk for IHS, but it was based on 4 cases and only 2 were medically recorded. In addition two other pills (Ovidon^{R} and Rigevidon^{R}) with the same components did not increase the risk of IHS.

Finally the total figure of these hormonal treatments was also significantly higher in the mothers of cases, than in the mothers of population controls.

Among maternal variables, the use of pregnancy supplements was also analyzed because it is a sensitive indicator of preconception/prenatal care and may have some effect for birth outcomes as well. In Hungary only one kind of folic acid tablets containing 3 mg was available during the study period. The daily dose of folic acid was between 3-9 mg, the estimated daily dose was 5.7 mg in the study. In general the onset of folic acid use was the first visit in prenatal care clinics and most pregnant women continued it until the end of pregnancy. The use of folic acid by 3,038 case mothers was the lower (48.5%) than in the mothers of 24,814 population male controls (13,509; OR with 95% CI: 0.79, 0.73-0.85). The use of folic acid-containing multivitamins was rare, but the lowest in the group of cases.

Only 203 mothers of cases were visited at home, 42 (20.7%) smoked cigarettes during the study pregnancy. The proportion of smokers was 19.0% and 22.2% in 800 population and 2,640 malformed control mothers, respectively. The combined rate of regular and hard drinkers together was 1.0% and 1.9% in population and malformed control mothers while 2 regular and 1 hard drinkers, together 3 (2.0%) were revealed during the study pregnancy in case mothers.

Discussion

The Interpretation of Data Connected with First Aim

This study confirmed the increasing recorded annual birth prevalence of cases with IHS in Hungary during the last 50 years. However, the Hungarian experiences show the importance the critical evaluation of reporting systems of cases with CA including IHS because the differences in the recorded annual IHS rates in the HCAR could be explained by the different attitudes of medical doctors who reported cases with IHS and the effort of the

staff in the HCAR within the study period and these differences associated with different completeness of ascertainment and reporting.

At the evaluation of ISH is worth differentiation birth prevalence of minor/mild IHS (including glandular and coronal ISH without necessary medical/surgical intervention) and severe IHS (including others types of ISH), however, we could not perform this analysis in the total of cases with IHS without the personal check-up of reported diagnoses. We were able to analyze different subgroups in 52% of our material. There was robust difference on the type distribution of IHS in the three Hungarian studies, because the examination technique was not the same (Table III).

The aim of this analysis was to answer the question whether the changing trend of recorded annual rates of IHS was caused by the more complete ascertainment/reporting of cases or it was true increase due to the contribution of changing maternal variables, new etiological factors, e.g., endocrine disruptors. Our recent analysis showed that the increasing rate of recorded IHS in the HCAR was not a real increase because can be explained partly by changing reporting activity, partly by higher reporting rate of mild coronal subgroup in the second part of 2000s.

The birth prevalence of hypospadias in boys shows extreme wide range, e.g., these rates of different CA-registries varied by a factors of about ten [Källen et al.,1986]. There are many explanations for this extremely wide range. First the complicated diagnostic criteria should be mentioned, e.g., the inclusion or exclusion of complex and/or syndromic hypospadias, in addition the inclusion or exclusion of mild forms of IHS. Secondly, different source of cases based on the reporting of cases by different doctors in the CA-registries or hospital-based data based on surgical intervention. Thus, the completeness of reporting depends on the attitude of different doctors, the activity of staff in the CA registries, and the underreporting is characteristic mainly for mild subgroups of IHS. Thirdly the age of examination is also important, e.g., in Denmark the hypospadias rate was 4.6% at the age of 3 years, whereas only 1% at birth [Boisen et al., 2005]. Many glandular forms of IHS can be detectable only when the physiological phimosis disappears, i.e., not at birth. In general patients receive surgery in their second year of life, but hospital discharge datasets include only surgically treated severe IHS. Obviously there are phenotypic/racial/population differences as well in HIS [Källen et al., 1986; Paulozzi et al., 1997]. Another problem is the frequent lack of controls for confounders, e.g., the occurrence of IHS depends on birth weight, because it is higher in newborns with low birthweight (less than 2500 g). Thus, unfortunately CA-registries are not appropriate for the estimation of true birth prevalence of ISH [Czeizel at al, 1993; Toppari et al., 2001].

The major message of this analysis is that if we detect a cluster or permanent increase in the recorded birth prevalence of certain CAs, it is necessary to check first the possible technical/methodological factors and we have to know the true birth prevalence of the given CA. Real changes mean significant increase or decrease compared to the true prevalence of CAs such as IHS as a gold standard.

We reported the Hungarian monthly distribution of births of cases with IHS [Czeizel et al., 1979; Czeizel and Tusnády, 1984], because previously Wehrung and Hay [1970] in the USA and Roberts, Llyod [1973] in South-Wales Jin et al., [2010] in southest China observed an interesting seasonality of the birth of cases with hypospadias. The sexual differentiation of the fetus is controlled by hormones and seasonal differences in daylight length are known to influence female hormones produced in ovary due to effects on the pineal gland [Kaupilla et

al., 1987]. The production of pituitary gonadotrophins depends on the duration of daylight and the shortest daylight are around December 21 in Hungary, while the observed higher rate of IHS births was between January and April.

A higher rate of twins in cases with IHS was found in the previous Hungarian study [Czeizel et al., 1979] but only a somewhat higher rate of twins was confirmed in this recent study.

The birth outcomes of cases with IHS in the HCCSCA were agreement with the results of our previous study [Czeizel et al., 1979; Czeizel sand Tusnády, 1984] and of some reports from other countries [e.g., Akre et al., 1999; Toppari et al., 2010]. The birth weight depends of gestational age, but the analysis of gestational age specific birth weights also showed smaller weight than in appropriate controls indicating intrauterine fetal growth restriction.

Interpretation of maternal socio-demographic data as the second aim of our project

We evaluated of socio-demographic variables of mothers whether associate with the risk of IHS, particularly with their changes during the study period. The mothers of cases with HIS Were somewhat elder than the mothers of controls, and advanced maternal age was found in Other studies [e.g., Angerpointer, 1984; Fisch et al., 2001] though other data sets resulted in controversial patterns [Källen et al., 1986]. Thus in general the maternal age does not seem to be an important confounder in the origin of ISH [Källen and Winberg, 1982]. However, our study showed a higher proportion of primiparous women among case mothers as first Chen and Woolley (1971) described and confirmed by others [Angerpointer, 1984, Källen et al., 1986]. The explanation may be the higher maternal level of free estradiol in the first compared to second pregnancy [Bernstein et al., 1986], though this association was not found in other studies [e.g., Roberts and Lloyd, 1973]. However, the more important maternal characteristic was the lower socio-economic status of case mothers with a lower use of folic acid in this study [Puho et al., 2005].

Interpretation of Maternal Risk Factors As the Third Aim Our Project

The most plausible explanation for IHS on the basis of family and twin studies is the multifactorial-threshold model [Czeizel et al., 1979; Driver at al., 1994; Fredell et al., 2002; Radpour et al., 2007; Van der Zanden et al., 2011, 2012] because the recurrence risk of IHS for brothers of patients is about 10% and the concordance rate of IHS in monozygotic twins in much higher than in dizygotic twins. Many candidate genes were indentified in the origin of hypospadias, the common variants in DGKK are strongly associated with the risk of hypospadias [Van Zanden et al., 2011]. Unfortunately patients with IHS and syndromic hypospadias were not differentiated in the previously mentioned descriptive epidemiological studies.

The main objective of the study was to evaluate the pregnancy complications, acute and chronic maternal diseases with related drug treatments and pregnancy supplement in the mothers of cases with IHS compared to the mothers of matched and population controls. Previously mainly progestins and endocrine disrupting chemicals with oestrogenic and anti-androgenic properties, including phytoestrogens were found as possible etiological factors in the origin of hypospadias.

A higher risk for preeclampsia and gestational diabetes while a lower risk for severe nausea and vomiting in pregnancy were found in the mothers of cases with IHS. Thus our findings were agreement with the results of some previous studies [Aberg et al., 2001; Porter et al., 2005; Carmichael et al., 2012]. Previous studies showed that the lack of nausea and

vomiting associated with a higher rate of early embryo loss [Weigel and Weigel, 1989], CAs [Czeizel et al., 2006] and preterm birth [Czeizel and Puho, 2004], i.e., this pregnancy complication seems to have a protective effect for fetal development [Weigel et al., 2006] due to a more favorable hormonal milieu due to the strong placenta. This recent study showed the association of severe nausea and vomiting in pregnancy with lower risk for IHS.

A higher risk for IHS was not found in the children of pregnant women with acute maternal diseases.

Among chronic maternal diseases, epilepsy occurred more frequently in the mothers of cases than in the mothers of population controls. However, this association could be explained by antiepileptic valproate, thus our study confirmed a higher risk of IHS after valproate treatment during pregnancy [Rodriguez-Pinilla et al., 2008; Jetnik et al., 2010;]. This IHS inducing effect of valproate may be connected with its gonadotropin-releasing hormone agonism. In general antiepileptic drugs induce characteristic CA-syndromes such as fetal hydantoin or valproate syndrome/effect [Czeizel and Bánhidy, 2010] but valproate may induce isolated CAs such as IHS as well.

A previous Hungarian study showed that cervical erosion associated with a higher risk IHS [Bánhidy et al., 2010] and it was confirmed in this study. This association cannot be explained by related drug treatments or by other factors but cervical erosion related higher estrogen level may contribute to the origin of IHS.

Our study confirmed the previously found association of oral nystatin use with higher risk of IHS [Czeizel et al., 2011b]. Nystatin is a polyene natural substance synthesized by bacteria (Streptomyces species) and defends against fungi due to the interaction with the element of the cell wall (ergosterol molecules) which leads to a reduction of permeability.

Our study provided further data for the debated teratogenic effect of progestins [Scially, 1988]. In the 1970s a higher risk for hypospadias was found in the sons of pregnant women after progestin treatments [e.g., Aarskog, 1979]. Previously Hungarian pregnant women were treated with allylestrenol very frequently (about 30%), and our first study showed a higher risk for IHS after their use in early pregnancy [Czeizel et al., 1979]. However, in a second study the infertility and threatened abortion of mothers were considered as confounders, and the previously found association disappeared [Czeizel and Huiskes, 1988]. If only medically recorded allylestrenol treatments were evaluated in this study, i.e., recall bias was excluded, there was no higher risk for IHS.

The use of hydroxyprogesterone during pregnancy in the mothers of cases with CA and their matched controls did not show significant association with the risk of IHS (OR with 95% CI: 1.2, 0.6-2.3) in a previous study [Dudás et al., 2006] and this finding was confirmed in this study.

However, this study detected the possible teratogenic effect of lynestrenol in the origin of IHS. The use of oral ethynilestradiol, oral Limovan^{R} and parenteral oil injection Limovanil^{R} also associated with a higher risk for IHS. Previously a hypothesis was generated that increased estrogen exposure for male fetuses during pregnancy may associate with a higher risk for CAs of male genital organs [Sharpe, 2003]. This hypothesis was supported by experimental investigations, e.g., the supraphysiological doses of synthetic estrogen during pregnancy induced hypospadias in 50% of the male fetuses [Kim et al., 2004]. Our study emphasizes the importance of the dose-effect phenomenon [Czeizel, 2009], because large doses of ethynilestradiol associated with a higher risk of IHS, while contraceptive pills containing low doses of estrogen-derivatives, did not. On the other hand it is necessary to

stress that the results of available human studies did not provide solid evidence that endocrine disruptors are risk factors for IHS [Martin et al., 2008; Van Zanden et al., 2012].

Our study suggested that high doses of folic acid alone might be associated with a lower risk for IHS. Unfortunately documentation of folic acid supplementation in the prenatal maternity logbook was often incomplete. Obstetricians are obliged to record all maternal diseases and related drug treatment in the prenatal maternity logbooks, but several obstetricians did not record pregnancy supplements in the prenatal maternity logbooks**.** However, prospectively and medically recorded folic acid use in the prenatal maternity logbook was associated with a significant reduction of IHS both in general and particularly between the second and fourth gestational month (i.e., in the critical period of IHS) in the mothers of cases compared to the mothers of controls. In addition, the lowest use of folic acid was found in the mothers of cases with severe IHS suggesting a dose-response relationship.

Among cases with incomplete morphogenesis, a possible schisis or midline CA-association was delineated [Czeizel, 1981, Bower and Stanley, 1991]. This association included the failure of (1) closing of the neural-tube (such as anencephaly and spina bifida aperta), (2) of closure of the lip (cleft lip), (3) of fusion of the maxillary palatal shelves (cleft palate), (4) of closure of the pleuroperitoneal canal (diaphragm defect), and their association was not random. Hypospadias is a developmental arrest of urethral fusion, i.e., failure of closing the urethral groove, thus the urethral opening is displaced along the ventral side of the penis. Thus hypospadias may have some similarity with schisis-type CAs.

The Hungarian randomized controlled trial showed the preventive effect of a folic acid (0.8 mg) containing multivitamin during the periconceptional period for neural-tube defects [Czeizel and Dudás, 1992], in addition for congenital heart defects and CAs of urinary tract [Czeizel, 1996] but not for IHS. Similar findings were observed in another Hungarian intervention trial [Czeizel et al., 2004a]. Thus the results of this study were in agreement with the previous intervention trials because multivitamins containing low doses of folic acid did not show any preventive effect for IHS.

Previous analysis of the HCCSCA data did not demonstrate the preventive effect of high dose folic acid for IHS, although the reduction was near to the level of significance [Czeizel et al., 1996]. We are aware of only one previous study with risk reduction for IH after folic acid supplementation [Ormond at al., 2009]; however, other studies have not seen a preventive effect of folic acid for IH [Van der Zanden et al., 2012]. In this study we evaluated high doses of folic acid, whereas previous studies have evaluated the effects of low doses.

Our study showed an association of high doses of folic acid with the reduction of IHS.

There was only one kind of folic acid tablets in Hungary during the study period, which contained 3 mg. The general recommendation was 1 tablet per day for pregnant women from 1978, but several obstetricians suggested 2 or 3 tablets. The results of MRC Vitamin Study [MRC Vitamin Research Group, 1991) showed the efficacy of 4 mg folic acid in the reduction of recurrent neural-tube defects. This publication was well-known in Hungary because nearly half of the participants in the MRC Vitamin Study were Hungarian, thus 2 tablets (i.e., 6 mg) became a widely used practice. Unfortunately Hungarian obstetricians did not know or accept the international recommendation of 0.4 mg folic acid for healthy pregnant women during the 1990s.

Previously many studies showed an association between drinking habit of pregnant women and characteristic pattern (fetal alcohol syndrome/effect) of CAs in their children (Jones et al., 1973, Vitéz et al., 1984). Our study did not show an association of maternal

regular and/or hard drinking during pregnancy with a risk of ISH in their son, though it was based only on a subsample of our material. The rate of smokers among case mothers during the study pregnancy was also not higher in the mothers of cases with IHS, thus our data do not support the previously described weak association between hypospadias and maternal smoking (OR with 95% CI: 1.19, 1.06-1.33) (Akre et al., 1999).

The strengths of our study are connected with the large population-based data set of the HCAR including 11,498 cases with IHS between 1962 and 2011 and the HCCSCA including 3,038 cases with IHS and their 4,981 matched controls, in addition 24,814 population male controls without CAs between 1990 and 1996 in the ethnically homogeneous Hungarian (Caucasian) population. The validity of IHS diagnoses was good because cases were reported by medical doctors in the HCAR, about one-third of cases were physically examined by the coworkers of the HCAR and the diagnosis was confirmed 3.5 $\pm$ 2.1 months later based on recent medical documents in the HCCSCA. This study included all pregnancy complications, acute and frequent chronic maternal diseases and related drug treatments, in addition pregnancy supplements particularly folic acid and these exposure data were based on multiple sources including prenatal maternity logbooks which provided prospective medically recorded data. The exposure time and potential confounders were known. Birth outcomes were also medical recorded and potential confounders were known.

However, there were some weaknesses of our study. Acute maternal diseases, drug treatments and pregnancy supplementations were based partly on retrospective maternal information burden by recall bias [Rockenbauer et al., 2001]. However, we accept these associations if were confirmed by prospective medically recorded data in the critical period of IHS and by the comparison of matched and population controls. Unfortunately our data were not appropriate for the evaluation of environmental disruptive chemicals due to the limited number of these exposures. The subgroups of IHS were known only in 52% of cases and we were not able to check these subgroups in two-third of cases by physical examination. In addition lifestyle factors, such smoking habit and alcohol drinking were known only in a subsample of mothers visited at home. However, these data were collected through a cross interview of mothers and their close family members, excluding the very unreliable maternal self-reported information [Czeizel et al., 2004b]. Another weakness of our study is that cases born between 1980 and 1996 were evaluated, thus the results of recent medical progress (e.g., IVF) could not be analyzed.

Conclusion

I. The critical evaluation of cases with IHS recorded in the HCAR during the last 50 years did not indicate a significant increase in the real birth prevalence of IHS in Hungary. The male cases with ISH had a higher proportion of first born, the higher rate of preterm birth and particularly low birthweight, i.e., intrauterine growth restriction of fetuses. The mild seasonality of birth with IHS, the somewhat higher rate of twins, and the higher rate of low birthweight due to intrauterine growth restriction of fetuses indicates the role of placental dysfunction in the origin of IHS. However these factors did not show drastic effect for the trend of birth prevalence of

IHS. Consequently, IHS is not a sign of testicular dysgenesis syndrome, as previously supposed [Aschim et al., 2004], at least in Hungary.

II. The changing maternal socio-demographic variables did not associate significantly with the birth prevalence of IHS.

III. There was a higher incidence of gestational diabetes and preeclampsia in the mothers of cases. Preeclampsia with the mild seasonality of birth with IHS, the somewhat higher rate of twins, and the higher rate of low birthweight due to intrauterine growth restriction of fetuses indicates the role of placental dysfunction in the origin of IHS. However these factors did not show drastic effect for the trend of birth prevalence of IHS. The rate of severe nausea and vomiting in pregnancy was lower in the mothers of cases with IHS. Our findings also suggested that maternal cervical erosion, oral treatment of valproate, nystatin, linestrenol and ethynilestradiol during the critical period of IHS associated with a higher risk for IHS. Our findings suggested an association of high doses of folic acid with the risk reduction of IHS. This analysis was based on observational data, therefore needs confirmation.

Undescended Testis

Among structural birth defects, i.e., congenital abnormalities (CAs), undescended testis (cryptorchidism) is an important but debated CA.

Undescended testis is the consequence of maldescended testes [Scorer, 1955; 1962; 1964; Scorer and Farrington, 1971] and this group of testis maldescent includes

i. anorchia and monorchia (the so-called vanishing one or both testes);
ii. ectopic testis (testis is to be found in the perineum at the base of the penis or elsewhere: interstitial, femoral, gluteal, transversal, pubic position, i.e., outside its normal pathway of movement);
iii. obstructed (or superficial inguinal ectopia);
iv. dystopic testis (abdominal, canalicular, emergent, high scrotal).

However, congenital inguinal hernia (because patent processus vaginalis is present in 95% of cases, and about 25% are associated with hernia), hydrocele and structural abnormalities of the testicular appendage (i.e., epididymis being lengthened and the gubernaculum often abnormally attached) may be associated with testis maldescent, therefore it may be part of undescended testis.

Introduction

Hospital-based reports showed an increasing trend in the occurrence of undescended testis in England and Wales [Chilvers et al., 1984; John Radcliffe Hospital Study Group, 1986] confirmed by the national statistics in England and Wales, 1964-1983 [Matlai and Beral, 1985], in addition by an *ad hoc* study in Lithuania [Preiksa et al., 2005].

The epidemiological variables of 689 cases with isolated undescended testis (IUT) recorded in the HCAR between 1970 and 1976 were evaluated [Czeizel et al., 1981a; Czeizel, 1993], and an obvious underreporting of cases with IUT was found.

The first aim of our study was the critical evaluation of cases with recorded undescended testis in the dataset of the HCAR between 1962 and 2011, i.e., 50 years.

Our previous epidemiological study based on 689 cases with IUT recorded in the HCAR between 1970 and 1976 [Czeizel et al., 1981a, Czeizel and Tusnády, 1984] suggested that undescended testis diagnosed at birth showed a high rate of preterm birth and twins with characteristic monthly distribution of their birth. Prematurity and/or intrauterine growth restriction associates frequently with IUT in boys [Berkowitz et al., 1993; Virtanen and Toppari, 2008; Acerini et al., 2009] and the major part of IUT diagnosed at birth has a postnatal descensus [Scorer, 1955; 1962; Hadziselimovic, 1983; Hutson et al., 1997]. Thus a hypothesis was generated that boys with undescended testis diagnosed at the third postnatal month differ – beyond the lack of postnatal testis descensus – in their birth outcomes and maternal variables from boys with undescended testis diagnosed at birth.

Thus the second aim of this study was to confirm or to reject this hypothesis generated in 1981 by the examination of maternal socio-demographic effect in cases with IUT diagnosed at birth and true IUT (ITUT) diagnosed at the third postnatal month with low birthweight and birth weight over 2,500 gram in the data set of the population-based Hungarian Case-Control Surveillance of Congenital Abnormalities, 1980-1996 [Czeizel et al., 2001].

The etiology of isolated undescended testis is not known appropriately [Virtanen and Toppari, 2008]. Thus the third aim of our project was to evaluate the maternal risk factors in the origin of ITUT also in the population-based Hungarian Case-Control Surveillance of Congenital Abnormalities (HCCSCA), 1980-1996 [Czeizel et al., 2001].

Results

I. The first aim: the critical evaluation of cases with IUT in the HCAR, 1962-2011.
At the evaluation of IUT, there are three exclusion criteria:

I. CA-syndromes including undescended testis due to major mutant genes (e.g., Aarskog's or Noonan's syndromes) or chromosomal aberrations (e.g., trisomy 13 or 18) or unidentified multiple CAs with the component undescended testes were excluded from the analysis.
II. The so-called GAM (genital anomalies of males) complex including undescended testes and hypospadias with or without congenital inguinal hernia and other CAs of the genital organs [Czeizel, 1987] was also excluded.
III. Boys with retractile testes due to hyperactive cremaster reflex were excluded from this study.

Thus only cases with well-defined diagnoses of IUT at birth were evaluated in the HCAR. However, the differential diagnosis among different manifestation of testis maldescent was difficult because the reported diagnoses in general did not mention the types of IUT, therefore these types were evaluated together in this study.

The annual numbers of live-births in Hungary and recorded annual numbers and live-birth prevalence of cases with CA, and among them IUT in the data set of HCAR, 1962-2011, are shown in Table II. As we summarized in the chapter of hypospadias, four stages can be separated in the live-birth prevalence of total CAs between 1962 and 2011.

All cases with IUT were diagnosed in live-born infants. The recorded annual live-birth prevalence rates of IUT in the HCAR are also shown in Figure 2. There was also an increase after 1970 and reached a plateau between 1980 and 1995 with a maximum of 2.22 per 1000 in 1983 followed by a decrease with the minimum 1.01 per 1000 in 1996 and a recent increase in the 2000s with a new maximum of 2.32 per 1000 in 2005.

However, the critical evaluation of recorded rate of cases with IUT needed the so-called reference rate of cases with IUT as reference. In a previous clinical-epidemiological Hungarian study, 10,203 newborn infants - among them 5,211 boys - born in the first half of 1973 in 7 leading Hungarian obstetric departments were examined from the first day of life until the 6th postnatal day by obstetricians and neonatologists to diagnose IUT on the previously defined diagnostic criteria [Pazonyi et al., 1975; Czeizel and Tusnády, 1984]. The live-birth prevalence of cases with IUT was 6.86 $\pm$ 0.62 per 1000 live-births (13.43 per 1000 boys).

Thus the recorded birth prevalence of cases with IUT in the HCAR did not indicate a real increase during the study period. The recorded maximum figures of IUT were also much lower than the expected reference rate (6.86 per 1000) based on the so-called active search of newborn infants with IUT in our previous clinical-epidemiological study.

II. The second aim: the classification of true IUT in the HCCSCA, 1980-1996.

Cases with undescended testis were reported by medical doctors to the HCAR, but HCCSCA provided an opportunity to check this diagnosis 3.5 $\pm$ 2.1 months later on the basis of recent available medical documents. If boys with IUT diagnosed at birth had postnatal descensus, they were classified as birth IUT. If IUT was confirmed at the third postnatal month, these cases were reclassified as true IUT (ITUT). If this diagnosis was not clear, we had a correspondence with mothers to clarify the status of their children. However, (i) if these cases were not found, (ii) the ITUT diagnosis was not confirmed, or (iii) mothers refused to collaborate, they were excluded from the study.

There were 4,685 live-born cases with IUT diagnosed at birth in the HCAR in 1980-1996; however, we focused our analysis for 2,052 cases with ITUT diagnosed at the third postnatal month in the dataset of the HCCSCA. The difference (i.e., 2,633 cases) was explained mainly by the late descensus of testes in 2,402 cases (91.2%) after birth, particularly in the first postnatal month. In addition, 91 cases were reported after the third postnatal month, 111 case mothers did not respond and/or had unknown new addresses, the diagnosis was not correct in 18 cases and 11 families refused to collaborate. Thus the proportion of late descensus was 53.9% in cases with known data (2,402/4,454).

There were 2,138,151 live-births in Hungary between 1980 and 1996, thus 38,151 population controls represented 1.8% of all Hungarian newborn infants, but only 24,814 male newborns were evaluated in the study. The group of 2,052 cases with ITUT had 3,252 matched controls. However, the variables of matched controls and population controls did not show significant differences, therefore only the data of population controls are shown in this chapter as controls.

Of 2,052 cases with ITUT, 1,816 (88.5%) were unilateral, while the rest: 236 had bilateral manifestations. Of 1,816 unilateral ITUT, 1,090 (60.0%) occurred on the right side due to chronological differences in the descent of testes.

The birth outcomes of cases and controls based on medical records are shown in Table X. Of 2,052 cases with ITUT, 30 (1.5%) were twins, this rate was somewhat but not significantly higher than the twin rate of controls. The mean gestational age was 0.4 wk shorter in the mothers of cases than in the mothers of controls, while the mean birth weight of cases was smaller by 212 grams compared to controls. The rate of preterm births and low birthweight was 1.5 and 2.5 fold higher in the group of cases than in the group of controls, respectively. The proportion of large birthweight (4500 g or more) was similar in cases with ITUT and controls (16; 0.8% vs. 188; 0.8%). Of 257 cases with ITUT and low birthweight, 124 (48.2%) were born after 37th gestational week, i.e., they were not preterm. Of 1,239 controls with low birthweight, 469 (37.8%) belonged to this subgroup. Thus, if we calculate boys with small-for-gestational age, this rate is 6.04% in cases with ITUT and 1.89% in controls, the difference is 3.2 fold. Thus the well-known higher rate of preterm births and low birthweight was confirmed in cases with ITUT, but our data indicate particularly intrauterine growth restriction of these fetuses.

Table X. Live-birth outcomes of male cases with isolated true undescended testis (ITUT) and to male controls without any defect

Live-birth outcomes	Male cases (N = 2,052)		Male controls (N = 24,814)	
Quantitative	Mean	S.D	Mean	S.D. t = p =
Gestational age (wk)*	39.0	2.4	39.4	2.0 8.56 <0.001
Birth weight (g)**	3,111	591	3,323	514 17.7 <0.001
Categorical	No.	%	No.	% OR 95% CI
Twins (triplets did not occur)	30	1.5	263	1.1 1.39 0.95-2.03
Preterm birth (less than 37 completed weeks)*	256	2.5	2,073	8.4 1.56 1.36-1.80
Low birthweight (less than 2,500 g)**	257	2.5	1,239	5.0 2.72 2.36-3.14

*Adjusted for age, parity (birth order) and employment status of mothers.

** Adjusted for the age, parity (birth order), employment status of mothers and gestational age of newborn babies.

Bilateral ITUT is more severe manifestation of this CA than unilateral. Of 1,816 unilateral cases, 212 (11.7%) were preterm birth and 194 (10.7%) had low birthweight, while of 236 bilateral cases, 44 (18.6%) were preterm birth and 63 (26.7%) had low birthweight.

The monthly rate of cases with ITUT was calculated on the basis of their monthly number expressed in all monthly live-births, and there was no seasonality in the distribution of monthly rates. (These data are not shown here.)

The maternal socio-demographic variables are shown in Table XI. Uni- and bilateral cases with ITUT did not show significant differences; therefore these maternal data were combined. The data of control mothers were considered as reference. The distribution of age groups in the mothers of cases and controls showed significant difference because of the larger proportion of young (less than 20 yr: 8.6% vs. 10.2%) and older (over 30 yr: 21.5% vs. 18.9%) case mothers. The mean maternal age of case mothers was somewhat higher but this value was only near to the level of significance due to the larger proportion of two ends of maternal age groups. The mean birth order (parity) was higher in the mothers of cases than in

the mothers of controls because of the larger proportion of 3 or more previous births. The interval between mean birth order and pregnancy order (birth + miscarriages) was similar in case and control mothers (0.2), and it is against a higher rate of fetal deaths in previous pregnancies.

Table XI. Main socio-demographic variables of mothers of controls as reference and of cases with ITUT, in addition to mothers of cases born with low (less than 2500 g) and other (2,500 or more g) birth weight

Variables	Mothers of controls (N = 24,814)		Mothers of cases (N = 2,052)			Mothers of cases with low birth weight (N = 257)		Mothers of cases with other birth weight: 2,500 g or more (N = 1,795)		
Quantitative	No.	%	No.	%	Comparison	No.	%	No.	%	Comparison
Maternal age (y)										
-19	2,141	8.6	220	10.7		29	11.3	191	10.6	
20-29	17,991	72.5	1,392	67.8	$x^2 = 26.7$ p<0.0001	158	61.5	1,233	68.7	$x^2 = 26.2$ p<0.0001
30-39	4,482	18.1	413	20.1		58	22.6	355	19.8	
40-	200	0.8	28	1.4		12	4.7	16	0.9	
Mean ± S.D.	25.4	4.9	25.6	5.3	t = 1.8 p = 0.0775	26.3	6.2	25.5	5.1	t = 2.3 p = 0.0224
Birth order										
1	11,880	47.9	938	45.7	$x^2 = 76.7$ p < 0.0001	108	42.0	830	46.2	$x^2 = 29.6$ p < 0.0001
2	9,318	37.6	670	32.7		61	23.7	609	33.9	
3 or more	3,616	14.6	444	21.6		88	34.2	356	19.8	
Mean ± S.D.	1.7	0.9	1.9	1.2	t=9.4 p<0.0001	2.3	1.6	1.9	1.1	t=5.1 p<0.0001
Pregnancy order										
1	10,645	42.9	838	40.8	x^2=49.6 p<0.0001	90	35.0	748	41.7	x^2=30.3 p<0.0001
2	8,806	35.5	636	31.0		58	22.6	578	32.2	
3 or more	5,363	21.6	578	28.2		109	42.4	469	26.1	
Mean ± S.D.	1.9	1.1	2.1	1.4	t=7.7 p<0.0001	2.6	1.8	2.1	1.3	t=5.5 p<0.0001
Categorical No. % No. % No. % No. %										
Unmarried	944	3.8	127	6.2	x^2=28.2 p<0.0001	21	8.2	106	5.9	x^2=2.0 p=0.1585
Employment status										
Professional	2,902	11.7	158	7.7		14	5.4	144	8.0	
Managerial	6,736	27.1	448	21.8		43	16.7	405	22.6	
Skilled worker	7,701	31.0	545	26.6		54	21.0	491	27.4	
Semiskilled worker	3,943	15.9	408	19.9	x^2=209.4 p<0.0001	49	19.1	359	20.0	x^2=32.4 p<0.0001
Unskilled worker	1,455	5.9	186	9.1		35	13.6	151	8.4	
Housewife	1,515	6.1	206	10.0		43	16.7	163	9.1	
Others	562	2.3	101	4.9		19	7.4	82	4.6	

The rate of unmarried mothers was higher in the group of cases than in the group of controls. The proportion of professional and managerial mothers was lower (29.5%) in the mothers of cases than in the mothers of controls (38.8%), while the proportion of semi- and unskilled workers and housewives was larger in case mothers (39.0%) than in control mothers (27.9%). Most Hungarian housewives belong to the poor women. Thus the distribution of maternal employments showed the lower socioeconomic status of case mothers.

We differentiated the socio-demographic status of mothers in the subgroup of 257 cases with ITUT with birth weight less than 2,500 g (i.e., low birthweight) and in the subgroup of 1,795 cases with birth weight more than 2,500 g (Table XI). The mean maternal age was higher by 0.8 yr in the mothers of cases with low birthweight due to the larger proportion of older (particularly over 40 yr) women and it associated with a higher mean birth order due to 1.75 fold larger proportions of women with 3 or more previous births. The proportion of professional and managerial (43.2% vs. 57.9%) and semi- and unskilled workers housewives (37.7% vs. 22.1%) showed robust difference, thus the low socio-economic status is much more important in cases with low birthweight.

III. The third aim: maternal risk in the origin of ITUT in the HCCSCA, 1980-1996

The dataset of the HCCSCA, 1980-1996, included 2,052 live-born male cases with ITUT, among them 266 cases (13.0%) were physical examined by us. The number of male controls was 24,814 and this population control group represented 2.4% of all Hungarian male births between 1980 and 1996.

The incidence of acute maternal disease groups (influenza, common cold, urogenital infections, etc) was not more frequent either during the study pregnancy or in the critical period of ITUT in the mothers of cases than in the mothers of population controls.

Table XII. The rarely used fertility related hormonal products during the study pregnancy in the study groups

Medicinal products	Case mothers (N = 2,052)		Population control mothers (N = 24,814)				
	No.	%	No.	%	OR	95% CI	
Oestradiol (Akrofollin^{R})	2	0.09	15	0.06	1.61	0.37	7.06
Ethinylestradiol + ethisterone (Limovan^{R})	1	0.04	21	0.08	0.58	0.08	4.28
Diethylstilbestrol (Syntestrin^{R})	1	0.04	7	0.02	1.73	0.21	14.05
Lynestrenol (Orgametril^{R})	5	0.24	3	0.01	**20.20**	**4.82**	**84.59**
Norethisterone (Norcolut^{R})	1	0.04	35	0.14	0.35	0.05	2.52
Progesterone (Glanducorpin^{R})	2	0.09	13	0.05	1.86	0.42	8.25
Dihydrogesterone (Duphaston^{R})	5	0.24	22	0.08	**2.75**	**1.04**	**7.28**
Chorionic gonadotrophin (Chorion^{R})	3	0.14	73	0.29	0.50	0.16	1.58
Clomifene (Clostilbegyt^{R})	3	0.14	69	0.27	0.53	0.17	1.67
Ethinylestradiol + ethynodiol diacetate (Bisecurin^{R})*	2	0.09	11	0.04	2.20	0.49	9.93
Ethinylestradiol + levonorgestrel (Rigevidon^{R})*	2	0.09	6	0.02	4.03	0.81	20.00
Ethinylestradiol + levonorgestrel (Ovidon^{R})*	2	0.09	15	0.06	1.61	0.37	7.06
Ethinylestradiol + levonorgestrel (Anteovin^{R})*	2	0.09	13	0.05	1.86	0.42	8.25
Ethynodial diacetate (Continuin^{R})*	3	0.14	26	0.10	1.40	0.42	4.62
Contraceptive pills together	11	0.53	71	0.28	1.88	0.99	3.55
Total	34	1.65	329	1.32	1.25	0.88	1.79

** Bold numbers show significant associations.

The evaluation of chronic maternal diseases was based on the prospective and medically recorded data in the prenatal maternity logbook. Endometriosis in the mothers associated with a higher risk for ITUT in their children compared to the mothers of population controls (39 and 1.9% vs. 197 and 0.8%, OR with 95% CI: 2.42, 1.71-3.42). Endometriosis was diagnosed before the conception of the study pregnancy and associated with infertility, thus needed treatment. Of 39 case mothers, 21 were treated with oral danazol, 6 by parenteral medroxyprogesterone and 3 contraceptive pills and 10 had surgical excision.

In general the treatment was stopped before conception, but 3 women ended danazol and one medroxyprogesterone treatment after the diagnosis of pregnancy in the second gestational month.

The rarely used fertility-related drugs were also evaluated separately (Table XII). Lynestrenol and dihydrogesterone associated with a higher risk for ITUT at the comparison of mothers of cases and population controls. Of 5 lynestrenol treatment, 3 were medically recorded, and if only these pregnant women were evaluated, this association disappeared. However, all dihydrogesterone treatments were medically recorded in the prenatal maternity logbook during the first half of pregnancy.

Discussion

First Aim

The distribution of recorded live-birth prevalence rates of IUT in Hungary showed two waves with the peak in 1983 and 2005, respectively. The increase of recorded birth prevalence of cases with IUT between 1972 and 1984, in addition between 2004 and 2011 was impressive though there was a drastic drop in the rate of cases with IUT between 1987 and 2003. The question is whether these two increases were real ones or were connected with the change of reporting of cases with IUT.

The reply for the above question is based on the reference rate of cases with IUT. Thus the increasing trends in the recorded birth prevalence of cases with IUT in some years of the study period can be explained by the more complete reporting of cases with IUT. The completeness of reporting of mild CAs such as IUT depends on the diagnostic criteria of IUT, the time of diagnosis, the activity and expertise of medical doctors who examine newborn boys and the co-workers of CA registries. Consequently, IUT is not a sign of testicular dysgenesis syndrome, as previously supposed [Toppari et al., 2010], at least in Hungary.

The very wide range of birth prevalence of reported undescended testis in different countries (0.4-4.2 per 1000) [ICBDMS, 1991; Toppari et al., 2001] indicates that CA-registries are not appropriate for the estimation of real birth prevalence of IUT due to different diagnostic criteria and examination techniques. The data of this analysis confirmed this conclusion. In addition, the reporting of this mild CA (though later frequently associated with infertility and testicular cancer) is poor into CA-registries, thus, Finland, for example, stopped the registration of IUT [Toppari et al., 2001]. Another important point of evaluation of IUT is the age of boys because the major part of IUT have late testicular descensus thus the rate of IUT is much lower at the third postnatal month than at birth and much higher in low birthweight (less than 2500 g) newborn infants because the testis descent occurs between 1,300 and 2,700 g in fetal development [Scorer, 1955; 1962; 1964; Scorer and Farrington, 1971; Mavrogenis et al., 2014c].

Thus the measurement of IUT rates needs special clinical-epidemiological studies with well-defined age and diagnostic criteria. The Hungarian live-birth prevalence of boys with IUT (6.9%) is in the upper region of rates found in other countries (between 2% and 9% in boys) [Boisen et al., 2004; Berkowitz et al., 1993; Virtanen and Toppari, 2008]. However, the significant differences are explained by population and/or geographical differences as well, e.g., it was fourfold higher in Denmark than that in Finland though the study was performed

in close collaboration with the same methodology in these two Nordic countries [Boisen et al., 2004].

The Second Aim

We decided to test our previous hypothesis, namely that there are two different nosological entities of IUT diagnosed at birth and at the third postnatal month. Previously boys with undescended testis diagnosed at birth were evaluated [Czeizel et al., 1981a, Czeizel and Tusnády, 1984], while cases with ITUT were analyzed in this study, thus we can compare their variables here.

Our findings showed a robust effect of maternal age, birth order and employment as indicator of socioeconomic status in the origin of cases with ITUT, i.e., diagnosed at the third postnatal month, if they were low birthweight newborns.

The rate of postnatal descensus in boys with IUT was 54% in this study, while this figure was 75% in two Nordic countries [Barthold et al., 2012]. A higher rate of twins (2.9%) was found among live-born cases with IUT in the previous Hungarian study [Czeizel et al., 1981a, Czeizel and Tusnády, 1984] but the rate of twins (1.5%) was not significantly higher in cases with ITUT in this recent study. Thus twins with IUT at birth have a large proportion of postnatal testicular descensus.

The rate of low birthweight in cases with ITUT (12.5%) in the HCCSCA was in agreement with this rate in cases with IUT at birth (12.2%) in our previous study [Czeizel et al., 1981a, Czeizel and Tusnády, 1984]. Thus, cases with IUT/ITUT and low birthweigh have limited chance for postnatal descensus. However, the rate of preterm births (17.7%) was higher in cases with IUT at birth [Czeizel et al., 1981a, Czeizel and Tusnády, 1984] than in cases with ITUT (12.5%) in this study, indicating a larger proportion of spontaneous descensus in preterm babies.

The mothers of cases with ITUT were somewhat older with higher rate of mean birth order. The older maternal age was found in other studies as well [Jones et al., 1998; Toppari et al., 2010]. However, the more important maternal characteristic was their lower socio-economic status in agreement with the results of our [Czeizel et al., 1981a, Czeizel and Tusnády, 1984] and other previous studies [Berkowitz et al., 1995; Akre et al., 1999; Jones et al., 1998; Weidner et al., 1999].

The main findings of this study are connected with the obvious difference of cases with ITUT born with low and other birth weight. Cases with ITUT born with lower birth weight had older mothers with higher birth order. In addition their higher pregnancy order suggests more frequent miscarriages in their previous pregnancies. Finally lower socio-economic status was particularly robust in cases with ITUT who were born with low birthweight. Thus cases with ITUT with low and other birth weight seemed to be different from epidemiological aspect.

Previous studies showed - beyond the higher rate of preterm birth - the higher rate of low birthweight aa well in patients with undescended testis [Berkowitz et al., 1993; Virtanen and Toppari, 2008; Acerini et al., 2009; Boisen et al., 2004 John Radcliffe Hospital Cryptorchidism Study Group, 1992; Berkowitz et al., 1995; Akre et al., 1999; Jones et al., 1998; Weidner et al., 1999]. However, low birthweight naturally associated with preterm birth, therefore later gestational age-adjusted low birth weight was calculated, and it appeared that SGA (small-for-gestational-age) is most strongly and consistently associated with ITUT

as gestational age and this finding indicates the importance of intrauterine growth restriction [Berkowitz et al., 1995; Akre et al., 1999; Jones et al., 1998; Weidner et al., 1999].

We attempted to focus our previous hypothesis regarding two nosological entities of IUT by the following arguments:

1. Lack of testis descensus is the main characteristic of ITUT diagnosed at third month. Obviously undescended testis is more frequent in preterm births because testis descensus occurs in fetuses with weight between 1,300 and 2,700 g [Scorer, 1955; 1962; Hadziselimovic, 1983; Hutson et al., 1997], undescended testis therefore is not a pathological phenomenon in boys born in gestation weeks 23-35. Thus preterm cases with undescended testis at birth had frequent postnatal descensus as the continuation of physiological descensus of testis, while it does not occur in cases with ITUT.
2. In addition a higher rate of twins (2.9%) was found among live-born cases with IUT diagnosed at birth in the previous Hungarian study [Czeizel et al., 1981a, Czeizel and Tusnády, 1984] but the rate of twins (1.5%) was not significantly higher in cases with ITUT in this recent study. In general twins are preterm babies, their higher rate of postnatal descensus is also an argument for our hypothesis.
3. High rate of low birthweight is characteristic for ITUT diagnosed at third month. The rate of low birthweight in cases with ITUT was 12.5% in this study and this rate was in agreement with the rate in cases with IUT at birth (12.2%) in our previous study [Czeizel et al., 1981a, Czeizel and Tusnády, 1984]. Thus, cases with undescended testis and low birthweight have a lower chance for postnatal descensus. The birth weight depends on gestational age, but the rate of gestational age specific low birth weights was 3.2 fold higher in cases with ITUT than in controls in this study indicating intrauterine growth restriction. This phenomenon was also shown previously, e.g., Akre et al., [1999] found a greater-than-additive effect of undescended testis in boys born with SGA. Thus intrauterine growth is characteristic for ITUT.
4. There was no seasonality in the birth of boys with ITUT in this study though the seasonality in birth of boys with IUT diagnosed at birth was found in our previous study [Czeizel et al., 1981a, Czeizel and Tusnády, 1984].
5. ITUT associated with characteristic maternal variables such as older maternal age with higher birth order. The older maternal age was found in other studies as well [Toppari et al., 2010; Swerdlow et al., 1983]. However, the more important maternal characteristic was their lower socio-economic status. The data of other previous studies were in agreement with our finding but IUT diagnosed at birth and ITUT diagnosed at the third months were not differentiated in these studies [Jones et al., 1998; Hjertkvist et al., 1989; Swerdlow et al., 1983; Moller et al., 1996]. These maternal factors contribute to the origin of intrauterine growth restriction in cases with ITUT, because pregnant women with lower socio-economic status associate with advanced maternal age and higher number of children in Hungary [Czeizel et al., 2007]. Other findings of this study showed difference of cases with ITUT born with low and other birth weight.

However, these socio-demographic factors cannot explain the origin of ITUT as a whole. Recent report showed association of some gene polymorphisms with fetal growth and birth weight [Freathy et al., 2010], thus we may suppose a common origin of fetal growth restriction and ITUT.

The main message of our studies is that IUT diagnosed at birth and IZUT diagnosed at third postnatal month would be worth differentiating among boys. Our hope is that this observation may help clinicians to find more appropriate medical treatment for these patients.

Third Aim

The most plausible explanation for IUT on the basis of family [Czeizel et al., 1981b; Schnack et al., 2008], twin [Jensen et al., 2010] and molecular genetic [Radpour et al., 2007] studies is the multifactorial-threshold model [Czeizel and Tusnády, 1984], while most syndromic undescended testes are caused by mutant major genes, chromosomal aberrations and teratogens [Czeizel et al., 1988]. However, the role of triggering environmental factors in the origin of ITUT is not known appropriately, therefore the maternal risk factors in the origin of ITUT were analyzed in the population-based HCCSCA, 1980-1996 (Czeizel et al., 2001).

Cases with ITUT were selected from cases with previously reported IUT at birth in the HCAR without spontaneous descent of testis until the third postnatal month due to the available medical documents in the HCCSCA. Each CA has a critical period when its development happens therefore this period is the most sensitive and vulnerable for environmental agents. The critical period of ITUT is estimated between 26-40 gestation weeks calculated from the first day of the last menstrual period, i.e., between the sixth and ninth gestation month (Czeizel, 2008; Czeizel et al., 2008).

The possible association of maternal diseases during pregnancy with the risk of undescended testis in their sons was studied rarely and ITUT was not differentiated within this CA-group [McBride et al., 1991; Berkowitz et al., 1995; Jones et al., 1998; Akre et al., 1999; Weidner et al., 1999; Virtanen and Toppari, 2008].

Among pregnancy complications, only threatened preterm delivery showed an association with the higher risk for ITUT and this finding is in agreement with the higher rate of preterm birth of boys affected with IUT and ITUT.

Acute infectious diseases did not associate with a higher risk for ITUT in our study.

Among chronic diseases, the possible association of maternal endometriosis with the higher risk for ITUT was observed. Endometriosis is an estrogen-dependent inflammatory disease in women of reproductive age, with an estimated prevalence of approximately 5 to 10% [Bulun, 2009]. This prevalence was between 0.8-1.9% in our study but recorded during the study pregnancy in the maternal prenatal logbook. The direct cause of endometriosis is the transplantation of endometrial tissue from the uterus to ectopic location inducing infertility [Wheeler, 1989]. Our case mothers had symptomatic endometriosis with related treatment before the conception of the study pregnancy; the question is whether this pathological condition may have a postconceptional effect. Estrogen production plays a key role in endometriosis. The inhibition of estrogens by GnRH analogues, oral contraceptives, progestins and aromatase inhibitors reduces pelvic diseases and pain in women with endometriosis (Olive, 2008). Prostaglandins, locally produced hormones involved in inflammation and pain, and progesterone resistance are important in the pathogenesis of endometriosis [Bulun, 2009g.

The role of sex hormones is well known in spontaneous descent of testes in the fetuses at the end of pregnancy [Scorer, 1962]. The testis develops from the urogenital ridge and can be identified at 7-9th weeks of gestation due to the testis-determining gene (SRA) on the Y chromosome in the location of embryonic kidney. Testicular descent includes two phases [Hutson et al., 1997]. Testes descend to the inguinal region in the first transabdominal phase during 9-15th weeks. The cause of this movement is the thickening of the gubernaculum provoked by insulin-like factors 3 secreted by Leydig cells. The second inguinoscrotal phase started from 25th gestation week "when the gubernaculums bulges beyond the external inguinal ring and descends to the scrotum, while simultaneously it is hollowed out by a peritoneal diverticulum called the processus vaginalis" [Hutson et al., 1997]. The processus vaginalis allows the exit of testes from the intraabdominal cavity due to effect of androgens and the increase of abdominal cavity pressure. Thus, gubernaculums pulls the testis down to the scrotum in the second stage, and after this the inguinal canal is closed.

Thus, our study revealed possible association of maternal endometriosis and dihydrogesterone treatment and a higher risk for ITUT in their sons. The role of endocrine dysfunctions was shown in the pathogenesis of endometriosis [Wheeler, 1989; Olive, 2008; Bulun, 2009], thus it would be necessary to find an association of these factors with the risk of ITUT. However, endometriosis with related treatment occurred before the study pregnancy in this study and the critical period of ITUT is during the last trimester of the study pregnancy. Thus direct association can be excluded; our hypothesis therefore is based on some common route of endometriosis and ITUT.

Our study identified only one drug: dihydrogesterone used during pregnancy with higher risk for ITUT, however, this association was based on only 5 pregnant women. In addition the use of this drug was in the first trimester, though the critical period of ITUT is at the end of pregnancy. However, these treatments were based on prospective medically recorded data due to threatened abortion of pregnant women. Dihydrogesterone belongs to the group of progesterone/progestin and a higher risk of CAs was reported in the children of mothers with the treatment of synthetic progestins [Katz et al., 1985; Ressegute et al., 1985; Check et al., 1986] but without mentioning undescended testis. However, the use of medroxyprogesterone did not associate with a higher risk of CAs [Yovich et al., 1988], but maternal progestin intake and risk of hypospadias was reported [Carmicheal et al., 2005].

We found some other possible associations as well but burdened by recall bias. The birth of an infant with CA is a serious traumatic event for most mothers who therefore try to find a causal explanation such as maternal disease, drug uses, etc during pregnancy for CA of their babies. This does not occur after the birth of a healthy newborn infant. Thus the comparison of retrospective self-reported maternal information of population controls and cases is distorted by recall bias and it might inflate an increased risk for CAs up to a factor of 1.9 in OR [Rockenbauer et al., 2001]. However, we can limit the recall bias by the use of prospective and medically recorded exposure data as a reference standard; therefore only medically recorded exposures were accepted in the study.

Our previous study showed first the importance of maternal factors in the origin of IUT [Czeizel et al., 1981b]. The mean age of menarche was 13.6 yr in the mothers of cases and 13.3 yr in the mothers of matched controls, but the proportion of mothers of cases and their matched controls with late menarche after the age of 14 years was 21.7% and 10.9%, and this difference was significant ($X^2_6 = 25.03$, $p = 0.0001$). In addition the mothers of cases had shorter menses. Thus, these data suggested the role of maternal hormonal status in the origin

of IUT due to the possible hypogonadism of women such as pituitary/placental gonadotropin deficiency. The data of cases with ITUT were not appropriate for the re-evaluation of the above variables, but we suppose that they are characteristic mainly for ITUT.

The strengths of our project are connected with the large population-based data set of the HCAR, 1962-2011 during 50 years in the ethnically homogeneous Hungarian (Caucasian) population. The IUT-diagnoses was based on medically documented CA reported by medical doctors and these recorded IUT cases were controlled by the coworkers of the HCAR [Czeizel, 1997; Czeizel et al., 2014]. In addition the large population-based data set of the HCCSCA, 1980-1996 included 2,052 cases with ITUT and 24,814 male controls without. The validity of ITUT diagnoses was good because only cases with medically recorded IUT diagnosed at the third postnatal month were evaluated in the study. The data of birth outcomes were based on medical records and potential confounders were measured. The collection of exposure data was based on multiple sources but finally only prospective medically recorded data in the prenatal maternity logbooks were evaluated. The exposure time and potential confounders were known.

However, there were some weaknesses in our study. Several cases with undescended testis were failed to be ascertained in the HCAR [Czeizel, 1993], similarly to other CA-registries [Toppari et al., 2001]. Thus the major weakness of our project is the underreporting of IUT in the HCAR. Our cases in the HCCSCA were selected from the HCAR, thus cases with ITUT for the HCCSCA are burden by selection bias. However, the low ascertainment of cases with ITUT did not distort maternal data [Czeizel et al., 2007]. Most maternal diseases and related drugs treatments were reported by mothers retrospectively, but finally only medically recorded exposures were evaluated. In addition we did not evaluate some other factors in the origin of intrauterine growth restriction in cases with ITUT, e.g., smoking [Czeizel et al., 2001].

Conclusion

I. The live-birth prevalence of recorded IUT did not show real increase between 1962 and 2011 in the HCAR, the observed changes can be explained by different completeness of reporting.
II. Cases with IUT can be differentiate into two subgroups: undescended testis diagnosed at birth mainly in preterm boys with frequent postnatal testis descensus and ITUT without postnatal testis descensus with frequent intrauterine growth restriction, older mothers with higher birth order and low socio-economic status.
III. Our findings showed the possible association of preconceptional maternal endometriosis and dihydrogesterone treatment in early pregnancy with the risk of ITUT. This preliminary analysis was based on observational data therefore these associations need confirmation.

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Chapter 2

Alleviating Premenstrual Syndrome (PMS) Symptoms Using a Natural Factor – Negative Air Ions

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Abstract

There is hardly any other element of the physical environment which has caused so much confusion and controversies as negative air ions (NAI). Despite more than 100 years of study in the field, the knowledge on ion specter is still in an embryonic stage.

However, NAI have found to have good or excellent results, as therapeutic, as well as a prophylactic factor. We used a therapy of moderate doses of negative air ions emitted by a generator, for up to 14-20th days to treat the symptoms of discomfort in Premenstrual Syndrome (PMS). On female students with PMS, the results were very significantly different from the witness group. The premenstrual intense pain decreased from 38% to 9.5% ($p < 0.001$), and generally pain completely disappeared in 45% of cases. Acne decreased from 76% to 43%, and irritability from 76% to 19%.

It is worth mentioning that the premenstrual irritability completely disappeared in 75% of students. Before the experiment 81% of the cases used pain killers, while after the experiment only 24% were still addicted to medication, especially as a preventive measure. Another effect was the regulation of the menstrual cycle, in 89% of the cases with irregular periods. All of these improvements lasted in half of cases up to one year after the treatment (no other known medicine has such a long effect). The items referring to the energetic basis of the neuro-psychic tonus have an ascending trend, except the well-being sensation and self confidence, which were situated from the very beginning at the higher levels of the scale. Negative air ions are not a universal panacea, but they could be considered a physiological factor, very cheap, with no side effects, not

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addictive, with very few contraindications, and which do not bring any foreign substance inside the organism.

In conclusion, considering the advantages, the artificially ionized air might be a potentially effective biologic factor, which, if properly harnessed, controlled, and utilized, may become a valuable adjunct to other forms of therapy.

2. Premenstrual Syndrome

2.1. Introduction

Throughout history and in all cultures, the woman's reproductive body caused much fascination, but also some fear (Cosgrove L.B.R., 2003; Fausto-Sterling A., 1992; Foucault M., 1989, 1998; Gear R., 2001; Usher J.M., 2005). Frequently occurring disorders related to menstruation (dysmenorrhoea, premenstrual syndrome) have been investigated by many researchers over the years. Dysmenorrhoea is more common in adolescents, in the first 2-3 years after menarche, and would be associated with immaturity of the hypothalamic – pituitary- ovarian axes (Dănilă-Muster, 1968; Dorofteiu M., 1992; Marshal J.C., and Kelch R.P., 1986; Steiner M., et al., 1980). This disorder may be caused by the accumulation of prostaglandins (PG) in the endometrium, becoming a source of discomfort.

Prostaglandins PGF_{2alpha} and PGE_2 produced by the endometrium, stimulate myometrium in contact with it, induce incoordinate hyperactivity of the uterine muscle resulting in uterine ischaemia and producing the sensation of pain (Pearlstein T.B., 2004).

Adolescents suffering from dysmenorrhoea have high levels of prostaglandins, showing an improvement in symptoms by treatment with prostaglandin synthase inhibitors (naproxen-sodium, mefenamic acid) (Mira M., et al., 1986). Dysmenorrhea may be, most often primary, and usually disappears after the first pregnancy (Edincott J., and Halbreich U., 1981; Goldstein D.P., et al., 1980), or secondary, as a consequence of structural abnormalities of the cervix or uterus, the presence of foreign bodies (intra- uterine device) or endometriosis/ endometritis processes (Chatman D.L., and Ward A.B., 1982).

Premenstrual disorders occur in most women of reproductive age (Reid R.L., and Yen S.C.C, 1981) and can be severe and long enough to produce subjective distress and interfere with social and occupational functions (Borenstein J. E., et al., 2003; Chawla A., et al., 2002; Johnson S., 1987; Lee S., 2002; Lete I., et al., 2011). Premenstrual syndrome (PMS) is a universal event in women’s life, a complex of behavioral signs and symptoms that occur cyclically after ovulation, during the second half of the menstrual cycle (luteal phase) and which usually can be solved with start of menstrual flow (onset of follicular phase), but can extend during menstruation (Mayo J.L., 2004). This would support the idea that SPM is not immutable linked to an imbalance foliculino-lutein, which would occur at this time. Seems plausible intervention of other hormones, extra-ovarians - PMS occurs both in the ovulatory and anovulatory cycles, differing from dysmenorrhea, which is associated with ovulatory cycles (Howard R., et al., 1992; Spitzer R.L., and Severino S.K., 1989; Uchino E. et al., 1990).

The diagnosis of PMS is based on the existence of a sufficiently severe symptomatology as to have a negative impact on the woman's possibility to properly "work" at home, at job or in personal relationships (Mayo J.L., 2004). PMS affects women of any cultural or socio-

economic level, but the type and level of discomfort symptoms differ from woman to woman and may have cultural differences (Chandra P.S., and Chaturvedi S.K., 1989; Dan A.J., and Mongale L., 1994).

The notions used for premenstrual experiences have evolved within the scientific literature: premenstrual changes (PMC); premenstrual experiences; premenstrual tension (PMT) (Abraham G., 1983); premenstrual symptoms; premenstrual syndrome (PMS); premenstrual syndromes; Late Luteal Phase Dysphoric Disorder (LLPDD); and Premenstrual Dysphoric Disorder (PMDD). The many terminologies are dues to insufficient definitions of this experience. The scientific studies in domain are very different, and for general people, the differences between the many notions used are subtle or insignificant.

The term "PMC" is used for the mild and non-distressing situations, the most frequent experiences. The term "PMT" originally used by Frank (Frank R., 1931), has been replaced by "PMS" because this stage includes much more symptoms (so, it's a syndrome) than "premenstrual tension." Often, terms PMS and PMDD are used interchangeably (Sundström I., et al., 1997). However, according to Steiner (Steiner M., et al., 2006), the notion PMS should be used for the most part of the women, with mild peri-menstrual experience, such as headache, bloating, mastalgia and minor mood changes. The PMDD (A.P.A., 1994, 2000) also known as late luteal phase dysphoric disorder (LLPDD) is a severe form of PMS, occurs in a smaller number of women (5-10%) and can be accompanied by very severe symptoms which could affect a women's functioning: anger, irritability, dysphoria, mood changes, anxiety or tension are especially prominent and interfere with their life style (Davydov D., et al., 2004; WHO, 2004). The women's physical condition and behavior surrounding the menses have been described (mostly negative) as early as the time of the ancient Greeks. About 2500 years ago, in his early writings, Hippocrates provided a description of observed premenstrual mood changes. In 1931, this disorder was officially recognized by the medical community, when American neurologist Dr. Robert Frank coined the term Premenstrual Tension for the seizures and mood changes premenstrually. Twenty two years later Dr. Katarina Dalton introduced the term Premenstrual Syndrome (PMS) and established the first clinic in Britain to treat this condition.

The most advanced state, originally defined in The Revised Third Edition of the Diagnostic and Statistical Manual for Mental Disorder (Widiger T.A., 1993) and used by the American Psychological Association from 1987, was the term LLPDD, then PMDD (APA, 2000). Later, an interdisciplinary group proposed a more precise classification – cyclic perimenstrual pain and discomfort (CPPD) – encompassing cyclic pelvic pain and mood and physical discomfort (McFarlane J., et al., 1988; ACOG, 2000, 2004).

Despite more than 80 years since Frank (Frank R., 1931) called the term "premenstrual tension" are still controversies in defining the syndrome, scientifically studied especially since the 80's (Ussher J.M., 2003a, 2003b; Ussher J.M. et al., 2000).

2.2. Epidemiology

PMS is a "syndrome" not a biological process, which is defined as a mental, emotional, and physical disorder linked to menstruation (Mishell D.R., 2005). Up to 90% of women experience one or more symptoms during the days before menstruation (Campagne D.M., and Campagne G., 2007), sometimes in their life. Very often the incidence of PMS has been

overestimated by considering all subjects who present any kind of symptoms, physical or emotional, during the second half of the menses. It can be state that the moderate to severe forms of PMS, which could lead to a loss of women function, affect 20% to 30% of women.

The most severe variant of PMS (PMDD) occurs in 3% to 8% of women, in their 4th decade of life. The number of women who suffer from PMS depends on the correct definition of this syndrome (Dean B.B., et al., 2006). WHO estimates the presence of 199 million women with PMS in 2010 (5.8% of the female population worldwide) (Vos T. et al., 2012). Since 80% of sexually active women has at least one symptom that can be attributed to PMS, the prevalence may vary between 3% (NIH Press, 1998) and 30% (Dean B.B. et al., 2006).

There are between 150 and 300 symptoms that were proposed to be included in the PMS (Dickerson L.M., et al., 2003; Singh B.B., et al., 1998; Tempel R., 2001) many of which being identified by different researchers in the last 50 years (Rubinow D.R., and Roy-Birne P., 1984; Dalton K., 1994; Spangenberg J.J., and Venter E., 2003; Steiner M., et al., 1980, 2003).

Among the symptoms that characterize PMS can be mentioned emotional, behavioral and physical symptoms, the most common being irritability, emotional instability, restlessness, fatigue, headache, anxiety, difficulty sleeping (hypersomnia/insomnia), acne, low ability to concentrate, depression, pain and turgid breasts (mastalgia), abdominal bloating (due to fluid retention), abdominal pain, oversensitivity, and exaggerated mood swings. There were also described disorders of appetite (increase appetite or different cravings, particularly for sweet and salty foods), back pain, weight gain, tearing, and sometimes tendency toward violence (Dalton K., 1994; Dell D.L., 2004). Many symptoms of PMS, including physical and emotional aspects can be manifested in a different register, from mild to severe. Depending on the number and intensity of these symptoms, quality of life of people affected or their partners could be seriously impaired (Dean D.B., and Borenstein J.E., 2004; Lustyk M.K.B., et al., 2004; Lustyk M.K.B., et al., 2006; Lustyk M.K.B., et al., 2007; A.P.A., 2000). Due to the partial overlap between many of these symptoms and affective disorders, many researchers investigated whether menstrual disorders represent an exacerbation of the affective disorders, or if they occur in the absence of preexisting psychopathology (Casson P., and Reid R.L., 1990; Herter L.D., and Spritzer P.M., 1993; Hurt S.W., et al., 1992; Kirsch J.R., and Geer J.H., 1988; Lustig R.H., and Fishman J., 1988; Lustyk M.K.B., et al., 2011; Steege J.F., et al., 1985). There is controversy whether physical symptoms should be included; if should be considered only symptoms that completely disappear for the rest of the cycle; if the severity of symptoms or simply their presence should be assessed; if the functional impairment can be a criterion; if positive premenstrual changes should be considered. It seems that SPM includes a number of overlapping syndromes rather than a single separate entity (Gath D.H. et al., 1987; Goldberg D.P. and Huxley P., 1991). In literature start two different orientations in interpreting SPM. The first utilizes retrospective questionnaires that ask women to report on their latest cycle, or on a "typical cycle" of last year, the second uses daily assessment (prospective) during the menstrual cycle, of symptoms occurred during one or more several menstrual cycles. Each of these two options could be used, each with its shortcomings, some using them both (Hargrove J.T., and Abraham G.E., 1982; Harrison W., and Endicott J., 1989; Hart W.G., and Collman G.J., 1987; Hurt S.W., et al., 1992).

2.3. Etiology

Despite considerable research effort, the Premenstrual Syndrome (PMS) is a controversial phenomenon, the term PMS is still not well understood, because the diagnose is difficult to make, and the symptoms are many and varied (Halbreich U., 2003).

At the present time, the basic physiology and symptomatology of the menstrual cycle are well understood, but the exact etiology of PMS has not been fully elucidated and may be complex and multi-factorial. Some physiologic mechanisms that underlie PMS have been proposed, and some theories have been formulated to explain the etiology of PMS.

These theories focus mostly on sex hormones and their metabolites, as well as their interactions with neurotransmitters (e.g., serotonin), their influences on kidney sodium regulation, and their ability to be metabolized in the liver (Tempel R., 2001; Ussher J.M., 1996; Foucault, M., 1978; Foucault, M., 1989; Ussher J.M., 2005).

The explanation of the premenstrual experience has progressed from a supposed hormonal imbalance (Rubinow D.R., and Schmidt P.J., 1992) to psychological (Bailey J.W., and Cohen L.S., 1999) and to a more recent multi-causal origin (Campagne D.M., and Campagne G., 2007; Hulstein P.L., 2009; Yonkers K., et al., 2008; Reiber C., 2008).

Most theories suggest that PMS is produced as a result of sex hormones (estrogen and progesterone) and their metabolites changes. PMS most often occurs in women with normal menstrual cycles which last 24-35 days. During the normal menstrual cycle, hormone levels change. Estrogen is made by the ovaries throughout the entire menstrual cycle, reaches its highest levels during the second weeks (the preovulatory phase) and third weeks and declines during the last week. During the last two weeks of the cycle (the luteal/the secretory/the postovulatory phase) the ovaries also make progesterone.

The main role of these hormones is to prepare the uterus (endometrium) for pregnancy. An increase of estrogen facilitates the development of an egg cell from the ovary. Both estrogen and progesterone levels drop off just prior to menstruation, perhaps causing PMS.

Some research has found estrogen treatments reduce PMS symptoms, but other studies have not found a significant difference between subjects receiving estrogen treatments and subjects receiving a placebo (Pearlstein T.B., 2004).

Other researchers believe that PMS symptoms occur because the sex hormones interact with brain chemicals, the neurotransmitters (such as serotonin, endorphin, norepinephrine).

Serotonin, made by the body from the amino acid tryptophan, decreases during the second phase of the menstrual cycle (when estrogen level goes down, also) and may cause depression and carbohydrate cravings. Another neurotransmitter is GABA (gamma-amino-butyric acid), important in feeling calm (Kalueff A.V., and Nutt D.J., 2007).

Endorphins, important in the experience of pain and pleasure, are related to mood, which may explain the shift to a more negative, irritable mood prior to menstruation in some people. One hypothesis is that PMS is caused by a significant decrease in serum levels of beta-endorphin just prior to menstruation (Chuong C.J., et al., 1994; Straneva P.A., et al., 2002; Turner C.E., et al., 1990). Some studies suggest that about 40% of women with PMS have this drop in beta-endorphins (Giannini A.J., et al., 1984; Gianini et al., 1985; Girdler S., et al., 2007; Girman A. et al., 2001). Beta- endorphin is a natural opioid, a neurotransmitter that has an affinity for the same receptor as heroin or other opioids. Thus, some researchers have noted similarities between the symptoms of PMS and those suffering from opiate withdrawal symptoms (Turner C.E., et al., 1990). With the possible exception of lower plasma levels of

beta- endorphin in the premenstrual phase (Chuong C.J., et al., 1985; Fachinetti F., et al., 1987), no other consistent biological marker could be revealed in PMS.

Norepinephrine and epinephrine are two neurotransmitters that influence mood, and play a role in blood pressure and heart rate.

In another study of 71 women with PMS were found elevated levels of serum pseudocholinesterase, enzyme that can be considered as a possible marker to characterize anxiety (Gianini A.J., et al., 1985).

Some authors confirm the idea issued by Keye (Keye W., and Trunnell E., 1986) that SPM can be a neuro- biological predisposition, a perhaps genetically determined (Hausfater G., and Skoblick B., 1985; Hennig J., et al., 1994). Despite the many studies including those provided by Rubinow D.R., et al., (1984, 1992), the literature on genetic etiology still remain unsatisfactory. From the 1971', first explored empirically was the possibility that PMS appears in family, finding highly significant correlations between mother and daughter about many menstrual variables, including "premenstrual tension" (correlation 0.24; $p < 0.001$). It is therefore possible a genetic component to the etiology of PMS (Jardine R. et al., 1984; Brown M.A., and Zimmer P.A., 1986) demonstrating significant genetic influences on traits of "neuroticism." Other authors argue scientifically for a predominant psychological etiology of PMS, in which genetic factors may play a less direct role (Jardine R., et al., 1984; Huo L., et al., 2007; Treloar S.A., et al., 2002; Watts S., et al., 1980).

2.4. Risk Factors

Although several risk factors for SPM are identified, data are not consistent. In general, following factors show a positive association with menstrual problems: age over 30 years; childlessness; single woman/unmarried status; lack of use of oral contraceptives; job; marital discords and other distresses of life; polycystic ovary syndrome; sexual dissatisfaction and sexual function disorders; anxiety, intermittent depressive disorders or postpartum depression (Halbreich V., and Endicott J., 1985); and emotional disorders (Fachinetti F., et al., 1990; Hallman J., 1986; Hallonquist J.D., et al., 1993; Lovestone S., 1992; Mcleod D.R., et al., 1993; Morris-Yates A., et al., 1990; Reid R.L., 1991; Severino S.K., and Moline M.L., 1989; Sioban D., and Matanoski G., 1991; Warner P., and Bancroft J., 1988).

PMS is not caused by stress, but is often associated with stress. Women may experience more stress prior to menstruation, and they may handle stress differently during this time, but it is not the cause of PMS. Excessive stress can, however, make PMS symptoms worse, and a stress free life or developing better means to deal with stress can be important (Perkonigg A., et al., 2004). PMS is not associated with any kind of personality factors or any personality types. All of these mentioned theories are waiting to be proven, as well as the specific treatment, and the scientific basis is to be enriched.

2.5. Treatment

Despite the broad range of scientific interest about the management of symptoms of discomfort in PMS, there is still no real treatment for PMS and PMDD at the moment. There are however, many options in managing the unpleasant experiences around the menses. Once

the diagnosis was accurately established, it's a priority to identify other medical or psychological conditions, and to treat them. The many variants in therapeutic modalities have been shown to be effective; so that the symptoms did not prevent women have a healthier and productive life.

Over time, PMS treatments have ranged from the dangerous ovarian irradiation to the ridiculous theory of hiding in one's room (Taylor D., 2005). Until recently, the focus on singular, usually pharmacologic, therapy has dominated the treatment for PMS. Clinical research now suggests that combination of treatments is more beneficial than are single treatments (Low Dog T., 2001; Taylor D., 2005).

New models of symptom management, which combine self-help, social support, medical therapies, and psychological strategies applied to specific conditions, have shown promising results (Freeman E., et al., 1990, 1995; Taylor D., 2005).

There are many strategies for PMS treatment (Cronje W.H., et al., 2004; Mira M., et al., 1986; Rapkin A.J., et al., 1997, Studd J., 2006), like conservative treatment: exercise (Stoddard J.L., et al., 2007), relaxation (Hernandez-Reif M., et al., 2000; Morse C.A., et al., 1991), stress management (Bhatia S.C., and Bhatia S.K., 2002; Choi P.Y.L. and Salmon P., 1995), religious or spiritual support (Ornitz A.W., and Brown M.A., 1993); self-disclosure (Warren C.J., and Baker S., 1992); non-pharmacological treatments including evening primrose oil (Budeiri D., et al., 1996); dietary modification (Johnson S.R., 2004), client education (Seideman R.Y., 1990), positive reframing (Morse G.G., 1997; 1999), light therapy (Parry B.L., et al., 1993; Lam R.W., et al., 1999) and cognitive-behaviour therapy (CBT) (Blake F., 1995; Blake F. et al., 1998, Hunter M.S., et al., 2002a, 2002b; Kirkby R.J., 1994; Ussher J.M, 2002; Usher J.M., et al., 2002). All of these strategies of treatment have been proven to be inconsistent, ineffective or uneven.

Other scientific researches indicate selective serotonin reuptake inhibitors (SSRIs), which act by indirectly increasing the brain serotonin levels, so correcting serotonin imbalance and stabilizing emotions (Rapkin A., 2003; Wyatt K.M., et al., 2002).

The many options of treatments for PMS and PMDD depend on the symptoms and their severity. General management refers to a healthy lifestyle like exercise (can provide emotional support from family and friends during the relevant cycle time); avoid salt during the second half of the menstruation; reduce caffeine intake (Rossignol A.M., 1985); quit smoking; reduce alcohol intake; and reduce intake of refined sugars. These options of management have been proven to be helpful in some women.

For mild cases, treatment includes vitamins (E, B_6, and B_5) and minerals (magnesium and calcium), diuretics, and pain killers (Burnet R.B., et al., 1991; Kashanian M., et al., 2007; Thys-Jacobs S., 2000).

For more severe cases of PMS and PMDD, a lot of medications could be used: antidepressant throughout the cycle, or only during the latter half of the cycle (Cohen L.S., et al., 2004; Fachinetti F., et al., 1989; Freeman E.W., 2005; Freeman E.W., 2011; Steiner M., et al., 1995), hormone treatment/oral contraceptive pills (Yonkers K.A., et al., 2005), diuretics, pain killers, drugs that suppress ovarian function (Hahn P.M., et al., 1995), or (only in extreme cases) surgery to remove the ovaries. Antidepressants pills act by increasing the brain level of serotonin; an alternative means of increasing serotonin levels is to eat more carbohydrates. Hormone treatment (oral contraceptives), as well as removal of the ovaries, prevent ovulation and the changes in hormones that accompany ovulation.

Phytoestrogens and natural progesterone from many skin creams or plant-derived compounds like tofu, represent an alternative treatment that can aim some physical symptoms of PMS by both affecting serotonin and hormone responses (Tamborini A., and Taurelle R., 1993). In addition, an increased consumption of phytoestrogens may reduce the risks of osteoporosis, cancer, and heart disease (Bancroft J., 1993).

These kinds of treatment are pretty effective, and a diet low in sugars and fats and high in phytoestrogens and complex carbohydrates, along with lifestyle changes can improve overall health and thereby can lead to the reduction of symptoms. Other methods, like over-the-counter pain relievers or prescription medications could be successfully used to alleviate some physical symptoms (cramps, headaches, backaches, and breast tenderness): Ibuprofen, Ketoprofen, Naproxen, Aspirin, and so on. Evidence suggests that exercise can help relieve some of the symptoms of PMS in young women and adolescents. Physical activity improves general health, helps relieve nervous tension and anxiety, and release endorphins (which is responsible for a euphoric feelings, known as the "runner's high" experienced after prolonged exercise). Endorphins are chemical messengers for nerves (neurotransmitters) that affect mood, perception of pain, memory retention and learning. Although estimates vary, most researchers think that only about 10 percent of women experience incapacitating symptoms (Gottlieb A., 1988); and no treatment works for every woman. More research is needed to understand better the cause and evaluate treatments of this condition.

3. Negative Air Ions (NAI)

3.1. Introduction

In the chemical composition of air there are a number of elements; majority of them are neutral, but some constituents are carriers of electric charges (ions). Ions are molecules that have gained or lost an electrical charge (negative or positive). They are created in nature as air molecules break apart due to sunlight, radiation, and moving air and water, but their concentration is quite low (Yaglou C.P., and Benjamin L.K.C., 1933) and varies widely in outdoor air, depending on geographical area, season, and pollution. There are also artificial negative air ions generators (like ELANRA, produced by Joshua Shaw in 1992), producing a larger number of ions/cm^3 of air, and wich strengthen the immune system by increasing level of Immunoglobulin-A (Chorny K., 2000). In natural areas, negative and positive air ions are in balance and give the feeling of fresh air, "country air" (Baldwin B.E., 2004). Air ions are classified according to the charge (negative and positive); size (small: 10^{-7}cm, medium: 10^{-6}cm, large: 10^{-5}cm), mobility (in inverse ratio to their size, higher at negative ions), lifetime (short in closed rooms, long overseas and oceans).

Once formed, the fate of small air ions in the air is different: it can combine with each other or with large ions, they can be adsorbed and form large ions, join conductors and give (loose) electrical charge, broadcasts by thermal agitation (Nagato K., 1999; Nakane H., et al., 2002). Small positive ions are generated outside by friction between air-to-ground, air-to-metal, air-to-air or air-to-particles. Indoor, small positive ions are produced in front of computers, copiers, through the use of air conditioning systems, or synthetic clothing around, causing the so-called "positive ion poisoning" (Wakamura T., et al., 2004).

Small negative air ions (good ions, "happy ions") are produced outside in unpopulated natural areas with varied landscape rich in vegetation, near waterfalls, on mountain peaks with trees, on the promenade, near the swirling waters or after the storm/rain (trillions/cm^3). They are created by friction caused by waterfalls, winds in the mountains, waves tumbling on a beach, rain slicing through the air, radiation from the sun and the earth's surface. Inside, the best source of negative ions, are shower and decorative fountains.

The normal concentration of small ions in natural areas varies between 500 and 3,000 pairs ions/cm^3 air, the values are different depending on the season, time of the day, the relative humidity (decrease), and altitude (increase). The most frequent ion count in fresh country air is 2,000 to 4,000 negative ions per cubic centimeter (about the size of a sugar cube). At Yosemite Falls, you'll experience over 100,000 negative ions per cubic centimeter. On the contrary, the concentration is far below 100 per cubic centimeter of Los Angeles freeways during rush hour. In therapeutic or prophylactic applications the literature indicates the use of unipolar ions (either negative, either positive); there are no data on bipolar ionization, although all artificial generators emit both types of ions, but some of them (usually the positive) are detained by devices called ion separators (Deleanu, M., et al., 1992).

Positive air ions therapy is used in rare cases (e.g., Calcium deficiency in hemophilia, or mice with cancer, in which positive ions inhibit the occurrence of tumors and prolong the life of mice). Small negative air ions (NAI) also called vitamins of the air, are odorless, tasteless, and invisible molecules that we inhale in abundance in environments like mountains, waterfalls, and beaches.

Unlike positive air ions, NAI have a beneficial effect on the body, both in therapeutic and prophylactic applications (Pino O., and La Ragione F., 2013; Soyka F., 1977; Sulman F., et al., 1978; Sulman F., 1980; Suzuki S., et al., 2008; Yamada R., et al., 2005).

Negative ions increase the flow of oxygen (negatively charged) to the brain, resulting in higher alertness, decreased drowsiness, and more mental energy. They protect against germs in the air, resulting in decreased irritation due to inhaling various particles that make us sneeze, cough, or have a throat irritation (air conditioning depletes the atmosphere of negative ions). The exhaustion of NAI in polluted air, in closed and conditioned rooms, near television and computer monitors leads to disturbance of health (Fletcher L.A. et al., 2007).

3.2. Mecanisms of Action

However, the primary physicochemical and biochemical mechanisms of beneficial biological action of NAI are still obscure. There are several theories to explain air ions action. One of them is the theory of lung/tissue electrical change: air ions are taken up by the respiratory tract and part of them reaches the lungs; as oxygen and water aerosols are mostly ionized, they are taken up by the erythrocytes and thrombocytes at the alveoli site together with normal oxygen and water. The negative charges are transferred to plasma/erythrocyte colloidal proteins and that will enhance their colloidal stability, improving our blood circulation, stimulating our nervous system and endocrine organs (Vasiliev L.L., 1953).

Another theory is the neuro-reflex one: negative air ions influence (inhibit) the respiratory center and consequently the breath is calm, easily and tranquil, and the respiratory pause is longer (Deleanu M., and Stamatiu C., 1985; Deleanu M., 1988; Deleanu M., et al., 1992). The serotonin hypothesis (Krueger A.P., 1968, Krueger A.P., and Reed E.J., 1976)

sustain that serotonin, a powerful neurohormone, could be affected by the polarity and concentration of inhaled air ions. Negative ions act to reduce serotonin levels in the respiratory system, blood and brain by converting Serotonin to 5-hydroxyindolacetic acid - a physiologically inactive metabolite. The reduction in serotonin level might be involved in the relief of pain, whilst positive ions increased serotonin levels.

Negative air ionization appears to reduce serotonin via enhancement of monoamine oxidize activity. Serotonin (5-hydroxy-tryptamine or 5-HT) can have neurovascular, endocrinal, and metabolic effects, influencing various processes (sleep, mood, transmission of nerve impulse). Reduced serotonin levels result in a mentally relaxed state and reduction in feelings of depression (Goel N., et al., 2005; Hiroshi I., et al., 1972).

In 1984, Wehner suggested that endorphins, enkephalins and interferon could mediate the action of air ions (Boscaro M., et al., 1990; Chuong C.J., et al., 1985; Cold E.W.D., et al., 1981; Donevan R.H., and Anrew G.W., 1987; Quartu M., and Del Fiaco M., 1994; Schwarz L., and Kinderman W., 1989; Wehner A.P., 1984). Endorphins and enkephalins are opioid neuropeptides having morphinelike activity.

They affect human thoughts, emotions and behavior and act as painkillers by stopping the pain signal to the brain. Enkephalins block calcium channels in the membrane of the pre-synaptic neurons. They block the synaptic transmissions, so the message doesn't reach the brain. Endorphins are released from the pituitary gland to control pain. They are carried to the brain and bind to pain receptors and block the release of the neurotransmitter that is used to transmit pain signals to the brain (Anntka T., et al., 1987).

NAI have good or excellent results in many diseases (the greater the level of physical stress, the greater the effect ion treatment seemed to have, rebalancing the energetic state of the body). As a therapeutic factor, the negative air ions moderate, reduce or normalize many respiratory illnesses- bronchial asthma (Khan M.A., et al., 2006), cardio-vascular, digestive (Bordas E., and Deleanu M., 1989), neuro-psychic diseases (Goel N., et al., 2005), as well as burns (ionized air has analgesic properties; healing is often more rapid and complete) (Kornblueh I.H., 1968).

As a prophylactic factor, the negative air ions increase the physical and mental efficiency and performance (Buckalew L.W., and Rizzuto A., 1984; Hawkins L., and Barker T., 1978; Laza V., 2000, 2007; Ogungbe A.S., et al., 2011); prevent some meteorology sensitive diseases and increases the general resistance (Deleanu M., et al., 1981; Deleanu M., 1988; Kornblueh I.H., et al., 1958). In healthy people, the negative air ions act by strengthening the immunity organs and preventing the disease (Temnov A.V., et al., 2000). The finding at LaTrobe University from Melbourne shown that exposure to NAI results in significant increases in the level of IgA–Immunoglobulin A, an important immune factor, effect mediate by the action on serotonin metabolism (Chorny K., 2000).

Many of researches made on human beings have been demonstrated that NAI have a normalizing effect on a lot of diseases (the more the disturbance, the best the effect of NAI), and a stimulating effect, increasing the physical and mental efficiency and the general resistance of the organism (Iwama H., 2004). Among the main results on students, the enhancement of the physical and mental efficiency could be noted (Laza V., et al., 1998).

As a result of extensive trials conducted by clinicians and physicians, air ion therapy has become a useful method of treatment in many countries (Beardwood C.J., and Jordi P.M., 1990; Morton L.L., and Kershner J.R., 1990; Deleanu and Bordas, 1991); in other countries, this kind of intervention is either unknown or viewed with frank skepticism. Many scientists,

from different countries all over the world, have brought sufficient evidence on biological effect of air ions (Livanova et al., 1999b).

Despite this evidence, we must admit, however, that at the moment there is not enough evidence about the basic mechanisms of biological action of ions. Kornblueh (1973) noted that the reactivity of various individual to negatively charged air ions depends on constitutional differences: responsiveness varies from person to person, depending on physiological, biochemical differences between the individuals.

Some people do not react at all under the influence of negative air ions; most people respond to negative ions, few people only react to positive ions. That's because the electrical sensitivity largely differs from individual to individual (Kornblueh I.H., 1973).

The most sensitive to air ions are some categories: children, elderly and sick people, and persons under stress (Livanova L.M., et al., 1999).

Other studies conducted on burned patients showed that the negative air ions treatment determined an increase in mental alertness (Beasley V.R., 1975; Kornblueh I.H., 1973). Kornblueh (1973) also revealed that in enclosed and crowded spaces, the number of negative ions rapidly decreases, leading to a predominance of positive ions. The accumulation of positive ions is responsible for the uncomfortable sensations of people. The introduction of negative ions is resulting in better environment and better feelings of crowded people.

4. Using Negative Air Ions to Alleviate PMS

4.1. Aims of Research

Favorable therapeutic action of negative ionization was valued over time in many diseases. Based on the encouraging results obtained from histological reproductive system in animals (Laza V., et al., 1998), a research has conducted to assess the effect of negative ionization on menstrual cycle disorders (premenstrual syndrome, dysmenorrhea) at a group of young students from the Faculty of Medicine, Cluj-Napoca, Romania.

The effects of the treatment in time as well as the duration of favorable effects and progression of neuro-psychiatric features under the influence of negative ionization, were followed for many months after the treatment was done.

Premenstrual disorders can be considered as a complex syndrome that also involves the psychic sphere. It is well recognized today that any somatic disorder includes in her morbid complex also psychological changes with echo on mood, on general mental reactivity and especially on work capacity (Bornstein R.F., and Gold Stephanie H., 2008; Predescu V., et al., 1968).

This condition can be addressed through the psychosomatic (induced by an accentuation of peculiarities of psychic structure specific to women), and somatic approach (organic pain or endocrine peculiarities emphasizes some psychological traits of personality).

The mainly affective life in women is determined by biological and social factors. More intense reactions to emotional trauma, more frequent sub-neurotic and neurotic states in women are just two aspects that support this female psychosomatic preponderance.

Somatization tendency of effects is more intense in women, and affective processes are accompanied by concomitant somatic ones. Somatic expressions could be: tachycardia, tachypnea, increased maximum blood pressure, or organs'spasms.

Detecting less apparent mental disorders, remained in the normal range (emotional instability, anxiety, or pronounced nervous reactivity), somato-psychic or psychosomatic related to dysmenorrhea, as well as the possibility of influencing them by negative aeroionotherapy (NAIT), is one of the goals of our research.

Assuming that the existence of affective disorders and some hiperemotivity states are obligatory accompanied by vegetative disorders varied in shape and intensity, we watched some of these disorders and their evolution under the influence of negative ionization.

Another objective of the research aimed the evolution of some components of neuropsychiatric tonus (the energetic support of formation and information processing) under the influence of negative air ions.

As the duration of received treatment was different, we were concerned about how this could influence the evolution of mental exercise capacity (by sustained concentration of attention) during treatment with negative air ions (Yonkers K.A., et al., 1997).

4.2. Participants and Study Design

Eligible for participation was a randomly selected sample of healthy women (n = 29), students from the University of Medicine and Pharmacy, Cluj-Napoca, Romania, 19-26 years of age, not-pregnant, sexually-active. Data were collected with a 17-items retrospective questionnaire (daily health diary) adapted from the Premenstrual Assessment Form (PAF) (Halbreich V., et al., 1982). The questionnaire is assessing menstrual cycle experiences (Endiccott J., et al., 2006; Mitchell E.S., et al., 1991) in most menstrual cycles (in a "tipical"cycle) during the past year, in order to detect the prevalence of PMS.

This instruments includes a symptom checklist with general discomfort symptoms frequently experienced by female students during the luteal phase or during the very first days of the menstrual flow: premenstrual pain, dismenorhoea (pain during the follicular phase), irritability, acne, breast tenderness or swelling (breast congestion), sensation of "bloating," nausea, edema, asthenia, diarrhea, insomnia, loss of appetite, vomiting, constipation, drowsiness, tenesmus, headaches.

The symptoms were scored from zero (minimum score) to four (maximum score). Participants were asked to rate the severity of the 17 symptoms daily on a 4-point scale: 0 (not present), 2 (mild), and 4 (intense). The total maximal possible score for symptoms ranges from 34 (17 x 2) to 68 (17 x 4). The subjects were considered as reporting severe premenstrual changes (SPMC) if they (a) scored intense (4 points) on at least two symptoms; (b) had a mean symptom severity score on the PCL greater than 16; and (c) said that the symptoms in the week before menses affected relationships or function in some way.

The girls were considered as reporting mild premenstrual changes if they scored mild (2 points) on at least two symptoms, reported symptoms relief at the onset of menses and reported that symptoms in the week before menses did not affect relationship or function in any way. From an initial batch of 29 students, 21 showed menstrual cycle disorders (at least two symptoms of discomfort). They received a negative air ions treatment starting with the 14^{th} day of the menstrual cycle (the beginning of the second phase of the menstrual cycle), the

total number of daily sessions, corresponding to a case varying between 4 (in cases of irregular and short menses, of 19 days), and 20 (in cases of irregular and long cycles, of 34 days).

Negative ionization was performed with an artificial ion generator (BION-90), which allows simultaneous exposure to more people, in a clean, well ventilated room, free from dust, cigarette smoke; air temperature and relative humidity fits into the norms of comfort (well-ventilated and meticulously clean rooms are a pre-requisite).

Exposure to negative air ions was daily performed, starting from the 14^{th} day until the last day of the menstrual cycle, gradually: 15 minutes in the first day, 20 minutes the next day and 25 minutes each of following days.

In the last two days of treatment, the exposure time was reduced by 5 and 10 minutes respectively, avoiding abrupt discontinuation of treatment. During sessions of air ions treatment, subjects breathe normally, but at the beginning of each session, and then every 10 minutes, they performed 10 deep breaths. The distance to the ions generator was 70-80 cm, the trunk being slightly bent backwards so that the flow of air ions is designed to be perpendicular to the frontal region. Negative air ions concentration emitted by the device was about 17,000 small negative ions/cm^3 of air, at a distance of 70-80 cm.

After the negative air ions therapy (NAIT) girls were investigated again by prospective daily ratings (during at least two consecutive symptomatic cycles), about the subjective symptoms occurred over the menstrual cycles, over a year, noting both the disappearance and mitigation of symptoms and duration of recorded remission/improvement.

No remuneration for participating in the study was provided to the participants. Knowing that dysmenorrhea can be associated with a neuro-endocrine imbalance or genital malformations (infantile or malformed uterus), the hormonal citovaginal and pelvic exams were performed on 6 students.

Cytologic examination was performed after the hormonal vaginal technique known in the literature: on 14^{th} and 21^{st} day of the menstrual cycle, in the laboratory of Obstetrics and Gynecology Clinic of Cluj-Napoca; gynecological examination was performed in the same clinic. Both exams were performed in the previous cycle, preceding that in which the treatment was done. Psychological effects of air ions have been reported for more than 80 years in the media and scientific literature (Perez V., et al., 2013). To highlight how some neuropsychological features evolved under the influence of negative air ions, 17 students were individually examined. Subjects were tested under the same conditions before and after treatment with negative ions.

Eysenck's Personality Inventory (EPI) was used for registration of personality traits (Crăciunescu M., 1991; Creţu R.Z., 2005; Dafinoiu I., 2007; Eysenck H.J., 1950, 1957, 1958, 1970; Eysenk H.J., and Eysenk S.B.G., 1975; Eysenk S.B.G., et al., 1985), thus revealing two basic dimensions of personality: extraversion-introversion and emotional stability-instability (neuroticism or emotionality). Inventory was applied only once, before treatment, to assess the subjects' personality type.

Nervous reactivity was determined by the Maudsley questionnaire (Eysenk H.J., 1959; Minulescu Mihaela, 1996) comprising a total of 40 questions following a complex of symptoms which express individual adaptability to stressful situations. The result is evaluated by counting the positive responses.

A subject who give over 18.0 ± 1 affirmative replies is considered as a persons with increased nervous reactivity. The questionnaire was applied before and after treatment,

cautioning the subjects to report (subjectively) which of the symptoms mentioned in the questionnaire underwent changes that could be attributed to treatment.

To discover the existence of the diencefalo-vegetative syndrome and therefore the affective disorders (hyperemotivity and sub-nevrotic moods) a questionnaire comprising six questions about the most obvious and constant signs of vegetative disorders was used.

These signs were: plantar and palmaris hyperhidrosis, Rosenbach sign (upper eyelid tremors), distal fingers trembling, tachycardia, acrohipotermia (cool extremities) and skin vasomotor disturbances.

Relationships about the evolution of such signs under the influence of negative ionization were asked at the end of treatment. Rosnebach's sign, which indicates unusual tremor of the eyelids, is often associated with various diseases involving high levels of thyroxine.

A self-assessment scale was also used to follow the evolution of neuropsychiatric tonus during treatment. The subject must describe the "state" that is, in terms of five factors (A-E): A and B factors represent components that result considering energy fund itself from different angles; C factor expresses affective tonus; D factor refers to the presence or absence of painful events that sometimes accompany fatigue (headache and so on) and E factor aimed self-confidence. For each factor, the subject indicate a value between zero (minimum rate) and 100 (maximum rate), depending on the level of mobilizing its energy that time. The neuropsychological tonus is determined by many factors whose boundary is difficult to accomplish, but was followed however the evolution of obtained curves based on averages from the 5 factors during treatment.

Using Kraepelin's mental arithmetic test, the ability of sustained mental effort with focused attention under the influence of AIT was determined, the working speed and quality indicating the efficiency. The test was applied in 4 steps of 4 minutes each so, a total of 16 minutes, before and after negative ionization. Acquiring of unitary working technique was provided by explanations and experiments. Kraepelin's test results were quantitatively analyzed by adding the correct answers, and qualitatively analyzed assessing errors and omissions. Statistical significances between the means for pair' samples, were calculated based on "t" test (Student). For the other tests, the results were assessed by calculating the percentage of frequency and the arithmetic average.

4.3. Results

In 16 out of the 21 symptomatic cases, the results were interpreted as "favorable" and in 5 as "partially favorable" (Table no.1). We noted that the result does not depend strictly on the number of performed sessions of treatment, favorable results being obtained, for example, in subjects who performed 4, 5 or 6 sessions of air ionization. The same positive effect of negative ionization is found in subjects who performed a greater number of sessions (between 11 and 20). Of the 17 accused symptoms present before NAIT (the number varies from one case to another), 4 symptoms completely disappeared after treatment (insomnia, vomiting, drowsiness, and tenesmus) and the others were reduced in number (e.g., premenstrual pain, dysmenorrhea, irritability, acne, and breast congestion). A significant reduction of the symptoms' frequency was found. Before treatment, the number of symptoms per subject ranged between 2 and 8 (an average of 5.23 symptoms/subject), and after air ionization decreased to an average of 3.3 symptoms per person (Tables no. 2 and 3). Total score

decreased from an average of 19 points per person to an average of 6.95 points per subject (that means a score reduction of 64.4%). It's worth noting that in two cases (no.4 and no.20) symptoms completely disappeared. In other subjects, some symptoms have disappeared and others were attenuated. Premenstrual pain was present in 15 subjects (71.5%) before treatment (Tables no. 3 and 4), while after treatment was evident only in 8 cases (38.1%). Dysmenorrhea was accused by 18 students (85.7%) before air ionization, and after air ionization was present only in 12 subjects (57.1%). Acne and irritability were present at 16 cases (76.2%) before air ionization. After AIT sessions, acne was noted in 9 cases (42.8%), and irritability has been accused only by 4 cases (19%).

Breast congestion decreased in a significant proportion from 10 cases (47.6%) before treatment, to 4 cases (19%) after treatment; bloating present in 9 cases (42.8%) before the treatment, decreased to 4 cases (19%) after treatment. Insomnia, tenesmus, vomiting, and drowsiness, completely disappeared in all subjects.

Table 1. Characteristics of the participants and study design

Cases/age/ menarche	Cycle lenght (days)		No. of simptoms		No. of seances	Medications		Duration of remission	d*
	before	after	before	after		before	after		
1. PI/22/14	24-25[a]	28[b]	4	1	8	No	No	4 months	
2. PD/24/14	24[a]	28[b]	5	1	9	Yes	No	3 months	
3. BD/22/15	28	28	4	1	12	Yes	No	2 months	
4. PD/24/14	28	28	6	0	12	Yes	No	9 months	
5. DC/23/13	28	28	8	7	9	Yes	Yes	-	
6. PC/2214	28	28	5	4	9	No	No	10 months	
7. PM/2214	25-30[a]	27[b]	6	3	4	Yes	No	1 year	
8. TC/24/12	28	28	4	3	5	Yes	No	2 months	
9. SC/23/14	24-28[a]	28[b]	2	2	14	Yes	No	10 months	
10. DC/22/14	28	28	5	3	6	Yes	No	12 months	
11. CD/22/12	29-57[a]	30[b]	6	3	12	Yes	Yes	12 months	
12. SB/24/13	15-24[a]	25-26[b]	6	5	10	Yes	Yes	12 months	
13. PN/19/14	28-29	28-29	7	6	14	Yes	Yes	2 months	Yes/yes
14. TF/23/14	19-28[a]	28-30[b]	5	2	7	Yes	No	4 months	Yes/yes
15. SD/23/13	28	28	3	2	7	Yes	Yes	4 months	
16. CA/22/13	28-42[a]	28-42[c]	5	3	20	No	No	4 months	Yes/yes
17. CM/23/14	28-29	28-29	6	2	6	Yes	No	3 months	Yes/yes
18. CC/22/12	35	35	7	2	6	Yes	No	3 months	No/yes
19. RC/24/12	28-31[a]	28[b]	8	1	11	Yes	No	2 months - 12months	Yes/yes
20. FF/26/13	27-28	27-28	4	0	10	No	No	6 months	
21. SD/23/13	30-31	30-31	4	1	14	Yes	No	1 months	

a = irregular cycles (9 out of 21) before negative ions treatment. b = cases with regulation of the cylce lenght (8 out of 9) after ions therapy. c= cases with irregular cycles after NAIT. d* = presence of either endocrine imbalances at citovaginal examination (absolute or relative hiperfoliculinemia by progesterone failure, or hipofoliculinemia)/or organic substrate (uterine hiperanteversion-flexion or hiperretroversion-flexion) in case of gynecological exam; both exams were performed before NAIT.

Table 2. Number of symptoms and scores per case, before and after ionisation

Cases/age/menarche	No. of simptoms/score		Score reduction	No. of seances
	before	after		
1. PI/22/14	4/8	1/2	75%	8
2. PD/24/14	5/18 (4/4+1/2)	1/2	89%	9
3. BD/22/15	4/16	1/2	87%	12
4. PD/24/14	6/12	0	100%	12
5. DC/23/13	8/32	7/28	12,5%	9
6. PC/2214	5/20	4/8	60%	9
7. PM/2214	6/24	3/6	58.33%	4
8. TC/24/12	4/16	3/6	62.5%	5
9. SC/23/14	2/8	2/4	50%	14
10. DC/22/14	5/20	3/6	70%	6
11. CD/22/12	6/24	3/6	75%	12
12. SB/24/13	6/24	5/20	16.66%	10
13. PN/19/14	7/28	6/24	14.3%	14
14. TF/23/14	5/20	2/4	80%	7
15. SD/23/13	3/12	2/8	33.33%	7
16. CA/22/13	5/10	3/6	40%	20
17. CM/23/14	6/24	2/4	83.33%	6
18. CC/22/12	7/28	2/4	85.7%	6
19. RC/24/12	8/32	1/2	93.75%	11
20. FF/26/13	4/8	0	100%	10
21. SD/23/13	4/16	1/2	87.5%	14
TOTAL	110/400	52/146	65.42%	

It's also interesting to note how symptoms of discomfort evolved in intensity. Before AIT, 6 out of 21 cases showed no premenstrual pain, 7 cases have had moderate pain and 8 cases had intense pain. Of the 15 cases with premenstrual pain before treatment, in 7 cases (46.6%) pain completely disappeared, in 5 cases (33.3%) pain was attenuated, and for the other 3 cases (20%) pain remained uninfluenced, so, after negative AIT, the number of painless rose to 13, 6 cases had moderate pain, and intense pain has been accused of only 2 cases (Table no.4). Before AIT, 3 (14.3%) out of group of 21 cases did not have dysmenorrhea, 15 (71.4%) had intense pain, and 3 had moderate pain. In 6 (33.3%) out of 18 cases with dysmenorrhea before treatment, the pain completely disappeared, in 8 cases (44.4%) pain diminished and in 4 cases (22.2%) was unaffected.

After exposure to negative air ions, 9 cases (42.9%) have never had pelvic pain, 6 cases (28.6%) had moderate pain, and the remaining 6 cases had intense pain. The number of subjects with severe pain decreased to less than half (from 15 to 6 cases).

Reported to the entire lot, it can be noted that before treatment, out of the 21 cases studied, 5 persons did not present acne, in 9 cases the acne was broadcast and in 4 cases acne was discreet. In 7 (43.8%) out of 16 patients with acne before NAIT, acne disappeared, in 3 cases (18.8%) acne was discreet, and in 6 cases (37.5%) the acne was unaffected. After the sessions of air ionization, 12 out of the 21 cases have no longer acne, 8 cases have discreet acne, and only one case (4.8%) has diffuse acne.

Table 3. Subjective symptoms before and after air ionization as presented by students

Symptoms		Cases																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Premenstrual pain	Before		X		X	X	X	X	X		X	X	X	X	X			X	X		X	X
	After					X	X		X		X		X	X	X			X				
Dismen-norrhea	Before		X	X	X	X		X	X	X	X	X	X	X	X	X	X		X	X	X	X
	After		X			X		X	X	X	X	X	X	X	X	X						X
Irritability	Before	X	X	X		X		X			X	X	X	X	X		X	X	X	X	X	X
	After		X									X	X			X						
Acne	Before	X	X	X	X		X	X	X	X			X	X	X	X	X	X	X	X		
	After	X					X	X	X	X							X	X	X	X		
Breast congestion	Before	X		X	X	X	X					X				X		X	X			X
	After					X	X					X				X						
Bloating	Before	X			X	X	X	X			X	X	X								X	
	After					X	X	X					X									
Nausea	Before				X	X							X	X						X		
	After					X							X	X								
Edema	Before					X									X		X	X	X			
	After					X								X			X					
Asthenia	Before											X					X			X		
	After											X										
Diarrhea	Before					X	X															
	After					X																
Insomnia	Before		X															X				
	After																					
Loss of appetite	Before													X						X		
	After													X								
Vomiting	Before													X								
	After																					
Constipation	Before										X											
	After										X											
Drowsiness	Before																			X		
	After																					
Tenesmus	Before			X															X			
	After																					
Headache	Before																					
	After																		X			
TOTAL	Before	4	5	4	6	8	5	6	4	2	5	6	6	7	5	3	5	6	7	8	4	4
	After	1	1	1	0	7	4	3	3	2	3	3	5	6	2	2	3	2	2	1	0	1

It was noted also, that 16 out of 21 cases before air ionization showed premenstrual irritability, and after treatment, their number reduced to four. Of 16 cases with premenstrual irritability, 12 cases (75%) have had no longer irritability, in one case (6.25%) this symptom was attenuated and in 3 cases (18.75%) remained uninfluenced. Prior air ionization, 17 out of the 21 cases, was using medication. After air ionization, out of 17 cases using symptomatic medication, 12 (70.6%) persons no longer need symptomatics, and 5 cases (29.4%) have required the administration of symptomatic therapy; they still continued medication either as needed or as taking drugs preventive, for pain recurrence fear.

Table 4. Evolution of discomfort symptoms after the NAIT

Symptoms		Without % (n)	Moderate % (n)	Intense % (n)
Premenstrual pain	Before 71.4 (15)	28.6 (6)	33.3 (7)	38.1 (8)
	After 38.1 (8)	61.9 (13)*	28.6 (6)	9.5 (2)**
Dismenorhoea	Before 85.7 (18)	14.3 (3)	14.3 (3)	71.4 (15)
	After 57.1 (12)	42.8 (9)*	28.6 (6)	28.6 (6)**
Acne	Before 76.2 (16)	23.8 (5)	33.3 (7)	42.9 (9)
	After 42.8 (9)	57.1 (12)*	28 (8)	4.8 (1)**
Irritability	Before 76.2 (16)	24 (5)	76 (16)	
	After 19 (4)	81 (17)	19 (4)	
Medicines	Before	19.05 (4)	80.95 (17)	
	After	76.2 (16)	23.8 (5)	

*statistically significant differences using the chi^2 test ($p < 0.05$) between the results obtained before and after NAIT.

**statistically very significant differences using the chi^2 test ($p < 0.01$) between the results obtained before and after NAIT.

Prior exposure to air ions, of the 21 cases, 12 (57.14%) had regular cycles, and 9 (42.8%) had irregular periods. After the ions therapy, 20 cases (95.24%) had regular cycles and one single case (4.76%) had irregular cycle. In conclusion, regulation of menses was achieved in 8 (88.88%) out of the 9 cases ($p < 0.001$). Related to the duration of occurred improvement and remission after NAIT (in terms of subjective symptoms of discomfort, of giving up treatment or menses regulation), students being tracked as mentioned, one year after completion treatment, can be noted that premenstrual and menstrual pain has improved starting with the first menstrual cycle in 2 cases, since the next cycle in 3 cases, and this improvement lasted for 3 and 4 months in 4 cases. In most of the cases (7) pain relief lasted 6 and 6+ months. Acne was influenced by NAIT and improvement lasted for 2 months in 3 cases, for 3 and 4 months in 2 cases and 6 or 6+ months in 3 other cases. Premenstrual irritability was favorably influenced only the first cycle in two cases, for a period of 2 months in 4 cases, for 3 months in 2 cases and for a period of 6 or more than 6 months in 4 cases. Regulation of the menstrual cycle lasted for two months in one case, three months in one case, four months in 2 cases, and 6 or 6+ months in 4 cases, proving that the favorable effects of negative air ions might be persistent over time. Cyto-hormonal vaginal examination results show that 5 out of the 6 cases investigated proved endocrine imbalances: hiperfoliculinemia (absolute or relative by insufficient progesterone) or hipofoliculinemia, which justify the presence of dysmenorrhea. The gynecological examination showed the organic support (uterine hiper-anteversion-flexion or hiper-retroversion-flexion), present at all the 6 cases, which could explain the affective concomitances during the menstrual cycle.

Personality tests have revealed the presence of emotional instability and increased nervous reactivity in a considerable proportion, the results in both tests being significant and consistent. Eysenck questionnaire (Table no. 5) shows that emotional instability (characterized by emotional lability, difficulty in restoring sanity after emotional shocks, diffuse somatic disorders, anxiety) is present in 41% of subjects. These people can still adequately fit the demands of life, but at the cost of increased efforts. More than half of the subjects included in the study (59%), shows a high degree of integration and self-control, and is emotionally stable. The most commonly personality type of the study group is the introvert-emotionally stable (41%) followed by the type introvert-emotionally unstable (29%). Lowest frequency has extrovert-unstable type (18%) and those extrovert-stable (12%).

All the cases with emotional instability exhibited an increased nervous reactivity as showed the Maudsley questionnaire. One single subject, introverted emotionally stable, only reached a minimum (17 affirmative answers) indicating an increased nervous reactivity.

Symptoms revealed by Maudsley questionnaire show that most strongly influenced would seem to have been the nervous exhaustion declared by 8 subjects (47%) before negative ionization and by only 2 subjects (12%; $p < 0.001$) after air ionization; 13 subjects (76%) had dizziness, their number decreasing to 9 (53%) after negative air ions therapy. Mental instability and anxiety were improved in 2 subjects, the percentage dropping from 65% to 53% for mental lability and from 53% to 41% for anxiety. One single subject out of 3 never accused headache during treatment.

The other symptoms of nervous reactivity, although common in the investigated subjects (hiperemotivity in situations of stress, dissatisfaction, diarrheal episodes in tense situations, timidity and distrust itself), were unchanged during ions therapy, some additionally requiring psychotherapeutic approach.

Among vegetative disorders (Table no. 6) detected by the questionnaire, the largest proportion was presented by skin vaso-motor disorders (82%), indicating the presence of hiperemotivity in those subjects. All of those disorders completely disappeared after negative air exposure. The influence of negative air ions therapy on the other disorders was subjectively highlight by their tendency to reduce in 2 or 3 cases: acrohipotermia was recorded in 71% of cases (12 subjects) and improved under the influence of negative air ions in 3 cases, the percentage decreasing to 53%. The questionnaire revealed the existence, in a high percentage (47%), of fingers distal tremor; it's frequency decreasing after negative aeroionotherapy to 35%, thus improving in 2 cases.

Tachycardia, sweating palmar and plantar (hyperhidrosis), upper eyelid tremors were found in a lower percentage, these disorders having a tendency of reduction (in 1 or 2 cases) under the action of negative ionization.

Components of proper energy fund of neuropsychic tonus (the feeling of fullness forces – A factor, and vivacity-freshness – B factor) had a clear upward trend during treatment, from an average of 57 points on the first day of treatment (A factor), and 63 points (B factor), to 83 and 86 points respectively on the last day of treatment (Table no.7).

Table 5. Distribution of personality traits revealed by Eysenck test

Trait	n	%	Trait	n	%
Emotional unstables	7	41	Introversion	12	70
Emotional stables	10	59	Extraversion	5	30

Table 6. Frequency of vegetative disorders

Symptoms	Before NAIT (%)	After NAIT (%)	Reductions %
1.Skin vaso-motor disorders	82	0	100
2. Acrohipotermia	71	53	25,4
3. Fingers distal tremor	47	35	25.6
4. Tachycardia	29	12	58.7
5. Palmar and plantar hyperhidrosis	24	18	25
6. Upper eyelid tremors	18	12	33.33

Table 7. The trend of neuropsichic tonus during the treatment

Days Components	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A. Feeling of fullness forces	57	67	55	65	75	73	79	73	82	69	83	81	78	83
B. Feeling of vivacity - freshness	63	68	67	62	68	70	76	77	79	74	78	81	77	86
C. Affective tonus	59	70	65	70	72	74	76	78	80	75	78	80	77	90
D. Feeling of physical wellbeing	83	75	70	74	80	67	77	75	78	65	70	75	77	79
E. Self-confidence	80	82	75	80	85	83	88	80	90	75	82	90	85	92

Table 8. Quantitative yield at mental exercise test before (x_1) and after (x_2) negative air ionization

PHASE	$x_1 \pm SD_1$	$x_2 \pm SD_2$	t Student	p value
I. 4 min	$130,88 \pm 50,05^a$	$175 \pm 66,86^a$	2,17	< 0,05
II. 8 min	$123,35 \pm 35,09$	$151,23 \pm 55,42$	1.74	NS
III. 12 min	$135,47 \pm 44,04$	$144,64 \pm 43,74$	0,60	NS
IV. 16 min	$103,17 \pm 41,27$	$77,35 \pm 56,21$	1,52	NS
TOTAL 16 min	$492,88 \pm 99,87$	$548,29 \pm 96,59$	1,64	NS

*SD = standard deviation before (SD_1) and after (SD_2) NAIT.
a) statistically significant differences using the "t" Student test ($p<0.05$) between the results before (x_1) and after (x_2) NAIT.
NS = not-significant.

An upward trend showed also the affective tonus (C factor): mood, appetite for life, appetite for work, from the average of 59 points in the first day at 90 points on the last day of treatment. Less marked was the increase in the sense of physical wellbeing (D factor) and self-confidence (E factor), which ranged from the beginning to the higher levels of the scale (83-80 points respectively). We emphasize that for all the five factors, in the last two days of therapy, chart had an upward appearance. Quantitative yield at mental exercise test (Table no. 8) showed an increasing trend under the influence of negative ionization, statistically significant only at 4 minutes of work (first stage).

Table 9. Qualitative yield at mental exercise test before (x_1) and after (x_2) negative air ionization

PHASE	$x_1 \pm SD_1$	$x_2 \pm SD_2$	t Student	p value
I. 4 min	$2,47 \pm 2,87^a$	$0,76 \pm 0,96^a$	2,32	≈ 0,02
II. 8 min	$1,52 \pm 1,80$	$1,29 \pm 1,35$	0,42	NS
III. 12 min	$2,11 \pm 2,23$	$1,70 \pm 1,85$	0,58	NS
IV. 16 min	$1,58 \pm 1,50$	$1,35 \pm 2,17$	0,35	NS
TOTAL 16 min	$7,70 \pm 6,18$	$5,11 \pm 4,35$	1,41	NS

*SD = standard deviation before (SD_1) and after (SD_2) NAIT.
a) statistically significant differences using the "t" Student test ($p < 0.02$) between the results before (x_1) and after (x_2) NAIT.
NS = non-significant.

The smaller average of the 4th stage (16 minutes) after NAIT ($x_2 = 77.35$) is explained by the fact that most subjects performed most of the calculations in the first 3 stages of work.

And in terms of quality (Table no. 9) the mental exercise test showed a trend towards improvement of the results, also statistically significant at the first stage of work (after 4 minutes).

4.4. Conclusion

Based on the results obtained by applying negative air ions on a group of students, some conclusions could be highlighted:

1. After negative ionization all the 21 cases investigated showed favorable results, none has a total failure.
2. Premenstrual pain gave up in half of the subjects, and in third cases was much attenuated.
3. Dysmenorrhea gave up in 1/3 of cases and in almost half of them slowed.
4. Acne has not appeared or has been very discreet in more than half of the cases.
5. Premenstrual irritability disappeared in 75% of studied subjects.
6. Out of 17 cases using symptomatic medication before air ionization, only 5 cases have left after treatment; the remaining 12 cases need no longer medication.
7. Setting the interval between two menstrual cycles occurred in 8 out of 9 cases with irregular cycle.
8. Favorable effects of negative air ionization on presented disorders, were maintained in most cases, more than the duration of treatment, at some students persisted about a year.
9. About half of the subjects investigated shows emotional instability and increased nervous reactivity with trends of improvement under the influence of negative air ionization. This improvement is most obvious in the case of symptoms such as nervous exhaustion, dizziness, neuro-psychic lability, anxiety.

10. Except for skin vaso-motor disorders, diagnosed subjective symptoms tended to reduce (subjectively related) during treatment, more pronounced in the case of acrohipotermia and tachycardia.
11. Neuro-psihic tonus registered a growing trend, more pronounced in case of proper energetic support and in his affective component.
12. The yeald of mental activity with sustained attention request was even better as the number of sessions was higher, something that is required to be verified in the future.
13. Although, due to the small number of cases, the results presented in this chapter should be regarded as indicative, they sustain the beneficial effect of negative air ions on symptoms of discomfort around menstruation.

Conclusion

There is hardly any other element of the physical environment which has caused so much confusion as ionized air and which created so much controversies. Despite the over 100 years of study, the knowledge on ion specter is still in an embryonic stage. All of the quoted effects in this chapter were obtained with low-medium concentration of negative air ions per cm^3 (17,000) at the distance of 70-80 cm, in daily applications for 4 to 20 days. The proper dosages are still not fully established. Most of the researchers have achieved positive results with very high doses of negative ions (Ryushi T., et al., 1998; Terman M., et al., 1998, Terman M., and Terman J.S, 2006), and after a prolonged exposure to negative ions, while others obtained the same improvements after the very first treatment. The minimal number of daily recommended treatments is 10 or more, depending on the condition of the patient. In other situations, when patients receive other forms of treatment alongside (mineral or sea water bath and physical therapy), NAIT is limited to every other day. Ill people respond faster than healthy individuals to the negative ions. The explanation could be that the disease is a kind of deficiency, an imbalance in the bioelectric energy of the body, which may be recovered under the influence of negative air ions (Beasley V.R., 1975).

Several factors in the area of negative air ionization can wrap the entire field of negative ionization in an aura of uncertainty and ambiguity: there isn't a standard procedure; there were used ions of both polarity (or, positive ions are deleterious for health); studies were very heterogeneous; sometimes the doses were very high; the exposure was sometimes too long; some devices were sold without emitting ions or emitting O_3 or NOx; we do not know the maximum and minimum doses of a favorable action and the interaction with other factors; physical parameters of the air (temperature and relative humidity) were not monitored; given that experimental subjects were not grounded, their external surfaces developed high electrostatic charges and in consequence, repelled ions (Fletcher L.A., et al., 2007; Hawkins L.H., 1985).

In a study on chicken eggs (Laza V., and Bolboaca S., 2008) we have tried to elucidate some methodological issues related to ions density, the optimal duration of application, and the best time of the ontogenetic development where AIT could be used. The preliminary results prove that neither excessive doses nor prolonged therapy is beneficial for hen chicks development.

Clinicians trying to evaluate the ions as a therapeutic method are responsible for all or some of the errors, and most of all, have neglected to use the double blind cross over technique for negative ions applications (Goel N., et al., 2005). Therefore, clear evidence of the role of negative ions as physiological mediators or as therapeutic factor, has been slow to emerge and hardly found. Moreover, the effects of NAI therapy depend on many individual factors like individual tolerance, resistance/sensitivity to the atmospheric electricity, age (the very young, and the old are more sensitive), health status (those most severely stressed and those with compromised immune system), and sex (female are more responsive than males to negative-ion depletion or enrichment). There are few contraindications for negative ions therapy: bronchial asthma associated to emphysema, severe cardiac failure, hypertension, cardio-vascular diseases, and nasal mucous alteration (Hawkins L., and Barker T., 1978).

In conclusion, it can be said that moderate doses of negative air ions seem to be indicated in a large specter of situations. They are not a universal panacea, but they could be considered a physiological factor, very cheap, with no side effects, not addictive, and which do not bring any foreign substance inside the organism (Khan M.A., et al., 2006).

However, much work remains to be done before effective minimum and maximum dosages (i.e., number of ions per cm^3 of air in a given period of time) are finally determined. Until then, any attempt to make ion generators directly accessible to the lay public without previous tests must be discouraged (Wakamura T., et al., 2007).

In the future, each unit should clearly state the density and quality of ions produced, and the public must be warned not to exceed the daily optimum of the inhalation prescribed. The increased tempo in research and widening of the circle of investigators, plus constructive criticism, should soon produce answers not yet available.

The main limitations of the results presented in this chapter were the small number of cases, which does not allow generalization of results; were studied only students whom worked with in classes and practical work (as mentioned, students volunteered, did not receive any remuneration for participation in the study), age between 19 and 26 years, not-pregnant, sexually-active. Therefore, future research should include a larger number of subjects (adequate group size) in several age groups, professional affiliations, and framing group "premenstrual syndrome" should be made based on more accurate and more elaborate criteria. Yet, based on the evidence surveyed in this chapter, and considering the advantages, it appears that NAI therapy could be a potentially effective biologic factor, which, if properly harnessed, controlled, and utilized, may become a valuable adjunct to other forms of therapy. As more information is acquired about the mechanisms, doses, and environmental factors which could interfere with the action of negative air ions, people should be able to appreciate more clearly the importance of NAI in nature and assess their potential for therapeutic and prophylactic applications.

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Chapter 3

Neutralization-Enhancing RF Antibodies

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Abstract

Monomeric specific high-affinity IgG antibodies arising during infection process have low avidity to multimeric structures of the pathogen. Moreover, in the case of viruses such antibodies may sometimes enhance FcR-mediated infection of monocytes.

Multistage generation of antibody complexes with high affinity and avidity are important for efficient neutralization of the pathogen. Multimeric IgM or IgA rheumatoid factor (RF) antibodies may specifically bind Fc parts of IgG molecules complexed with pathogen and thus enhance the neutralization activity of pathogen-specific monomeric IgG. Such neutralization-enhancing RF antibodies amplify IgG response via increased aggregation and clearance of antibody-pathogen immune complexes.

Neutralization-enhancing RF antibodies (NeRFa) might be induced via prolonged repeated immunization with protein antigens. Induction of NeRFa would be an important task for creating effective therapeutic and prophylactic vaccines against HIV, malaria, Ebola and other pathogens. Single-administration vaccine technology with pulsatile release of protein antigens from biodegradable polymeric microspheres opens new possibilities to achieve such a goal.

The idea that specific induction of NeRFa can be a universal approach to eradicate any pathogen, including cancer-associated viruses, will be put forward in this review article.

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Introduction

Antibodies play a central role in immune response against pathogens. However, pathogens prepared a surprise for the researchers. There is a constant race between mutating pathogen and mutating antibodies. What to do with a virus if its envelope glycoprotein mutates via the same mechanism as do antibodies (Suslov, 2004)?

Therapeutic efficacy of naturally appearing neutralizing antibodies (NAbs) against viruses can be very high (Yu et al., 2008). Antibodies may differ in neutralizing activity and may be not neutralizing at all. Could we also imagine that antibodies may not neutralize, but instead increase infectivity of the virus (Takeda et al., 1988)? Moreover, the preliminary presence of antibodies may promote "early death" of the organism after subsequent challenge with an infectious agent (Barrett & Gould, 1986; Gould et al., 1987; Prabhakar & Nathanson, 1981; Weiss & Scott, 1981). Finally, what will be the benefit from the vaccination if it may cause "early death" phenomenon (Blancou et al., 1980)?

These questions imply that we may underestimate the power of antibodies. The other question is how to direct this power against the pathogen? There is still no effective vaccine against HIV. There is still no cure for cancer. Because the mutational diversity of HIV is so high (Klinman et al., 1991), it is very difficult to obtain antibodies able to simultaneously neutralize a broad range of viral isolates. The existence of antibody-dependent enhancement (ADE) of viral infection (Takeda et al., 1988) with consequences for HIV pathogenesis (Homsy et al., 1990) makes the development of an effective HIV vaccine based on induction of neutralizing antibodies even more problematic. All these aspects favor "back to basics" approach, when we need to make several steps back to understand better the virus, its pathogenesis and interaction with antibodies for the future benefits for vaccine design.

In this chapter, the action of neutralization-enhancing RF antibodies (NeRFa) (Suslov, 2014) and their potential for vaccines against pathogens will be reviewed. NeRFa can both enhance the potential of neutralizing antibodies and convert the function of infection-enhancing antibodies into neutralizing antibodies. Induction of NeRFa not only holds the potential to improve the efficacy of both prophylactic and therapeutic vaccines against different pathogens, but also is promising for spontaneous regression of cancer by means of eradication of cancer-associated viruses along with cancer tissue.

There will be also a short place in this review for the reader to take a "coffee break" and to think about antibodies, HIV and cancer from the evolutionary point of view.

Pathogens and Antibodies

Antibodies protect the host organisms from invading pathogens. Antibodies bind surface antigens of the pathogen to halt its pathogenic activity via different neutralization mechanisms (Crowe et al., 2001; Dowd & Pierson, 2011; Reading & Dimmock, 2007). High affinity binding correlates with better neutralization capacities of the antibodies (Dowd & Pierson, 2011; Torán et al., 1999; Yu et al., 2008). The increase in the valency of antibodies automatically increases their "functional affinity" (avidity) and neutralization activity (Deyev & Lebedenko, 2008; Wolbank et al., 2003)

IgM antibodies, appearing as an early response to the foreign pathogen, have low affinity. This effect is partially compensated by the high avidity of IgM antibodies, which are pentameric and have 10 antigen-binding sites (Crowe et al., 2001). During the immune response to the infectious agent, antibodies undergo affinity maturation and isotype switching processes (Victora & Nussenzweig, 2012). As a result, high affinity IgG antibodies appearing later have low avidity to multivalent structures of the pathogen, because IgG is monomeric with just two antigen-binding sites. Thus, low avidity of IgG is the first problem of these antibodies pretending to be the main players in neutralization of foreign pathogens.

Can one antibody molecule have both high affinity and avidity? Dendritic antibody supramolecules (DAS) contain pentameric IgM immunoglobulin, carrying ten IgG immunoglobulins (Harada et al., 2003). DAS molecules possess both high affinity and avidity. For example, DAS with IgM and ten anti-gp120 IgG immunoglobulins might be constructed and tested for anti-HIV neutralizing activity (Suslov, 2014).

Antibody-dependent enhancement (ADE) is the second problem of IgG antibodies, which arises from the fact that these antibodies may enhance infection of cells by viruses through FcR-mediated mechanism (Peiris et al., 1981; Takada & Kawaoka, 2003). ADE plays a key role for pathogens like dengue virus (Flipse et al., 2013; Guzman et al., 2013), West Nile virus (Diamond et al., 2008), yellow fever virus (Barrett & Gould, 1986), rabies virus (Prabhakar & Nathanson, 1981; King et al., 1984), HIV (Homsy et al., 1990; Takeda et al., 1988) and other viruses (Phillpotts et al., 1985; Takada & Kawaoka, 2003; Weiss & Scott, 1981). This implies the paradoxical suggestion that viruses can exploit antibodies for their own benefit (Suslov, 2014).The power of antibodies should not be underestimated. Sometimes antibodies can even cause death, according to brilliant research reported in one-page article (Prabhakar & Nathanson, 1981). This “early death” phenomenon can even appear after prophylactic vaccination of both animals and humans (Blancou et al., 1980; Gould et al., 1987). What would be the solution to prevent the ADE process? The answer would be to “close” or to “cover” the Fc-part by something that will not allow the Fc-FcR interaction between corresponding antibody and receptor on the cell to take place.

Both “problems” of IgG, i.e., low avidity and ADE, can be solved with an aid of rheumatoid factor (RF) antibodies, which enhance neutralization of the pathogen (Suslov, 2014).

Neutralization-Enhancing RF Antibodies

Rheumatoid factors (RFs) are autoantibodies, which bind constant (Fc) part of the immunoglobulin G molecule (Chen et al., 1988; Nemazee, 1985; Tarkowski et al., 1985). Along with anti-idiotypic antibodies, RFs represent a type of anti-immunoglobulins, which may play a beneficial physiological role in the immune system (Almeida & Griffith, 1980; Carson et al., 1981; Chen et al., 1988; Fehr et al., 1997; Nemazee & Sato, 1983; Tarkowski et al., 1985). Term “rheumatoid factor” is rather a historically fixed name for the anti-immunoglobulins produced in rheumatoid arthritis (RA). Surprisingly, RFs were originally discovered in nonrheumatoid states (Bartfeld, 1969).

RFs can be divided into two groups: pathological and physiological (Carson et al., 1981; Thompson, et al., 1994; Van Esch et al., 2001). Alternatively, RFs can be called pathologic

and nonpathologic (“natural”) rheumatoid factors (Stewart et al., 1997). Pathological RFs represent hypermutated high-affinity antibodies of IgG isotype continuously produced and able to self-associate with further formation of complexes leading to pathologic events such as local inflammation and vascularization of the joint in autoimmune disease rheumatoid arthritis (Natvig & Munthe, 1975; Pope et al., 1975; Stewart et al., 1997). The presence of IgG RF and IgA RF, but not IgM RF was correlated with disease activity in RA (Silvestris et al., 1985). In rheumatoid arthritis, IgM RF can bind IgG-IgG aggregates for subsequent formation of large immune complexes able to fix complement (Carson et al., 1987). Physiological RFs are low-affinity multimeric antibodies of IgM or IgA isotype transiently appearing as a part of an immune response during the acute phase of infection and leaving no adverse effects to the host (Almeida & Griffith, 1980; Jarvis et al., 1993; Tarkowski et al., 1985; Van Esch et al., 2001). It has also been suggested to use the term “rheumatoid factor” only for pathological RFs in rheumatoid arthritis and to use the term “infection related antiglobulin”(IRAG) for antiglobulins produced during normal physiological response to infection (Almeida & Griffith, 1980).

Binding of the pathogen by antibody (Ab) may sometimes directly lead to neutralization. In the ADE process, antibodies may enhance infectivity of the virus through the formation of infectious virus-Ab complexes. Rheumatoid factor antibodies will then be able to neutralize these complexes. The resulting process can be summed up by the following scheme (Daniels, 1975):

Virus + Ab → infectious Virus-Ab complex ("sensitized" virus)
Infectious Virus-Ab complex + RF → Neutralized Virus

The first stage in this scheme represents the so-called “sensitization” stage, when infectious virus-antibody complex is formed (Notkins et al., 1966). At the second stage, sensitized virus will be neutralized by rheumatoid factor (anti-gamma-globulin) (Coutelier & Van Snick, 1988; Notkins et al., 1966) with a possible role of complement (Ashe et al., 1971).

From the classical point of view, the interaction between the pathogen and an antibody might be enough for neutralization of the former. In the reality, as we can see from the fundamental work by Prof.A.L.Notkins and colleagues, multistage generation of antibody complexes possessing both high affinity and avidity for the foreign pathogen can be required for neutralization. IgG antibodies, directly binding the pathogen, constitute the first order antibodies. Second order antibodies (IgM, IgA RF antibodies) bind Fc-parts of the first order antibodies complexed with pathogen. In turn, other antibodies and complement molecules can represent the third and further orders, if they exist.

Second order antibodies (multimeric IgM and IgA RFs) can enhance neutralizing properties of the first order antibodies (monomeric IgG). Therefore, as for terminology in analogy to infection-enhancing antibodies (Montefiori et al., 1990; Takeda et al., 1992), and taking into account the discovery of the "enhancing"(strengthening Ab-antigen interaction) properties of RFs (Nemazee & Sato, 1982), the second order antibodies can be called neutralization-enhancing RF antibodies (NeRFa) (Suslov, 2014). Despite the apparent distinction between neutralizing and infection-enhancing antibodies (Takeda et al., 1992), NeRFa are expected both to enhance the potential of neutralizing antibodies and to transform infection-enhancing antibodies into neutralizing.

Multistage generation of immune complexes containing pathogen, first order antibodies, second order antibodies (NeRFa), third order antibodies, complement molecules etc. is a sophisticated process in which every element plays a role. First order antibodies (monomeric high-affinity IgG) provide specificity for binding a particular pathogen and each contribute locally to the further overall avidity of the whole antibody construction. Second order antibodies (multimeric IgM and IgA RFs) give the whole construct high avidity (Harada et al., 2003), further enhance Ab-antigen interaction (Nemazee & Sato, 1982), favor efficient aggregation of viral particles (Almeida & Griffith, 1980), increase the sedimentation rate and clearance of the formed immune complexes (ICs) (Van Snick et al., 1978). NeRFa may efficiently amplify the specific immune response mediated by the first order antibodies even in the case of low amount of IgG distributed over the pathogen's surface (Almeida & Griffith, 1980; Carson et al., 1981; Clarkson & Mellow, 1981; Dresser & Popham, 1976). Third order antibodies, complement and other molecules even more enhance the key effect mediated by the second order antibodies (NeRFa).

RFs can be induced in various infectious processes as a part of an immune response to a particular pathogen (Bartfeld, 1969; Clarkson & Mellow, 1981; Dresner & Trombly, 1959; Greenwood et al., 1971; Hook et al., 2003; Houba & Allison, 1966; Kawabata et al., 1984; Langenhuysen, 1971; Lehman et al., 1972; Svec & Dingle, 1965; Tomaras et al., 2008; Williams & Kunkel, 1962). RFs efficiently mediate neutralization of infectious virus-antibody complexes (Ashe et al., 1971; Coutelier & Van Snick, 1988; Gipson et al., 1974), enhance neutralization of viruses (Douvas et al., 1996) and protozoa (Clarkson & Mellow, 1981; Green & Packer, 1984; Hook et al., 2003; Stuart & Green, 1990).

The level of RF is elevated during pregnancy and postnatal period (Clarkson and Mellow, 1981; Meurman et al., 1978; Pope et al., 1982). The function of RF in such a vulnerable period of life might be the efficient maternal protection of the babies against infection (Clarkson and Mellow, 1981). The mechanisms, by which RF is induced at pregnancy and lactation, represent a special interest and yet to be discovered (Clarkson and Mellow, 1981).

Secondary immunization with various antigens or immunization with immune complexes may lead to the induction of RF (Nemazee & Sato, 1983; Nemazee, 1985; Van Snick & Coulie, 1983). RFs can be induced as a result of vaccination (Aho et al., 1962; Almeida & Griffith, 1980; Svec & Dingle, 1965; Welch et al., 1983). Rheumatoid factors (IgM RFs) display high specificity to the new antigenic determinants created in Fc-part of IgG upon IgG-antigen complex formation and able to "enhance" IgG-antigen interactions (Nemazee & Sato, 1982).

Rheumatoid factors (Table 1) can be induced after single immunization of animals with bacteria and may enter into idiotypic network as antibodies bearing an internal image of the Fc-binding part of bacterial Fc-binding proteins (Carson et al., 1987; Johnson et al., 1985; Johnson & Smalley, 1988; Nardella et al., 1987; Oppliger et al., 1987; Williams et al., 1972).

Vaccines Based on Induction of NeRFa

Most of the vaccines against HIV failed (Hu & Ho, 2013) and only one clinical trial based on prime-boost approach showed partial protection with an efficacy of 31% (Montefiori et al., 2012). What might be the reason for such failures? The answer is antibody-dependent

enhancement (ADE) of viral infection (Homsy et al., 1990; Takeda et al., 1988). Induction of neutralizing antibodies (NAbs) against HIV gp120 envelope glycoprotein is a promising approach, but this may not be enough to stop the virus. Indeed, viral particles may already be covered by infection-enhancing antibodies, which determine disease progression to AIDS (Homsy et al., 1990). Appearance of neutralizing antibodies might not change the situation. Simultaneously, HIV particles covered by infection-enhancing antibodies represent "sensitized" virus, which can be neutralized by NeRFa with possible subsequent involvement of complement. NeRFa will also enhance neutralization of viral particles covered by NAbs.

Although multimeric IgM and IgA RFs are detected in some of the HIV-infected patients (Jackson et al., 1988; Jarvis et al., 1993; Jendis et al., 1988; Procaccia et al., 1987; Tomaras et al., 2008), the level of RF during acute phase of infection is unstable with a tendency towards a decrease (Tomaras et al., 2008). The question is how to maintain the level of RFs constantly high?

Induction of NeRFa during HIV vaccination could be achieved via prolonged repeated immunization (e.g., vaccinations every 2-4 weeks depending on the level of induced RF in patients) with gp120 glycoprotein (Suslov, 2014). The approach can be tested on animal models. Single-administration vaccine (SAV) technology (Cleland et al., 1998; Cleland, 1999) allows repeated immunizations to be performed in one shot via pulsatile release of the therapeutic protein from biodegradable polymeric microspheres.

The scheme for the patient-specific therapeutic HIV vaccine (Suslov, 2014) will be as follows:

(i) The only HIV variant formed after the process of HIV population homogenization (Learn et al., 2002) at the beginning of the asymptomatic period will be isolated from the patient. HIV *env* gene will be sequenced, amplified and expressed. Corresponding gp120 will be purified, according to the methodology (Jones et al., 1995).
(ii) Resulting therapeutic gp120 glycoprotein will be used for prolonged pulsatile release from biodegradable polymeric microspheres every 3 weeks under SAV technology platform (Cleland et al., 1998; Cleland, 1999).

The scheme for the prophylactic HIV vaccine (Suslov, 2014) will be the following:

(i) Several circulating strains of HIV, which are the most representative in the particular geographical region, will be isolated. The set of corresponding HIV *env* genes will be sequenced, amplified and expressed. Gp120 glycoproteins will be purified, according to the methodology (Jones et al., 1995).
(ii) Resulting therapeutic gp120 glycoproteins will be released in a sequential way (Klinman et al., 1991) from biodegradable polymeric microspheres every 3 weeks for prolonged period under SAV technology platform (Cleland et al., 1998; Cleland, 1999).

Germinal centers of the secondary lymphoid organs represent a major reservoir for HIV persistence during the long asymptomatic period (Fox et al., 1991; Pantaleo et al., 1993; Tenner-Rácz et al., 1989). Within germinal centers, HIV particles covered by antibodies bind fragments of C3 complement for attachment to complement receptors on follicular dendritic

cells (Bánki et al., 2005; Bánki et al., 2006; Kacani et al., 2000; Stoiber et al., 2005). Intriguingly, IgM RF may prevent attachment of C3 to aggregated human IgG bound to cells (Bolton et al., 1982). Thus, the induction of NeRFa in patient-specific therapeutic HIV vaccines may hold potential not only to neutralize the free virus, but also to completely detach viral particles from follicular dendritic cells. In this case, germinal centers reservoir might be cleared of HIV particles, which then will be neutralized by NeRFa.

Induction of NeRFa might be a perspective method against different pathogens, including *Plasmodium falciparum* (Green & Packer, 1984; Hook et al., 2003; Stuart & Green, 1990) and Ebola virus (Wong et al., 2014). Chain-like multistage formation of antibody complexes with a key role of NeRFa might in the future allow researchers to perform neutralization process of any pathogen in a rational way.

Evolutionary Line: tRNA–prions–Viruses–Antibodies–HIV–Cancer–Human of the Future

During this short "coffee break," let us have a look to the viruses, antibodies, cancer and HIV in the context of the Evolution.

According to Virus-Prion theory (Suslov, 2005), viruses are composed of prion-like proteins with a nucleic acid, which encodes them. Virus is a powerful "machine" due to prion-like properties of its proteins. At some point of the evolution, prions or prion-like proteins from the Prion-like World could have assembled into viruses (Suslov, 2005). Bioinformatics analysis of viruses (Suslov, 2007a) and functional studies of viral proteins (Lee et al., 2006) further support Virus-Prion theory. Normal cellular prions may control the expression of the endogenous retroviruses during the immune response (Lötscher et al., 2007). Intracellular prion-like proteins may participate in innate immune response to viruses (Hou et al., 2011). Morphology of the extracellular viral particles may change from the shape of the lemon to the particles with very long tails (Häring et al., 2005). It remains to be determined whether such a formation of viral fibrils involves prion-like transitions in viral proteins (Suslov, 2007b).

Analysis of sequence alignments suggests that the presence of tRNA-like elements encoded in the genes is a "hallmark" of prion-like properties of corresponding proteins (Suslov, 2007a). This concept is very compatible with "genomic tag hypothesis" (Maizels et al., 1999; Weiner & Maizels, 1999) and presence of tRNA-like elements in the viruses (Niller et al., 2004). It follows from this that tRNAs are probably among the most important elements, which in the ancient RNA World gave birth to the viral-like elements, prions, viruses, antibodies etc. Intriguingly, loop structures of the tRNA-like EBERs in EBV virus RNA genome contain the same somatic hypermutation signature motifs (RGYW) as do immunoglobulin genes (Niller et al., 2004).

Antibodies have a function to protect the organism from invading pathogens, which in turn include viruses, bacteria, protozoa etc. Nevertheless, the virus such as HIV may use complex mechanisms to avoid or even exploit antibodies. Interestingly, HIV might use the same mechanism (i.e., AID-mediated somatic hypermutation) for mutation as do antibodies (Suslov, 2004; Suslov, 2010). Recent experimental data (Balin et al., 2008) support this view.

Another aspect is antibody-dependent enhancement (ADE) of HIV infection (Takeda et al., 1988; Homsy et al., 1990). In other words, HIV with its antibody-like gp120 glycoprotein both mutates like antibodies and exploits antibodies to infect the cell.

What is the origin of the process, which diversifies antibodies? The similarities between RAG1/RAG2-mediated V(D)J-recombination process and retroviral integration is striking (Agrawal et al., 1998; Brandt & Roth, 2004; Dreyfus et al., 1999; Fugmann et al., 2000; Jones & Gellert, 2004; van Gent et al., 1996; Zhou et al., 2004). Is it possible that contemporary (V), (D) and (J) segments in immunoglobulin genes are the remnants of the ancient retroviruses, which at some point of evolution assembled together to create an adaptive immune system? Does it mean that long time ago retroviruses jumped into the genome and gathered into effective system which can protect the host from other viruses? Can adaptive immune system have a viral origin? Could an ancient immune system be a virus? Pentameric IgM might have been a part of the envelope of the ancient "immune virus" (Suslov, 2005).

Human immunodeficiency virus (HIV) could be an element of the ancient immune system. Indeed, HIV envelope glycoproteins gp120 and gp41 have multiple sequence homologies with members (HLA I, HLA II, CD4, TCR V regions, IgV regions) of immunoglobulin superfamily (Metlas & Veljković, 1995; Metlas et al., 1995; Silvestris et al., 1995). From the schematic representation of HIV gp120 in the article (Silvestris et al., 1995) we can see that viral protein looks like an "ancient antibody" with the most variable region (V3 loop) located on the top and with conserved regions homologous to CH1 of IgG2/ IgA2 on the bottom ends of the molecule. Two immunoglobulin recombinant elements in the HIV-1 *env* gene are located apart from each other on the same distance as in immunoglobulin gene (Veljković & Metlas, 1992; Metlas et al., 1991). Finally, all current experimental data are consistent with the hypothesis that AID enzyme may mutate HIV *env* gene (Suslov, 2004) in the same way as immunoglobulin genes.

There are some possibilities how HIV, a possible remnant of an ancient immune system, became activated in the contemporary human immune system (Suslov, 2005). In contrast, HIV may be not an element of the ancient immune system, but instead may represent a part of the more advanced immune system of the Human of the Future. Indeed, HIV gp120 glycoprotein includes many of the elements of the contemporary immune system, including parts of HLA, TCR, IgV etc (Silvestris et al., 1995). HIV is able to replicate and kill the cells, but does not demonstrate any potential to recognize or kill pathogens. Antibodies are the key elements of the contemporary immune system that are not able to replicate, infect or kill the cells.

Human placenta, autoimmune disorders, cancer and AIDS can be viewed in terms of expression of HIV antigens, as reviewed in (Suslov, 2005). HIV-like virus is responsible for cancers of the reproductive system and the breast cancer (Rakowicz-Szulczynska et al., 1998). Outstanding articles (Logothetou-Rella, 1993; Logothetou-Rella, 1994; Logothetou-Rella, 1995; Logothetou-Rella, 1996a; Logothetou-Rella, 1996b) by Prof.H.Logothetou-Rella about "nuclear vlimata" and its role in malignancy represent an invaluable experimental material for understanding the nature of cancer. Embryo–like growth has been discovered in hematological malignancies (Logothetou-Rella, 1996b). Surprisingly, both embryonic morphogenesis and tumor metastasis are regulated by the same transcription factor Twist (Yang et al., 2004). Can we expect that something real to be born in human malignancies? Does it mean that cancer in humans may represent an unsuccessful attempt of the Evolution to give birth to the new human organism - Human of the Future? Can HIV be a part of the

advanced immune system of the Human of the Future? Intriguingly, both HIV and cancer metastases intensively exploit the same CXCR4/SDF-1alpha axis (Hirbe et al., 2010; Otsuka & Bebb, 2008; Wang et al., 2009; Zhang et al., 2007).

Table 1. Functions and therapeutic potential of RF antibodies in the organism

Functions and potential of RFs	References
RFs and nonrheumatoid states	Bartfeld, 1969
Pathological RFs and rheumatoid arthritis	Carson et al., 1987; Natvig & Munthe,1975; Pope et al.,1975; Stewart et al., 1997
Physiological RF and defense against infection	Almeida & Griffith, 1980; Carson et al., 1981; Jarvis et al., 1993;Tarkowski et al., 1985; Van Esch et al., 2001
Kinetics of RF induction	Nemazee & Sato, 1983; Nemazee, 1985; Van Snick & Coulie, 1983
Immune networks and RFs	Carson et al., 1987; Johnson et al., 1985; Johnson & Smalley, 1988; Nardella et al., 1987; Oppliger et al., 1987; Williams et al., 1972
RFs strengthen Ab-antigen interactions	Nemazee & Sato, 1982
RFs and clearance of immune complexes	Van Snick et al., 1978
RFs and aggregation of viral particles	Almeida & Griffith, 1980
RF-mediated neutralization of sensitized virus	Ashe et al., 1971; Coutelier & Van Snick, 1988; Daniels, 1975; Gipson et al., 1974; Notkins et al., 1966
RF enhances neutralization of HIV	Douvas et al., 1996
RF and enhanced clearance of *Plasmodium falciparum*	Green & Packer, 1984; Hook et al., 2003; Stuart & Green, 1990
RFs and maternal protection of babies from infection	Clarkson and Mellow, 1981
Elevation of RF during pregnancy and lactation period	Clarkson and Mellow, 1981; Meurman et al., 1978; Pope et al., 1982
Induction of RF after vaccination	Aho et al., 1962; Almeida & Griffith, 1980; Svec & Dingle, 1965; Welch et al.,1983
RF and incidence of warts: inverse correlation	Johansson et al., 1977
RF positive plasma promotes complement-dependent cytotoxicity against cancer cells	Twomey et al., 1978
IgM RF level and better survival from cancer	Jónsson et al., 1992
Induction of NeRFa and HIV vaccines	Suslov, 2014
Induction of NeRFa for cancer regression	Suslov, 2015 *

*This chapter

Cancer cells in the particular cellular "context" may become normal cells (Illmensee & Mintz, 1976; Martin, 1980; Mintz & Illmensee, 1975; Rosen, 1981). The Human of the Future will be composed of normal cells. Now, at the current stage of the Evolution, the human Soma presumably does not provide an optimal "context" for the new Organism to be born. The cancer and metastases are the parts of the new Organism, which dies. Probably it would not die, but the human Soma dies from incompatibility with the new "baby." The Evolution is doing experiments on human beings. The Evolution tries to create something perfect, even by the cost of the human being.

How the Human of the Future will look like? Nobody knows… Probably it will be an immortal Organism with high regenerative potential of all parts of the body for the unlimited ability to acquire different shapes and forms. However, this will be another story that would rather constitute a scenario for a Hollywood Film. What would happen if we will go against the Evolution? Of course, we will think about the Evolution of ourselves. Now we need to solve current tasks to find a cure against cancer. These aspects will be considered in the last part of the Chapter.

Induction of NeRFa and Spontaneous Regression of Cancer

Spontaneous regression of cancer has been known for a long time during the history of human civilization and has been often associated with an acute infection (Hoption Cann et al., 2002; Hoption Cann et al., 2003). Acute infections in both children and adults significantly reduced the risk of subsequent cancer development (Hoption Cann et al., 2006; Nauts, 1989). At the end of the 19th Century William Coley, a brilliant researcher and bone surgeon from the New York City, found in the notes of the New York Hospital the case of the patient in which an accidental attack of erysipelas after a surgical operation led to complete regression of the tumor (Coley, 1893; Hoption Cann et al., 2003). The following case of spontaneous regression of sarcoma described in the article (Coley, 1893) is so intriguing that would deserve the attention of Sherlock Holmes, the hero of the famous stories by the writer and physician Sir Arthur Conan Doyle. So, in the classical paper (Coley, 1893) we find:

"While collecting the cases of sarcoma treated at the New York Hospital during the past fifteen years, I found a case that, to my mind, had convincing evidence that erysipelas possessed a powerful curative principle antagonistic to sarcoma. (Figure 1). This was one of round-celled sarcoma of the neck, occurring in a German, aged thirty-one years. Five operations had been performed by Dr. W. T. Bull within a space of three years. At the last operation it was found impossible to remove all of the tumor, and the case was considered hopeless. Two weeks after the operation a severe attack of erysipelas occurred, followed by a second attack shortly after the first had subsided. During the progress of the erysipelas the remains of the sarcoma entirely disappeared, the wound rapidly healed, and the patient was seen both by Dr.Bull and myself seven years afterward, at which time the photograph appended was taken. The diagnosis in this case had been repeatedly confirmed by well-known pathologists, and there was no possibility of attributing the cure to any other cause than the erysipelas."

Erysipelas is a cutaneous infection usually caused by group A *streptococci*, although other groups of *streptococci* as well as *staphylococci* can also be involved (Krasagakis et al., 2006). William Coley first used live bacteria for inoculation directly into the tumor mass of the patient, but then switched to the mixture of heat-inactived *Streptococci* from erysipelas and *Bacillus prodigiosus* (Coley, 1910). Live bacteria in general gave better effect, but the use of the heat-inactivated bacteria was safer for patients. Comprehensive review of the success of the Coley's method was presented in the article (Nauts et al., 1946). A brilliant experimental model providing the evidence for the anti-cancer effect of group A *streptococci* has been developed (Jordan et al., 1958).

Although more than one hundred years left since William Coley introduced into practice his method to cure patients from cancer, there is still no description of the molecular mechanism, which stands behind such a phenomenon as spontaneous regression mediated by live or heat-inactivated bacteria. This may be one of the main reasons, why Dr. Coley's approach became a "hidden treasure" (Hoption Cann et al., 2002). Indeed, it is impossible to make an effective drug against cancer while not knowing the exact molecular mechanisms of its action. In addition, more promising methods at that time such as radiotherapy and chemotherapy gradually replaced Coley's approach (Hoption Cann et al., 2002; Hoption Cann et al., 2003). Ironically, the high level of RF in cancer patients was noted after chemotherapy and irradiation by means of the increase of the cancer-associated antigens released from the tissue (Twomey et al., 1976). Dr.Coley emphasized the role of the repeated inoculations of bacteria: although the first inoculation decreased the size of the tumor, the second inoculation apparently played the key role and led to disappearance of the tumor (Coley, 1891). This distantly reminds us an effect of both the secondary immunization and repeated vaccinations on the induction of rheumatoid factor (RF). Interestingly, the investigation of the large cohorts of participants revealed that better survival from cancer is associated with an elevated level of IgM RF sustained before cancer development (Jónsson et al., 1992).

There are still many unanswered questions (not only for Sherlock Holmes), which might have practical significance for the treatment of cancer. For example, what is the nature of "auxiliary antibody" and "non-specific neutralizing factor" described in the article (Shwartzman, 1931)? What the proteins or cascades of proteins are responsible for curative effect of Shwartzman phenomenon in experimental models of tumor regression (Jacobi, 1936)?

Keeping in mind the induction and protective role of RF antibodies in trypanosoma infections (Clarkson & Mellow, 1981; Harboe, 1988), it is of interest to note the inhibition of cancer growth and even cancer regression by *Trypanosoma cruzi* (Kallinikova et al., 2001). Further experiments have shown that antibodies produced in mice after immunization with *Trypanosoma cruzi* can have protective effect against tumor growth and may lead to complete tumor regression (Kallinikova et al., 2006; Kallinikova et al., 2008). It would be of interest to detect RF levels and to find any correlations with their potential oncoprotective effects in such an *in vivo* model of antibody-mediated regression of cancer.

Group A *streptococci* as well as *staphylococci* have so-called Fc-binding proteins, which bind IgG molecules at the same location in Fc-part as do rheumatoid factors (Nardella et al., 1987; Oppliger et al., 1987). It has been shown that RFs contain the internal image of the Fc-binding proteins, because antibodies against Fc-binding proteins bind to idiotypic determinants on human RFs (Oppliger et al., 1987). Immunization of animals with different bacteria including *streptococci* and *staphylococci* leads to production of RFs (Williams et al.,

1972). Production of RF-like antibodies was also noted after immunization of animals with peptidoglycan-polysaccharide (PG-PS) complexes from streptococcal cell wall (Johnson & Smalley, 1988). Interestingly, mice antibodies against human RF also react with peptidoglycan-polysaccharide (PG-PS) from group A *Streptococcus pyogenes* cell wall (Johnson et al., 1985). It might be suggested that heat-inactived *Streptococci* from erysipelas may induce RF production in cancer patients via a similar idiotypic network mechanism. *Bacillus prodigiosus* (*Serratia marcescens*) is a gram-negative bacterium. Gram-negative bacteria contain LPS in their outer membrane. Lipopolysaccharide induced strong RF response both in mice *in vivo* (Dresser & Popham, 1976; Izui et al., 1979) and in human PBMs *in vitro* (Németh et al., 1985). LPS-mediated induction of RF could be another possible mechanism involved in the mysterious curative effect of Coley's toxins.

The idea is that induction of RF might be the key phenomenon, which destroys cancer cells and eradicates the cancer-associated virus. Indeed, the viral etiology of human osteosarcomas has been supported by many facts, as reviewed in the article (Morton et al., 1969). It should be noted that human sarcomas were among the cancer cases treated most successfully in a systemic way by Coley's toxins (Coley, 1910). Many other cancer types such as melanoma (Morton et al., 1969), breast and gynecological cancers (Rakowicz-Szulczynska et al., 1998), leukemia (Blayney et al., 1983; Coffin et al., 1981; Dietz et al., 1977), carcinoma (Hudachek et al., 2010) are also associated with a virus. The interplay between the viral titer, antibody-mediated neutralizing activity against the virus and regression of cancer has been elucidated (Dietz et al., 1977; Whitfill et al., 1982). Among the possible mechanisms of RF-mediated regression of cancer tissue might be two, which in turn can act in synergy. The first includes NeRFa-mediated clearance of cancer-associated viruses. The second, as we will see further, represents NeRFa-mediated complement-dependent cytotoxicity to cancer cells.

Sometimes observations in autoimmune diseases lead to discoveries. It has been shown that serum from MCTD patients contains RF playing a crucial role in enhancement of neutralization of HIV (Douvas et al., 1996). Another example comes from patients with SLE, in which statistically significant inverse correlation between the presence of RF and incidence of warts has been found (Johansson et al., 1977). Authors concluded that RF might play a protective role against wart virus (Johansson et al., 1977). Modern data show that the wart virus is HPV (Bravo & Félez-Sánchez, 2015; Vartanian et al., 2008). Cutaneous warts represent bening lesions with a potential for subsequent malignant conversion (Bravo & Félez-Sánchez, 2015; Cubie, 2013).

RF positive plasma from RA patients mixed with normal plasma from healthy individuals in the presence of complement was cytotoxic to malignant melanoma cells (Twomey et al., 1978). By themselves, neither RF negative plasma nor RF positive plasma showed cytotoxicity to melanoma cells (Twomey et al., 1978). Authors concluded that different types of antibodies (IgM and IgG) when appear together may cause fixation of complement to malignant cells. Authors also suggested that antibodies from normal plasma bind directly cancer-associated antigens on malignant cells. Unfortunately, the data from latex fixation test (LFT) might have misled authors in interpretation of their results and led them to conclude that not RF in RF positive plasma is apparently responsible for cytotoxic effect (Twomey et al., 1978). Indeed, plasma from RA patients may contain both free IgM RF and IgM RF bound to IgG RF-IgG RF complexes (Carson et al., 1987). Probably, only free IgM RF will be adsorbed and subsequently recognized by complement on malignant cells coated with IgG

from normal plasma. This may explain why there is no correlation between RF titer in RA plasma and cytotoxicity, because in different RA plasmas there will be a different ratio between free IgM RF and IgM RF bound to IgG RF-IgG RF complexes. Similarly, adsorption with tumor cells may remove all free IgM RF, but the remaining fraction will give positive LFT test due to the ability of IgM bound to IgG RF-IgG RF complexes still to agglutinatate latex particles. Indeed, within pentameric IgM molecule several IgM momomers may bind IgG RF-IgG RF complexes, while the rest monomers due to free valencies can participate in agglutination reaction.

More sensitive, reproducible and allowing to distinguish isotypes of RF methods such as ELISA (Maiolini et al., 1978; Hermann et al., 1986) and RIA (Yamagata et al., 1979) can be applied to confirm that exactly RF, but not other component from RA positive plasma is responsible for complement-mediated cytotoxicity to melanoma cells. When IgG-containing serum from melanoma patient was mixed with RF positive serum from RA individual there was an IgM RF binding to melanoma cells, while there was no effect when each serum was taken alone (Giuliano et al., 1979). This supports the view that melanoma cell-IgG-IgM RF-Complement is a complex responsible for cytotoxicity against this cancer.

Pregnancy and lactation period is characterized by the elevated level of RF (Clarkson and Mellow, 1981; Meurman et al., 1978; Pope et al., 1982). Normal pregnancy serum had a cytotoxic effect against cancer cells (Bolande et al., 1989; Bolande & Mayer, 1990; Bolande, 1990). This effect is mediated through IgM antibody activating complement on the surface of cancer cells (Bolande et al., 1989; Bolande & Mayer, 1990; Bolande, 1990). Intriguingly, pregnancy sera in the second and third trimester (which actually coincides with an elevated RF level) were found to be more toxic against cancer cells than sera from nonpregnant females (Bolande et al., 1989). There are also cases of spontaneous regression of cancer following pregnancy (Luosto et al., 1974; Sewpaul et al., 2014). It would be of interest to elucidate whether IgM RF in pregnancy sera is responsible for these phenomena.

To test the hypothesis that IgM RF plays a key role in complement-mediated cytotoxicity against cancer cells more experiments with IgM RF using *in vitro* system (Twomey et al., 1978) and *in vivo* model (Jordan et al., 1958) are required. Additional *in vitro* experiments similar to classical (Ashe et al., 1971) might show whether IgM RF-mediated complement-dependent neutralization mechanism can be active in the case of cancer-associated viruses.

To sum up, NeRFa may potentially participate simultaneously in two mutually enhancing mechanisms leading to destruction of cancer tissue:

(i) Cancer-associated virus coated by specific IgG antibodies will be neutralized by IgM NeRFa with subsequent involvement of complement.
(ii) Cancer cells bearing on their surface cancer-associated antigens bound by specific IgG antibodies will be recognized by IgM NeRFa and destroyed by subsequent involvement of complement.

Only after the thorough and successful experiments/tests on animal models, possible patient-specific therapeutic approaches against cancer might be developed by means of the search for the optimal bacterial therapeutic proteins (e.g., Fc-binding proteins or other bacterial proteins) and can be realized in two ways:

Approach (I) includes the following stages:

(i) Rabbits will be immunized with different strains of bacteria, according to the approach (Williams et al., 1972). Corresponding immunization-induced IgM RFs will be isolated. Isolated rabbit RFs are able to interact with human IgG (Williams et al., 1972).
(ii) Isolated rabbit IgM RFs will be mixed with IgG-containing patient's serum and with patient's cancer cells in the presence of complement to test anti-cancer cytotoxic activity according to the *in vitro* method described in (Twomey et al., 1978). Those RFs, which gave the best result in cytotoxicity tests, will indicate the bacterial strain from which the Fc-binding protein or another protein might be isolated to have the best therapeutic potential.

Approach (II) includes stages:

(i) Isolation of the IgM RF from the patient with cancer. Immunization of rabbits with these RFs.
(ii) Isolation of anti-idiotypic (anti-IgM RF) antibodies from rabbits and testing them for binding to different strains of bacteria. Bacterial strains, which showed the best binding, will be chosen for isolation of bacterial proteins with therapeutic potential.

The first approach is preferable, because it exploits direct cytotoxicity test in contrast to the second approach, which is based on the fact that RFs carry the internal image of Fc-binding parts of bacterial Fc-binding proteins (Oppliger et al., 1987). The second approach has limitations and might be applied only if IgM RF already exists in the cancer patient.

Perspective therapeutic proteins from bacteria should go through the thorough control on safety to the human organism. To sum up, there is no need in Coley's toxins, which contain many bacterial components with unknown mechanism of action and thus representing many concerns about the safety for the use in humans. It will be a concrete therapeutic protein from bacteria able to induce NeRFa with the highest potential for subsequent regression of a particular type of cancer in the particular patient. The task would be to find such a Therapeutic Protein, which might be indeed a "hidden treasure" if it eventually will lead to the development of an efficient patient-specific cancer immunotherapy.

Conclusion

Neutralization-enhancing RF antibodies (NeRFa) may enhance neutralization of viruses due to the higher avidity of the resulting antibody complexes possessing better ability for aggregation and subsequent clearance of viral particles. NeRFa may not only enhance neutralizing capacity of neutralizing antibodies (NAbs), but also can convert infection-enhancing antibodies into NAbs. Viral particles covered by infection-enhancing antibodies represent "sensitized" virus to be neutralized by NeRFa. In the case of HIV, which exploits antibody-dependent enhancement (ADE) mechanism, induction of NeRFa might be the only way to neutralize the virus. Induction of NeRFa can be achieved via prolonged puslatile

release of therapeutic proteins from biodegradable polymeric microspheres and may be a general approach to eradicate different pathogens. Induction of NeRFa by therapeutic proteins might be a promising method for triggering spontaneous regression of cancer.

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Chapter 4

Advances in the Diagnosis, Assessment, Management and Outcome of Takayasu's Arteritis: A Narrative Review

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Abstract

Takayasu arteritis (TA) is a rare, chronic large-vessel vasculitis (LVV) characterized by granulomatous inflammation of the vessel wall with an unknown etiopathogenesis. TA predominantly affects young females during the 2nd or 3rd decades of life and mainly involves the aortic arch and its major branches, ascending aorta, thoracic descending aorta and abdominal aorta. A physical examination is the first step for disease assessment. Systemic inflammatory response does not always show a positive correlation with inflammatory activity in the vessel wall. Imaging modalities are very important for establishing the diagnosis of TA. Conventional angiography, the gold standard for initial diagnosis, seems to be replaced with the new imaging modalities such as magnetic resonance angiography, ultrasonography, computerized tomography and, 18F-fluorodeoxyglucose positron emission tomography in recent years. Prognosis is possibly getting better with lower mortality in recent years. The most commonly used agents include corticosteroids and conventional immunosuppressive agents such as methotrexate, azathioprine, micophenolate mofetil and leflunomide. However, in resistant and/or intolerant patients, biologic drugs including anti-TNF agents (mostly infliximab), rituximab and tocilizumab seem to be promising. Antiplatelet treatment may also lower the frequency of ischaemic events in TA. Endovascular interventions (balloon angioplasty or stent graft replacement) or by-pass surgery may be useful for critical arterial occlusions. There is a clear need to develop a validated set of outcome measures in TA, such as measures of disease activity, health-related quality of life and disease-related damage. The OMERACT Vasculitis Working Group has taken on this task and aims to develop a core set of outcomes for LVV.

Keywords: Takayasu arteritis, large vessel vasculitis, disease assessment, outcome

Introduction

Takayasu arteritis (TA) is a rare, chronic large-vessel vasculitis (LVV) characterized by granulomatous inflammation of the vessel wall of an unknown etiopathogenesis [1]. The pathology of TA is characterized by the involvement of all arterial layers (i.e., panarteritis) with a variable inflammatory infiltrate including acute exudative inflammation, chronic and granulomatous inflammation situated mainly in the media and adventitia while hyperplasia and neovascularization are observed in the intimal layer [1]. Vasa vasorum is considered the portal entry of inflammatory cells in TA and the cellular infiltrate comprises macrophages, CD4+ T cells, CD8+ T cells, γδ T cells, natural killer cells and neutrophils [1, 2]. Ex vivo studies have suggested a role for self-reactive leukocytes producing mediators (TNF-α and IL6) which are thought to play a critical role in the pathogenesis of LVV [3]. Inflammatory lesions in TA eventually evolve to diffuse and/or nodular fibrosis in the arterial wall [4].

TA predominantly affects young females during the 2nd or 3rd decades of life and mainly involves the aortic arch and its major branches, ascending aorta, thoracic descending aorta and abdominal aorta, as well as subclavian, carotid and the pulmonary arteries [5, 6].

Early in the disease course or during relapses, TA patients may present non-specific inflammatory complaints such as fever, malaise, anorexia, weight loss, myalgia or arthralgias which can be associated with vascular pain (e.g., carotidynia). Later, inflammation of the involved arteries progresses, resulting in segmental stenosis, occlusion, dilatation and/or aneurysm [7]. This may cause extremity pain, claudication, light-headedness, bruits, absent or diminished pulses, and loss of blood pressure. Heart failure may develop as a consequence of hypertension, coronary heart disease and/or aortic regurgitation. Transient ischemic attacks, stroke and mesenteric ischemia are other ischemic manifestations of TA, but these events are rare in TA [6]. TA is reported to be potentially life-threating, reflected in mortality rates as high as 35% 5 years after diagnosis, similar to that seen in malignancies [8]. Factors that contribute to significant morbidity in TA include heart failure and neurological ischemic events such as stroke and transient ischemic attacks [9]. Moreover, patients with TA present more risk factors for cardiovascular disease than controls [10, 11] and subclinical accelerated atherosclerosis is well documented in TA as well being potential factors for ischemic complications [12].

While many patients with TA have been found in Asian countries, this disease is reported from all over the world. TA is, however, seen more commonly in Indian subcontinent, Japan, China, Korea and south east Asian countries amongst the APLAR nations as well as in Turkey, Brazil, Mexico and south Africa. Reports from the Western World including Iceland and USA also do exist in literature [13-16]. The guidelines for the management of vasculitis syndrome by the Japanese Circulation Society (JCS 2008) reported that more than 5000 patients with TA were registered in the MHLW registry, so the prevalence was more than 0.004% [17]. Moreover, the distribution of vascular involvement reportedly differs among regions [18]. In Japan and South America, cervical and thoracic arterial lesions are prevalent, whereas abdominal lesions are more common in Israel and other Asian countries [19].

The HLA-B52 haplotype is well known to be closely associated with TA not only in Japan, but also in other countries [20-22]. In Japan, the prevalence of the HLA-B52 allele in healthy controls is higher compared with that in other countries, which might be responsible for the high prevalence of TA and the higher prevalence of HLA-B52 in patients with TA than in other countries.

A study of a large number of Japanese patients with TA showed a significant female predominance (the female to male ratio was eight to one), and some reports from other countries indicated the same results. However, a report comparing the characteristics of patients with TA in Japan and India revealed a significantly lower ratio of female to male patients with TA in India than the ratio in Japan [23].

The aim of this article is to review the current advances in the diagnosis, assessment, management and outcome of TA.

Diagnosis

A proper diagnosis of TA is an important issue since delays may result in significant morbidity. Until 1988 no diagnostic or classification criteria were available for TA, when Ishikawa proposed a set of diagnostic criteria [24].

The terms disease definition, diagnostic criteria and classification criteria are important for disease nomenclature, especially in systemic vasculitis. Disease definition describes the condition itself in a concisely, diagnostic criteria help to distinguish the vasculitis from normal controls out of the general population and from similar mimicking conditions and are meant to be used in clinical practice whereas classification criteria help to distinguish one form of vasculitis from another and should be used for the enrollment of patients in studies [25, 26]. Characteristic signs and symptoms of TA $\geq$ 1 month duration included in the obligatory criterion of Ishikawa's criteria are described on Table 1.

Modifications on Ishikawa's diagnostic criteria for TA were suggested by Sharma et al., in 1995 [27]. This modification showed in Table 2.

Table 1. Characteristic signs and symptoms of TA included in Ishikawa's criteria for TA [24]

Manifestations
Cardinal limb signs or symptoms
Pulselessness
Differences in pulses in the arms
Unobtainable blood pressure
Significant blood pressure differences in the arms
Easy limb fatigability or pain
Minor signs or symptoms
Unexplained fever or high ESR ($\geq$20 mm in the first hour, by the Westergren method), or both
Neck pain
Transient amaurosis or blurred vision or syncope
Dyspnea or palpitations or both, or hypertension or aortic regurgitation

ESR: erythrocyte sedimentation rate.

Table 2. Ishikawa's diagnostic criteria for TA modified by Sharma et al., [27]

Criteria	Definition
Obligatory criterion	
Age ≤40 years	Age ≤40 years at diagnosis or at onset of "characteristic signs and symptoms" of 1 month duration in patient history.
Two major criteria	
1) Left mid subclavian artery lesion	The most severe stenosis or occlusion present in the mid portion from the point 1 cm proximal to the left vertebral artery orifice to that 3 cm distal to the orifice determined by angiography.
2) Right mid subclavian artery lesion	The most severe stenosis or occlusion present in the mid portion from the right vertebral artery orifice to the point 3 cm distal to the orifice determined by angiography.
Nine minor criteria	
1) High ESR	Unexplained persistent high ESR≥20 mm/h (Westergren) at diagnosis or presence of the evidence in patient history
2) Carotid artery tenderness	Unilateral or bilateral tenderness of common carotid arteries by physician palpation; neck muscle tenderness is unacceptable.
3) Hypertension	Persistent blood pressure ≥140/90 mmHg brachial or ≥160/90 mmHg popliteal at age ≤40 years, or presence of the history at age ≤40 years.
4) Aortic regurgitation or Annuloaortic ectasia	By auscultation or Doppler echocardiography or angiography. By angiography or two-dimensional echocardiography.
5) Pulmonary artery lesion	Lobar or segmental arterial occlusion or equivalent determined by angiography or perfusion scintigraphy; or presence of stenosis, aneurysm, luminal irregularity or any combination in pulmonary trunk or in unilateral or bilateral pulmonary arteries determined by angiography.
6) Left mid common carotid lesion	Presence of the most severe stenosis or occlusion in the mid portion of 5 cm in length from the point 2 cm distal to its orifice determined by angiography
7) Distal brachiocephalic trunk lesion	Presence of the most severe stenosis or occlusion in the distal third determined by angiography.
8) Descending thoracic aorta lesion	Narrowing, dilation or aneurysm, luminal irregularity or any combination determined by angiography; tortuosity alone is unacceptable.
9) Abdominal aorta lesion	Narrowing, dilation or aneurysm, luminal irregularity or any combination and absence of lesion in aorto-iliac region consisting of 2 cm of terminal aorta and bilateral common iliac arteries determined by angiography; turtuosity alone is unacceptable.

ESR: erythrocyte sedimentation rate.

A high probability of TA is considered when two major criteria are present or one major and two minor criteria or four minor criteria are present. The sensitivity and specificity of the modified Ishikawa's diagnostic criteria for TA was 92.5% and 95.0%, respectively.

Table 3. The 1990 American College of Rheumatology Classification Criteria for Takayasu arteritis [29]

Criteria	Definition
Age at disease onset < 40 years	Development of symptoms or findings related to TA at age < 40 years
Claudication of extremities	Development and worsening of fatigue and discomfort in muscles of 1 or more extremity while in use, especially the upper extremities
Decreased brachial artery pulse	Decreased pulsation of 1 or both brachial arteries
Blood pressure difference >10 mmHg	Difference of >10 mmHg in systolic blood pressure between arms
Bruit over subclavian arteries or aorta	Bruit audible on auscultation over 1 or both subclavian arteries or abdominal aorta
Arteriogram abnormality	Arteriographic narrowing or occlusion of the entire aorta, its primary branches, or large arteries in the proximal upper or lower extremities, not due to arteriosclerosis, fibromuscular dysplasia, or similar causes; changes usually focal or segmental

For purposes of classification, a patient shall be said to have TA if at least 3 of these 6 criteria are present. The presence of any 3 or more criteria yields a sensitivity of 90.5% and a specificity of 97.8%.

In 1990, the ACR published the classification criteria for seven systemic vasculitides including TA. The objective of the ACR classification criteria was the identification of a homogeneous group of patients to be evaluated in studies on epidemiology and therapy rather than to be used in clinical practice as a diagnostic tool [28]. Six criteria were proposed for TA (Table 3) and patients were classified as TA in the presence of at least three out of six criteria with 90.5% sensitivity and 97.8% specificity [29]. The main criticism towards Ishikawa's diagnostic criteria and to the 1990 ACR classification criteria for TA includes the age restriction for the disease onset (< 40 years) and their limitation in TA patients from areas with predominant aortic involvement such as India [27].

The 1994 Chapel Hill Consensus Conference (CHCC) determined names and definition for common systemic vasculitides in order to standardize vasculitis nomenclature. The definition of ten vasculitic syndromes was made based on clinical and histological criteria whereas all vasculitic syndromes were categorized according to the predominant size of involved vessels [30]. The 2012 updated definitions based on the improved understanding of etiology, pathogenesis, demographics and clinical manifestations of vasculitic syndromes. TA was categorized as a large vessel vasculitis together with GCA and defined as an arteritis, often granulomatous, predominantly affecting the aorta and/or its main branches with onset usually in individuals younger than 50 years. However, the CHCC authors emphasize that the definitions should not be used as diagnostic criteria or as a classification system [31].

Regarding children with TA, classification criteria for the more common childhood vasculitides were proposed by the European League Against Rheumatism (EULAR), the Pediatric Rheumatology European Society (PRES) and by the Pediatric Rheumatology International Trials Organization (PRINTO) in 2005 [32] and subsequently validated in 2008 [33]. These criteria are referred as EULAR/PRINTO/PRES criteria and are meant to be used

in patients younger than 18 years [33]. The criteria for childhood TA (c-TA) include angiographic abnormalities in the aorta or its main branches and pulmonary arteries as a mandatory criterion and five additional features of c-TA (Table 4). c-TA is classified when the patient presents the mandatory criterion and at least one of the five other features. The EULAR/ PRINTO/PRES criteria for c-TA had a 100% of sensitivity and 99.9% of specificity [33].

A global project, The Diagnostic and Classification Criteria in Vasculitis Study (DCVAS) is an international effort that is under way to develop a single classification system and a validated set of diagnostic criteria for systemic vasculitides using data driven methods [28]. The study population aims to include 2000 patients with systemic vasculitis and 1500 patients presenting conditions that mimic vasculitis. This novel classification system and diagnostic criteria is meant to be used in clinical practice and in clinical trials. So far, over 2500 patients have been recruited in 101 sites [34].

Assessment

Physical Examination

Physical examination is the first step for disease assessment in TA. The bruit audible on auscultation over 1 or both subclavian arteries or abdominal aorta, decreased or absent brachial artery pulse, or blood pressure differences are evaluated as physical signs. However, the limitations of physical examination were recently shown in a study comparing physical signs with imaging data [35]. Monitoring and control of blood pressure may be difficult in cases with absent or reduced pulses in some extremities. Blood pressure measurements should be made in the unaffected extremities. In some patients with unreliable measurements, the presence of hypertensive retinopathy may be a warning sign for the clinician. Hypertension were seen in 33-83% of patients [5], generally reflecting renal artery stenosis. In the presence of treatment-resistant hypertension, the possibility of renovascular hypertension should be considered, which may be treated with endovascular interventions or surgery [36]. Other characteristic features were:

a) Aortic regurgitation resulting from dilatation of the ascending aorta, separation of the valve leaflets, and valve thickening in 20-24% TA patients [37].
b) Congestive cardiac failure associated with hypertension, aortic regurgitation, and dilated cardiomyopathy.
c) Neurological features secondary to hypertension and/or ischaemia, including postural dizziness, seizures, and amaurosis.
d) Pulmonary artery involvement in 14-100% of patients, depending on the method used to assess pulmonary vasculature. Oligaemic lung fields on plain chest *x* ray correlate with pulmonary vasculopathy in approximately a third of cases [38].
e) Other symptoms include dyspnoea, headaches, carotodynia, myocardial ischaemia, chest wall pain, and erythema nodosum.

Table 4. The EULAR/PRINTO/PRES criteria for childhood Takayasu arteritis [33]

Criterion	Definition
Angiographic abnormality (mandatory criterion)	Angiography (conventional, computed tomography, or magnetic resonance imaging) of the aorta or its main branches and pulmonary arteries showing aneurysm/ dilatation, narrowing, occlusion or thickened arterial wall not due to fibromuscular dysplasia, or similar causes; changes usually focal or segmental
Pulse deficit or claudication	Lost/decreased/unequal peripheral artery pulse(s) Claudication: focal muscle pain induced by physical activity
Blood pressure discrepancy	Discrepancy of four limb systolic blood pressure >10 mmHg difference in any limb
Bruits	Audible murmurs or palpable thrills over large arteries
Hypertension	Systolic/diastolic blood pressure greater than 95th percentile for height
Acute phase reactants	Erythrocyte sedimentation rate > 20 mm per first hour or C-reactive protein any value above normal (according to the local laboratory)

TA is classified when the mandatory criterion is present plus any other criteria.

Variable disease presentation between different populations is well illustrated by Moriwaki et al., in their study of Indian and Japanese patients [23]. The Japanese patients (n = 80) were predominantly female (96%), presenting with dizziness, vertigo, pulselessness, more prolonged and severe inflammation, and more aortic regurgitation, reflecting involvement of the aortic arch and its main branches. This contrasted with the Indian patients (n = 102), 37% of whom were male. They tended to present with headache, hypertension, and left ventricular hypertrophy as a result of vasculitis affecting the abdominal aorta and renal vessels.

Laboratory

Systemic inflammatory response does not always show a positive correlation with inflammatory activity in the vessel wall. In one study, active disease was present in the setting of normal erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in 23% of the patients with TA [39], and this called *vice versa*. Similarly, ESR was elevated in only 72% of patients considered to have active disease and was still high in 44% of patients considered to be clinically inactive [40].

TA patients presented higher levels of platelet P-selectin and plasma thromboxane B2 (TXB2) and lower levels of 6-keto-PGF1α, specially in those with an active phase who in turn had an enhancement in platelet aggregation in response to collagen [41-43]. Also, TA patients had higher plasma levels of thrombin-antithrombin III complex, fibrinopeptide A and D-dimer than healthy controls, indicating that other mechanisms besides isolated platelet activation may be responsible for the hypercoagulable state observed in TA [44]. Serum autoantibodies such as anti-endothelial antibodies, serum biomarkes such as IL-6, IL-8, IL-17, IL-18, matrix metaloproteinase-9, and pentraxin 3 [45] have been suggested to be related to active disease in TA.

Imaging in TA

Imaging modalities are very important for establishing the diagnosis of TA, determining the distribution of lesions and monitoring disease activity [46]. Conventional digital subtraction angiography is the "gold standard" for detecting the stenosis, occlusions, and aneurysms that characterize the latter stages of TA, but is the least sensitive method for visualizing wall thickness. It may miss minor, non-occlusive lesions. Assessment of pulmonary vasculature by angiography is not universally recommended, being reserved for patients with symptoms of pulmonary hypertension [5].

Takayasu arteritis can be divided into the six types based on angiographic involvement. New angiographic classification of TA based of the International TA Conference in Tokyo 1994 shown in Table 5 [23].

Recently, non-invasive methods such as magnetic resonance angiography (MRA), colour Doppler ultrasound (CDU), computerized tomography angiography (CTA), positron emission tomography (PET) with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) and ^{18}F-FDG PET/CT can detect vessel wall thickening.

Table 5. New angiographic classification of Takayasu arteritis, Takayasu conference 1994 [23]

Type	Vessel involvement
Type I	Branches from the aortic arch
Type IIa	Ascending aorta, aortic arch and its branches
Type IIb	Type IIa region plus thoracic descending aorta
Type III	Thoracic descending aorta, abdominal aorta, and/or renal arteries
Type IV	Abdominal aorta and/or renal arteries
Type V	Combined features of types IIb and IV

According to this classification system, involvement of the coronary (C) or pulmonary (P) arteries should be designated as C (+) or P (+), respectively.

Computerized Tomography Angiography/Magnetic Resonance Angiography

Contrast-enhanced MRA or CT angiography (CTA) allows noninvasive imaging of the aorta and its major branches. Recently, it has became popular for the diagnosis of TA. MRA is performed to determine location, extent, and situation of vessel lesions. On T1-weighted image, vessel wall thickness and lumen diameter were measured. On T2-weighted image, aortic wall and periaortic tissue may appear bright due to inflammatory edema. With the use of gadolinium as delayed contrast medium, the aortic wall is identified and increased signal intensity in the aorta compared with that of back muscle (erector spinae) indicates increased vascularity. The enhanced aortic wall on delayed contrast-enhanced T1-weighted images, along with the high intensity of T2-weighted image, indicates activity of TA [47]. Compared to invasive angiography, three-dimensional MRA can effectively show vessel wall thickening, including mural edema and increased mural vascularity. Another advantage of MRA is the lack of iodinated contrast material [48]. MRA is extensively investigated in the

current literature for evaluating vascular inflammation and has increasingly replaced invasive angiography [47] but 2% of stenotic arteries were wrongly portrayed as occluded.

In a study of 24 patients by Tso et al., [49] MRA scans of 94% of the patients revealed vessel wall edema during periods of unequivocally active disease, 56% also showed them during apparent clinical remission. This study enlightens some of the problems of imaging-only based vessel-wall assessment for TA, suggesting that other clinical features should also be considered.

Colour Doppler Ultrasonography

Ultrasonography is a noninvasive and effective method for evaluating the carotid arteries in patients with TA. Also, may be a useful method for follow-up of anatomic and hemodynamic changes in response to therapy [50].

The ultrasonographic features of TA involving the carotid arteries have been reported as long-segmental or diffuse circumferential thickening with isoechogenicity or hyperechogenicity of the arterial wall [50, 51]. In transverse sections, the circumferentially thickened arterial wall was termed the "macaroni sign" by Maeda et al., [52]. This thickening had a clear-cut boundary between involved and noninvolved areas [50]. Raninen et al., [53] showed that CDU can detect stenosis in carotid arteries with high sensitivity (90%) and specificity (91%). However, the acoustic window of US is limited by the fact that the sound beams cannot penetrate the bony profile; therefore, many arteries involved in TA like the arcus and thoracic aorta cannot be visualized by ultrasound.

Positron Emission Tomography (PET) with ^{18}F-Fluorodeoxyglucose (^{18}F-FDG)

^{18}F-FDG PET is a non-invasive imaging method that measures ^{18}F-FDG, which accumulates in hypermetabolic, activated inflammatory cells infiltrating the vessels. The method of assessing ^{18}F-FDG uptake varies according to the study.

Some studies used semiquantitative analysis comparing the ^{18}F-FDG uptake of a vascular region of interest with that of the liver. The severity of large vessel ^{18}F-FDG uptake was visually graded using a four-point scale. 0 = no uptake present, I = low-grade uptake (uptake present but lower than liver uptake), II = intermediate-grade uptake (similar to liver uptake), and III = high-grade uptake (uptake higher than liver uptake) [54].

Other studies quantified ^{18}F-FDG uptake using method such as standard uptake value (SUV). Kobayashi et al., [55] was the first to establish a cutoff for max SUV. In a study of 14 patients they reported a sensitivity of 90.9% for detecting active disease and a specificity of 88.8% given the SUV cutoff point of 1.3. In that study, patients with active disease were defined as those requiring corticosteroids to clinically control the disease, and patients with inactive disease were those who did not need corticosteroids for ≥ 2 years.

In contrast to this study with high sensitivity and specificity, Arnaud et al., [56] found a lack of correlation between FDG uptake and biologic or radiologic assessment of disease activity and a trend toward an association between FDG uptake and clinical activity. They shown that the ESR and CRP values as well as the proportion of patients with elevated ESR

or CRP values did not correlate with abnormal vascular uptake on FDG-PET scans. Also, they pointed out that previous studies used various invalid criteria for active TA and may therefore be biased. They depended exclusively on clinical symptoms without markers of inflammation when assessing clinical disease activity and reported that the ^{18}F-FDG PET scan had a sensitivity of 69.2% and specificity of 33.3% for clinically active TA. Karapolat et al., [57] found out that^{18}F-FDG PET findings were mostly consistent with clinical disease activity, having sensitivity and specificity values of 100% and 88.9%, respectively.

In a very recent study, Alibaz-Oner et al., studied 14 TA patients. Patients were clinically inactive (according to the definition of activity by Kerr et al.), while categorized as having "persistent" disease activity by physician's global assessment due only to acute phase response. They are observed increased ^{18}F-FDG uptake in the majority of TA patients with an increased acute phase response, but clinically silent disease. ^{18}F-FDG-PET/CT showed the presence and localisation of active inflammation in the aorta and its branches. Although specificity of observed lesions are not clear, ^{18}F-FDG-PET/CT imaging may influence physician's assessment of clinical activity and treatment choices in TA [58].

In conclusion, ^{18}FDG-PET seem to be useful for the assessment of clinical activity in TA. However, it needs to be validated in larger groups for cost-effectiveness and sensitivity to change.

Outcome Measures in Takayasu's Arteritis

Measures of Disease Activity in TA

The simple definition of "active disease" was the first reported in a study from the National Institute of Health by Kerr et al., [5]. According to the Kerr criteria, the presence, recent occurrence or deterioration of at least two of the following four criteria shows active disease: (1) systemic features like fever and arthralgia that cannot be explained by other reasons, (2) elevated ESR, (3) findings of vascular ischaemia and inflammation and (4) typical angiographic findings.

In 2005 the Disease Extent Index-Takayasu (DEI-Tak) was defined for the follow-up of TA by assessing only new clinical findings within the past 6 months without the requirement for imaging techniques or acute phase reactants [59]. The DEI-Tak was shown to be a practical and valuable tool to assess disease activity and progression in a Turkish TA series [60]. In this study, patients with active or persistent disease had higher DEI-Tak scores compared to patients in remission. Physician's global assessment (PGA) and DEI-Tak scores had modest agreement (68%). Sixty-nine percent of subjects with slow progression of disease demonstrated no change in the DEI-Tak. Further, 31% of patients deemed inactive by DEI-Tak had "active/persistent" disease according to the PGA. In contrast, 18% of the patients with a DEI-Tak ≥ 1 (active) were considered inactive by the PGA score.

In 2008, a new version of DEI-Tak, the Indian Takayasu's Arteritis Score (ITAS), was introduced [61]. ITAS has only six systems, and scoring is weighted for vascular items (0–2). In 2008, ITAS has been used in a clinical trial of tocilizumab in TA [62].

Recently, the Indian Takayasu arteritis consortium proposed Inidian Takayasu Clinical Activity Score (ITAS2010), a novel method of evaluating TA disease activity [63]. They also

expanded ITAS2010 to ITAS2010-A by incorporating acute-phase reactants. This Indian study is the largest study following patients with TA and assessing disease activity. Standardization of composite measures to assess disease activity in TA would make clinical examinations easier in a multi ethnic manner. It should be noted that there is no evidence concerning the usefulness of the novel markers and composite measures for improving prophylaxis of patients with TA [64].

The OMERACT Vasculitis Working Group also performs a Delphi exercise for the assessment of disease activity in LVV to develop a core set of validated outcome measures [65]. As the gold standard for disease activity assessment, new vessel involvement was favored by 84%, as determined by either clinical examination or imaging, whereas physician's global assessment was found suitable by only 13%. A scalable index was supported over a dichotomous outcome by 89% of participants and weighting of items was strongly endorsed (87%). However, 80% accepted that it is not clearly possible to differentiate "low" versus "high" disease activity or damage versus activity (83%) in TA.

Alibaz-Oner F et al., [66] included 51 patients with TA and showed significantly increased IL-6, IL-8 and IL-18 levels in patients with TA compared to healthy controls. Only IL-18 level was significantly higher in active patients assessed by ITAS2010. IL-18 was also the only cytokine in their study that correlated with CRP. These findings suggest that cytokines associated with neutrophilic, pro-inflammatory responses such as IL-6, IL-8 and IL-18 can be potential biomarkers for the assessment of disease activity in TA and warrant further studies in larger series.

Recently, two teams from Japan and Italy identified pentraxin 3 (PTX-3) as a promising serum marker for TA to follow its activity [67, 68]. The Italian team reported that PTX-3 provided better area under curve in receiver operating curves to detect active patients with TA. The Japanese group reported six out of eight patients presented increased levels of PTX-3 without any increase in CRP levels. PTX-3 might serve as a marker to follow patients who develop progressive occlusion of the aorta in spite of negative CRP cases.

Measuring Health-Related Quality of Life in TA

Disease-related damage and treatment toxicity in TA can severely impact patients' quality of life and functional status. Thus, it is important to measure the health-related quality of life (HRQoL) in patients with TA and determine the effect of treatments on this domain of illness. It has been shown that patients with vasculitis judge the importance of various disease manifestations differently from how physicians rate the same problems [69]. Incorporation of HRQoL measurements with a generic instrument such as the Medical Outcomes Study Short-Form 36 (SF-36) will likely add to the content validity of outcome measures for LVV. Measurement of HRQoL through the use of the SF-36 has been evaluated in 2 studies of TA. These studies demonstrated that patients with TA had reduced SF-36 scores, similar to other chronic inflammatory disorders such as rheumatoid arthritis and ankylosing spondylitis [70, 71].

In a study from Turkey, when patient-reported outcomes such as Health Assessment Questionnaire (HAQ), Multidimensional Assessment of Fatigue Scale (MAF), SF-36, and Hospital Anxiety/Depression Scales (HADS) were investigated, SF-36 physical component score was observed to be significantly lower and HAQ score was significantly higher in

patients with TA compared to healthy controls. No differences were present in MAF, HADS, and SF-36 mental component scores between TA and controls [72]. The discrepancy between SF-36 and MAF suggests that chronic inflammation in TA has possibly a lower impact on general fatigue compared to other inflammatory disorders such as rheumatoid arthritis or systemic lupus erythematosus.

Management of TA

Management of TA is not easy. First, early diagnosis is difficult and requires clinical awareness and suspicion. Second, and even more important, is the lack of standard and reliable parameters reflecting disease activity. Thirth, is the low level of evidence of TA. The relative rarity of TA and the lack of ideal outcome measures are barriers in conducting placebo-controlled, randomized clinical trials in TA. Current evidence reflects the results of open studies, case series and expert opinion [73].

General Principles

Patient education and cooperation between the doctor and the patient are essential. The rationale of the medical treatment is to suppress systemic and vascular inflammation using corticosterois (CS) and immunosuppressive (IS) agents. When the CS dose cannot be lowered and conventional IS agents remain ineffective, or when these agents can no longer be used due to adverse events, biologic agents may be tried. In selected cases, endovascular interventions or bypass surgery may be useful for the treatment of critical arterial occlusions [74].

Supportive Measures

Similar to other inflammatory diseases, atherosclerosis risk is also increased in TA, and preventive measures should be considered [75]. Statins were prescribed when serum low-density lipoprotein (LDL) levels exceeded 130 mg/dl [76]. Diet, low salt intake, calcium and vitamin D supplementation and regular exercise are essential to reduce the metabolic side effects of CS agents [74].

The other important medical issues relate to the management of hypertension and the prevention and treatment of thrombosis. Hypertension can be particularly difficult, and worsened by the use of steroids with their fluid retaining side effects. The use of angiotensin converting enzyme inhibitors requires careful monitoring in view of the frequency of renal artery stenosis [77].

Inflammation, accelerated atherosclerosis and modification of laminar blood flow due to distortion of vessel wall anatomy may all contribute for the occurrence of arterial thrombosis and ischemic events in TA patients [45]. Chronic antiplatelet therapy has been recommended in TA patients provided that aspirin is capable of inhibiting platelet aggregability irrespective of soluble CD40 ligand levels (a proinflammatory and prothrombotic marker) and in TA

patients it was shown that a daily dosage of 80 mg of aspirin is effective for the suppression of TXB2, but not of prostacyclin in surface-damaged blood vessels [78, 79]. Recetnly, Souza et al., [76] suggested that antiplatelet therapy with aspirin dosage between 100-200 mg/day reduces the risk of acute ischemic events in patients with TA.

Corticosteroids

In the presence of active disease, standard initial treatment of TA is high-dose (1 mg/kg/day) prednisolone or its equivalents. Generally, two-thirds of the total daily dose is given early in the morning and the rest of the dose in the evening after meals. The response to high dose prednisolone is generally favourable, but relapses may occur while gradually tapering the dose and adverse effects of long-term treatment can cause problems.

Therefore many physicians tend to start conventional IS agents together with the initial CS treatment or while tapering the CS dose [74, 80]. Th17 and Th1 pathways contribute to the systemic and vascular manifestations of TA. Glucocorticoids suppressed Th1 cytokines and spared Th17 cytokines in TA [81].

Early data suggested little benefit [82], with six of eight patients treated showing no improvement. Data from the USA in 1985 [83] from 29 steroid treated patients demonstrated a reduction in ESR, a reduction of inflammatory symptoms, and eight of 16 patients with absent pulses were shown to have a return of a pulse after a delay of several months. In a later study, of 48 treated patients, remission was achieved at least once with steroids alone in 60% [5]. It is now accepted that approximately half of patients treated with steroids will respond [84]. Petrovic-Rackov et al., [85] reported 16 patients with TA treated with CS. The majority of patients (11, 69%) required other IS agents. This lack of universal success and the side effects associated with steroid use have led to a search for a more effective treatment.

Conventional IS Agents

In TA there is no randomized study comparing the efficacy of different IS agents, therefore there is no evidence showing which IS agent is superior in the treatment of TA. Kerr et al., [5] studied 25 steroid unresponsive patients receiving cytotoxic medications including cyclophosphamide, azathioprine, or methotrexate, although not concurrently. The overall remission rate was 33%. Twenty three per cent of all treated patients in their study never achieved remission.

Methotrexate (MTX) is the first choice of many physicians. The frequency of MTX use in TA is variable in diferent studies. An early report of MTX [86] suggested that it was a clinically useful, well tolerated drug. A follow up study of 16 steroid unresponsive patients treated with MTX and steroid demonstrated remission in 81% [87]. However, seven of 16 relapsed as they were weaned off of steroids. Overall, eight patients sustained remissions of four to 34 months and four of these were able to discontinue treatment altogether. Three of 16 progressed despite treatment.

A Brazilian study included 12 patients treated with MTX and prednisolone 58% of them had a good response [88]. No reference to MTX use is given in the Indian cohort study

whereas it is added to corticosteroid treatment in 16.3% of Mexican patients, in 20.3% of Korean patients and in 25.6% of French patients with TA [89-92]. The frequency of MTX use in TA is higher in the US (43%) and Turkish (63%) cohort studies [93, 94].

In 52 patients with TA who were on regular follow-up at the Vasculitis Unit of Universidade Federal de São Paulo between 2003 and 2009 MTX is widely used to treat TA [95]. It was prescribed for 76.9% of patients at diagnosis and for 82.7% along follow-up. Nonetheless, the rate of MTX discontinuation in this study was 41.9% mainly due to inefficacy based on clinical criteria for disease activity.

Azathioprine (AZA) is another IS agent widely used for the treatment of TA. Besides case reports [96, 97], there is only one open study from India [98]. In this study, 65 TA patients who had not received any IS agent previously were given 2 mg/kg/day AZA in addition to CS treatment for 1 year. Acute phase responses were significantly reduced, no adverse events occurred and control angiography showed no progression. However, long-term follow-up of these patients was not reported.

Cyclophosphamide (CYP) is generally used for the treatment of systemic vasculitis in the presence of severe life and/or vital organ threatening conditions (retinal vasculitis, pulmonary artery involvement with or without aneurysm, severe aortic regurgitation or myocarditis) [99, 100]. In a prospective study in TA, seven patients resistant to CS treatment were additionally given 2 mg/kg/day oral CYP [84]. After a mean period of 27.5 months, no clinical or radiological progression was observed in these patients. Haemorrhagic cystitis developed in two patients, herpes zoster in one and oligomenorrhoea in seven. In another open study, eight patients with myocardial involvement were reported to have clinical haemodynamic and morphological improvement using CS plus CYP treatment [101]. There is also a case report of a resistant TA patient treated with autologous stem cell transplantation with CYP [102].

Micophenolate mofetil (MMF), which is widely used for the treatment of lupus nephritis, is also a promising agent in TA. In a single case series of three TA cases resistant to CS plus MTX, MMF treatment (2 g/day) for at least 1 year prevented both clinical and radiological progression, steroids were tapered or discontinued, and no toxicity was observed [103].

In the first open MMF study, 10 patients with treatment-resistant TA were given MMF for a mean period of 23 months, resulting in significant reductions in acute phase proteins [104]. Recently the data of 21 consequent Indian TA cases using MMF for 9.6 ± 6.4 months were reported [105]. Eleven patients were on steroids alone at baseline while ten patients had received AZA prior to administration of MMF. Improvement in disease activity was shown using ITAS and physician global assessment. The CS requirement was also reduced from 36 (±16) mg/day at baseline to 19 (±14) mg/day at last follow-up ($p < 0.001$). The only adverse event was skin rash in a single patient. This study is notable in that it reflects MMF data for the largest TA series with favourable efficacy and safety profiles. Larger studies will be necessary to confirm these findings and establish the place of this drug in the treatment of TA.

Cyclosporine A (CSA) [106-109], **tacrolimus** [110] and **leflunomide** (LEF) [111, 112]. were also tried in selected cases with successful results. CSA may also be effective in some cases in the treatment of pyoderma gangrenosum complicating TA (107-109). In a prospective open-label study of LEF, 15 TA patients with treatment-resistant active disease were given 20 mg/day LEF with a mean follow-up of 9.1 months. The short-term results

showed a favourable clinical response in 12 (80%) of the patients. No patients discontinued therapy due to adverse effects. However, two patients developed new angiographic lesions in the follow-up MRA [113].

Biologic Agents

In the presence of refractory disease in the resistant and/or intolerant patients, biologic drugs including anti-TNF agents (mostly infliximab), rituximab and tocilizumab seem to be promising. In previous studies refractory disease was accepted if disease activity increased following reduction of the CS dose or persisted despite use of at least one conventional IS agent [87]. The Turkish TA Study Group defined refractory disease [114] as angiographic or clinical progression despite treatment or the presence of any of the following characteristics: (1) prednisolone dose > 7.5 mg/day after 6 months of treatment, despite administration of conventional IS agents; (2) new surgery due to persistent disease activity; (3) frequent attacks (more than three per year) and (4) death associated with disease activity.

Anti-TNF Agents

Serum TNF-α levels are increased in TA and T cells from patients with active TA had higher TNF-α production compared with those in remission or healthy controls [115, 116]. Therefore anti-TNF agents, mostly infliximab (IFX), were tried in refractory TA patients. In 2008 the same group retrospectively reported 25 cases with refractory TA from a single centre [117]. Treatment duration was up to 7 years. CS treatment was discontinued in 15 patients and was successfully tapered to < 10 mg/day in 7 patients. Adverse events were seen in four patients.

The results of long-term follow-up of anti-TNF treatment were reported in another case series of 20 refractory TA patients from a single centre [118]. In this study, 17 patients (85%) received infliximab, 2 patients (10%) received adalimumab, and 1 patient (5%) received etanercept. The median duration of treatment with TNF inhibitors was 23 months. Treatment with TNF inhibitors resulted in disease remission in 18 (90%) of 20 patients and sustained remission in 10 patients (50%). Ten (83%) of 12 patients were able to taper prednisone below 10 mg and 7 patients discontinued prednisone. However, 6 of the 18 patients achieving remission experienced relapse while receiving TNF inhibitors. Eleven patients (55%) discontinued TNF inhibitors for the following reasons: relapse, persistently active disease, lack of corticosteroid-sparing effect, adverse effects (4 patients), and other reasons (4 patients). These autors concluded that treatment with TNF inhibitors induced remission, including sustained remission in patients with refractory TA. However, 33% of patients experienced disease relapse while receiving TNF inhibitors and 20% discontinued treatment because of adverse events.

In 2012, Comarmond et al., [119] reported five new patients and reviewed the data of 79 patients previously reported in the literature. Most patients received IFX together with MTX or AZA. While 37% of patients achieved complete remission, 53.5% showed a partial response. CS treatment could be discontinued in 40% of the patients. However, < 10% of

patients remained resistant and side effects were observed in 20% of patients, including mainly infections and hypersensitivity reactions.

In another recent study, IFX was reported to show a sustained clinical improvement in the long-term in TA, with significant benefits in HRQoL. Also, radiological disease activity was assessed in 13/15 during IFX therapy, with evidence of improvement in 2/13, stable disease activity in 9/13, and worsening in 2/13 [120].

Some studies did not report the frequency of relapses. Recently, in 11 case series of 75 TA patients treated with IFX, 74.7% (56/75 patients) achieved remission and 32% discontinued CS therapy during follow-up. Of the patients that achieved remission, 28.6% (16/56 patients) developed a relapse [121]. Reduction of CS dose results from the case series were not pooled because they were too clinically heterogeneous. The follow-up periods in the case series ranged from 6 to 101 months.

Etanercept (ETN) was also not effective in maintaining remission in TA. In two case series, 4 TA patients receiving ETN, remission and reduction of CS doses were achieved in 3 patients, but 2 of them relapsed within the follow-up periods. The follow-up period for TA patients treated with ETN was 4 to 82 months [117]. Two patients initially treated with ETN were not controlled on ETN monotherapy and were switched to IFX in order to control their disease [122].

Tocilizumab

Since IL-6 is highly expressed within inflamed arteries and serum levels correlate with disease activity, blocking IL-6 may be effective in TA [111, 115]. Unizony et al., [111] reported four case series with 11 patients with TA who received tocilizumab (TCZ) for a mean period of 11months (range 4–41 months). One case was treatment naive and received TCZ monotherapy, and eight patients were refractory to concomitant prednisone (mean dose 23mg/day; range 5-40 mg/day) and other IS (MTX, n = 5; AZA, n = 3; MMF, n = 3; CYC n = 2; CSA n = 1; IFX, n = 4; and adalimumab n = 1). All patients achieved disease control, and those on CS were able to either discontinue or significantly taper prednisone after 3-6 months of TCZ therapy. Serial imaging in eight patients (MRA, n = 2; CT, n = 2; PET/CT, n = 4) showed improvement of vasculitic features in seven patients, evidenced by decreased vascular ^{18}fluordeoxyglucose uptake (n = 4), decreased vascular paramagnetic contrast enhancement (n = 2) or decreased aortic wall thickness (n = 1). One patient was shown to have a decrease in the thickness of the aortic wall following TCZ treatment, but progressive narrowing of the lumens of the renal, subclavian, and vertebral arteries was also observed. One patient relapsed after 8 months of treatment while still receiving TCZ 8 mg/kg every 4 weeks. A second patient relapsed within 3 months of discontinuing treatment.

Seitz M., et al., [123] were treated by TCZ 5 consecutive patients with giant-cell arteritis and 2 with TA. All patients achieved a rapid and complete clinical response and normalisation of the acute phase proteins. Remarkably, prednisone dosage could be reduced within 12 weeks to a mean of 2.5 mg/day (range 0-10 mg/day). No relapse and no drug-related side effects were noted.

Rituximab

Two case series examined rituximab (RXB) for TA patient (n = 5). Galarza et al., [124] reported good clinical response to rituximab, evidenced by improvement in clinical signs and symptoms, in one of two patients with TA refractory to MTX and TNF-α inhibitors. Hoyer et al., [125] reported three patients with refractory TA despite prednisone, MMF, CSA and adalimumab, who responded to RXB. Of note, anti-CD20 therapy normalized the number of peripheral plasma cell precursors, which subsequently increased during relapse in two patients that were successfully retreated with B-cell depletion.

No studies reported data on abatacept, adalimumab or ustekinumab in TA.

The adverse effects for each biological agent are summarized in Table 6. IFX was associated with more adverse effects, particularly infusion reactions some of which resulted in cessation of treatment. Two TA patients from case series developed malignancies-breast and pancreatic cancers. The patient who developed pancreatic adenocarcinoma was previously on AZA, possibly an underlying risk [118]. Patients treated with ETN had higher rates of infection; one patient developed heart failure [122].

Table 6. Summary of adverse effects in TA patients treated with biological agents

Biological agent	Number of Patients	Number of Studies	Number pts. (%) with AE	Cessation rate*	Adverse Effects	Miscellaneous effects
					Infections	
IFX	85	12	23/85 (27%)	11/85 (15.2%)	11/23 (47.8%)	steroid psychosis, breast CA transaminitis, allergic rash, allergy, serum sickness, pancreatic CA (all 1 pt) 4 infusion reactions
ETN	12	3	3/12 (25%)	1/4	2/4	N and HTN (1 pt in total)
TCZ	11	4	0	N/A	N/A	N/A
ADA	3	2	NR	NR	NR	NR
RXB	5	2	NR	NR	NR	NR

Abbreviations: A/E (adverse effects), TCZ (tocilizumab), IFX (infliximab), ETN (etanercept), ADA (adalimumab), RXB (rituximab), N (nausea), V (vomiting), HTN (hypertension), N/A (not applicable), NR (not reported),

* Cessation rate - discontinuation secondary to adverse effects.

Results from case series of patients with TA suggested that TCZ may be of some benefit for the maintenance of remission, and for the reduction of CS use. Case series results also suggested that IFX may be beneficial in the maintenance of remission and possibly reducing the amount of CS use in TA patients. IFX may be associated with increased risk of complications such as infections in TA patients.

An inherent problem with studying LVV and its management is the variable definition of disease remission. Many studies defined LVV remission as the absence of symptoms, and normalization of inflammatory markers (ESR and CRP). However, inflammatory markers are not completely reliable [126] especially when TCZ inhibits IL-6 from binding to its receptor and IL-6 is required for CRP synthesis from the liver [67]. This is highlighted in one of the studies using TCZ where one patient was noted to be in remission; however, autopsy results suggested active disease [111]. Moreover in TA, remission should not solely be monitored using inflammatory markers or clinical outcomes as these parameters may not reflect radiographic progression [127, 128].

In a minority of the studies (9 out of 22), remission was defined using the combination of clinical parameters, inflammatory markers, and the absence of new radiographic changes suggesting disease activity during the follow-up periods. Only well-designed trials will begin to answer which the cost/benefit of biological agents with their efficacy and adverse effects.

Surgery and Invasive Inetrventional Radiology

As a general rule, both endovascular interventions and surgery should be done in the quiescent phase of the disease. It has been shown that the rate of restenosis is higher when the surgery is performed during the active phase of the disease when compared to those with stable disease. Moreover, post-procedure therapy also plays an important role for the prevention of restenosis and the combination of steroids with immunosuppressive agents being more effective than steroids alone or no therapy [129].

Percutaneous transluminal angioplasty (PTA) was widely used for relief of short-segment arterial stenotic lesions, and initial reports revealed excellent results ranging from 81 to 100% [130-132]. However, restenosis occurring in up to 77.3% of the procedures in the long term appeared to be a major problem with PTA [133]. Therefore PTA is not cost effective and may be better used only in selected cases.

Stent grafts are better than uncovered metal stents or PTA in terms of the patency period and occurrence of restenosis in TA patients. Since the inner layers of the vessel wall derive nutrition from the luminal blood flow, placement of a stent graft may disturb luminal blood flow, leading to a decrease in chronic inflammation and less severe fibrotic reaction on the luminal side, with a lower incidence of restenosis [74, 105]. In a retrospective study analysing the outcome of endovascular interventions including stent replacements performed in the inactive stage of TA, the restenosis rate was reported as 17% after a mean follow-up period of 23.7 ± 18.4 months [134].

Antiplatelet treatment recommended used before and after endovascular interventions in TA, because decrease the occurrence of restenosis. Some authors [135, 136] administer loading doses of 300 mg of aspirin and clopidogrel 12h before the procedure, then continue with aspirin (100 mg/day) indefinitely and clopidogrel (75 mg/day) for 4 weeks after the intervention.

Indications for surgery in TA include critical cerebrovascular or coronary artery ischaemia, extremity claudication and severe renal artery stenosis. Progressive aneurysm enlargement with a tendency for dissection or rupture, severe aortic regurgitation and aortic coarctation also require surgery [74].

Surgical bypass was widely used for long-segment stenosis with extensive periarterial fibrosis or occlusion. Occlusion or restenosis after bypass grafting occurs in 8-31% of cases after a follow-up period of 3-6 years [19]. Maksimowicz-McKinnon et al., [39] were retrospectively studied 75 patients with TA. Both angioplasty and vascular surgery were initially successful, but recurrent stenosis occurred in 78% of angioplasty and 36% of bypass/reconstruction procedures.

Saadou et al., [137] in a retrospective, multicentre study analysing the results and outcomes of 79 consecutive patients with TA who underwent 104 surgical and 62 endovascular procedures, the frequencies of complications were 37.5% and 50%, respectively, after a follow-up of 6.5 years. Freitas et al., [95] showed that surgical treatment was performed in 34.6% of 52 TA patients and restenosis was observed in 13.5% in a median time of 11 months after surgery.

Since TA patients are generally immunosuppressed and often obese as the result of chronic CS therapy, surgical procedures carry additional risks. In particular, surgery for aortic aneurysms has a high morbidity and mortality. Surgical complications such as restenosis, graft occlusion and anastomotic site aneurysm may be related to the progressive inflammatory nature of TA. Anastomotic detachment may occur anytime in the long term, however, the use of synthetic suture material was reported to reduce this complication [74].

In conclusion, it is necessary to strictly control the disease activity before and after the surgical treatment using immunosuppressive agents to reduce the complication of the treated arteries.

Pregnancy

Because Takayasu arteritis predominantly affects women of reproductive age, the issue of pregnancy is important. Kerr et al., [5] reported five pregnancies in their series of 60 patients, all of whom had normal deliveries of a normal live infant. Only one patient had disease exacerbation during pregnancy. A study from Hong Kong in 1983 [138] reported on 13 women who had experienced a total of 30 pregnancies. Apart from hypertension, there were no major obstetric problems and no maternal deaths directly related to pregnancy. Fetal outcome could be predicted on the basis of maternal vessel involvement (abdominal aorta and renal), severity of maternal hypertension, superimposed pre-eclampsia, and timing of adequate blood pressure control.

Sharma et al., [139] studies 124 patients with TA over a period of 20 years (1979-1999). In this group 12 female patients (mean age 23.6 ± 3.6 years) experienced 24 pregnancies. The presenting features during pregnancy were severe hypertension (11 patients), congestive heart failure (two patients) and unequal pulses (one patient). Aortography revealed that abdominal aorta was involved in 11 patients and renal arteries in nine patients. Of 17 live babies born, intrauterine growth retardation was present in five babies and premature deliveries were encountered in four patients. Pregnancies resulted in abortion in two patients and intrauterine death in five patients. Maternal complications included superimposed pre-eclampsia in four patients, congestive heart failure and progression of renal insufficiency in two patients each and post partum sepsis in one patient. All patients with poor perinatal outcome had abdominal aortic involvement and a significant delay in seeking medical attention.

Recently, Alpay-Kanitez et al., [140] reported 37 TA patients who had a total of 84 pregnancies. The mean age of patients ranged 24.5 ± 6.6 years. Subclavian arteries (86%) were the most frequently involved vessels. They were able to complete the follow-up of ten patients who had a pregnancy after diagnosis during the period of pregnancy. Two patients who had renal artery involvement and active disease in third trimester suffered from preeclampsia and a worsening of hypertension. In one of them, disease flared up in the third trimester. There was no active disease in the postpartum sixth month. Maternal heart failure, cerebrovascular accident, death or cerebral hypoperfusion at the time of delivery, asphyxia and newborn anomalies were not seen in any of these patients. Very recently, Soma-Pillay et al., [141] reported a case of a 35-year-old para one gravida two patient with TA (group III disease) complicated by chronic hypertension and a severely dilated ascending aorta.

Tanaka et al., [142] analysed 27 pregnancies in 20 women with TA in Japan. None of the pregnancies showed TA activity. The obstetric events were pre-eclampsia in four pregnancies (15%), fetal growth restriction in one (4%), and placenta abruption in one (4%). Three pregnancies involved a steroids dose increase. There were no cardiovascular events. Eighty percent of the pregnancies that included an obstetric event also involved the mother's chronic hypertension. All listed autors agree that good blood pressure control during pregnancy is an important measure in reducing obstetric morbidity. Also, fertility is not adversely affected, pregnancy per se does not appear to exacerbate the disease, but management of hypertension is essential. Hypertension in the second stage of labour is a risk factor for cerebral haemorrhage; shortening this stage by use of low forceps delivery or vacuum extraction appears to be a reasonable solution.

Outcomes in TA

Amnong different series, mortality seems to range between 3 and 15% without obvious causes [45]. Ohigashi et al., [143] first showed a decrease in the mortality rate of 2.8% during the follow-up period of 2000-2010. Also showed that the time from onset to diagnosis was significantly shortened in the past decade (5,2 ± 6.1 years to 1.2 ± 2.34 years) which may be related to the development of noninvasive diagnostic imaging tools such as ultrasound, CTA, MRA, and PET. Also, showed that the frequency of occlusion in aortic arch branches and the complication of moderate or severe aortic regurgitation had decreased, and the maximum dose of prednisolone (22.4 ± 14.4 mg to 38.9 ± 14. 6 mg) and the use of immunosuppressive agents (7.0 to 42.9%) had increased. Although the vascular involvement has become less severe in the past decade, the number of patients who require surgical treatment has not changed (22.5%, 22.8% respectively), which may be related to recent technical advances in vascular surgery.

Conclusion

The prognosis of TA patients has improved in the past decade, and this may be because of early diagnosis owing to the development of noninvasive diagnostic imaging tools and improved medical treatment.

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Chapter 5

Macronutrients and Premenstrual Syndrome

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Abstract

Premenstrual syndrome (PMS) has few established risk factors and an unknown etiology. Effective treatment options are limited and most have substantial limitations. It is important to identify modifiable risk factors for PMS including dietary aspects. A number of recommendations concerning macronutrient intake have been made regarding prevention or treatment of PMS. For example, the American Congress of Obstetricians and Gynecologists and the Association of Reproductive Health Professionals recommend consuming regular meals with adequate protein and complex carbohydrates, with reduced fat and sugar intakes to alleviate PMS. However, these recommendations are not evidence-based and largely unsubstantiated.

Potential physiological effects of fats on PMS include inflammation and altered hormonal milieu. Saturated fats act as pro-inflammatory factors and increase estradiol and luteinizing hormone levels, whereas polyunsaturated fats like omega-3s act as anti-inflammatory factors. To date, two cross-sectional studies have examined fat intake and PMS symptom severity, and five small clinical trials have tested whether supplements containing fatty acids are effective at relieving symptoms. Total, saturated, and monounsaturated fats have overall higher symptom scores, particularly pain but total fat intake is related to lower cravings and bloating. The treatment trials show that gamma-linoleic acid supplements reduce symptoms.

Potential effects of carbohydrates include increasing serotonin levels, regulation of blood glucose and insulin sensitivity, and reducing estrogen levels via whole grain fiber. Few studies have directly assessed how carbohydrates impact premenstrual symptoms. These studies have shown inconsistent relationships suggesting that higher carbohydrate intakes are associated with lower symptom experience, particularly for pain and negative affect. Women with PMS show increased intake of total carbohydrates primarily from

simple sugars, which are associated with higher symptom severity, whereas whole grains and fiber are not associated, except for breast pain.

Dietary protein may potentially affect premenstrual symptoms through action of the renin-angiotensin-aldosterone system, lowered calcium levels, negation of serotonin increases from carbohydrates, and altered hormonal milieu. Soy protein is related to lower estradiol and progesterone levels, whereas red or processed meats have increased steroid hormones and sex hormone binding globulin. To our knowledge, only three studies have examined the association of protein intake and premenstrual symptoms. It appears that while women with higher intakes of protein have fewer symptoms, there is no difference in severity. Low-fat vegetarian diets show reductions in symptoms and severity, though not related to soy intake.

Several major limitations exist among available literature. All studies have assessed macronutrients as a potential treatment rather than a causal factor of PMS. Among the observational studies, it is unknown whether observed associations are due to reverse causation; specifically, it is unclear whether macronutrient intake contributes to PMS or whether women with PMS have altered their diet to control their symptoms. Lastly, many studies have looked at each macronutrient separately and have not assessed the effects relative to each other, to kilocalorie intake, or to confounding factors. Future research should use prospective designs to determine the temporal relationship between macronutrient intake and PMS controlling for other macronutrients and confounders. This research has the potential to help women prevent PMS via modifiable risk factors or treat symptoms effectively and safely.

Introduction

Premenstrual syndrome (PMS) has few established risk factors and the etiology of premenstrual syndrome is still largely unknown. However, the disorder likely results from the interaction of hormonal, neural, genetic, psychosocial, and dietary factors [1]. Treatments focus on treating the symptoms rather than the syndrome overall and often they are only able to alleviate one or two symptoms with limited efficacy [2]. Additionally, these treatments are further limited by serious side effects [2]. Thus, it is important to prevent the development of PMS by identifying modifiable risk factors. Diet is one potential modifiable risk factor. Most of the previous research on how diet impacts PMS has focused on micronutrients and several have been evaluated extensively, such as calcium, vitamin D, B vitamins, magnesium, and manganese [2]. In comparison, relatively few studies have assessed how intake of macronutrients relates to PMS, though a number of recommendations concerning macronutrient intake have been made for the prevention or treatment of PMS. In particular, both the Association of Reproductive Health Professionals and the American Congress of Obstetrics and Gynecologists (ACOG) suggest eating frequent small portions of complex carbohydrates and reducing intake of sugar to treat PMS [3, 4]. The ACOG additionally recommends reducing fat intakes to alleviate PMS [3]. However, these recommendations are not evidence-based and largely unsubstantiated.

The purpose of this review is to examine the available literature to date regarding the association between intake of fats, carbohydrates, and proteins and premenstrual syndrome.

Fats

Dietary fats are the densest source of energy with 9 kilocalories per gram of fat [5]. On average, dietary fats contribute to 32-33% of kilocalorie intakes for women ages 12-50 years[6]. In this age range, saturated fats provide approximately 10-11% of kilocalories, while 11-12% are from monounsaturated fats, and 7-8% from polyunsaturated fats. Fats are important in the structure and function of biological membranes, as well as hormone precursors and cellular signals [5].

Physiology

Dietary fat intake may play a role in the development and treatment of PMS through three physiological pathways: 1) promotion of chronic inflammation; 2) alteration of the hormonal milieu; and 3) changes in prolactin concentrations due to a defect in the conversion between linoleic acid to GLA (Figure 1).

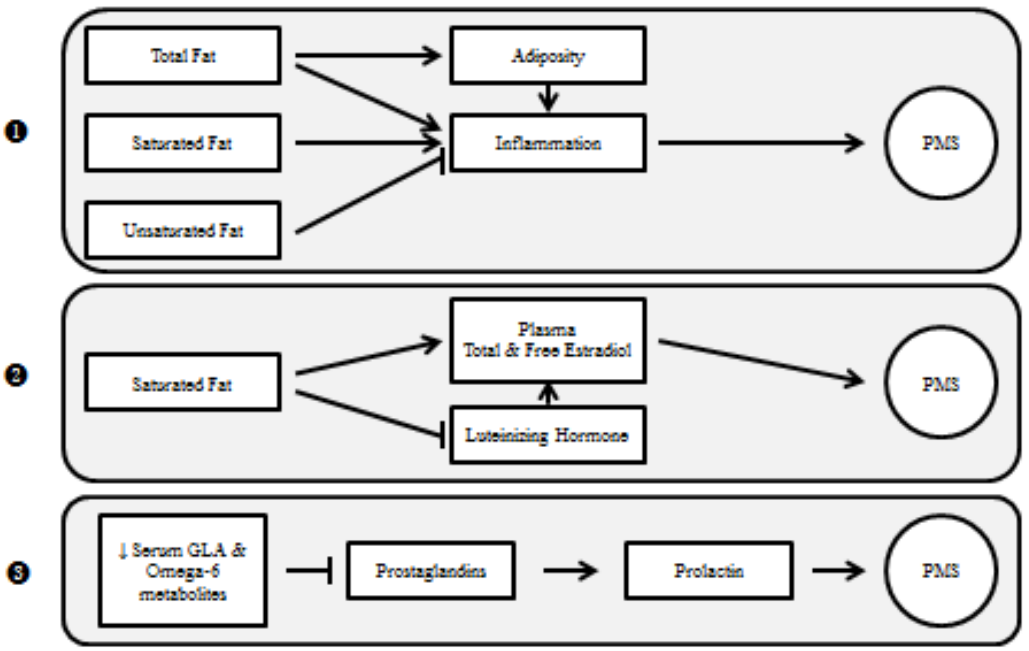

Figure 1. Suggested physiological mechanisms linking dietary fat intake and premenstrual syndrome (PMS). 1) Higher intake of total and saturated fat can lead to higher levels of inflammation, whereas unsaturated fat leads to lower levels of inflammation. Additionally, total fat can lead to increased adiposity which leads to higher levels of inflammation. High levels of inflammation is potentially associated with increased risk of PMS. 2) Saturated fat increases plasma concentrations of total and free estradiol and lowers levels of luteinizing hormone. Higher levels or sensitivity to changes in estrogens are associated with increased risk of PMS. 3) Low levels of gamma-linoleic acid (GLA) and subsequent omega-6 metabolites lead to lower levels of prostaglandin production. Low levels of prostaglandins lead to increased sensitivity to prolactin. Increased prolactin concentration and sensitivity has been suggested to increase risk of PMS.

Recent studies have assessed whether PMS is associated with chronic inflammation, as a number of psychiatric and somatic disorders that share common features with PMS have been found to be associated with inflammation [7, 8]. Additionally, cytokine expression has been found to occur in the endometrium, ovarian tissue, and granulosa cells [9-11] and varies during the menstrual cycle [10, 12-17]. A study by Puder et al., (2006) evaluated the relation of physiological and physical symptoms, as assessed by the Freeman Daily Symptom Record, and changes in serum concentrations of C-reactive protein (CRP) across the menstrual cycle [14]. Independently of weight status, they found that the total number of symptoms was positively associated with CRP concentrations. Symptom groupings that were associated with higher CRP concentrations included mood, behavior, and physical symptoms. Looking at PMS as a syndrome rather than general premenstrual symptoms, a study by Bertone-Johnson et al., (2014) assessed the association with several serum markers of inflammatory markers [18]. Interleukins (IL)-4, IL-10, IL-12, and interferon (IFN)-γ were all significantly higher in PMS cases versus controls. Additionally, IL-2, IL-4, IL-10, and IL-12 were significantly associated with higher symptom scores, with IL-2 associated with higher affective symptoms scores and IL-4 with higher physical symptom scores.

Dietary fat and saturated fats have been shown to act as pro-inflammatory factors increasing CRP concentrations [19, 20], while the unsaturated omega-3 fatty acids have been shown to act as anti-inflammatory factors decreasing CRP and IL-6 concentrations [21-23]. Additionally, increased adiposity is associated with increased inflammation [19] and has been shown to be a risk factor for PMS [24]. Therefore, dietary fat intake leading to inflammation may play a role in the etiology of PMS.

Secondly, fat intake has been associated with hormonal alterations. Hormone levels have been long implicated in the etiology of PMS due to its cyclical nature of symptoms that occur in the luteal phase when progesterone predominates, estradiol is waning, and gonadotropins are low [25]. Additionally, the cyclicity of symptoms disappears during anovulatory cycles [25]. While the etiology is still unclear, the hormones implicated in symptom development or severity include increased estrogen, sensitivity to falling estrogen, sensitivity to changes in estrogen-progesterone ratios, increased progesterone levels, and excess prolactin [4, 25].

Among premenopausal women, higher intakes of saturated fat are associated with higher plasma total and free estradiol levels and lower concentrations of luteinizing hormone (LH) [26]. Dietary trials of low-fat diets compared to usual diet suggest the potential for modification of fat intake to change hormone levels, but results have been conflicting. A randomized controlled trial by Gann et al., (2003) comparing a low-fat, high-fiber diet containing approximately 20% kilocalories from fat and 25g of fiber to usual diet found that the low-fat, high-fiber diet was associated with a non-significant decrease in serum total estradiol concentrations [27].

A crossover study by Barnard et al., (2000) comparing a low-fat, vegetarian diet to usual diet found that the low-fat, vegetarian diet increased serum concentrations of sex-hormone binding globulin (SHBG) but did not alter concentrations of estrogens [28]. It is unclear whether the results are due to the reduction of dietary fat or due to other factors such as higher fiber or vegetarian diets, and these other dietary factors may account for the differences in findings between the two dietary trials. Overall, it appears that dietary fat intake may alter hormone concentrations, particularly estradiol, playing a role in the etiology of PMS.

Thirdly, high dietary fat may contribute to PMS in women with excess prolactin concentrations due to a defect in the conversion of linoleic acid to gamma-linolenic acid

(GLA). A study by Brush et al., (1984) found that serum concentrations of alpha-linolenic acid (ALA), a precursor to omega-3 fatty acids and all subsequent omega-3 metabolites (e.g., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), were non-significantly elevated in women with PMS compared to those without PMS [29]. Additionally, serum concentrations of linoleic acid, the precursor to omega-6 fatty acids, were elevated but GLA and all subsequent omega-6 metabolites (e.g., arachidonic acid) were lower in women with PMS compared to women without PMS. The higher concentrations of linoleic acid indicate no intake deficiency, while lower GLA and subsequent omega-6 metabolite concentrations may suggest a defect in the conversion of linoleic acid to GLA in women with PMS. Furthermore, several of the omega-6 metabolites (i.e., dihommogammalinolenic acid, arachidonic acid, adrenic acid) are precursors to prostaglandins, which stimulate progesterone synthesis. Lower concentrations of prostaglandin E1would lead to excess prolactin, as prostaglandin E1 at normal levels attenuates the effects of prolactin. Therefore, lower concentrations of these omega-6 metabolites resulting from a defect in conversion from linoleic acid to GLA could lead to lower prostaglandins, which in turn could lead to lower progesterone levels and higher sensitivity to prolactin; both of which have been linked to PMS [30]. Progesterone has been implicated as a potential agent in PMS since progesterone can induce premenstrual-like symptoms, however it does not appear that levels are different in women with PMS versus those without but sensitivity may be different in women with PMS [30]. Additionally, prolactin injections have induced premenstrual-like symptoms [31], though prolactin levels do not appear to be different in most studies [30].

Epidemiology

Evidence suggesting a relationship of dietary fat with PMS comes from observational studies of dietary fat and premenstrual symptoms and clinical trials of fatty acid supplements to treat PMS. No studies to date have examined the association of dietary fat and the development of PMS. Two cross-sectional studies have examined dietary fat intake and premenstrual symptoms [32, 33].

Nagata et al., (2004) evaluated the relationship of dietary fat intake and premenstrual symptoms among 189 Japanese women aged 19-34 years [32]. Dietary intakes of total fat, saturated fat, monounsaturated fat, and polyunsaturated fat were assessed using a food frequency questionnaire (FFQ). A Japanese translated Moos Menstrual Distress Questionnaire (MMDQ) was used to assess 47 symptoms items during menses (menstrual), the week before menses (premenstrual), and over the remainder of the menstrual cycle (follicular), rating the severity of each symptom between 1 “no experience of symptom” and 6 “disabling or incapacitating.” The change in symptoms from the follicular phase to the premenstrual phase was used to calculate a total symptom percentage change score and the following sub-symptom percentage change scores: pain, concentration, behavioral change, autonomic reactions, water retention, negative affect, and arousal. After controlling for age, marital status, exercise, smoking status, age at menarche, and number of bleeding days, higher intakes of total fat, saturated fat, and monounsaturated fat were significantly correlated with greater change in the total symptom score ($r = 0.15\text{-}0.17$, $p < 0.05$) and pain sub-symptom score ($r = 0.16\text{-}0.21$, $p < 0.05$). Additionally, higher saturated fat intake was significantly

correlated with greater change in water retention ($r = 0.16$, $p = 0.04$) and negative affect ($r = 0.15$, $p = 0.04$) sub-symptom scores.

A second cross-sectional study, conducted by Gold et al., (2007) evaluated the relationship of dietary fat intake and premenstrual symptoms in a multi-ethnic group of 3302 US premenopausal women aged 42-52 [33]. Total fat consumption in grams and the % kilocalories from fat was assessed using a FFQ. The presence of eight premenstrual symptoms that ceased within 1-3 days from the start of the menstrual cycle were assessed though interviews. The eight symptoms were condensed into five symptom complexes: 1) anxiety/jitter/nervous and mood changes, 2) abdominal cramps and back/joint/muscle pain, 3) increased appetite/craving and weight gain/bloating, 4) breast pain/tenderness, and 5) headaches. The authors found that higher intakes of total fat in grams was significantly associated with lower craving and bloating symptom complex scores (OR = 0.56, $p = 0.024$) and non-significantly associated with lower anxiety and mood change (OR = 0.71, $p = 0.32$), breast pain (OR = 0.91, $p = 0.68$), and headache (OR = 0.92, $p = 0.66$) symptom complexes. Additionally, increasing % kilocalories from fat was associated with lower symptom complex scores but not significantly so.

In addition to the two observational studies described above, there have been five clinical trials assessing whether supplements containing fatty acids are effective at relieving premenstrual symptoms [34-38].

One clinical trial evaluated krill oil and fish oil, which led to significant reduction in premenstrual symptoms [34]. Another evaluated a supplement containing 200mg gamma linoleic acid, 175mg of oleic acid, 345mg linoleic acid, 250mg of other polyunsaturated acids, and 20mg of vitamin E [35]. Women ($n = 120$) were randomized to take a 1-gram dose, a 2-gram dose, or a placebo for 6 months. The women in the 1-gram and 2-gram dose had significant reduction in symptoms after 3 and 6 months compared to the placebo group, with the 2-gram dose providing the greatest symptom improvement.

Three trials evaluated various dosages and timing of Evening Primrose Oil supplementation [36-38], of which one showed supplementation with Evening Primrose Oil led to significantly alleviated premenstrual symptoms [36] and 2 found no difference in premenstrual symptoms between supplementation and placebo [37, 38]. The dosage, timing, and length of follow-up among all studies varied, which may explain the inconsistencies among the studies. The one study that found an association only supplemented during the luteal phase of the menstrual cycle [36], whereas the other two supplemented during the entire menstrual cycle [37, 38].

Total dietary fat intake may be associated with premenstrual symptoms; however, the two observational studies conflict in the direction of this association [32, 33]. The differences may be due to the populations examined, one was a younger population of Japanese women [32], and the second was an older population of American women [33], which may relate to the amount of saturated fat in the diet. The Japanese women consumed an average of 19.3g of saturated fat [32], whereas American women tend to on average consume higher amounts of saturated fat [6]. Only one of the two evaluated subtypes of fat, suggesting that higher saturated fat intake was specifically associated with pain, water retention, and negative affect symptoms [32]. While the one observational study found no association with polyunsaturated fats [32], the clinical trials appear to show improvement of premenstrual symptoms with higher intakes of omega-3 and omega-6, particularly GLA. However, it is unclear whether the results for individual symptoms would be similar for PMS overall.

Carbohydrates

Dietary carbohydrates contribute 4 kilocalories per gram of carbohydrate to total energy [5]. On average dietary carbohydrates contribute to 50% of kilocalorie intakes for adult women [39] and are the primary source of energy [5].

While the recommended Adequate Intake (AI) for dietary fiber is approximately 14 grams per 1,000 kilocalories per day, women consume on average only 8.1 grams per 1,000 kilocalories per day [40]. Foods items accounting for most of the United States' fiber intake comes from grains (44%), vegetables (21%), fruits (13%), and 10% from beans, peas and other legumes [40]. While the American Heart Association (AHA) recommends limiting consumption of added sugar to approximately 100 kilocalories per day [41], women on average consume 239 kilocalories (13.2% of total kilocalories) from added sugar daily [42].

Physiology

Dietary carbohydrate intake may play a role in the development and severity of PMS through three physiological pathways: 1) production of serotonin, 2) regulation of blood glucose and insulin sensitivity, and 3) alteration in hormone milieu (Figure 2).

Dietary carbohydrate intake may be associated with PMS through regulation of the neurotransmitter serotonin. Women with PMS have been shown to have lower whole-blood serotonin (5-HT, 5-hydroxytryptamine) levels during the late luteal phase in one study [43]. Carbohydrates are part of a feedback loop, where lower carbohydrate intake is associated with lower tryptophan levels [44]. Tryptophan is an amino acid precursor to serotonin; lower tryptophan levels lead to lower levels of serotonin resulting in increased dietary preference of carbohydrates. Upon consumption of carbohydrates, the resulting release of insulin stimulates the uptake of large neutral amino acids such as leucine, isoleucine, and valine into the muscle, thereby lowering serum levels of large neutral amino acids that compete with tryptophan for receptor-mediated transport across the blood-brain barrier [45, 46]. With less competition, higher levels of tryptophan are transported across the blood-brain barrier and converted into serotonin, leading to increased serotonin availability [44]. However, even small amounts of concurrent protein ingestion with carbohydrates have been shown to inhibit this effect [45]. Therefore, low intake of carbohydrates may be associated with lower serotonin concentrations thereby contributing to PMS.

Dietary carbohydrate intake may be associated with PMS through regulation of blood glucose and insulin sensitivity. Decreased insulin sensitivity has been reported during the luteal phase in symptom-free women; in PMS-susceptible women, a decline in insulin sensitivity may be a contributing factor to PMS [45]. Serotonin regulates glucose and estradiol levels that affect insulin resistance and blood glucose levels [47]. Zarei et al., (2013) found lower levels of blood glucose and insulin resistance among women with PMS during the luteal and follicular phases compared to women without PMS [47]. However, among women with PMS there was no difference between insulin levels measured in the luteal versus the follicular phase. The reduction in insulin sensitivity may depend on the increased level of progesterone, as both insulin resistance and progesterone levels increased during the luteal phase.

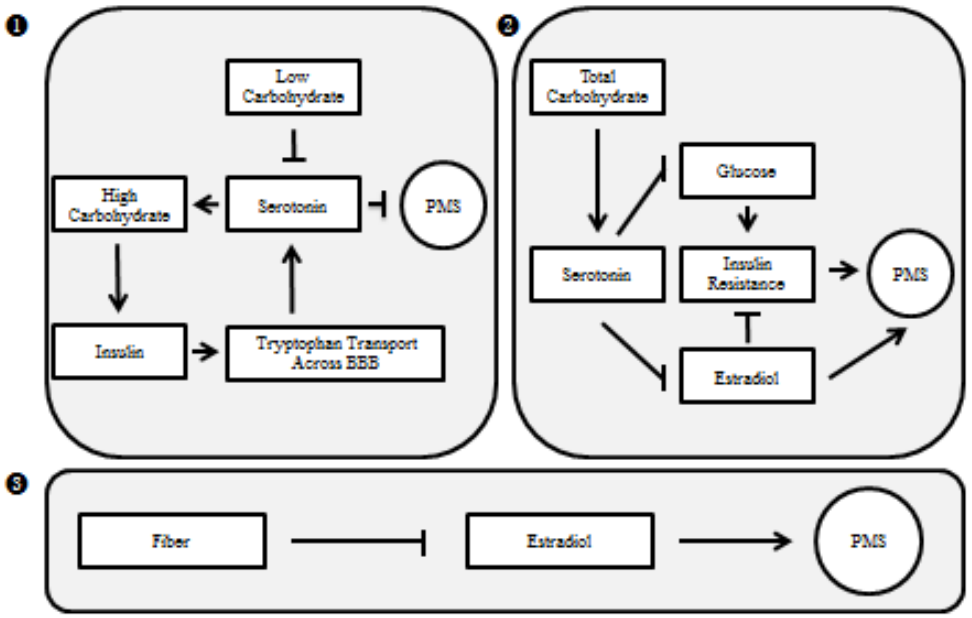

Figure 2. Suggested physiological mechanisms linking dietary carbohydrate intake and premenstrual syndrome (PMS). 1) Low intake of carbohydrate leads to lower insulin levels, and low insulin levels lead to increased carbohydrate preference. High carbohydrate consumption then increases insulin release. Increased insulin reduces competition for transport of tryptophan across the blood brain barrier (BBB). High tryptophan levels lead to an increase in serotonin. High levels of serotonin may reduce risk of PMS. 2) High intakes of total carbohydrates increase serotonin levels via method in number 1. High serotonin lowers blood glucose and serum estrogen levels. High levels of blood glucose increases insulin resistance. Insulin resistance can lead to higher risk of PMS. Increased estrogen can increase PMS directly and indirectly through insulin resistance. 3) High intake of fiber reduces estradiol levels. Increased levels or sensitivity to changes in estrogens are associated with increased PMS.

Finally, higher intake of dietary fiber may reduce premenstrual symptoms via a reduction in serum estrogen levels [33]. An inverse relationship between fiber and serum estradiol (E2) was observed in a population of premenopausal American women [48], though other studies have not found an association [26, 49, 50]. It proposed that diets high in fiber may reduce serum estrogens in premenopausal women by enhancing the excretion of endogenous estrogens and altering the enterohepatic circulation of estrogens by speeding up intestinal transit and preventing estrogen metabolites from being reabsorbed [33]. While many studies do not find an association between estradiol levels and premenstrual syndrome, one study showed an association between estradiol levels and symptom severity among women with premenstrual syndrome [51].

Epidemiology

No studies to date have examined the association of dietary carbohydrates and the development of PMS. One observational study has examined the association of dietary carbohydrates with prevalent PMS [52] and five observational studies have examined dietary carbohydrate intake and premenstrual symptoms [32, 33, 44, 53, 54].

Cross et al., (2001) evaluated energy intakes during different phases of the menstrual cycle, including intake of carbohydrates, among women with PMS [52]. Among healthy women not taking oral contraceptives who completed screening for PMS during two menstrual cycles with a Steiner questionnaire, 88 women were classified as having PMS and 56 were classified as controls. These 144 women also completed 4-day diet records during the premenstrual and postmenstrual phases of both menstrual cycles. Postmenstrual was considered to be 5 days after the beginning of menses and premenstrual was the four days prior to the predicted onset of menses. The authors found significant increases in intake of total carbohydrates (44.6% vs. 45.6% of kilocalories, $p = 0.05$) and simple sugars (18.9% vs. 20.9%, $p < 0.001$) from the postmenstrual to the premenstrual phase among women with PMS but not among women without PMS. There was a non-significant decrease in intake of complex carbohydrates (25.6% vs. 24.6% of kilocalories, $p = 0.07$). Additionally there was a weak correlation ($r = 0.26$, $p < 0.05$) between symptom severity and change in carbohydrate intake among women with PMS that did not exist among women without PMS. Among women with PMS, there was a significant increase in energy intake from the postmenstrual to the premenstrual phase ($p < 0.001$) from cake and other desserts and high sugar foods.

Nagata et al., (2004) evaluated the relationship of carbohydrates and premenstrual symptoms among 189 Japanese women aged 19-34 years [32]. Briefly, carbohydrate intake was assessed using a validated, 169-semi quantitative food frequency, and a Japanese translated MMDQ was used to assess the presence and severity of symptoms during three time points: during menses, the week before menses, and over the remainder of the menstrual cycle. The authors compared percent change in symptom scores between the follicular phase and additionally assessed percent change in scores for the following symptom clusters: pain, concentration, behavioral change, autonomic reactions, water retention, negative affect, and arousal. After controlling for age, marital status, exercise, smoking status, age at menarche, and number of bleeding days, the authors found that women with higher total intake of carbohydrates had non-significantly higher changes in the total symptom score ($r = -0.12$, $p > 0.05$) in the premenstrual phase. However, they found that intake of cereals/potatoes/ starches was inversely correlated with change in the total symptom score ($r = 0.16$, $p = 0.04$), as well as for the sub-symptoms pain ($r = -0.17$, $p = 0.02$) and negative affect ($r = -0.16$, $p = 0.03$) in the premenstrual phase, after adjusting for age and total energy.

Johnson et al., (1995) assessed macronutrient intake in healthy, normally menstruating women ($n = 26$) without complaints of menstrual distress [44]. Women completed a disguised questionnaire for one menstrual cycle about symptoms and type and amount of food consumed. The authors found that percentage of kilocalories from carbohydrates was positively associated with negative affect ($r = 0.51$, $p < 0.01$) and behavior change ($r = 0.42$, $p < 0.05$). Additionally, appetite ($r = 0.30$, $p > 0.05$) and control ($r = 0.29$, $p > 0.05$) were positively correlated, and arousal ($r = -0.27$, $p > 0.05$) was negatively correlated with carbohydrate intake, however, these did not reach statistical significance.

Murakami et al., (2008) assessed the association between dietary glycemic index (GI), a measure of the rise in blood glucose levels due to food, and premenstrual symptoms in Japanese dietetic students ($n = 640$) aged 18-22 years [53]. Consumption of carbohydrates was assessed using a comprehensive, validated FFQ and premenstrual symptoms were assessed using a retrospective version of the MMDQ. The authors reported that dietary GI and total MDQ scores were negatively associated (p for trend = 0.016) after adjusting for confounders. Additionally, dietary GI was negatively associated with concentration

(p for trend = 0.042), autonomic reaction (p for trend=0.045), and water retention (p for trend = 0.024) MDQ subscales during the premenstrual phase. Dietary glycemic load (GL) was not associated with premenstrual MDQ scores. In contrast, there was no association of dietary fiber intake with any of the premenstrual symptoms assessed.

Rasheed et al., (2003) examined the relationship between sweet-tasting food items and PMS in a population of college-aged women (n = 464) in Saudi Arabia enrolled in health-related programs [54]. They observed a significant positive relationship between premenstrual symptom severity and consumption of sweet-tasting food items such as chocolates, cakes, and deserts. Among women with the highest premenstrual symptom scores, 61.5% ate these food items > 3 times/day and 36.5% consumed them less often ($p < 0.05$).

Gold et al., (2007) assessed fiber intakes and premenstrual breast pain in a population-based study of premenopausal Americans (n = 3302) [33]. They showed that fiber intake was positively associated with premenstrual breast pain (aOR = 1.39, p = 0.037).

In addition to the six observational studies, Sayegh et al., (1995) conducted a double-blind, crossover study in 24 women with premenstrual syndrome to test the efficacy of a carbohydrate-rich beverage on premenstrual symptoms prior to and 30, 90, and 180 minutes after consumption [46]. Three beverages were used: A) the experimental beverage containing simple and complex carbohydrates (dextrose and maltodextrin) shown to raise serum tryptophan to large neutral amino acid ratios, B) mixture of protein and carbohydrates (casein and dextrose) that did not alter amino acid ratios, and C) carbohydrates (galactose and dextrose) not altering tryptophan to amino acid ratios. The experimental beverage significantly decreased depression, anger, confusion, and carbohydrate craving 90-180 minutes after consumption. In summary, the relationship between dietary carbohydrate intake and premenstrual symptoms remains unclear. Several studies have observed that women with PMS have increased carbohydrate intake premenstrually, primarily from refined grains and simple sugars. Higher intakes of desserts containing refined grains and simple sugars are associated with higher severity of symptoms. However, this observation conflicts with the findings that consumption of high GI foods may decrease premenstrual symptoms, particularly concentration, autonomic reactions, and water retention, though glycemic load showed no association. Overall, complex carbohydrates and dietary fiber did not appear to be associated with premenstrual symptoms, except perhaps breast pain.

The epidemiologic findings appear to conflict with the physiological mechanisms, where epidemiological studies show higher severity of symptoms with increased carbohydrate intakes primarily from refined grains and sweet desserts and the physiology suggesting that increases in serotonin from consuming additional carbohydrates may alleviate symptoms. Since the epidemiological studies were cross-sectional, the temporality of the association is unknown and reverse causation may be a potential explanation for this conflict, where women with PMS may be consuming higher intakes of carbohydrates in order to alleviate the symptoms.

Protein

Dietary proteins contribute 4 kilocalories per gram [5]. On average, adult women consume 15.5% of their total kilocalories as dietary protein [39]. Functions of proteins

include enzymes, transcription factors, binding proteins, transmembrane transporters, hormones, immunoglobulins, motor protein, receptors, structural proteins, and signaling proteins [55].

Physiology

Dietary protein intake may affect the development and severity of PMS via several physiologic mechanisms, including 1) alteration of renin-angiotensin-aldosterone system function, 2) lowering of calcium levels, 3) effects on neurotransmitter metabolism, and 4) alterations to the hormonal milieu (Figure 3).

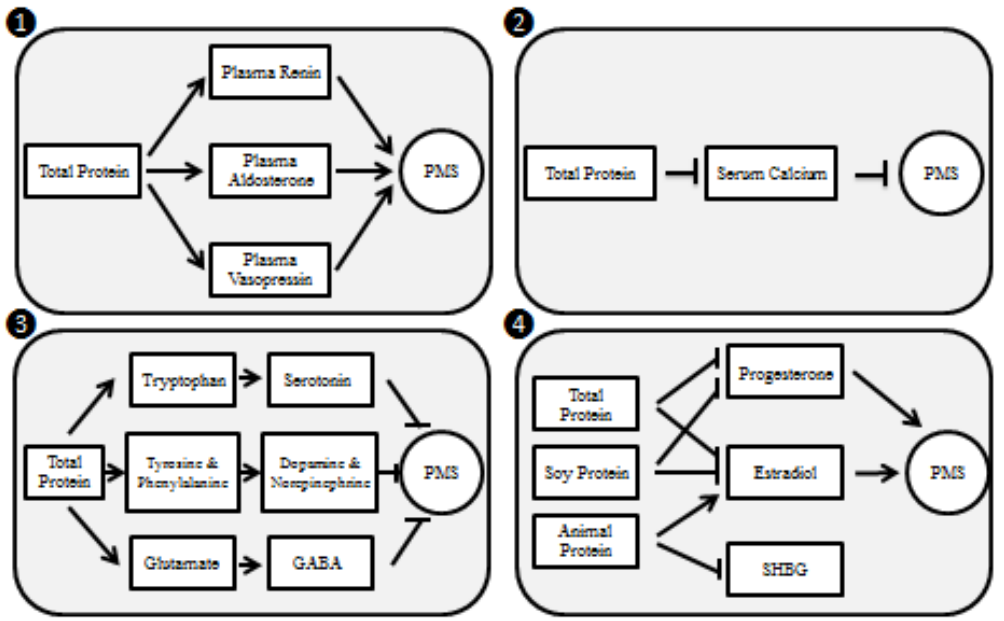

Figure 3. Suggested physiological mechanisms linking dietary protein intake and premenstrual syndrome (PMS). 1) Higher intake of protein increases plasma renin, aldosterone, and vasopressin, all of which have been suggested to increase the risk of PMS. 2) Higher intakes of protein could decrease serum calcium. Calcium deficiency has been linked with PMS. 3) Higher intakes of protein increases levels of amino acids. Higher tryptophan leads to higher serotonin, higher tyrosine & phenylalanine lead to higher dopamine & norepinephrine, and higher glutamate leads to higher γ-aminobutyrate (GABA). Serotonin, dopamine, norepinephrine, and GABA have been linked to decreased PMS. 4) Higher intake of total protein and soy protein lead to increased progesterone and estradiol levels. Higher animal protein intake leads to higher estradiol levels and lower sex hormone binding globulin (SHBG). Higher levels of progesterone and estradiol have been suggested to cause PMS.

First, protein intake is reported to increase serum levels of vasoactive hormones of the renin-angiotensin-aldosterone system (RAAS), including plasma renin, aldosterone, and vasopressin [56]. Function of the RAAS has been suggested to play a role in the etiology of premenstrual syndrome [57, 58]. Aldosterone is higher during the luteal phase of the menstrual cycle and is associated with potassium and sodium ratios [57]. Additionally, women with PMS were found to have higher aldosterone concentrations and increased plasma

renin activity during the luteal phase compared to women without PMS in one study [58]. These differences in the RAAS may explain premenstrual symptoms of ankle edema, bloating, and breast swelling.

Second, high intakes of protein could contribute to premenstrual syndrome via a lowering of calcium levels. Some studies, though not all, have suggested that high protein diets can cause increased calcium excretion [59]. Intake of calcium from dietary sources [60] and supplements [61-64] has been shown reduce the risk of PMS.

Third, protein intake provides amino acids, which are precursors to several neurotransmitters suggested to play a role in PMS [25, 65]. Neurotransmitters that have been implicated in the etiology of PMS include serotonin, γ-aminobutyrate (GABA), and catecholamines [4, 25]. Tryptophan is a precursor for serotonin, and tyrosine and phenylalanine are both precursors for the catecholamines dopamine and norepinephrine [66]. Glutamate (glutamic acid) is the main excitatory neurotransmitter in the central nervous system and is a precursor to GABA, the main inhibitory neurotransmitter in the central nervous system [67]. Additionally, as described above, tyrosine and other large neutral amino acids such as leucine, isoleucine, and valine compete with tryptophan for receptor-mediated transport across the blood-brain barrier for neurotransmitter synthesis [45, 46]. Consumption of small amounts of protein along with carbohydrates in a single meal have been shown to negate the increase in serotonin levels caused by the consumption of carbohydrates via an insulin mediated mechanism. A clinical trial by Sayegh et al., (1995) described earlier examining the effect of a tryptophan-depleting drink on amino acid levels and premenstrual symptoms among women with PMS (n = 24) found that at baseline, there were lower tyrosine concentrations during the luteal phase, and tryptophan depletion increased intensity of premenstrual symptoms [65]. The women were randomized to either a carbohydrate drink designed to raise tryptophan levels, the carbohydrate drink with added protein, or a carbohydrate drink not designed to alter tryptophan levels. The tryptophan raising carbohydrate drink significantly reduced symptoms of depression, anger, confusion, and carbohydrate craving at 90 and 180 minutes after consumptions, whereas the other two drinks had no significant reductions in symptoms.

Fourth, dietary protein intake may be associated with PMS via alterations in sex steroid hormone concentrations. Soy protein, one source of vegetable protein, has been inversely associated with ovarian hormone levels, particularly 17-beta-estradiol and progesterone, in a number of studies, potentially due to the high contents of the isoflavones, daidzein and genistein [68]. Additionally, it is hypothesized that proteins from animal sources may contain exogenous hormones that could contribute to circulating steroid hormone concentrations in women [69]. Diets high in animal products like red meat or processed meats have been associated with an increase in circulating steroid hormones including total and free estradiol and a reduction in SHBG in a number of studies [69].

Epidemiology

There have been no studies to date directly evaluating protein or amino acid intake and development of PMS. However, three observational studies have examined protein intake and premenstrual symptoms [32, 44, 52].

Cross et al., (2001) evaluated energy intakes during different phases of the menstrual cycle, including intake of protein, among women with PMS, as described previously [52]. Among healthy women not taking oral contraceptives who completed screening for PMS during two menstrual cycles via Steiner questionnaire, 88 women were classified as having PMS and 56 were not. These 144 women also completed 4-day diet records during the premenstrual and postmenstrual phases of both menstrual cycles. Postmenstrual was considered to be 5 days after the beginning of menses and premenstrual was the four days prior to the predicted onset of menses. Women with PMS consumed 78.7g of protein during the premenstrual phase and 65.6g during the postmenstrual phase, whereas the women without PMS consumed 74.5g during the premenstrual phase and 71.5g during the postmenstrual phase. Among women with PMS, there were significant increases (16.6% change, $p < 0.001$) in protein intake premenstrually compared to the postmenstrual phase. There was no significant change among women without PMS. As discussed previously looking at the epidemiology of carbohydrates and PMS, premenstrually there was increase intake of cereals, composite savory dishes, cake/desserts, high-sugar foods, and savory snacks contributing to increased protein intake. These foods also provide substantial carbohydrates. Additionally, correlations between symptom severity and change in protein intake premenstrually were not significant among women with PMS.

Nagata et al., (2004) looked at the correlation between protein intake and premenstrual symptoms among 189 Japanese women aged 19-34 years [32]. Neither total protein intake ($r = 0.11$, $P > 0.05$) nor soy intake ($r = -0.02$, $p > 0.05$) were correlated with symptom severity.

Johnson et al., (1995) assessed macronutrient intake in healthy, normally menstruating women ($n = 26$) without complaints of menstrual distress [44]. Women completed a disguised questionnaire for one menstrual cycle about symptoms and type and amount of food consumed. Results suggested that arousal symptoms specifically were negatively correlated with percent of kilocalories from protein intake ($r = -0.37$, $p < 0.05$) but correlations were not observed for other symptoms assessed.

Two clinical trials have assessed the ability of vegetarian diets [28] and amino acids supplements [65] to relieve premenstrual symptoms.

Barnard et al., (2000) used a crossover study in women with PMS ($n = 33$) to assess a low-fat, vegetarian diet for two months compared to usual diet with a placebo supplement for two months [28]. The low-fat vegetarian diet consisted of grains, vegetables, legumes, and fruits with no restrictions, whereas animal products, added oils, fried foods, avocados, olives, nuts/nut butters, and seeds were not allowed. The average intakes during the low-fat, vegetarian diet were 43.5g protein, 313g carbohydrates, and 16.6g of fat, which was lower in protein and fats but higher in carbohydrates than the usual diet which contained 67.2g protein, 293g carbohydrates, and 51.8g of fat. The low-fat, vegetarian diet showed significant reductions in symptoms and symptom severity compared to usual diet. However, it is unclear whether the reduction in symptoms and symptom severity was related to the reductions in dietary fat or the vegetarian diet.

Menkes et al., (1994) compared plasma levels of the amino acids tryptophan, isoleucine, leucine, phenylalanine, tyrosine, and valine across the menstrual cycle in 16 women with premenstrual syndrome. Results suggested significant decreases in tyrosine and non-significant decreases in total plasma tryptophan levels premenstrually [65]. Tyrosine levels were 87.3mM during the follicular phase and 74.4mM in the premenstrual phase ($p = 0.002$)

and total tryptophan levels were 61.8mM and 54.1mM respectively ($p = 0.16$). The authors then randomized the women to receive either a plasma tryptophan depletion drink or a placebo drink. Women with depleted tryptophan levels had increased symptom intensity compared to controls, particularly for withdrawn, restless, irritable, and mastalgia. Additionally, the symptom magnitude corresponded to the decrease in tryptophan.

In summary, dietary protein does not appear to be associated with overall level of premenstrual symptoms, but may decrease symptoms of arousal. Vegetarian diet may reduce premenstrual symptoms, but it remains unclear whether the effect is due to the vegetarian diet or the low-fat diet. Additionally, intake of soy does not appear to be associated with PMS risk. Lastly, lower levels of the amino acids tyrosine and tryptophan may increase premenstrual symptoms.

Conclusion

The available literature to date examining how macronutrients contribute to PMS is relatively scant, and many questions remain to be answered. First, no studies have assessed how macronutrient intake may be etiologically related to the development of PMS. The available literature has primarily assessed whether modifying macronutrient intake may be useful for treating premenstrual symptoms or PMS. Additionally, as discussed above, all of the observational studies have been cross-sectional and it thus remains unknown whether the findings of these studies may be attributable to reverse causation. Specifically, it is unclear whether macronutrient intake contributes to PMS or whether women with PMS have previously altered their diet to control their symptoms. Additionally, underlying deficiencies that are causing cravings and diet changes premenstrually such as part of the carbohydrate feedback loop may not be the same as the underlying deficiency etiologically leading to PMS. For example, women with increased carbohydrate cravings may be self-medicating and treating symptoms rather than increased carbohydrates etiologically related to PMS. The use of randomized controlled trials and prospective studies of incident PMS should be used to tease this apart.

Secondly, much of the work in this area has evaluated premenstrual symptom severity in healthy populations rather than evaluating how dietary factors are associated with PMS as a syndrome. The associations present for specific individual premenstrual symptoms may not hold when looking at the aggregate of PMS for two reasons. First, it is unclear whether the relationships for low-grade symptoms hold for more severe symptoms of clinically significant PMS. Second, individual symptoms can have different etiologies and the subtleties can be lost when looking at PMS as a disorder with heterogeneous symptom experiences across women.

Lastly, the majority of studies conducted to date have considered the independent effects of each macronutrient separately and have not assessed the effects relative to each other. Because individuals in energy balance maintain a relatively consistent intake of kilocalories over time, high consumption of one macronutrient directly leads to lower consumption in another macronutrient. For example, increased intake of carbohydrates is often associated with decreases in protein intake. Therefore, any effects seen may not be due to increases in the macronutrient assessed but the changes in the other macronutrients in the diet.

Additionally, the physiological mechanisms suggest interactions of macronutrients, particularly carbohydrate and protein intake, in the development of PMS.

With these limitations, recommendations on macronutrient intake related to PMS appear to be premature. Future research should use prospective study designs to assess the temporal relationship between macronutrient intake and PMS to verify that macronutrient intake leads to PMS. Secondly, the research should use validated measures of PMS rather than individual symptoms. Lastly, studies should include potential confounders and assess the possibility of the effects due to decreases in other macronutrients. If macronutrients are found to play a role in the etiology of PMS, future clinical intervention studies should assess which of the suggested physiological pathways are likely to be involved. This research has the potential to help women prevent PMS via modifiable risk factors or treat symptoms effectively and safely.

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Chapter 6

Pressurized Intraperitoneal Aerosol Chemotherapy (PIPAC): Occupational Health And Safety Management

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Abstract

Pressurized IntraPeritoneal Aerosol Chemotherapy (PIPAC) is an innovative drug delivery system applying chemotherapy as a pressurized therapeutic aerosol into the abdominal cavity. PIPAC has superior pharmacological properties and first clinical results concerning efficacy and safety are promising. However, applying chemotherapeutic substances as a toxic aerosol is a challenge for occupational health and safety management. A risk assessment of PIPAC was performed to meet legal requirements. It was not possible to replace chemotherapeutic drugs with other substances since platin-based drugs and/or anthracyclines are the drugs of choice in many peritoneal cancers. However the dose of toxic substances could be reduced by 90%. Exposure of health workers to the chemotherapeutic drugs can be cutaneous (liquids) or respiratory (aerosol). Wearing chemotherapy gloves, protective clothing and glasses was effective for preventing cutaneous or ocular exposure, since the drugs applied are not absorbed through the skin. A three-level confinement system was designed to prevent any inhalative exposure of health workers. The first level is the closed abdomen itself, which

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tightness is controlled before chemotherapy application. The second level is the volume of the operating room together with air exchange through high-flow ventilation. The third confinement is the physical operating room, since the PIPAC procedure is remote-controlled and the team leaves the room during application. At the end of the procedure, the toxic aerosol is exhausted through a Closed Aerosol Waste System (CAWS) over a special line into the external environment, using the same system used for eliminating narcosis gases. Repeated environmental measurements in two different hospitals according to NIOSH protocols at the potential working places of the surgeon and of the anesthesiologist detected no traces of platin in the air. Biological monitoring in the blood of 5 surgeons after 500 procedures showed no traces of platin or doxorubicin. Mathematic modelling of the worst case scenario (immediate release of the toxic aerosol into the working environment) showed a potential respiratory uptake lower than 1/100'000 of a usual systemic chemotherapy dose. Over 650 procedures, to minor incidents related to disconnection in the tubing system were reported in the Critical Incident Reporting System (CIRS). No severe incident, in particular no leakage of the toxic aerosol, was recorded. No pressure- or technology-linked patient complication was noted. Work place measurements remained below the tolerance margin. The safety measures and conditions as defined above are sufficient. PIPAC can be used safely in the clinical setting if the conditions specified above are met. For the drugs tested, PIPAC is in compliance with European Community working safety law and regulations.

Introduction

Local drug administration has been used as a therapeutic modality for many years and for a broad spectrum of indications. Drugs can be delivered locally as powder (e.g., talcum into the pleural space), as a liquid (e.g., intraperitoneal chemotherapy) or as an aerosol (e.g., nasal spray). Among these possibilities, delivering drugs as an aerosol has significant benefits, including ease of use, patient comfort, dose reduction, greater selectivity, and the potential to decrease side effects [1].

As a consequence, aerosol formulations have found wide application, in particular in pulmonary medicine for treating asthma, chronic obstructive lung disease, allergies, etc. A further indication for aerosols in pulmonary medicine is lung cancer [2]. Several chemotherapeutic agents have been tested in this indication in vitro or in vivo (reviewed in 3).

Recent phase I studies have demonstrated the feasibility and safety of aerosol delivery of doxorubicin [4] and gemcitabine [5] in lung cancer patients. In the abdomen, the use of intraperitoneal drug delivery in the treatment of malignant disease confined to the peritoneal cavity is based on the potential for increased exposure of the tumor to antineoplastic agents leading to improved cytotoxicity.

In patients with tumors confined to the peritoneal cavity, there is established pharmacokinetic and tumor biology-related evidence that intraperitoneal drug administration is advantageous. Clearly, intraperitoneal drug delivery is an important adjunct to surgery and systemic chemotherapy in selected patients [6]. However, there are practical and theoretical concerns about intraperitoneal chemotherapy with liquids including [7]:

1. Adequacy of drug distribution throughout the entire peritoneal cavity
2. Limited direct penetration of drugs into tumor or normal tissue

3. Decrease in the delivery of drug to the tumor by capillary flow (through the systemic circulation) after regional delivery
4. Unique toxic effects associated with local delivery—e.g., abdominal pain, bowel perforation, infection, and obstruction
5. Added time, inconvenience, and cost associated with the specific requirements of regional delivery—e.g., catheter placement

These concerns explain why, despite the positive effect of intraperitoneal chemotherapy on progression-free and overall survival in selected patients with small volume peritoneal carcinomatosis, this management strategy is not broadly accepted and finds only limited use in clinical practice.

Pressurized IntraPeritoneal Aerosol Chemotherapy (PIPAC)

A new way of intraperitoneal chemotherapy is the application of cytotoxics in form of a pressurized aerosol into the abdominal cavity. We first described the principle of Pressurized IntraPeritoneal Aeorosol Chemotherapy (PIPAC) 15 years ago [8]. PIPAC relies on logical physical principles: local administration into the body cavity to improve therapeutic ratio, gaseous form to achieve homogeneous drug distribution, pressure application to enhance convective drug uptake into tumor nodes, and minimally-invasive approach to minimize operative trauma. PIPAC allows repeated therapy cycles and objective tumor response assessment (reviewed in 9). PIPAC has superior pharmacological properties and first clinical results concerning efficacy and safety are promising [10].

PIPAC technology (Figure 1) uses a laparoscopic access via ports through the abdominal wall. In a first step, a normothermic (37°C) capnoperitoneum (CO_2) is established with a pressure of 12 mmHg. Using a specific high-pressure injector and a micropump, a cytotoxic solution is aerosolized into the abdominal cavity, and maintained for 30 min.

At the end of the procedure, the aerosol is removed through a closed suction system. Since its physical behaviour is similar to an ideal gas, an aerosol applied in the peritoneal cavity allows a homogeneous distribution of the chemotherapeutic agent within the closed abdominal space [11]. Furthermore, an artificial pressure gradient is generated that overcomes tumoral interstitial fluid pressure and results in higher tissue drug penetration compared to conventional intraperitoneal or intravenous chemotherapy [12-14]. At the same time the plasma concentration of the chemotherapeutic agent remains low [15]. However, delivering chemotherapy as an aerosol is a challenge for occupational health and safety management because it carries a potential risk of accidental exposure of health care workers as compared with other administration routes. This is due to the difficulty to control the spread of aerosols which in turn contributes to the risk of unwanted exposition. This problem is not new and technical solutions have already been proposed. For example, in pulmonary medicine, a tent system combined with HEPA filters has been proposed to reduce this risk to a minimum during aerosol chemotherapy.

This system was shown to be effective at containing any nebulized liposomal encapsulated cisplatin during patient treatment [16]. A similar challenge is raised by PIPAC.

To prevent any harm to health care workers, we have identified and evaluated potential hazards concerning occupational exposures during PIPAC performance.

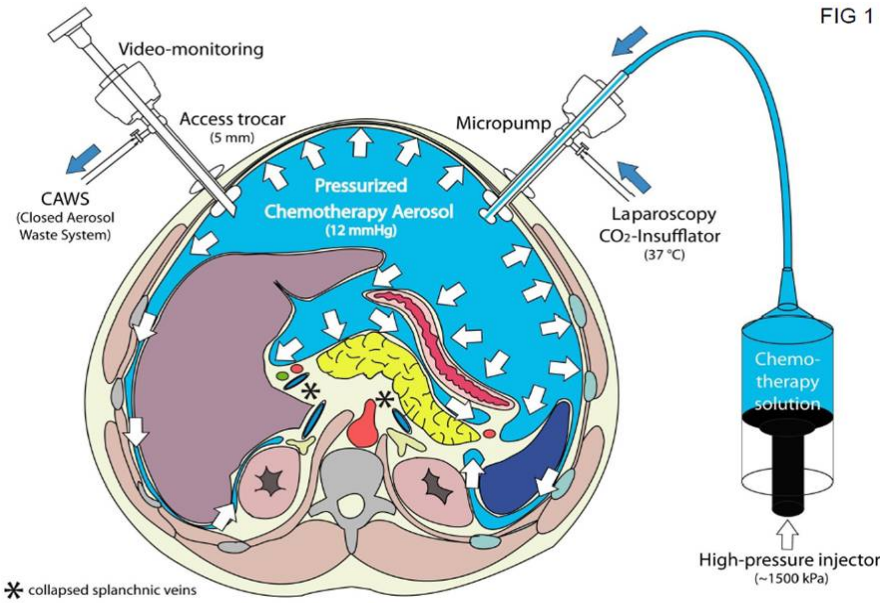


Figure 1. Technique of Pressurized IntraPeritoneal Aerosol Chemotherapy (PIPAC).
A capnoperitoneum of 12 mmHg is established as usual during laparoscopy. A chemotherapy aerosol is generated at the tip of a mechanical micropump introduced through a balloon trocar, and maintained for 30 min at 37°C. Then, the toxic aerosol is exsufflated over a secure system (CAWS: Closed Aerosol Waste System) into the outside environment.

In a second set of experiments, we have simulated PIPAC in the laboratory and in the operating room. Then, we have applied PIPAC in the human patient using chemotherapeutic drugs and measured contamination levels under real clinical conditions.

Later on, we have sampled body fluids, looking for the presence of traces of chemotherapy in healthcare workers. Finally, we have simulated accidental exposure by developing mathematical models in the worst case scenario.

Methods

Ethical, Legal and Regulatory Background

The study protocol was submitted to the Institutional Review Board (IRB, Common Ethics Committee of the Westfalian Wilhelms-University Münster and of the Westfalian Medical Chamber).

The IRB recommended performing the first PIPAC therapy with volunteers, which were extensively informed and trained in the PIPAC procedure. PIPAC application was performed within the framework of regulatory studies (PIPAC-OV1; PIPAC-GA1; PIPC-OV2) and as off-label use according to German Drug Act (AMG).

Methodology

The following steps were defined: identification of hazardous substances and dose; identification of possible exposure ways; simulation of the PIPAC procedure with non-toxic aerosols and smoke; redaction of Standard Operating Procedures (SOP); 2nd simulation according to the SOP; informing and training of the health care workers; performance of the first two PIPAC procedures with chemotherapeutic substances and workplace measurements under real conditions.

These steps were determined together with an external company specialized in occupational health safety in the chemical industry (DEKRA Industrials, Stuttgart, Germany). After successful workup in the first institution (Evangelic Hospital Bielefeld, Germany), we repeated the measurements in a second hospital (Marien Hospital Herne, Ruhr-University Bochum, Germany) under supervision of a second independent company (Dräger Safety, Lübeck, Germany).

After 500 PIPAC procedures, blood samples were taken from the 5 surgeons and gynaecologists having performed these procedures. Incidents were recorded prospectively with a Critical Incident Reporting System (CIRS).

Finally, mathematical simulation allowed to determine theoretical contamination levels in the worst case scenario (complete release of the toxic aerosol into the working environment).

Aerosolizer

The micropump (MIP®, Reger Medizintechnik, Rottweil, Germany) has been described elsewhere [17]. In brief, it consists of several components, including a shaft and a nozzle. The nozzle has a diameter of 0.2 mm. A pressure of up to 20 bar is delivered through a high-pressure line upstream of the micropump, using an industry-standard injector for vascular surgery (Mark 7 Arterion, Bayer Healthcare, Berlin, Germany), including a remote control device.

Operating Room Characteristics

PIPAC procedure was performed within an operating room (OR) equipped with laminar air flow. Volume of the OR was approximately 168 m^3. Air flow was 1.8 x 10^6 L/h. Room temperature was 22.3-22.6°C. Relative humidity was 36-37%. Atmospheric pressure was 994 hPa. Vacuum was generated with a negative pressure of -0.85 bar (Dräger, Lübeck, Germany).

Chemotherapy

We have focused on the application of two chemotherapeutic agents*:* cisplatin and doxorubicin. Chemotherapy was applied as following: nebulization over 3-6 minutes of 7,5 mg cisplatin/m^2 body surface followed immediately by the aerosolization of 1,5 mg doxorubicin/m^2 body surface into the abdominal cavity filled with CO_2 at a pressure of 16 to

20 mbar (12 to 15 mmHg) at a temperature of 37°C followed by 30 min steady-state before exsufflation.

Experimental Protocol

PIPAC procedures were performed in peritoneal cancer patients. Between the procedures, the room was cleaned according to the hospital's standard hygiene and surface cleaning protocols. Each procedure was structured into 4 consecutive phases:

- **Phase 1:** CO_2 insufflation over an industry-standard trocar (Kii Access System, Applied Medical, Darmstadt, Germany), with a target pressure of 16 mbar (12 mmHg). The access system was secured with an intra-abdominal balloon and an extra-abdominal obturator, ensuring tightness of the abdomen and steadiness of the pressure. Two 5- mm working trocars are inserted.
- **Phase 2**: Introduction of a micropump (MIP®, Reger Medizintechnik, Rottweil, Germany) through the access trocar and aerosol formation of the chemotherapy solution into the abdominal cavity using the high pressure injector.
- **Phase 3**: The system was kept in steady-state for 30 minutes at a constant pressure and temperature. The abdomen is hermetically sealed and the total gas flow is minimal.
- **Phase 4:** At the end of the procedure, the gas from the abdomen was released directly into the hospital's air waste system over one of the trocars and an aerosol/smoke filter (pores 0.027 μm, model 03110-10, MTP, Neuhausen ob Eck, Germany).

Mathematical Simulations

Modelisation was performed with MatLab® software (MathWorks, Natick, MA, USA).

Risk Assessment

Risk assessment was performed beforehand, before first PIPAC application in the human patient. Following steps were taken:

Identification of Hazardous Substances and Dose

For PIPAC, drug choice was derived from the protocol of the German Society for General and Visceral Surgery (DGAV) for CytoReductive Surgery (CRS) and Hyperthermic IntraPeritoneal Chemotherapy (HIPEC) [18].

Cisplatin

Cisplatin is highly poisonous. It can provoke anaphylactic reactions; irritates eyes and skin, has no transdermal absorption. Cisplatin irritates airways, has a cumulative toxic effect on kidney, bone marrow and the inner ear. It is probably carcinogenic to humans. Cisplatin toxicological characteristics are summarized in Table 1.

Table 1. Toxicological characteristics of cisplatin

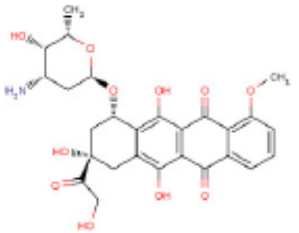
Parameter	Value
CAS-/EG Number	15663-27-1 / 239-733-8
Formula	
Molecular weight	300.06 g/mol
Melting point	270°C; dark yellow powder at room temperature
Boiling point	Not applicable
Steam pressure	Not applicable
Water solubility	2.530 g/L [25°C¨]
LD50 oral	20 mg/kg (rat) 32 mg/kg (mouse)
NOAEL[1]	No data
Important toxicological details	Acute toxicity: very toxic Skin and eye irritation No evidence for transdermal absorption Cumulative damage of kidney, bone marrow and inner ear No evidence for carcinogenity in human Evidence for carcinogenity and teratogenity in mouse and rat Level of carcinogenity: 2A Anaphylactic reactions reported Sensibilisation of skin and airways
Total amount of applied cisplatin	15 mg in 150 ml NaCl 0.9%
Concentration of applied solution	0.1 mg/ml = 0.1 g/L = 0.01%
Total applied amount of cisplatin	150 ml
Workplace exposure limits	Germany: not available Netherlands: 0.00005 mg/m^3

Doxorubicin

Doxorubicin is hazardous to human health by provoking mucosal inflammation, leucopenia as well as dilative cardiomyopathy. Additionally, it induces DNA mutation and is carcinogenic to humans. Doxorubicin toxicological characteristics are summarized in Table 2.

The total dose applied during PIPAC (s. above) is approximately 10% of a usual systemic chemotherapy dose. There is no legal exposure limit for either of these two substances in Germany. However, in the Netherlands, the maximally allowed air concentration for cisplatin is < 0.00005 mg/m^3.

Table 2. Toxicological characteristics of doxorubicin

Parameter	Value
CAS-/EG Number	23214-92-8 / 245-495-6 25316-40-9 / 246-818-3 (Hydrochloride)
Formula	
Molecular weight	543.52 g/mol
Melting point	205°C (Degradation); crystallinered powder at room temperature
Boiling point	Not applicable
Steam pressure	Not applicable
Water solubility	0.0928 g/L [25°C¨]
LD50 oral	570 mg/kg (mouse)
NOAEL1	No data
Important toxicological details	Acute toxicity: harmful Dilatative cardiomyopathy Inflammation of mucosa Leucopenia Evidence for carcinogenicity in animals Evidence for mutagenicity in animals Level of carcinogenicity 2A
Total amount of applied doxorubicine	3 mg in 150 ml NaCL 0.9% solution
Concentration of applied solution	0.02 mg/ml = 0.02 g/L = 0.002%
Total applied amount of doxurubicine	150 ml
Legal upper limit for working place	Germany no upper legal limit

Identification of Possible Exposure Ways

Identified exposure ways are ocular, dermal and inhalative exposition. Other possibilities were reasonably excluded. The preparation of the chemotherapeutic agents in the hospital pharmacy and their transport in adequate containers to the operating room is organized according to the German guidelines [19]. The hospital pharmacy is certified. Both agents are provided in a closed delivery system (special injection syringes filled with NaCl 0.9% solution) in a double envelope, together with patient identification tags and labels defining agent and dose. The syringes are hermetically sealed with special caps.

Simulations

First PIPAC Simulation with Physiological Solution Aerosols

Before performing the first clinical PIPAC application, the procedure was simulated in the operating room using a laparoscopy training phantom and non-toxic NaCl 0.9% aerosol.

Working steps were identified and a first operating protocol written. Based on this protocol, risk analysis was performed within an interdisciplinary team including physicians (surgeons and anaesthesiologists), scrub nurses, hospital technicians, the engineers having developed the PIPAC technology and occupational health experts in order to list a maximal number of safety issues, from a 360° perspective. A chronological protocol of the procedure was designed. Possible failures were defined at each step of the procedure, related to the personnel, the patient, the high-pressure injector, the infusion line, the CO_2 insufflator, the access trocars, the micropump itself, the laminar air flow system, the tightness of the abdomen and the exhaustion of the aerosol. For each issue identified, the expected frequency and severity were determined. Then, appropriate safety measures were defined step by step. On this basis, a standard operating protocol was established that served as the basis for the second simulation.

Second PIPAC Simulation with Smoke and an Artificial Leak

The second simulation was performed in the operating room under strict implementation of the standard operating procedures. The abdomen was simulated with a sealed plastic container of similar dimensions. An aerosol of CO_2 and smoke with the same pressure as during laparoscopy (12 mmHg) was installed and industry-standard technical instruments (access trocars, video camera, grasping forceps) were tested. We decided to choose double-balloon access trocars (Kii access port, Applied Medical, Düsseldorf, Germany) to guarantee complete tightness of the abdomen. We excluded CO_2 insufflators equipped with a recapture function in case of overpressure, in order to prevent any release of chemotherapy-loaded CO_2.

We were able to perform the complete procedure without any incident; in particular, the system remained tight. Then, a maximal leakage was simulated (an access trocar was fully opened). The smoke escaping from the leak was flowing downward to the floor and into the lateral outflow windows of at floor level of the operating room (Figure 2).

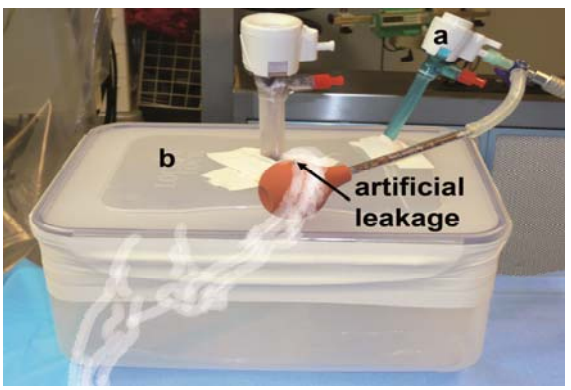


Figure 2. PIPAC simulation with smoke and artificial leakage: sealing access trocars (a) were introduced into a sealed plastic box (b) with the same volume dimensions as the human abdominal cavity. The box was pressurized with CO_2 and steam. Via an artificial leakage (open access trocar), the steam (white bold arrows) was observed to be directed to the floor and not randomly distributed within the operating room. This is caused by the laminar air flowing downwards from the ceiling to the floor.

Design of a Safety Concept

Based on the experience from both simulations, and together with the consulting engineers, we then designed a comprehensive safety concept taking into account all possible incidents and their prevention.

Safety Concept for Cutaneous Exposition

Cutaneous absorption of anthracyclines (such as doxorubicine) and platin-based chemotherapeutics has not been documented. However, local skin contact with chemotherapy solutions might lead to cutaneous irritation or even chemical burns.

Since PIPAC is applied within a closed system, the risk of skin contamination with chemotherapy is minimal. Under normal working conditions, a skin contact with chemotherapy is excluded. For such a contact to occur, a human or material failure has to be assumed, e.g., due to a manipulation error with the high pressure injector, or use of inadequate, low-pressure infusion tubing leading to explosion of the system, wrong line connections, etc.).

During PIPAC preparation, application and termination, the potential risk of skin contamination with chemotherapy solutions can be adequately met by wearing protective, water-proof surgical clothing and special chemotherapy gloves.

The risk of ocular lesions (keratitis, inflammation of the anterior eye chamber, scar formation, etc.) is satisfyingly met by wearing protecting glasses or transparent masks covering the entire face. This risk can be further reduced by providing sterile one-block systems (nebulizer and infusion tubing sealed together with secured, doubled connections), and by training and drilling the team in order to minimize human errors.

The floor of the operating room under the high-pressure injector and the connecting line is covered by a waterproof protection sheet. Commercial sets are available in order to remove chemotherapy solution in case of spillage, and are available in the OR.

The operating room has to be cleaned after each PIPAC procedure – as a standardized procedure by trained personnel. Tissues, tubes, lines and other devices such as operation drapes and sponges have to be disposed into special, sealed chemotherapy waste containers with adequate labelling.

Safety Concept for Respiratory Exposition

A safety concept consisting of three consecutive levels of confinement was designed in order to prevent any accidental respiratory exposition of the personnel to the chemotherapy aerosol. These 3 levels of confinement are (Figure 3):

- The closed abdomen
- The operating room and the ventilation system
- Remote-controlling of the procedure.

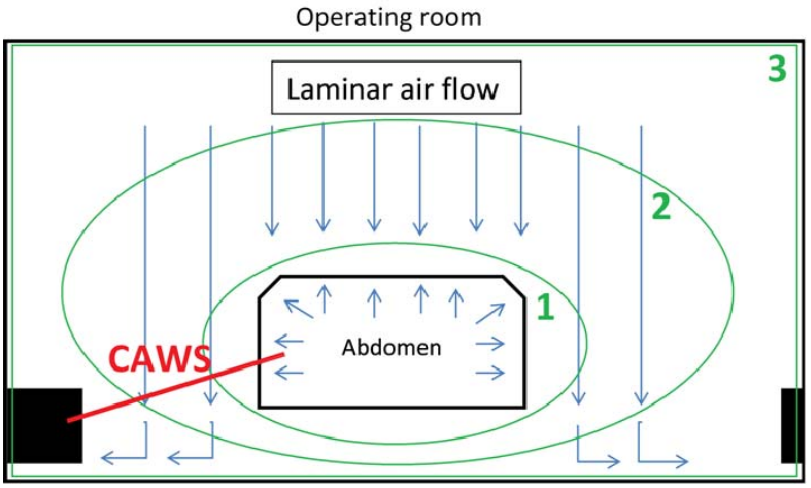


Figure 3. A safety concept consisting of three consecutive levels of confinement was designed in order to prevent any accidental respiratory exposition of the personnel to the chemotherapy aerosol. These 3 levels of confinement are: The closed abdomen, The operating room and the ventilation system and Remote-controlling of the procedure. At the end of the PIPAC procedure, a single person enters the operating room and opens the gas evacuation system (Closed Aerosol Waste System, CAWS) designed to discard the toxic aerosol without risk of exposition.

The tightness of the abdomen can be easily controlled by a zero-flux from the CO_2 insufflator. However, CO_2 is absorbed by the organism so that a flow rate of 0.1 L/min (max 0.2 L/min) is acceptable.

The second confinement level is more complex and consists of the operating room and the ventilation system together. A usual operating room has a volume between 120 and 150 m^3 so that a leakage of few litres CO_2 would be significantly diluted (by a factor of about 50'000). Modern operating rooms are equipped with high-flow ventilation systems ensuring rapid air exchange. The pre-treated air can be cooled,. warmed and/or humidified according to the need. Then, it is filtered through one or several series of filters placed in the ventilation tubing (in the rule so-called F-filters) and/or in the ceiling of the operating room (so-called H-filters). In some cases, a ventilator ensures a continuous air flow from the operating room ceiling in cases of pressure loss in the ventilation system. The air enters the operating room through a micro-perforated ceiling (ventilation) or through advanced systems generating a laminar air low (without turbulences). Aspiration of the air is provided through caption exits at the floor level, with anti-reflux valves generally equipped with pressure-regulating feed-back systems.

The principle of a typical ventilation system is described in Figure 4.

The detailed description of the various filters and corresponding ISO norm 14644 goes beyond the scope of this work but is available in the specialized literature [20]. Some ventilation systems can be programmed to function with negative pressure aspiration, which is advantageous for performing PIPAC because any air contamination of independent rooms can be completely excluded. The third level of confinement consists of the walls of the operating room, since the procedure is remote-controlled and the personnel is leaving the operating room just before application of the chemotherapeutic aerosol.

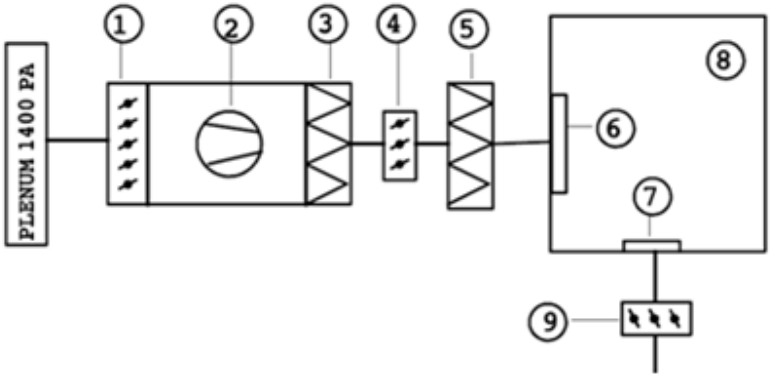


Figure 4. Example of a typical air-filtering system in the operating room. The air enters the system through a motorized valve system (1), is propulsed by a ventilator (2) through the first series of filters (e.g., F9 filters). Then, the air goes through a motorized disinfection valve system and a second series of filters (e.g., H12 filters) before entering the operating room (8) through a perforated ceiling (6). The air is aspirated through the exit openings (7) at the floor level and discarded through an anti-reflux valve system (9).

In case of emergency, it is possible for a surgeon or for an anaesthesiologist to enter the operating room for delivering drugs, etc. However, the presence time in the area at risk should be kept as short as possible. At the end of the PIPAC procedure, a single person enters the operating room and opens the gas evacuation system (Closed Aerosol Waste System, CAWS) designed exclusively to discard the toxic aerosol without risk of exposition (this is not the usual suction system used by surgeons in the operating room).

The CAWS consists of a closed line connecting the valve of a single access trocar with the wall suction plug routinely used by the anaesthesiologists to evacuate narcosis gases. Two micro-particle filters are placed in series to further limit the risk of contamination of the working environment with chemotherapy micro droplets. During the PIPAC procedure, the CAWS is closed by two valves in order to exclude any premature exsufflation of the abdomen.

Information and Training

On the basis of the successful simulations, it was decided to schedule two patients for the first PIPAC procedures. Information meetings allowing open, interactive discussion were organized since the planned procedures had raised emotional concerns, in particular among scrubbing nurses and cleaning workers.

On the basis of these discussions, we decided to restrict the first procedure to volunteers within the framework of a special shift under exclusion of other simultaneous surgical procedures. Before the first procedure, the team of volunteers received interdisciplinary training according to the standard operating procedures. Access to the operating room where PIPAC is applied was strictly restricted, the area was signalled with warning shields on each access door. These shields were turned on the backside at the end of the procedure.

Safety Assessment

In occupational settings, environmental monitoring of exposure to toxic aerosols seems to be superior to biological monitoring. It offers the possibility of simultaneous determination of components of mixtures, is simple to interpret, and evaluates short-term exposure to environmental irritants [21]. Thus, estimation of exposure under real conditions was an important step to provide a safe working environment during PIPAC.

First Environmental Assessment

The two first PIPAC procedures worldwide were performed on Nov. 5th, 2011 at the Evangelic Hospital in Bielefeld, Germany. The operating setting is shown in Figure 5.

The standard operating protocols were strictly implemented; in particular, nobody remained within the operating room during the PIPAC procedure, which was remote-controlled. The nebulizer functioned as expected and the system remained air tight. At the end of the procedure, the chemotherapy aerosol was exhausted into the air waste system of the hospital and released into the outside environment.

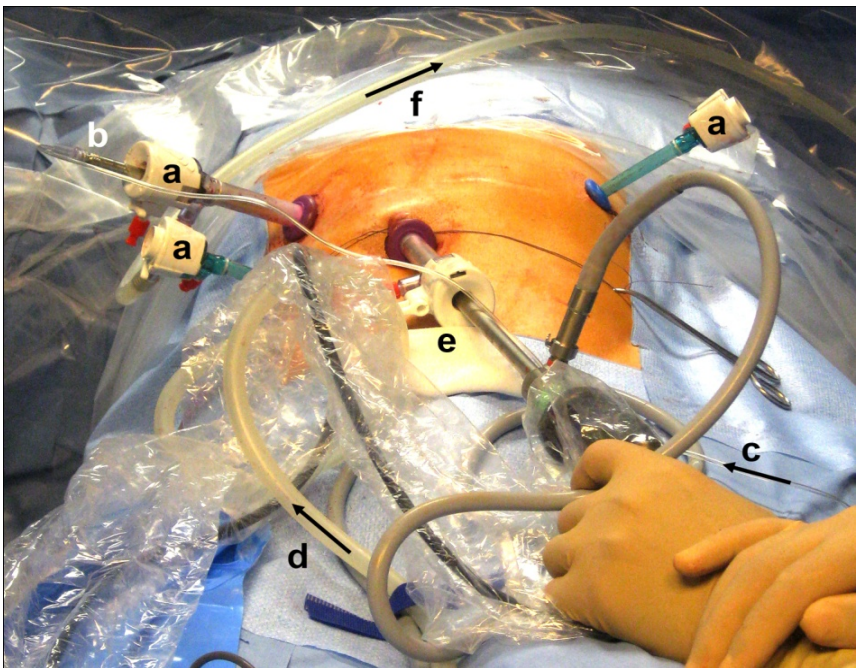


Figure 5. First PIPAC under real conditions: Access trocars (a) with the nebulizer (b) in situ. The chemotherapeutic agents were transported from the injector to the nebulizer via a high-pressure infusion line (c). CO2 was injected into the abdominal cavity via a standard gas line (d) and the trocar (e); (camera trocar). At the end of the procedure, the chemotherapeutic capnoperitoneum was exsufflated via closed line (f) over two serial microparticle filters into the air waste system of the hospital. Dark arrows indicate the flow direction of the gas and chemotherapeutics, respectively.
* = trocar sealing rings.

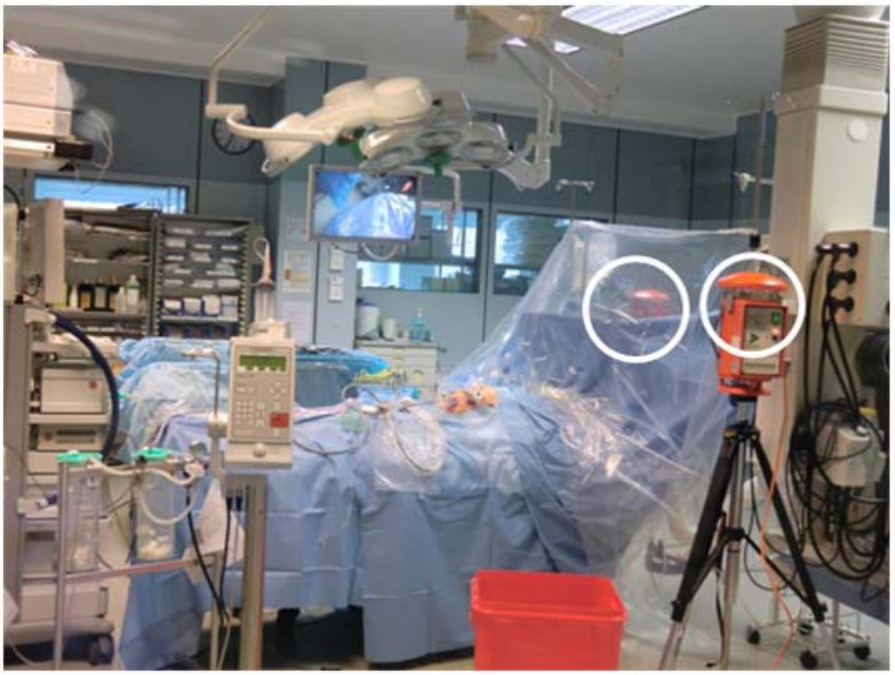


Figure 6. Operating room (OR) set up for first PIPAC and safety measurement. The OR is equipped with laminar air-flow. The abdomen is tight. The procedure is remote-controlled. Environmental air sampling was undertaken at the surgeon's (a) and anesthesiologist's (b) working positions. The pressure injector (c), sealing trocars and nebulizer in situ (d) and also the exsufflation line (e) are shown. To minimize any possible chemotherapeutic exposure of the anesthesiology crew, a transparent curtain dividing the laminar air flow was hanged vertically 50 cm above the access trocars, between the patient's head (position of the physician) and the abdomen (site of chemotherapy application).

The procedure was scheduled on a holiday and no other surgical procedure was performed at that time in any other room. The procedure was performed under the supervision of a safety officer and included workplace air measurements. Air was sampled during two consecutive PIPAC procedures. The operating room setting including safety measures and air sampling devices at the putative positions of the surgeon and of the anaesthesiologist are shown in Figure 6.

The probe sampling system used was a Gravikon VC25 device combined with a dust detector (Ströhlein, Kaarst, Germany). Air was collected on a cellulose nitrate filter with a diameter of 50 mm, with a flow of 22.5 m^3/h. Toxicological research analysis of cisplatin levels was performed according to a standard protocol (NIOSH 7300). The detection limit was 0.3 µg/sample. Sampling and analysis were performed by engineers of the Division for Hazardous Substances at the Laboratory for Environmental and Product Analysis of DEKRA Industrial GmbH in Stuttgart (Germany), an independent certification organization. Results are summarized in Table 3. Air analysis showed no traces of cisplatin, neither at the position of the surgeon nor at the position of the anaesthesiologist [22].

Table 3. Measurement of platinum concentration in the operating room

Measurement points	1	2
Probe N°	0511-01	0511-02
Date	05.11.2011	05.11.2011
Start [hh:mm]	12:15	12:15
End [hh:mm]	16:38	16:38
Duration [h]	2.4	2.4
Airpressure [hPa]	994	994
Temperature [°C]	22.5	22.5
Volume stream [m^3/h]	22.5	22.5
Partial gas volume [m^3]	54.2	54.0
Platinum in inhalable dust [mg/Pr.]	0.3	0.3
Limit of Determination (LoD) [mg/Pr.]	0.3	0.3
Relative LoD [mg/m^3]	0.000006	0.000006
Analysis [mg/Pr.]	< 0.3	< 0.3
Concentration [mg/m^3]	< 0.000006	< 0.000006
Platinum calculated as cisplatin Concentration [mg/m^3]	< 0.000009	< 0.000009

Nota:

(a) To allow a precise analysis of the limit of determination (LoD), probe sampling was performed during two operations but only when chemotherapeutic drucs were applied. In the meantime, sampling was paused. (b) Measurement locations:

[1] Anaesthesiologist's position- patient head, 150 cm above floor.

[2] Surgeon's position – patient abdomen, 150 cm above floor.

Second Environmental Assessment

A second set of environmental measurements was commissioned when PIPAC procedures were started in a second hospital (Marien Hospital Herne, Ruhr University Bochum, Germany). Evaluation was performed by Dräger Analytical, Lübeck, Germany in order to exclude a potential release of hazardous substances into the air during PIPAC therapy. Again, platinum concentration was used as a tracer for possible contamination of the circulating air. For the second assessment, another probe sampling system was used, consisting of a Gravikon PM 4.2 device combined with a dust detector (Ströhlein, Kaarst, Germany). Air was collected on a cellulose nitrate filter with a diameter of 50 mm, with a flow of 4 m^3/h. The absolute detection limit for platinum was 0.0005 mg, corresponding to 0.00013 mg/m^3 for 4m^3 air. Blinded analysis of the test and control samples was performed by Kiwa Control GmbH, Rostock, Germany, by flameless atomic absorption spectrometry (AAS), executed with inductive coupled plasma (ICP-OES).

All probes remained negative, meaning that no traces of platin could be detected in the air. Thus, this second assessment confirmed the absence of air contamination during PIPAC in a second institution with different technical setup in the operating room [23].

Biological Monitoring

In Germany, biomonitoring is required by law in the context of occupational health as a secondary prevention measure for the identification of individual load after exposure to hazardous substances. For many hazardous materials, including many chemotherapeutic drugs, the individually recorded load is quantifiable by biomonitoring and thus assessable. The aim of biomonitoring is to take appropriate measures (improvement of technical, organizational and personal prevention) to reduce the burden and the health hazard (reviewed in 24).

In particular, biomonitoring should be taken into consideration in activities (non-exhaustive list):

- where direct skin contact with hazardous substances is possible,
- for which exposure to hazardous substances with long biological half-life is present,
- when exposed to carcinogenic or mutagenic substances,
- when exposed to mutagenic substances,
- where the hazardous substances are difficult to measure in the air,
- in case of accidental exposition to hazardous substances.

Some of these conditions are clearly given for health workers administering PIPAC to patients. Therefore, after 500 PIPAC procedures, blood was sampled from all 5 surgeons and gynecologists performing PIPAC on a regular basis in our institution. Samples were taken by an independent physician. Serum concentrations of doxorubicin and cisplatin were determined by an independent laboratory. After obtaining consent of the physicians examined, we could collect all individual results: all samples remained negative, meaning that no traces of cisplatin or doxorubicin could be detected in the persons with the highest risk of potential exposure to toxic aerosols (data on file). Thus, the probability that other health workers have been exposed is extremely low, since no one of these individuals applied PIPAC in patients.

Mathematical Modelization, Worst Case Scenario

A further step in PIPAC risk assessment is to evaluate the worst case scenario, namely the case of complete release of the toxic aerosol into the working environment, in order to be able to determine the degree of exposition of the crew in such an extreme case. For this purpose, we performed mathematical simulations. The assumptions made on the basis of physiological, clinical and technical data available are given in Table 4. In addition, it was assumed that the aerosol behaves like an ideal gas, meaning that it is homogeneously distributed within the volume of the operating room after release. Finally, it was assumed that the volume of the abdomen remains constant because leakage is compensated by the CO_2 insufflator.

For calculating what would happen in the case of complete aerosol release, we first determined both extreme boundary scenarios (limits):

- all-at-once release of the aerosol (immediate, complete release at the beginning of PIPAC application)
- continuous release of the whole aerosol over time (slow release until completion of the procedure).

By definition, any leakage mode will lie between these both limits so that the real exposition can be assumed to lie between the boundaries calculated for these scenarios.

Immediate Release Scenario

The first scenario presupposes an all-at-once release of the whole toxic aerosol immediately after application. Mathematically this scenario can be described by an exponential function. The following function describes the trend of the concentration of the chemotherapeutics in the operating room depending on the time.

$$c_1(t) = c(t_0) * e^{-a*t} \; with \; c(t_0) = \frac{m_{start}}{V_{OR}}$$

where $c_1(t)$ = concentration over time, t_0 = concentration at time 0, m_{start} the administered dose of chemotherapeutics, V_{OR} the volume of the operating room, and a the air exchange rate through the laminar flow.

Slow Release Scenario

The second scenario describes the slow, continuous release of the whole aerosol over a period of 30 minutes. This scenario can also be described by a composition of exponential functions.

$$c_2(t) = c(t_0) * (1 - e^{-b*t}) * e^{-a*t} \; with \; c(t_0) = \frac{m_{start}}{V_{OR}}$$

where b is the rate of toxic agent leaving the abdominal cavity over time.

The curves for both scenarios are shown in Figure 7. Differences between both boundary scenarios are obvious. The immediate release scenario results in a about 50 times higher peak concentration of chemotherapeutic substances in the air of the operating room than the slow release scenario. Moreover, the timepoint of the maximal potential exposition is different, immediate in the first scenario and after about 3 minutes in the second scenario. In both scenarios, the concentration of toxic substances in the air can be considered to be insignificant after 12 to 15 minutes operating time.

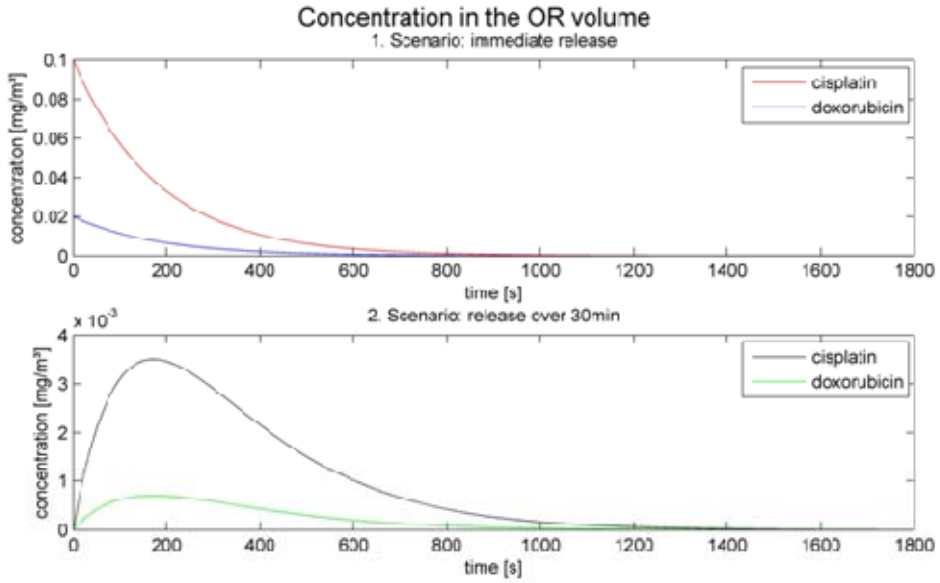


Figure 7. Concentration over time of cisplatin and doxorubicin in the air of the operating room (OR) after total leakage during PIPAC. Upper panel: immediate release scenario. Black curve: cisplatin; blue curve: doxorubicin. Lower panel: slow release scenario. Black curve: cisplatin; green curve: doxorubicin. The y-axis scale differs between panels by 3 orders of magnitude.

Table 4. Assumptions made for mathematical modelization of the worst case scenario (complete release of the toxic aerosol into the working environment)

Parameter	Value
Volume of the operating room	150 m^3
Volume of the abdomen	3 liters
Body surface	2 m^2
Application time	30 minutes
Air exchange rate	50 m^3/min
Breathing volume	5 liters/min
Administered doses: Cisplatin Doxorubicin	 15 mg 3 mg

Inhaled Dose

In a second step, to determine the inhaled dose, it is necessary to multiply the concentration with the minute breathing volume.

Then, an integer function supplies the dose inhaled over time. To get the integration constant it is necessary to know the context.

For the first scenario (immediate complete release), following function applies:

$$d_1(t) = \int_0^t c_1(\tilde{t}) * \dot{V}_{breathed}\, d\tilde{t} + \text{const}$$

Obviously it must be: $d_1(t=0) \stackrel{!}{=} 0 \Rightarrow const = \frac{c(t_0)*\dot{V}_{breathed}}{a}$

$$\Leftrightarrow\ d_1(t) = c(t_0) *\ \dot{V}_{breathed} * \frac{1}{a} * (1 - e^{-a*t})$$

where $V_{breathed}$ is the volume breathed/minute. In analogy, the same steps are necessary to calculate the inhaled dose for the second scenario (slow continuous release).

$$d_2(t) = \int_0^t c_2(\tilde{t}) * \dot{V}_{breathed}\, d\tilde{t} + \text{const}$$

$$d_2(t=0) \stackrel{!}{=} 0 \Rightarrow const = c(t_0) * \dot{V}_{breathed} * \left(\frac{1}{\mathrm{a}} - \frac{1}{\mathrm{a+b}}\right)$$

$$\Leftrightarrow\ d_2(t) = c(t_0) * \dot{V}_{breathed} * \left(-\frac{1}{a} * e^{-a*t} + \frac{1}{a+b} * e^{-(a+b)*t} + \left(\frac{1}{a} - \frac{1}{a+b}\right)\right)$$

By applying above functions, it is now possible to calculate the theoretical inhaled dose of chemotherapeutics assuming a continuous presence of a crew member over the whole application time (30 minutes).

Chemotherapeutics	**Inhaled dose over a period of 30 minutes**	
	Immediate release	Slow continuous release
Cisplatin (15 mg)	1,49*10^{-3} mg	1,36*10^{-4} mg
Doxorubicin (3mg)	2,99 *10^{-4} mg	2,72*10^{-5} mg

The chemotherapeutic dose applied during PIPAC is dependant of the body surface of the patient so that the results obtained can be directly compared to systemic chemotherapy doses.

Chemotherapeutics	**Dose delivered***	
	Typical systemic dose	Inhaled dose
Cisplatin	150 mg	1,49*10^{-3} to 1,36*10^{-4} mg
Doxorubicin	30 mg	2,99 *10^{-4} to 2,72*10^{-5} mg

*assuming 2 square meter body surface.

Thus, the maximal inhaled dose in the worst case scenario after PIPAC application is expected to lie in the order of magnitude between 1:100’000 and 1:1’000’000 of a usual systemic dose. Any leakage scenario is expected to lie between these extreme boundaries.

The maximal potential exposition of the crew in the case of release of the toxic aerosol is minimal under the conditions described above. It has to be noted that the numbers given above are conservative since:

- the crew leaves the operating room during the application
- simulations with smoke showed that the laminar airflow directs smokes to the floor of the operating room so that a standing human person standing within the "clean area" will be prevented to inhale any toxic substance
- the immediate release scenario (worst case) is not realistic since the aerosol cannot be released all-at-once, the flow resulting from a leakage and not from an explosion.

Taking together, mathematical simulation of any possible leakage situations during PIPAC is showing that significant inhalative exposure of the operating crew to chemotherapeutic agents is extremely unlikely to occur and can be in practice excluded, even if the complete toxic aerosol is released from the abdominal cavity into the operating environment.

Critical Incident Reporting System (CIRS)

Between Nov, 2011 and Jan, 2015, our surgical and gynaecological teams at the Ruhr-University Bochum have performed over 650 PIPAC procedures. All scrub nurses have accepted to participate to the PIPAC procedures and the initial resistance of some employees has vanished. A two-person, specific safety checklist has been implemented which is systematically used before chemotherapy application, according to the 4-eyes principle. This checklist includes each important step of the procedure, in particular checking the tightness of the abdomen, the functionality of the laminar air-flow and proper connection of the CAWS. The checklist has largely contributed to the safety and to the acceptance of the procedure.

Two intraoperative incidents were reported and analyzed, within the framework of our CIRS (Critical Incident Reporting System). Both incidents were classified as minor because they did not cause any exposure of the personnel, and were caused by an accidental disconnection of the high-pressure line between the injector and the micropump.

Following measures were implemented:

- a protection sheet (analogous to a sterile sheet protecting the camera during laparoscopy) has been added to the safety system.
- the manufacturer of the equipment was required to provide the micropump sealed together with a high-pressure tubing, in order to exclude such disconnection in the future.

No severe incident, in particular no significant leakage of the chemotherapy aerosol has been registered, and no harm was caused to any participant. No patient injury caused by material and/or pressure was reported.

Discussion

Chemotherapy is an essential component of modern, multimodal cancer therapy. However, many drugs used to treat cancer are known to be mutagenic, teratogenic and

carcinogenic. Great caution has to be taken in the manipulation of these substances in order to prevent any contamination of the environment and in particular of the team involved. In particular, special attention is traditionally given to the prevention of any chemotherapeutic aerosol. Therefore, it is all but a surprise that development of PIPAC has first raised concerns about the risk of occupational inhalation linked to the generation of toxic aerosols. To assess this problem, it was not possible to rely on existing safety standards, since PIPAC had not been performed before. However, we could compare – to some extent – intraperitoneal aerosol chemotherapy to aerosolized chemotherapy in lung cancer. In this latter setting, aerosolized chemotherapy had been delivered in a well-ventilated room with an air filtering system [19]. Alternatively, a mobile filter air cleaning system combined with a collecting tent has also been also effective in preventing propagation of aerosol during inhalation of nebulized liposomal cisplatin [16]. Chemotherapy concentration in the air remained below workplace exposure limits.

In fact, the problem of potential inhalative exposition of the operation room personnel is not specific to PIPAC at all. The operating room team is regularly exposed to toxic aerosols, in particular to toxic vapors generated by electrosurgery (reviewed in 25). The chemicals present in the greatest quantity in electrocautery smoke are hydrocarbons, nitriles, fatty acids and phenols [26]. Of these chemicals, carbon monoxide (CO) might be the most concerning: high levels of CO are produced during laparoscopic cholecystectomy [27]. CO production is of particular concern in laparoscopic procedures where smoke is trapped and concentrated in the peritoneal cavity. Electrocautery during laparoscopic procedures has been shown to increase intra-abdominal CO to 'hazardous' levels, leading to small yet significant elevations of carboxyhaemoglobin (COHb) [28]. Levels of CO in the intra-abdominal cavity at the end of a laparoscopic cholecystectomy have been found to be 100–1,900 parts per million (ppm) above the 35 ppm for a one-hour exposure set by the US Environmental Protection Agency (EPA) [29]. In addition, CO is readily absorbed from the peritoneum into the bloodstream, creating a route for systemic intoxication [30]

Other chemicals present in smaller quantities, but which are still of significant concern, include acrylonitrile, hydrogen cyanide, formaldehyde and benzene. Acrylonitrile is a colourless, volatile liquid that is absorbed easily through the skin and lungs and exerts its toxicity by liberating cyanide [31]. The Occupational Safety and Health Administration (OSHA) has set the upper limit of ambient exposure to this substance at 2ppm. Exposure levels of OR personnel have been shown to be 1–1.6ppm, just under the established limit [28]. Hydrogen cyanide is a toxic, colourless gas that is absorbed easily by the lungs, gastrointestinal tract and skin. It combines with ferric iron in cytochrome oxidase, thereby inhibiting cellular oxygen utilisation. In addition, it can act synergistically with CO in impairing tissue oxygenation. The US Department of Health and Human Services has set the short-term exposure limit at 10ppm. Levels in the ambient environment of the OR in one particular experiment were found to reach a mean of 5.7ppm and up to 10ppm, just at the allowed exposure limit [28]. Benzene has been proposed to be significantly responsible for the mutagenicity of electrocautery smoke.

The US NIOSH acknowledges the dangers of surgical smoke and recommends that smoke evacuation systems should be used where high concentrations of smoke and aerosols are generated. NIOSH has performed own investigations and bases recommendations on the finding of the mutagenicity of the airborne compounds collected during its evaluation and the acute health effects reported by OR personnel [32].

During PIPAC, a chemotherapy aerosol representing about 10% of a usual systemic dose is installed within the closed abdomen and removed through a closed suction system into the external environment. Our studies show that the risk of inhalative exposition of operating room personnel during PIPAC is reasonably low, since no traces of platin have been detected in repeated environmental analysis. Even in the worst case scenario, namely the complete release of the toxic aerosol into the environment, mathematical simulation shows a maximal inhaled dose of 1:100'000 to 1.1'000'000 over a period of time of 30 minutes. It has to be noted, however, that the effective inhaled dose in the worst case scenario lies in reality much lower than these numbers since:

- The repartition of the toxic aerosol is not homogeneous within the operating room, due to the direction of the laminar air flow down to the flow, so that a person standing next to the leakage point would be exposed only to a minimal fraction of the dose, if any
- Nobody is remaining within the operating room during the application period, since the procedure is remote-controlled.

In summary, this report shows that the risk of occupational exposure to chemotherapy during PIPAC has been reduced to a minimum so that the procedure is complying with German occupational safety regulations. In comparison with other surgical procedures – both open and laparoscopic surgery – where specific exposition of operating room personnel to toxic aerosols has been documented – PIPAC appears to be particularly safe. Thus, PIPAC can be applied on a regularly, daily basis in the clinical setting without exposing the personnel to cumulative doses reaching the long-term exposure limits. The safety conditions are given for performing Phase 2 and Phase 3 clinical trials in order to define the possibilities and limits of PIPAC in therapy of peritoneal carcinomatosis. However, following the implementation of all equipment, organizational aspects and procedures as described above, any other team starting with PIPAC should perform a toxicological work-place analysis. Environmental analysis should be scheduled at the very beginning of the routine application of PIPAC, and repeated in regular time intervals, in combination with biological monitoring.

Even though the data presented above are documenting a high level of safety during PIPAC, the potential risk of exposition of the personnel performing such procedures remains and would become real if the measures defined above are not implemented. As in other medical settings such as radioprotection, hygiene, etc., strict application of the standard operating procedures, repeated measurement of exposure levels and continuous education of physicians and nurses is necessary with the increasing use of this innovative and fascinating therapeutic strategy, in order to avoid any harm to the personnel.

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Chapter 7

The Control of MAO Expression: A Resource Underscored by the Pharmacology

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Abstract

Mitochondrial monoamine oxidases (isoforms A and B; MAO-A and MAO-B respectively) are ubiquitous enzymes located at the outer mitochondria membrane facing toward cytoplasm. This localization results strategic allowing products of enzyme catalysis distributing in cytoplasm as well as into organelles. In this respect, several studies indicate the deleterious role of the hydrogen peroxide produced by MAO catalysis in inducing oxidative damage of structural or mitochondrial proteins (Kaludercic et al., 2010), triggering tissue loss of function and senescence.

Interesting enough, experimental evidence indicate that MAO-A isoform over-activation occurs in degenerative pathologies including heart and kidney failure. These evidence suggested that MAO-A overactivation might be considered as a novel source of free radicals participating to unbalancing the cell redox state, a ground condition for the onset of several diseases.

Accordingly, inhibition of enzyme activity has been proposed as a novel antioxidant strategy to reduce tissue oxidative status. This approach has however, intrinsic limitations including the increase of the the sympathergic tone.

Bach et al., (1988) cloned MAO-A and MAO-B describing interesting differences and similarities between the two isoforms. In this respect they clearly indicated that the promoters of the two isoforms are controlled, as expected, by different regulatory factors. In particular, in addition to sex hormones, they reported that angiotensin-II (AT-II) selectively activates MAO-A promoter activity. This evidence, which can have clinical implications, has been neglected largely by pharmacologists.

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Drugs targeting AT-II and its intracellular cascade are considered gold standard treatments for cardiovascular diseases (CVD). The beneficial effects of such drugs may include the control of MAO-A over-activation, thus working indeed as "indirect antioxidants." This aspect may over-come the use of MAO inhibitors, which *di per se* are endowed of scavenging activity in respect of oxidants, and be considered an added value for drugs rescuing against the cardiovascular risk.

Introduction

Jumping throughout MAO History

Many scientists contributed to the identification and characterization of amine oxidases, including mitochondrial mono amine oxidases (MAO). The aim of this chapter however, was not to mention all of them but only the people who were important for the development of the "Italian branch" of the research around amine oxidases. We apologize for this limitation.

The history of amine oxidases began from the research of an illuminated scientist named Herman Karl Blasckho (1900-1993), a medical doctor who dedicated his life to the study of exogenous and endogenous amine metabolism. He was a physiologist, a chemist and a physician at the same time. The age of his maturation as a scientist coincided with a tragic historical period and, because of this, he was obliged to move from Germany to England. There, before in Cambridge and then in Oxford, we founded a school of biochemistry dedicated to the study of amine metabolism individuating the activity of oxidative de-aminating enzymes, named amine oxidases (AOs). He gave a strong contribution to the mechanisms of monoamine synthesis, uptake and metabolism as part of the complex regulatory system of the sympathergic neuron.

The school he found was attractive for many other young scientists of the time. Among them, we like to recall Franca Buffoni, the first woman to move from Florence, Italy, to England just for doing research. Nobody knew but a new era was just started… the research could not be confined within the border of a country. There, Franca Buffoni (1924-2011) first purified and then crystallized the AO from pig plasma endowed of histaminase activity. In 1968, Franca was back from Oxford and from that moment she was never again the same and she started dedicating her life to the study of the AOs (Figure 1). In addition to the biochemical characterization of AOs, she was a pioneer researcher intuiting these enzymes i) might be target of drugs, ii) could play toxicological roles and iii) could represent Phase I non microsomial drug detoxifying enzymes. Thanks to her work AOs studies started disseminating around Europe, new enzymes were purified and characterized producing the concept that AOs were a large and heterogeneous family of enzymes with different cell localization, substrate specificity and cofactor. A classification of AOs in respect of their substrate specificity resulted as enzymes degrading exclusively primary amines localized at mitochondria, monoamine oxidases (MAO), or at plasma membrane (semicarbazide-sensitive amine oxidases), or enzymes degrading polyamines, including diamine oxidase (DAO) and spermine oxidase, which were soluble enzymes.

Panel A: Franca Buffoni, a picture at the age of 80.
Panel B: Crystals of pig plasma benzylamine oxidase purified by Franca Buffoni (a picture from an experimental protocol).

Figure 1. Franca Buffoni, "a portrait" of pig benzylamine oxidase.

Nowadays, this classification appears outdated and are much more complicated by the identification of enyme polymorphysms, of "silent proteins" and of many other isoforms of AOs. Notwithstanding the complexity, all AOs, the old and the new, share the ability to degrade amines with the production of hydrogen peroxide, ammonia and aldehydes, thus impacting on the redox balance of the cell.

The physiological role of MAO, as part of the sympathergic system, made clear from the beginning the clinical importance of controlling their activity. In this respect, the potential of MAO inhibitors (iMAO) as antidepressant was early recognized (Garrone and, Dick, 1960; Bates and Douglas, 1961) even before the discovery that MAO activity resulted from two distinct isoforms namely MAO-A and MAO-B (Youdim et al., 1969).

Youdim's work around MAO helped to clarify enzyme kinetic properties and produced indications for identification of the chemical moiety to produce selective and irreversible inhibitors (propargylamines; Youdim and Bakhle 2006). The availability of such compounds were also of extreme importance as experimental tools to explore the physiopathological role of MAO-A and MAO-B.

With the explosion of the molecular biology, novel technologies and scenarios were offered to the scientific community and the cloning of MAO genes was possible. Bach et al., (1988), from Jean Shi labs,first cloned the two genes encoding for MAO type A and B and described the phenotype of selective and double knock out mice. The study of these mice revealed that the inactivation of type A and/or B MAO produces a number of functional and behavioural alterations, some of which may be harnessed for therapeutic aims (Bortolato et al., 2008).

In the new century, MAO research focused on the consequences of MAO over-activation (mainly MAO-A) and inhibition in extra neuronal tissues including the cardiovascular system. In this field, interesting and convincing evidence suggested a role for MAO in the generation of oxidative stress, one among the main determinant of heart failure, thus indicating the

potential usefulness of using iMAO to control the inflammatory ground which triggers cardiac hypertrophy and dysmetabolism (Kaludercic et al., 2011).

Furthermore, to confirm the importance of MAO in the cardiovascular system, Anderson et al., (2014) indicated MAO as an important determinant of redox balance in human atrial myocardium and a risk factor for atrial fibrillation in post cardiac surgery.

In aggressive prostate cancer, Wu et al., (2014) reported that the increased expression of monoamine oxidase A (MAO-A) in high-grade aggressive cancer exhibit correlated with worse clinical outcomes in patients.

MAO General Features

MAO activity catalyses the degradation of primary amines with the production of the corresponding aldehyde, hydrogen peroxide and ammonia. MAO-A and B show different substrate and inhibitor selectivity. Among MAO-A optimal substrates is serotonin and noradrenaline, while optimal endogenous substrate for MAO-B remains elusive if we exclude the trace amine b-phenylethylamine. Instead, dopamine and the exogenous trace amine tyramine are common MAO-A and B substrate. In addition, MAO-A and B also show different tissue distribution being MAO-A preferentially located in neurons and MAO-B expressed in glial cells as well as in periphery.

MAO-A and B are coded by different genes and share 70% homology in their primary sequence and both contain the obligatory cofactor flavin adenine dinucleotide (FAD) covalently bound through a thioether linkage to Cys406 in MAO-A and to Cys397 in MAO-B. Their promoter activity can be activated by different humoral factors. In particular, among MAO-A promoter activators are angiotensin-II, androgens and corticosteroids, estrogens for MAO-B.

From MAO cloning, knock out mice for each isoform but also double knock out mice have been produced (Shi et al., 2004). Despite the obvious limits of such models, their availability allowed to learn very much about MAO role in physiopathological processes (Godar et al., 2014).

Is MAO (mainly MAO-A) Over-Activation a Determinant or an Epiphenomenon of the Pathology under Investigation?

The ratio of MAO-A to MAO-B in the human brain is 25/75. Increased MAO-A density in several brain areas has been considered an important monoamine-lowering process during depressive episodes in major depressive diseases (MDD). In light of the pivotal role that MAO plays in the metabolism of neurotransmitters, MAO-A over-expression/activity was considered a drug target in a variety of neuropsychiatric disorders based on hyperactivity (Wu et al., 2009; Meyer et al., 2010).

Inhibitors of MAO activity (iMAO) are a large and heterogeneous family of compounds which include selective and not selective, reversible and irreversible inhibitors. Among the irreversible ones, namely propargylamines, deprenyl was the main studied and it was

indicated in sustaining standard therapies for treating Parkinson's disease, increasing dopamine levels, and for neurodegenerative diseases for its antioxidant features in respect of amylod plaque deposition.

However, the control of MAO activity might be only a part of propargylamines antioxidant features. In fact, propargylamines have documented scavenging activities and anti apoptotic features in virtue of their chemical structure, independently on MAO activity inhibition (Naoi et al., 2009). These features, of course, are not shared by non propargylamine iMAO, included those of third generation, thus sustaining the concept that antioxidant potential of iMAO differs in respect of the molecule used. To confuse the design, pro-inflammatory activity has been documented in glial cells after exposure to phenelzine (a first generation iMAO) (Chung et al., 2012). Even if non selective iMAO represented a milestone in the pharmacological treatments of depressive disorders, today their use is gradually abandoned, mainly owing to their potential for drug-drug and drug-food interactions and more selective MAO-A or MAO-B inhibitors have been developed with substantially reduced risks. Those drugs were approved for the treatment of depression and Parkinson's disease and some of them also show neuroprotective properties which could be of some beneficial in neurodegenerative diseases where the block of MAO-A activity rescues from ROS neurotoxic effects implicated in dendritic loss, cortical shrinkage, and neuronal apoptisis. However, the use of iMAO as psychotropic drugs is associated with sexual behavior, sleep dysfunctions and hormone levels modification (Slater et al., 1977; Landolt and de Boer, 2001).

However, it is important to remind that iMAO inhibit enzymes independently on their tissue expression levels and/or activity. This means that also "basal" MAO activity will be inhibited following iMAO treatment, a result which might not be irrelevant on cell signaling homeostasis, either in respect of amine substrate interaction at receptors or in term of levels of ROS produced by mitochondria, recognized as important regulators of cells functions and of MAO expression (Kumar et al., 2013). When using iMAO for treating depression, enzyme activity will be inhibited also in extra-neuronal tissues with implication in diet amine and in drug metabolism.

More than 100 studies evaluated the potential association between mood disorders and CVD, showing that depression is more prevalent (20-35%) in populations with any CVD and the presence of depressive disorders worsen the prognosis in patients with known coronary heart disease (e.g., doubling the risk of cardiac events). Among the possible causes of such negative impact, MAO activity might be a possible one. In particular, the increased sympathoadrenal system activity observed in mood disorders associated with enhanced excretion of noradrenaline, epinephrine and dopamine. Similarly, patients with mood disorders present often increased heart rate (Maes et al., 2002). Degradation of cathecolamines by MAO contributes from one side to reduce their levels, from the other to generate ROS, thus initiating a vicious cycle retro-controlling the activity of the sympathergic neurons. The use of iMAO would reduce ROS production but further increased catecholamine levels, with possible impact on heart rate . In addition, MAO-A is present in the myocardium of rodents and MAO-A over-activation was described in the failing heart of mice (Kaludercic et al., 2011). At this setting, increased intracellular metabolism of serotonin and of noradrenaline, by producing ROS, opens to receptor independent effects of amine substrates and contributes to unbalancing cell redox state (Villenueve et al., 2013). Experimental evidence indicate that the degradation of serotonin and noradrenaline by MAO-

A activates intracellular cascades involved in cell death, hypertrophy and mitochondrial dysfunction (Villenueve et al., 2013;Pchejetsk et al., 2007). Both serotonin and noradrenaline are pro-arrhythmic, hypertrophic and increased cardiac inotropism. Accordingly, cardiomyocytes exposed to high serotonin levels activate hypertrophic and apoptotic pathways (Bianchi et al., 2005). At the light of these evidencee cardiac MAO-A overexpression could represent a a pathogenic event, thus sustaining the beneficial of using iMAO in treating patients with mood and and cardiovascular disorders.

However, MAO-A actively participates in controlling cardiac serotonin levels and increasing its levels by using iMAO or by enzyme overactivation producesactivation of cardiac hypertrophy (Lairez et al., 2009). Accordingly, knock-out MAO-A mice present cardiac hypertrophy possibly due to increased serotonin levels at reecptors. Then, experimental models, suggest that blocking iMAO does not rescue from inducing cardiac hypertrophy. Then, we have to think abot using something else.

Over-activation of MAO-A has been described at several pathological settings including diabetes (Manni et al., 2012; Manni et al., 2013), hypertension (Pino et al., 1997). In the kidney of the diabetic rat, MAO-A was found overactivated generatingROS involved in nephropathy.Diabetes is a typical stress related diseases and there are evidence of co-morbidity between diabetes and depression (Warnock and Mutzig, 1998) In diabetes, indications for iMAO are limited because their interaction with glucose disposition possibly inducing hypoglycemia and because iMAO induce weight gain and required appropriate diets. In addition, iMAO activate the sympatherig tone, including corticosteroid release, impacting on vascular tone and behaviour possibly aggravating insulin resistance.

The question around iMAO use appropriateness in diabetes and/or in heart failure could be overcame entifying the mechanisms responsible for inducing MAO-A over-activation. From the pharmacological point of view this means that, using drugs controlling humoral factor(s) regulating MAO-A expression we could impact on MAO-A expression (ethiogenic approach) “up-stream.”

In this respect, Shi’s study on MAO promoter told us very much. She first described that MAO-A promoter can be activated by humoral factors including androgens, corticosteroids and AT-II. All these hormones, in particular AT-II, have a great impact on the homeostasis of the cardiovascular system. AT-II levels were found increased in hypertension, heart failure, diabetes and they are recognized among the determinants of hyperglycemia-induced complications. All the deleterious cardiovascular effects of AT-II are mediated by the activation of AT-II type 1 receptor (AT1R). In line with this, drug targeting AT-II and/or its receptor represent the gold standard treatments for primary and secondary prevention of CVD in risk patients, including diabetics.

While extensive experimental and clinical works were produced attesting cardiovascular beneficial effects of drugs targeting AT-II and its signaling cascade, whether the control of MAO-A activity is included effectiveness of such drugs it remains to be investigated.

Experimental evidences indicate that MAO-A is over-activated in the kidney and in the heart of the diabetic rat and that this activity contributes, by producing ROS, to diabetic nephropathy and cardiomyopathy. When animals received losartan, an AT1R blocker, MAO-A levels were lower than in untreated rats and kidney function improved. This evidence confirmed that, blocking AT-II activity, resulted in amelioration of kidney function, as already known, but, for the first time, indicated that losartan can have an additional, indirect, antioxidant features (Manni et al., 2012). Similar results were obtained in the diabetic heart

where MAO-A overactivation correlates with oxidative damage to cell proteins and with reduction of the antioxidant systems (Manni et al., 2013). Accordingly, hypertensive rats treated with angiotensin converting enzyme inhibitors (ACE i) presented increased cardiac cathecolamine levels resulting from reduced MAO activity (Raash et al., 2002). To note, MAO activity was found increased in cardiomyocytes from hypertensive rats (Pino et al., 1997). All these evidence suggest that the stimulatory role of AT-II on MAO-A promoter activity can be reproduced experimentally and that MAO-A over-activation is part of the pro-inflammatory and pro oxidant program induced by AT-II. Then, the cardiovascular protection offered by ACEi and AT1R blockers includes the control of MAO activity, adding value to their mechanism of protection. Another advantage of using ACEi or AT1R blockers , instead of iMAO, is that the ACEi and ATR1 blockers reduce rather than increase the sympatheric tone. In fact, AT-II exerts its pro inflammatory and pro-oxidant effects also stimulating the sympathergic tone. In this respect, MAO over-activation might represent a "compensatory" mechanisms opposing to AT-II stimulation. Then, if iMAO were used, cathecolamines will be free to stimulate their receptors sustaining further the vicious cycle between AT-II and the sympathergic system. Instead, blocking at the beginning the cause and/or the effect, namely over-expression of MAO-A, such vicious cycle is interrupted with cardiac (cardiovascular) beneficial. The clinical impact of controlling MAO-A expression by using ACEi is or ATR1 blockers are largely neglected by pharmacologists but all the evidence indicate that their use should be encouraged at the very early stage of CVD. At the moment is unknown if the beneficial, in term of reduction of MAO-A activity and then of oxidative stress, should also expected following b-blockers use.

Furthermore, AT-II is a main determinant of atrial fibrillation. Interestingly, MAO-A activity increase has been recognized recently as a predictor of the risk to develop atrial fibrillation in patients undergoing cardiac surgery (Anderson et al., 2014). MAO-A over-activation may be considered a "sub-clinical" event, together with other markers of inflammation. Again, drug targeting AT-II are first line treatments for atrial fibrillation and their use down regulate MAO-A expression.

MAO Activity Beyond Mood, Diabetes and Heart Failure

Among complications of heart failure is sarcopenia, a condition of comorbidity with cardiovascular diseases negatively impacting on the prognosis, the risk/safety of drugs used, and increasing patient frailty. Sarcopenia is defined as a reduced muscle mass associated with loss of function and performance. The impact of sarcopenia on the outcome of cardiovascular patients is rather underscored and appropriate clinical trials aimed to evaluate its incidence are still lacking. Less is even known on pharmacotherapies to counteract sarcopenia.

Clinical evidence indicate that the use of ACEi and/or AT1R blockers associate with a better conservation of physical performance of and cardiovascular patients (Carter et al., 2005) . In addition, losartan, an AT1R blocker, shows protection against disuse atrophy in humans with sarcopenia (Burks et al., 2011) and in mice with congenital muscular dystrophy

(Elbaz et al., 2012.) A possible key of lecture of the beneficial of ACEi and ATR1 blockers might relay on their effect in controlling MAO activity.

At the moment there are no evidence indicating that the sarcopenic muscle presents higher MAO activity than the healthy muscle. However, there are evidence indicating that AT-II reduces satellite cell growth and regeneration thus indicating that AT-II is a common determinant in cardiovascular and skeletal muscle diseases (Yoshida et al., 2014), Then, even if conclusive evidence of the link between AT-II, MAO and sarcopenia are lacking yet, this relation represents an interesting matter for future investigations. Actually, MAO-A over-activation has been documented in skeletal myocytes exposed to dexamethasone (Ou et al., 2006) a drug typically inducing sarcopenia. This evidence suggest that sarcopenia potentially includes MAO-A over-activation and that stress factor levels including AT-II and corticosteroids should be kept under control. Again, the use of an ethiogenic drug, would be helpful.

Interestingly enough, MAO-B induced oxidative stress was found a causal determinant of mitochondrial dysfunction and apoptosis in myoblasts from patients affected by collagen VI myopathies, conditions prevented by pargyline. If confirmed in vivo, inhibition of MAO (MAO-B) should be explored as a potential treatment for these diseases.

Controversial Role of MAO-A Activity in Cancer Diseases

Depression as well as altered MAO-A expression, were both found associated with a poor prognosis in cancers. MAO-A was found over-expressed in aggressive prostate cancer and in experimental breast cancer (Lizcano et al., 1991). Yet, in apparent contradiction, MAO-A mRNA was found down-regulated in breast and prostate cancers (Rybaczy et al., 2008).

Epidemiological studies associate usage of antidepressants, including iMAO, with breast cancer risk. Experimental studies aimed to solve this apparent paradox indicate that the use of drugs manipulating MAO activity might deserve much attention in respect of cancer.

Recent evidence also suggest a role for a silent form of the MAO-A protein and of a transition from mitochondria to membrane of MAO-A as indicative of tumor extravasion (Anderson et al., 2014). In this context, MAO-A inhibition by pargyline, an irreversible iMAO, triggers a mesenchymal-to-epithelial transition in breast cancer cells via a non-canonical mechanism. This potentially implicates an MAO-A-sensitive step in advanced breast cancer and should be borne in mind when considering pharmacological treatment options for co-morbid depression in breast cancer patients. Epidemiological studies would be necessary to ascertain the incidence of breast tumors and of their metastatization in patients taking iMAO.

The expression of MAO-A was also found induced following exposure to docetaxel, contributingto the resistance to the chemiotherapeutic used in prostate cancer, a condition impacting on clinical outcomes (Gordon et al., 2014). Prostate cancer is a typical androgen-dependent pathology and MAO-A promoter activity, as described by J Shi, is also susceptible to androgen activity.Then, in prostate cancer, a dual role for MAO-A might be postulated with possible beneficial effects of iMAO in reducing resistance to the chemiotherapy.

On the reverse, MAO-A was found down regulated in tissue from hepatocarcinomas closely correlating with cancer metastasis. In this case, stimulation of MAO-A promoter activity might represent a potential novel approach for therapy (Li et al., 2014).

Conclusion

Experimental and clinical evidence indicate MAO (MAO-A) activity dysregulation might represent a pathogenic event or a typical response of cells to stressing conditions (epiphenomenom). In both cases, MAO activity is a potential novel drug target. In this respect we have the opportunity to choose inhibitors of MAO-A activity or drugs controlling MAO-A expression depending on the role of MAO-A within the scenario of a certain pathology. This means that, in respect of MAO role, there could be the possibility to use "symptomatic, iMAOs, or aethiologic drugs which tagetting the heart of the pathology prevent MAO dysregulation

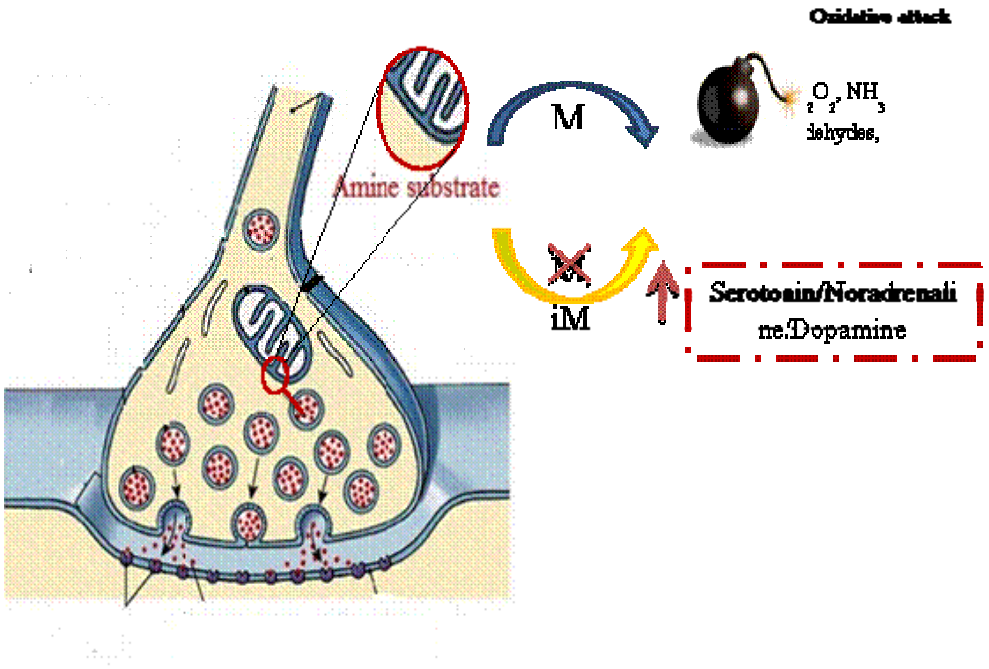

Figure 2. Monoamine oxidase activity triggers oxidative attack which is rescued by using enzyme inhibitors.

Mitochondrial monoamine oxidases scavenging biogenic amines produce reactive oxygen species including aldehydes and hydrogen peroxide. Both compounds triggers oxidative attack to cell proteins, lipids and DNA. The use of MAO inhibitors potentially rescues against MAO-induced oxidative attack.

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Chapter 8

iMAO: What We Know About

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Abstract

MAO inhibitors (MonoAminoOxidase) - iMAO, are a group of antidepressant agents acting through the blocking of the enzymatic pathway of the disintegration of the serotonin - of MAO enzyme decomposing serotonin. One distinguishes two isoenzymes: MAO-A and MAO-B differing in the location and action. Isoenzyme MAO-A is found in a nervous tissue and its action is based on controlling the level of affection, whereas MAO-B is located in non-nervous tissues and with its action it regulates the appropriate blood pressure. Also numerous side effects, which may appear in the course of therapy with MAO, are based on these relations, especially on nonselective ones.

iMAO action was revealed by chance, as these medicines were used in treating tuberculosis, and their influence on the mood was quickly connected with action on neurotransmitters. The entire group of MAO inhibitors are not only drugs used in the treatment of depression, and these are divided in three subgroups. The first, oldest group includes drugs restraining MAO permanently and not selectively. Isoniazid, applied up till today in curing tuberculosis is in this group. The following second group, entails medicines blocking MAO irremediably, but selectively, e.g., Selegiline (MAO-B) - used in therapy of Parkinson's disease. Last, most contemporary group, are selective and reversible medicines, e.g., moklobemid, (MAO-A) that is the most important antidepressant agent being a representative of iMAO entire group.

iMAO group is used universally worldwide, which does not mean that these medicines are deprived of side effects. Above all, an amount of interactions they can enter with other medicines, elevating the serotonin level is quite large. Effects of such combinations are very serious, including serotonin syndrome and death. A wrong diet, containing lots of the tyramine, also decomposed by MAO, has a similar effect. Other adverse effects characteristic of MAO inhibitors are a hepatotoxicity and hypertensive action.

MAO Inhibitors (MonoAminoOxidase) - iMAO constitute a narrow group of medicines acting by blocking of the enzymatic pathway of the disintegration of serotonin - MAO. For understanding the impact of these medicines, including their adverse effects, a few issues essential for later considerations will be discussed first. In this case biochemistry and physiology, i.e., neurotransmitters and the serotonin are the foundations for proper understanding of the subject. Noradrenalin belongs to the cycle of transformations of this group of medicines too, but in case in question it is exactly serotonin which is most important one of neurotransmitters, which the recalled group of medicines seems to have an influence on. Serotonin has repeatedly been described for ages on pages of specialist articles, as it shares the metabolic trail blocked by iMAO exactly with the noradrenalin. Discussing two transmitters of our interest is crucial for understanding not only the effect of the medicine, but also their interactions and adverse effects. Below, only most important notions of physiology and biochemistry will be quoted in the minimal scope, but they will hopefully allow understanding their significance. Additionally an issue of receptors, i.e., uptake points for neurotransmitters, enabling central and peripheral action will be discussed. What is more, typical clinical knowledge essential in applying pharmacotherapy will be shortly discussed.

The iMAO action was revealed by chance, as the drugs of this group were used at first in treating tuberculosis, and their impact on mood was quickly linked with action on neurotransmitters. The entire group of MAO inhibitors encompasses medicines administered and registered not only in the treatment of depression. All known pharmacological medicines restraining MAO, were divided in three subgroups. The first, oldest group includes long-lasting and nonselective blocking agents, the least safe in treatment. Isoniazid belongs to this group, never used for curing mood disorders, but applied up till today in curing tuberculosis. The following, second group, are medicines blocking MAO irremediably, but selectively, e.g., clorgiline - which blocks the subunit of MAO-A, and selegiline – subunit of MAO-B which is used in therapy of Parkinson's disease.

One should consider it that selegiline, in larger twenty-four hour doses, loses selectivity of its action and becomes a nonselective blocking agent. The newest group of drugs is selective and reversible medicines. A representative of this group of medicines – moklobemid, came into existence in the end of the 80s. It suppresses MAO-A isoenzyme, and at present moment, is the most important medicine being a representative of the iMAO entire group, as for psychiatric treatment. Broforamine belongs to the same group, but is definitely less popular than moklobemid.

Serotonin – Chief Neurotransmitter of Depression

Neurotransmitters are chemical substances, whose task is to transfer signals between individual nerve cells - which takes place in regions called synapses. The neurotransmitter is being freed under the influence of the electrical signal, which is, in fact, a wave of depolarization reaching the end part of the neuron. On account of the chemical structure, *modus operandi* and stimulated receptors, we divide neurotransmitters into 3 main groups. In first group we may find amino acids responsible for fast synaptic transmission. As for their action, we divide them into two subgroups: stimulating amino acids and suppressing amino

acids. The second form of neurotransmitters includes amines and the acetylcholine (ester). We talk here about catechol amines and serotonin, and the recalled already - acetylcholine.

The third class of neurotransmitters is peptide substances, also called neuropeptides. We can distinguish two subgroups here: IIIa and IIIb, the division takes into account their concentration in the brain. Majority of transmitters of the third group are regarded as neuromodulators, i.e., factors changing the effect of transmitters of the first and second class [Vetulani; 2010]. As it was recalled earlier, serotonin is classified in the second-class of neurotransmitters, and in reference to understanding the action of the discussed group of drugs, only the second group of neurotransmitters remains important.

Serotonin, determined as 5 HT (5 hydroxytryptamine), is sometimes called the hormone of happiness, as it is perceived as the neurotransmitter directly connected with the level of mood. The higher the level of serotonin, the mood further from "depressive." The general rule of operation of antidepressive agents consists in elevating serotonin level. Such a process never takes place without the influence on the level of other neurotransmitters, which triggers many adverse effects. Tryptophan is the precursor of serotonin - an amino acid, which after being absorbed into the blood-stream, penetrates the blood- brain barrier, and further - to serotoninergic neurons. The process of creation of serotonin from tryptophan is a two-stage process.

Serotoninergic Receptors

Generally, we divide the receptors into ionotropic and metabotropic ones. The first group includes receptors stimulated by classical neurotransmitters and plays a crucial role in generating the action potential. They are permeable for the specific group of ions: Cl, Na, K, Ca. Group of metabotropic receptors affects the neuronal metabolism; in the course of this process, secondary transmitters come into existence, and for the correct functioning of receptors of this group an activation of G proteins is essential, whereas every neurotransmitter acts through specific receptors. So far, 7 groups of serotoninergic receptors have been identified. Unlike 1st class of neurotransmitters, which are loosely dispersed in the brain tissue, in case of 2nd class of neurotransmitters specific neurotransmitters are grouped into centres, creating nerve routes.

The largest cluster of serotoninergic neurons is located in a dorsal nucleus of the suture. Also the fact that the influence is taking place through receptors, is characteristic of the 2nd class of metabotropic transmitters, although in case of serotonin, the receptor of 5HT3 subclass is ionotropic [Vetulani J.; 2010]. Receptor units both have different locations, and they present varied effects of action. For example: as a result of the activity of the 5HT1A receptor is reducing fear intensifying, depression or pain, but 5HT1D will be responsible for constricting blood vessels, in the process of triggering migraine. The excitation of the 5HT2A receptor can trigger hallucinations, whereas of 5HT3 - vomiting [Fitzgerald].

MonoAminoOxidase

MonoAminoOxidase consists of two isoenzymes: MAO-A and MAO-B differing in the location and action. MAO-A isoenzyme is found in the nervous tissue of the central nervous system, sympathetic terminals, digestive tract, liver and the skin. It mostly decomposes serotonin and noradrenalin, but also dopamine, tyramine, octopamine and tryptamine. Physiological effect of MAO-A isoenzyme is based on controlling the level of emotion. The other isoenzyme, determined as MAO-B will be encountered in the nervous tissue of the central nervous system, but also in the liver and the blood platelets. Proportions, in this case, are definitely different and the majority of this isoenzyme is found in "non-nervous" tissues. It mostly decomposes benzylamine and phenyloethyloamine, as well as dopamine, tyramine, tryptamine and methylohistamine. MAO-B action is based on a relevant regulation of the blood pressure.

As it results from the above, dopamine, responsible for the appropriate pressure, is decomposed at the participation of both MAO-A and MAO-B isoenzymes even though many competent authors regard the other isoenzyme as the acting force. These relationships, similarly to metabolic routes of the disintegration of the serotonin, are the underlying reason for understanding numerous side effects which can turn up at the course of therapy with MAO, especially nonselective ones. Mechanisms of action of monoamine oxidase, and actually- the kinds of this enzyme, also provide a possibility of understanding direction in which exactly this group of medicines develops [Ciraulo, Kaplan- Pharmacotherapy of psychic disturbances].

Innitially, from the iMAO group three preparations were derived: phenelzine, tranylcypromine, isocarboxazid, being irreversible blocking agents of subunits A and B. It meant that in case of the need for the exchange of the medicine into other antidepressant, a need to have the break between the end of therapy with one, and the beginning of the treatment with new preparation was needed. Non-selective tranylcypromine is absorbed quickly from the digestive tract, achieving the maximum plasma concentration already after 1 up to 2 hours after ingestion, at the time of the half life of 2 hours. In spite of this, the single dose can cause stopping enzyme production even for the week. The monthly therapy causes it that after stopping the administration the sensitivity to tyramine continues at least for a few days, whereas at some patients it can last longer. Additionally it embroils problems associated with supporting the specific model of the diet by the patient poor in tryptamine. In detail, these relations and clinical clues will be discussed further in this chapter.

Medicines never applied in therapy of depression come from the iMAO group, the examples being: pargyline used in hypotensive treatment, procarbazine applied in oncology, aforementioned isoniazid, i.e., the antituberculous drug. This group also contains furazolidone, -an antibacterial medicine. All mentioned above medicines are irreversible inhibitors of both subunits, which is not only connected with their clinical action, but above all with adverse effects reducing the possibility of the application in significant way [Ciraulo et al., 1999].

Clinical Action

Most effective groups of mood raising medicines (antidepressants) are, all at the same time, two oldest drugs - TCA - tricyclic antidepressant agents and MAO inhibitors (iMAO). Along with widely applied and popular worldwide - SSRI group (Selective Serotonin Reuptake Inhibitors), they create a group of classical antidepressive agents [Acting in depression].

Tricyclic medicines are generally nonselective medicines, and so they interact not only with serotonin, but also with other transmitters. MAO inhibitors act perhaps more selectively, but they suppress the whole chain of the transformations typical of serotonin at the certain stage through blocking of the decomposing enzyme, which is combined with the possibility of the onset of numerous adverse effects. Not only the clinical effective action, but also the aspect of adverse effects links these two groups of medicines: TCAs and iMAO drugs.

In the neurotransmitter scope, both groups of medicines raise, apart from serotonin, also a level of noradrenaline, provided TCAs have such an action by stopping the blocking of reuptake of NA, which clearly results in elevating its level [Sedno, Psychiatry]. At a great amount of NA, the second of the enzymes participating in the disintegration (COMT) isn't powerful enough. This mechanism is the underlying reason for a violent rise in pressure, as a result of blocking MAO. It is possible also in this way to explain the most sudden drug interactions which occur between "widely" acting clomipramine from TCA group, with some iMAO. Some adverse effects in TCA and iMAO groups coincide, and so at joining of these two groups they additionally become stronger. Numerous sexual dysfunctions often triggered by each of the groups of classical antidepressive agents can be an example (TCAs, iMAO, SSRI).

iMAO advantage is the fact that these drugs don't lower the convulsive threshold and unlike TCAs, they act definitely less cardiotoxic way [Acting in depression]. Interactions with other antidepressive agents, since the time of introducing medicines from the iMAO group for the treatment of depression, are still not entirely recognized and described. In literature it is possible to come across information that past experiences, pointing to the threat of associated therapy, were exaggerated. Even iMAO and TCA associated treatment was postulated in cases of drug-resistant depressions, because there were patients, at whom the combination therapy gave better results than the monotherapy [Ananth et al., 1977]. In this case appearance on the market of Moklobemid, as the reversible iMAO inhibitor (RiMAO), introduced the new quality in the safety of applying, but didn't lower the risk up to zero.

After data analysis from the literature we may find clues concerning conducting of the associated therapy, as well as information that joining Moklobemid or the more so of irreversible MAO inhibitors, with imipramine, desipramine and clomipramine and with SSRI is strictly contraindicated.

As for associated therapy, it is important to apply doses of combined medicines - smaller than in the monotherapy with each of them. One should administer medicines orally, if possible and it is more safely to begin therapy, than to attach TCA e.g., to already given iMAO [Ciraulo et al., 1999].

Unwanted Effects

Until recently, iMAO was divided into two groups, that is: classical - irreversible, unpredictable and burdened with the greater risk of unwanted effects (phenelzine, trancylpromine, isocarboxazid) and constituting the new group and the new quality - moklobemid, from the group of MAO reversible inhibitors (RiMAO). Currently, classical medicines are starting slowly to fall into disuse for the sake of the newer versions, although they are still authorized for sale. Generally, one should also divide undesirable symptoms in two groups, because their intensity will be different for iMAO and RiMAO.

iMAO group is used universally worldwide which doesn't mean that these medicines are deprived of side effects. Above all, we mean here- unpleasant to the patient - anticholinergic action and rise of the arterial pressure. Increasing these manifestations is dependent on the iMAO dose, but with time their intensity is clearly falling, to the complete absence. Manifestations for the patient are the irregularities in the body weight, sedation and disturbance of sexual functions, which already are disturbed as a result of depression [Acting in depression]. Undesirable symptoms are described below in detail.

Adverse effects are not only due to interactions between iMAO medicines, but even due to inappropriate diet. The foods rich in the tyramine, i.e., the precursor of serotonin, cause a sudden increase of the level of the transmitter which cannot be surrendered to further disintegration. Tyramine plays the role of the false transmitter of the noradrenergic system. For understanding of this mechanism, it is possible by way of analogy, to give the reaction of alcohol - anticol, when even little amounts of ethyl alcohol result in sudden and increased symptoms of poisoning, as a result of the lack of ability of the disintegration and the elimination. Similarly, this happens too with lot of the tyramine taken. Foods strictly forbidden when taking iMAO, containing lots of the tyramine are: mature cheeses, wines, beers, liqueurs, salted and smoked fish, bean, yeast, sauerkraut, bananas, figs and liver.

Theoretically, along with the appearance of moklobemid - RiMAO representative, as the safer iMAO alternative, one may assume that dietetic discipline with reference to the content of the tyramine in the diet does not have to be applied, as drastically as in the past. One allows consumption one or two meals per day containing certain amounts of the soy sauce, bananas, avocado, spinach, plums, raisins, tomatoes or yoghurt. These products admittedly contain tyramine, but in the dose, which is linked with RiMAO should not trigger adverse effects, especially in the comparison with the old inhibitor - tranylcypromine. Moklobemid is far less increasing the risk of a violent rise in pressure as a result of accepting tyramine, for comparison in the literature we will find data indicating 30 fold or even 50 fold increasing reaction to tyramine towards 4 fold, or 7 fold reaction in case of moklobemid. Concluding, some foods containing paucities of tyramine, in the course of therapy with moklobemid are not contraindicated to the patient. In such case, the patient must be precisely informed which foodstuffs are good for him and which are not, additionally patients must exhibit self-control and extreme care with that.

Additionally, respecting diet is necessary at least two weeks after ceasing therapy, which is until the total leveling of regaining enzymatic efficiency. The dietetic regime would bind itself also with the need for determining of the doses of the tyramine, i.e., weighing foods and their proper preparing. For example - green bananas are allowed for the consumption but only when are cooked in their skin. Taking into consideration all these problems and the risk of the

absence of co-operation on the part of the patient, for example with simultaneous alcohol dependence, the iMAO treatment appears to be very difficult and dangerous.

Numerous interactions which iMAO drugs can enter into with other medicines elevating serotonin level lie at the bottom of many undesirable symptoms. Effects of such combinations are very serious, inclusive with the serotonin syndrome and death. Of course most sudden effects will be caused by combining iMAO with other medicines raising the serotonin level, i.e., already discussed drug interactions, although in case of the discussed group of medicines for triggering similar action a wrong diet, containing to high doses of tyramine, also decomposed by MAO, can be a big problem. Standard, balanced diet contains about 40 mg of tyramine, a day, which should not cause, for example, an arterial rise in pressure at individuals treated with Moklobemid, therefore there is no need for rigorous preservation of the diet. One should, however, recall that every treated patient using iMAO should get maximum information concerning foods being the source of tyramine, similarly to diabetics, who must follow glycemic index.

Both mokolobemid, as the representative of safer, in this case, group of reversible iMAOs, as well as classical medicines cause the increase in the sensitivity to tyramine. Additionally, in case of irreversible inhibitors, this effect is present within a few weeks after ceasing the treatment. Perhaps the example may be Selegiline and Moklobemid. Change of treatment from Moklobemid to Selegiline requires only 2 days of break, as opposed to one or two 2 weeks. [Rzewuska, Curing psychic disturbances]. Other adverse effects characteristic of MAO inhibitors are mainly somatic: the hepatotoxicity and hypertensive action.

One should not also forget about the fact that moklobemid strongly restrains the chromosomal 1 - A2 CYP450 subunit and above all 2 - D6. This is another danger, associated with disturbing the metabolism of all medicines decomposed with this pathway. These are above all: TCAs, SSRIs, and from atypical medicines: maprotiline, venlafaxine, mianserine, mirtazapine and trazodon [Pużyński; 2003]. Dangers also arise because the recalled subunits participate in the metabolism of not only medicines applied in the psychiatric treatment. It is necessary to take everything into account, from antibiotics and chemotherapeutic agents, also medicines for cancer treatment. The recalled relation allows us to understand why iMAO combination with other safe drug, would seem to turn out to be dangerous. Unfortunately vast quantities of medicines prescribed worldwide, as well as bought without the prescription, are more and more often connected with the occurrence of completely unpredictable and sometimes fatal interactions.

IMAO poisoning triggers many undesirable symptoms. At first the period of intensification of biochemical changes and disorders lasts without any symptoms, and then it produces considerable excitement and the psychomotor acceleration. Coma is becoming a next stage with simultaneous hyperthermia, tachycardia, precipitating breath, the extension of pupils and with intensification of deep reflexes. These are the characteristic manifestations of the serotonin syndrome [Kaplan; 1998].

Interactions

Apart from undesirable iMAO effects, one should dedicate the separate chapter to their interactions. It is one of most important aspects which we must take into consideration at

deciding about the administration of a drug from this group. In my evaluation, one should take into consideration undesirable symptoms and precautionary measures, and apply them to the entire iMAO group. Describing and characterizing individually reversible and irreversible types can create erroneous image of almost complete safety of RiMAO applying and weaken our clinical vigilance. Of course on one side old, classical inhibitors (iMAO) are less safe than newer (RiMAO) ones, however the practice also teaches us that the individual changeability and the sensitivity to the effect of even the same medicine can be sometimes diametrically different at two patients. In case of the discussed group, these are all cases of different increasing of undesirable symptoms, which is explained by different activity of acethylising enzyme. Low activity manifests itself at the half of representatives of the Caucasian race and in even higher percentage of Asian people. It is clear that the caution as for the discussed group of drugs is strictly required.

Classical iMAOs enter into reactions with opiate medicines, especially meperidine which sometimes leads to coma. Here a possibility exists both of unspecified interactions, as well as potentialising the meperidine action. If the need occurs to take painkillers from the group of opioids, Moklobemid should be complemented simply with morphine, or - with phentanyl. Interactions with analgesic medicines can just turn out to be the biggest problem; for example in case of the need to perform the operation treatment. If the operation takes place under the scheduled procedure, putting away iMAOs for two weeks is recommended ahead of schedule, worse if the treatment takes place in emergency, as the life saving procedure. Then the resulting risk from joining these two groups of medicines is becoming quite real. Additionally, lengthening of the sleep in the course of anaesthetization evoked by medicines from the group of barbiturates, applied to the patient along with tranylcypromine was described. In the literature, it is also possible to find descriptions of action with succinylocholine applied mainly in the short anaesthesia at electro convulsive therapy (ECT). These procedures are planned though and designed as to minimize drug interaction problems.

Other Problems Associated with iMAOs

Ethyl alcohol doesn't demonstrate interaction when applied with iMAO, but drinks with the large content of tyramine can contribute to the uncontrolled increase of the blood pressure. This mechanism was already described above.

At simultaneous applying of iMAO and medicines lowering the level of the sugar, the increase of hypoglikemising action was being observed irrespective of, whether it was insulin, or sulphonylurea derivatives. The change of the demand for anti-diabetic medicines is an effect which also regards medicines from the SSRI group and in a similar manner should be taken into account at compounding the doses. Medicines from the TCA group can have the reverse action; however a search of the dose of medicines regulating the blood glucose level should become a joint effort.

Medicines controlling blood pressure: reserpine, clonidyne and propranolol in combination with iMAOs give completely unpredictable interactions, starting from uncontrolled changes of the arterial pressure, hallucinating or the sudden increase in the activity of the autonomous system with intense agitation. Such combinations, if possible at all, require special care and attention.

The neuroleptics along with iMAOs can cause the notable increase in manifestations of anticholinergic and extrapyramidal type. Therefore one should avoid their combinations. Increasing symptoms of the Parkinson's disease by iMAO can be an effect of such an action. It will be possible to expect similar iMAO effect towards TCAs with increasing symptoms of psychosis, especially not suppressed with antipsychotics. By analogy one should very cautiously apply iMAOs in patients with Bipolar Disease on account of the risk of freeing the manic episode. The carefulness in the dosage and therapy is necessary in these cases.

Anti-asthmatics can in combination with iMAOs give increasing anxiety manifestations or the tachycardia in the mechanism potentialising of adverse effects of anti-asthmatic medicines. The identical relation regarding anticholinergic medicines, including iMAO drugs occurs with a sudden increase of the effect of atropine or scopololamine.

Sympaticomimetic medicines can give unpredictable effects in combination with iMAOs - from a sudden increase of the blood pressure through a rise in temperature, excitement, all the way to convulsions and coma - similar effect as at iMAO overdosing.

In treatment with iMAO there can occur pyridoxine deficiency (Wit. B6), which, in such a situation, creates the need for quick supplementation. In case of deficiencies, patients start to feel muscle pains or paraesthesia. Vitamin B6, as water-soluble vitamin, can safely be given, provided the patient has healthy and well functioning kidneys [Kaplan; 1998].

The only groups of medicines recognized as safe enough in combination with iMAOs are benzodiazepines and lithium carbonate. If lithium seems to be a potentialiser of antidepressant effect of the discussed medicines, benzodiazepine are applied in order to eliminate growing fear, anxiety, or the sleeplessness which, as everybody knows, appears frequently in the course of the use of antidepressant medicines [Ciraulo; 1999].

Clinical Application of iMAOs

The use of medications from the iMAO group is not only limited to patients with episodes of depression, as such. Wide action and the possible usefulness result exactly from their *modus operandi*, but mostly from biochemical pathways. Recommendations for the iMAO treatment will be close to the ones applied for tricyclic medicines or four-cyclic ones. Below there will be cues mentioned for using these medicines in a typical practice and different clinical situations, but curing episodes of depression will always remain superior; these medicines are classified as antidepressants.

If this is the case, Moklobemid should be applied in clinical picture of depression with the dominance of: apathy, inhibition, and lack of motivation and with social withdrawal. Fear of great intensity, as the leading manifestation also constitutes the episode of depression pointing to applying Moklobemid which in this case exceeds the remaining iMAOs [Jarema; 2011]. In case of applying Moklobemid is given in doses from 150 up to 450 mg a day. The dose of 600 mg is being regarded as maximum, in addition - achieving the highest doses must always take place slowly. Such a dosage regimen allows securing against sudden appearance of unpredictable manifestations. Moklobemid has a short span of biological half life enough (1 - 2 h), which undoubtedly supports the safety of the treatment, but one the other side triggers the need of systematic taking.

Potential, antidepressant action of moklobemid is utilised in curing episodes of depression of different intensity and in different clinical situations. In the literature we will find information about the possibility of applying it in depressions of the advanced age, when it is recognized as the acceptable medicine and a comparatively safe drug [Parnowski; 2003]. One should consider the length of the treatment which in senile depressions should amount to at least 6 months after appearance of manifestations. We must take into account the cooperation with the patient and the regularity, as well as a general mental state and family circumstances. Lone, elderly man, without the supervision over medicine s administration will be potentially exposed to adverse effects. A 'drug holiday' and a lack of the clinical effect will take its toll. It isn't possible also to forget about numerous adverse effects resulting from the iMAO combination with other pharmacological preparations, and patients in the advanced age take, as a rule, great quantities of drugs. Additionally we never know if kidneys or the liver of such patients are efficient.

In case of the need of curing mood disorders at pregnant women, one should avoid giving preparations from the discussed group. They similarly aren't recommended in schemes of proceedings of administering iMAOs in converting depression (unipolar) of pregnant women, because their teratogenic action is not fully recognised, whereas the potential risk of the appearance of interaction with other medicines is great. Similarly, pregnancy and breast-feeding are recognized as contraindication against the treatment with Moklobemid, all iMAOs permeate to mother's milk. According to the majority of authors pregnancy and the period of the feeding constitute the contraindication against the treatment with any single medicine from the iMAO group.

Good effects of Moklobemid were registered in case of atypical depressions, with the dubious and sometimes daubed clinical picture. Data in literature point even at the higher effectiveness than the one of classical TCAs. Such situations concern depression with a little component of vegetative manifestations, with gluttony, sleepiness and fear. In such cases it one should consider advantageous action of older, irreversible iMAOs, but on account of numerous contraindications at present the one which solely remained as the safe one is Moklobemid.

Similar, positive details about the clinical efficacy concern the iMAO application in the course of dysthymia. In this case, however, there aren't any data pointing at particularly advantageous action of the discussed group, because the straight majority of antidepressive agents in the course of dysthymia bring improvement.

Curing paroxysmal fear is also done using iMAOs, however there are no clear-cut conditions deciding about the advantage over classically applied anxiolytics, TCAs or SSRIs. In such case Moklobemid is regarded as the medicine of ‘secondary action’. In case of the social phobia or the agoraphobia, positive clinical experiments date as far back as the half of 80-ties. Then they used to apply phenelzine, whereas years later Moklobemid turned out to be equally effective but safer [Pużyński et al. 2010]. In our times, many authors regard Moklobemid as the medicine of choice exactly in case of social phobias. In the above cases, Moklobemid is given in large doses (300 to 600 mg/d), which considerably increases the efficiency of action [Rzewuska et al. 2000].

Clinical Recommendations and Conclusion

Considering the entire iMAO group, both classical and new ones (RiMAOs), one should remember that tranylcypromine displays greatest activating action, one should start the treatment with 10 mg per day, phenelzine should be administered cautiously, starting from 15 mg a day. Powerful increase may take place when administering the initial dose, but it must be done in week's cycles. In fact, as it was mentioned above, Moklobemid is currently exceeding over all remaining medicines of this group. In case of the need of potentialising iMAO action, one should consider it after about 6 weeks of the absence of a clinical response, adding lithium carbonate cautiously, or tridotironine. Dosage of Moklobemid, interactions and potential hazards resulting from the iMAO joint administration and of other antidepressive agents were already described.

Admittedly rare, but still topical are news reports on the possibility of hepatotoxic action, which is prompts the need for periodic examining of the function of the liver (AST, ALT, GGTP). Curing people in the advanced age is acceptable and was described, but one should consider the possibility of appearing of adverse effects more often than in those patients at the young age. Moklobemid isn't applied at children patients.

Undoubtedly, iMAOs are less cardiotoxic and epileptogenic than classical antidepressive agents - the tricyclics or four-cyclic medicaments. Most adverse effects of IMAOs are: orthostatic drops of the blood pressure, an increase in the body weight, sleeplessness and disorders of sexual functions. Additionally difficult to eliminate swellings can appear. Sometimes sleepiness can accompany the night sleeplessness in a day. If this is the case, one should revise the dose of the medicine and the possible need for the change of the pharmacotherapy. If necessary, one should act symptomatically.

In case of poisoning, one should immediately cease taking the medicine; consultation and the neurological help are necessary, although if this is the case, curing is also confined to symptomatic action. A fact that souring urine considerably increases iMAO elimination can be an indication that in extreme cases a dialysis should be recommended.

One should remember the above- mentioned problems, however it should be stressed once again that administering iMAO drugs with due caution is extremely helpful in the everyday clinical practice.

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