Prague Medical REPORT

(Sborník lékařský)

Multidisciplinary Biomedical Journal
of the First Faculty of Medicine,
Charles University

Vol. 117 (2016) No. 2–3

Primary Scientific Studies

Smoking Prevalence and Its Clinical Correlations in Patients with Narcolepsy-cataplexy / *Peřinová P., Feketeová E., Kemlink D., Kovalská P., Chlebušová K., Nepožitek J., Ibarburu V., Králíková E., Nevšímalová S., Šonka K.* page 81

CCL5 rs2107538 Polymorphism Increased the Risk of Tuberculosis in a Sample of Iranian Population / *Kouhpayeh H. R., Taheri M., Baziboroon M., Naderi M., Bahari G., Hashemi M.* page 90

The Response of C_{19} Δ^5-steroids to ACTH Stimulation and Hypoglycemia in Insulin Tolerance Test for Adrenal Insufficiency / *Dušková M., Kolátorová Sosvorová L., Hill M., Šimůnková K., Jandíková H., Pospíšilová H., Šrámková M., Kosák M., Kršek M., Hána V., Stárka L.* page 98

The Effect of Infliximab on Intestinal Anastomosis Healing in Rats / *Karaköse O., Eken H., Ulusoy A. N., Topgül H. K., Bilgin M., Yürüker S. S., Gülbahar M. Y.* page 108

Case Reports

Thoracic Outlet Syndrome: A Significant Family Genetic Phenotypic Presentation / *Janák D., Novotný K., Roček M., Rohn V.* page 117

IgG4-related Diseases – A Rare Polycystic Form of Ormond's Disease / *Průcha M., Czinner P., Prokopová P.* page 124

Bone Metabolism of the Patient with a Malignant Melanoma during the Entry Examination and the Check-up of Whole-body Bone Scintigraphy / *Weissensteiner J., Babušíková E.* page 129

Instructions to Authors page 135

D
E
C
Fig. 7.
B
C
A
Fig. 6.
B
A
Fig. 8.
C
B
A
B
Fig. 2.
D
A
D
E
Fig. 9.
A
E

Prague Medical Report (Prague Med Rep) is indexed and abstracted by Index-medicus, MEDLINE, PubMed, and Scopus.

Abstracts and full-texts of published papers can be retrieved from the World Wide Web (http://pmr.lf1.cuni.cz).

Engraving overleaf: Laurentius Heister, Institutiones chirurgicae, Amsterdam 1750.
Illustration provided by the Institute for History of Medicine and Foreign Languages.

Smoking Prevalence and Its Clinical Correlations in Patients with Narcolepsy-cataplexy

Pavla Peřinová[1], Eva Feketeová[2], David Kemlink[1], Petra Kovalská[1], Karolína Chlebušová[1], Jiří Nepožitek[1], Veronika Ibarburu[1], Eva Králíková[3,4], Soňa Nevšímalová[1], Karel Šonka[1]

[1]Centre for Sleep and Wake Disorders, Department of Neurology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic;
[2]Department of Neurology, Faculty of Medicine, Pavol Jozef Šafárik University in Košice and Louis Pasteur University Hospital, Košice, Slovak Republic;
[3]Institute of Hygiene and Epidemiology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic;
[4]Centre for Tobacco-Dependent, 3rd Department of Medicine – Department of Endocrinology and Metabolism, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic

Received January 15, 2016; Accepted September 2, 2016.

Key words: Narcolepsy-cataplexy – Smoking – Nicotine – Etiopathogenesis – Stimulating effect

Abstract: Narcolepsy-cataplexy (NC) is a chronic neurological disease with suggested autoimmune etiopathogenesis. Nicotine stimulates central nervous system and smoking increases the risk of autoimmune diseases. Assessment of smoking habits and its correlation to clinical parameters among 87 adult NC patients (38 male, 49 female) included night polysomnography and multiple sleep latency test. In our sample, 43.7% NC patients were regular smokers, and 19.5% former smokers compared to 22.2%, and 12.6%, respectively, in the general population. Patients started to smoke in the mean age of 20.0 (SD ±6.0) years. 72.2% of NC smokers started to smoke before the onset of NC and the mean of the delay between smoking onset and NC onset was 9.1 (±5.8) years. We found

This study was supported by a grant by the Ministry of Health of the Czech Republic, NF 13238-4/2012, and a grant by Charles University, PRVOUK P26/LF1/4.

Mailing Address: Prof. Karel Šonka, MD., DSc., Department of Neurology, First Faculty of Medicine, Charles University and General University Hospital in Prague, Kateřinská 30, 120 00 Prague 2, Czech Republic; Phone: +420 224 965 568; e-mail: ksonka@lf1.cuni.cz

http://dx.doi.org/10.14712/23362936.2016.8
© Charles University – Karolinum Press, 2016

a direct correlation between smoking duration and the number of awakenings, duration of N1 sleep, REM sleep latency, and apnoea/hypopnoea index (AHI), and, on the contrary, indirect correlation between smoking duration and N3 sleep duration, showing that smoking duration consistently correlates with sleep macrostructure. Smoking is highly prevalent in NC and has relationship with clinical features of NC.

Introduction

Narcolepsy-cataplexy (NC) is a chronic neurological disease with a prevalence of approximately 0.045% in North America and Europe (Ohayon et al., 2002). The manifestations of NC are excessive daytime sleepiness (EDS) and cataplexy, and roughly half of patients experience hypnagogic hallucinations and sleep paralysis. Additionally, most patients have fragmented night-time sleep. Dysregulation of REM sleep is typical, with REM sleep occurring within the first 15 minutes of sleep onset, termed sleep onset REM periods (SOREMp), during the day and at night (Dauvilliers et al., 2007). The age at onset varies from childhood to approximately 60 years of age, with manifestation most often at 15–20 years of age, and a second peak between 35 and 40 years of age (Dauvilliers et al., 2001).

The pathologic basis of the disease is a deficiency of neurons in the lateral hypothalamus that produce hypocretin (Thannickal et al., 2000). Although the exact mechanism of hypocretin deficiency is unknown, evidence from the past 20 years strongly favours an immune-mediated or autoimmune attack, targeting specifically hypocretin neurons in genetically predisposed individuals (Liblau et al., 2015). The hypothesis that a targeted immune-mediated or autoimmune attack causes the specific degeneration of hypocretin neurons arose mainly through the discovery of genetic associations, first with the HLA DQB1*06:02 allele (Mignot, 1998) and then with the T-cell receptor α locus (Hallmayer et al., 2009). It is suggested that specific autoimmune process is triggered by different environmental stimuli like streptococcal infection or anti H1N1 vaccination or H1N1 infection itself in genetically disposed subjects and is time limited (Partinen et al., 2014).

Dependency on tobacco is one of the most common dependencies in today's society, including the Czech Republic and Slovak Republic, with prevalence of about 30% in the population 15–64 years (Sovinová and Csémy, 2015). Smoking as related to NC deserves our interest for several reasons. Nicotine exhibits stimulatory effects (Boutrel and Koob, 2004), so patients might use it in attempt to suppress their EDS. Some studies report that NC is associated with higher levels of impulsiveness and the so-called sensation-seeking behaviour in this condition (Dimitrova et al., 2011), which might explain increased tendency to smoke. Smoking is a risk factor for development and progression of multiple sclerosis (Carlens et al., 2010; Wingerchuk, 2012; Hedstrom et al., 2013) and other inflammatory diseases such as rheumatoid arthritis, Crohn's disease, ulcerative colitis, and

sarcoidosis (Carlens et al., 2010). It is thus possible to assume that smoking plays a role in the development of NC, even when smoking appears to cause multiple sclerosis in other ways than through the mediation of nicotine (Carlens et al., 2010).

This led us to find out about the proportion of our patients with NC who are smokers, and whether there is a relationship between smoking and the clinical parameters of NC.

Sample and Methods

Our study included 87 adult patients with NC we were able to contact over the course of 2015. All patients fulfilled the diagnostic criteria of the International Classification of Sleep Disorders, 2nd Edition (ICSD2; American Academy of Sleep Medicine, 2005). The records of subjects diagnosed before 2005 (the year of ICSD2 publication) were carefully checked and conformity with ICSD2 was verified. The study included 49 women and 38 men aged from 19 to 83 years, mean age of study participants was 46.6 (SD ±16.3) years. Mean age of onset of narcolepsy symptoms was 23.5 (±10.4) years. HLA DQB1*0602 genotyping was available for 83 patients 82 of whom were positive corresponding to commonly reported representation in NC (Liblau et al., 2015). Relevant clinical data including the results of polysomnography analysed according to American Academy of Sleep Medicine (AASM) guidelines (Iber et al., 2007) and Multiple Sleep Latency Test (MSLT) conducted according to AASM recommendations (Arand et al., 2005) and Epworth sleepiness scale (Johns, 1991) were completed based on patient medical records. Night polysomnography and MSLT were performed in patients who have had not been treated before or who were not taking drugs influencing sleep and mood ≥ 2 weeks.

The patients were asked about smoking according to our own structured questionnaire during their outpatient examination or in the form of phone interview. The questionnaire included the following items: classification into non-smokers (less than 100 cigarettes in life course), former smokers and current smokers (WHO, 2008). Former and present smokers were additionally subjected to targeted questions regarding age at the first cigarette, age of regular smoking onset, number of cigarettes smoked in one day and attempts to stop smoking.

All categorical data were compared using two sided chi-squared statistics. Since polysomnographic parameters are by their nature not normally distributed, we used for inter-group comparisons Mann-Whitney U-test, other parameter were compared using parametric T-tests. Correlations were calculated as Pearson's correlations coefficients.

All statistical analyses were conducted using STATISTICA (data analysis software system), version 12. www.statsoft.com, StatSoft, Inc. (2013).

This study was part of a large study on narcolepsy approved by the Ethical Committee of the General University Hospital in Prague and all patients provided signed informed consent with this study.

Results

Regular smokers (one or more cigarettes daily) represented 43.7% patients, while 19.5% were former regular smokers. Prevalence of smoking present in any period of life was thus 63.2% (55 patients). The patients smoked only cigarettes, no other tobacco product was recorded. Mean age at initiating smoking was 20.0 (±6.0) years and mean number of cigarettes smoked daily was 13.6 (±10.7). Forty (72.2%) smokers started smoking prior to their first symptoms of narcolepsy, and mean recorded latency of onset of narcolepsy since smoking initiation was 9.1 (±5.8) years. Mean age of NC onset in patients who started smoking prior to developing NC was 27.8 (±9.0) years, while patients who commenced smoking after developing NC or had never smoked developed NC at 20.5 (±10.2) years of age ($p<0.001$).

Table 1 – Clinical data on subjects suffering from NC under study expressed as mean (SD)

	All	Non-smokers	Smokers	P-value
Number (%)	87.0 (100)	32.0 (36.8)	55.0 (63.2)	NA
Age at smoking interview (years)	46.6 (16.3)	42.2 (13.8)	49.1 (17.2)	0.043
Age at NC onset (years)	23.5 (10.4)	21.8 (12.0)	24.5 (9.2)	NS
BMI	29.2 (5.1)	28.3 (4.5)	29.7 (5.4)	NS
Epworth sleepiness scale	18.0 (3.6)	17.0 (4.1)	18.5 (3.1)	NS
Number of patients with restless legs syndrome (%)	15.0 (17.2)	4.0 (12.5)	11.0 (20)	NS
Latency between night polysomnography and MSLT and smoking interview (years)	5.4 (5.2)	6.3 (6.9)	4.9 (3.9)	NS
Night polysomnography				
Sleep efficiency (%)	82.6 (9.9)	86.0 (7.8)	80.7 (10.2)	0.022
Sleep N1 duration (%)	11.9 (9.2)	9.0 (6.0)	13.6 (10.3)	0.034
Sleep N2 duration (%)	39.0 (10.8)	38.6 (9.6)	32.3 (11.4)	NS
Sleep N3 duration (%)	15.8 (8.2)	19.6 (7.0)	13.6 (8.2)	0.002
REM sleep duration (%)	19.5 (7.1)	21.0 (5.8)	18.6 (7.6)	NS
REM sleep latency (min)	40.1 (60.9)	30.3 (63.7)	45.6 (59.2)	NS
PLMI	17.8 (22.6)	9.3 (13.8)	22.5 (25.1)	0.016
AHI	8.8 (16.6)	3.8 (5.5)	11.8 (20.0)	0.041
MSLT				
Sleep latency – MSLT (min)	2.9 (2.2)	2.2 (1.6)	3.3 (2.4)	0.031
SOREM MSLT (number)	3.6 (1.2)	3.6 (1.2)	3.6 (1.2)	NS

Non-smokers are defined as individuals who have smoked less than 100 cigarettes in their whole life, and smokers are patients smoking at the time of questioning, taken together with those who have already quitted smoking (but had smoked more than 100 cigarettes in their life). SD – standard deviation; NA – non applicable; NC – narcolepsy with cataplexy; NS – nonsignificant; BMI – body mass index; MSLT – multiple sleep latency test; N1, N2, N3 – non rapid eye movement sleep stage 1, 2, 3 respectively; REM – rapid eye movement; PLMI – periodic leg movements index (number of periodic leg movements/1 hour); AHI – apnoea/hypopnoea index (number of apnoea/hypopnoea episodes/1 hour of sleep); SOREM – sleep onset REM period

Relevant clinical parameters for the whole patient group and the group of smokers (i.e. former smokers and those who smoked at the time of study interview of all actively smoking taken together) and non-smokers (i.e. individuals who had smoked less than 100 cigarettes in their whole life) including statistical comparisons are shown in Table 1.

We found direct correlation between age at NC onset and the latency between onset of symptoms of narcolepsy and the age of initiating regular smoking (0.822, $p<0.001$, $N=30$). Secondly we found negative correlation between age at NC onset and the time delay (both positive and negative) from smoking initiation to NC onset (–0.7936, $p<0.001$, $N=45$) in all NC patients.

Smoking duration correlates rather consistently with the parameters of night sleep macrostructure. We found correlation with the number of awakenings (0.5217, $p=0.001$, $N=39$), duration of NREM 1 sleep (0.3573, $p<0.015$, $N=46$), REM sleep latency (0.3511, $p<0.016$, $N=47$) and also AHI (apnoea/hypopnoea index) (0.5059, $p=0.001$, $N=43$). Smoking duration was negatively correlated with sleep efficiency (–0.5145, $p<0.001$, $N=47$), duration of NREM 3 sleep (–0.6142, $p<0.001$, $N=46$). No correlation was found between smoking duration and subjective or objective evaluation of sleepiness during the day.

The number of cigarettes smoked in one day did not correlate with any NC parameter of interest.

Discussion

There are 23.5% active regular smokers in the age group of those above 15 years in the Czech Republic (Sovinová and Csémy, 2015) while the rate recorded in our sample of patients with NC was 43.7%. Occurrence of active regular smoking in NC is thus twice as high as in general Czech population. A similar difference concerns the rate of former regular smoking, specifically 12.6% of former smokers in the whole Czech population against 19.5% of former regular smokers among patients with NC. Regarding the number of cigarettes smoked, our patients with NC (13.6 cigarettes daily) come close to this number in common population where regular smokers smoke around 15 cigarettes daily (Sovinová and Csémy, 2015). We assume that smoking habits are similar in Slovakia. The proportion of smokers in our NC population is about 50% higher than in an Italian study where, however, was the same percentage of smokers in the control group as is reported for the Czech population, so also the Italian study has shown higher frequency of smoking among patients with NC, the authors explain this higher rate with the stimulatory action of nicotine (Palaia et al., 2011). Higher proportion of current smokers in NC than in controls (37.2% vs. 21.7%) was also reported in recent large French study (Barateau et al., 2016).

Our data do not allow any conclusion regarding a possible relationship between smoking and the etiopathogenesis of narcolepsy, the differing ages of patients who started smoking prior to NC symptom onset and the age of other patients with

NC is rather due to the age at NC onset than to its relationship to smoking. The relationship between smoking initiation, albeit passive, and the development of NC is suggested by the finding that in individuals with HLA DQB1*0602 positivity passive smoking was a risk factor of narcolepsy. The authors of this study explain why passive smoking was a risk factor, unlike active smoking, with the fact that narcolepsy symptoms in many patients begin in their childhood where exposure to active smoking is negligible (Ton et al., 2009).

Our results showing worse quality of sleep in patients with NC suggest that nicotine worsens the quality of sleep in NC as well, in the same way as in general population (Jaehne et al., 2009). The results, however, have to be taken as only approximate in this respect as smokers in our sample were older than non-smokers, and smokers had higher AHI and PLMI (periodic leg movements index). Sleep apnoea and periodic limb movements during sleep participate in disturbing the quality of night sleep, and this is also the case in NC (Sansa et al., 2010).

In our sample, smoking duration correlated clearly with objective parameters of night sleep quality. To some degree, this might be explained with ageing – the changes described develop also as a result of ageing as such (Šonka et al., 1993). This finding might also suggest that smoking has a cumulative rather than immediate effect on quality of sleep which has not been described yet and should be tested by a more elaborated study.

The fact that the number of cigarettes smoked does not correlate with subjective or objective sleepiness, may be rather interpreted as suggesting that our patients do not use cigarette smoking as self-indicated drug against EDS but this relationship is far to be excluded. Shorter mean sleep latency in MSLT in patients – non-smokers may be interpreted only with difficulty as patients undergoing MSLT are prohibited to smoke (Arand et al., 2005) and smokers should thus rather have shorter latency of falling asleep as is the case in smoking abstinence in smokers in the general population (Prosise et al., 1994). More severe sleepiness of smokers in MSLT, however, might have been influenced by worse quality night sleep related to obstructive apnoeas and periodic limb movements that may accentuate sleepiness during the day by itself, though no clear evidence for this exists in NC, unlike in general population (Engleman and Douglas, 2004; Hornyak et al., 2006). Case reports suggest that nicotine may mask or relieve symptoms of narcolepsy, including EDS and even cataplexy (Krahn et al., 2009; Ebben and Krieger, 2012). Such an interaction is supported by limited data suggesting that nicotine addiction may be mediated by hypocretin pathways (Corrigall, 2009).

Nicotine exhibits antidepressant action (Tizabi et al., 1999), so higher-degree smoking might also be related to increased prevalence of several depressive symptoms (Vourdas et al., 2002; Fortuyn et al., 2010). Higher rate of smokers among narcolepsy patients is not connected to hypocretin deficiency because from animal models it seems that hypocretin system's role is to reduce drug seeking behaviours. Hypocretin knockout mice showed reduced signs of withdrawal from

nicotine (Plaza-Zabala et al., 2013). All other reasons possibly leading to smoking in NC should be tested by more sophisticated research.

Not negligible is certainly also the impact of smoking on the general health of a patient with NC. Smoking is a risk factor for many cardiovascular and metabolic diseases. Literary data and our unpublished results show that patients with NC suffer from higher rates of arterial hypertension, type 2 diabetes and obesity (Sonka et al., 2010; Jennum et al., 2013; Ohayon, 2013), which may also be related to higher rate of smoking in NC.

Although narcolepsy symptom relief may be viewed as a benefit of nicotine and may thus be a barrier to smoking cessation in narcoleptics, the act of smoking is itself of concern because of many reasons. First is the risk of falling asleep while smoking resulting in injury and damage (Krahn et al., 2009) and second is the already mentioned risk factor of many other diseases. Third reason is based on the fact that nicotine is a highly addictive substance (Benowitz, 2008), and this obviously applies to the narcolepsy population as well.

This study has several limitations that were mostly mentioned in the discussion above. The limitation is not only a small number of patients, but also the latency between complete clinical examination and interview regarding smoking, limited information about smoking and missing personality profiles of the respondents. Larger prospective studies of smoking in NC are certainly worth considering. In any case, patients with NC should be instructed not to start smoking, and if this has already happened, to quit smoking.

References

American Academy of Sleep Medicine (2005) *International Classification of Sleep Disorders: Diagnostic and Coding Manual*, 2nd Ed. American Academy of Sleep Medicine, Westchester.

Arand, D., Bonnet, M., Hurwitz, T., Mitler, M., Rosa, R., Sangal, R. B. (2005) The clinical use of the MSLT and MWT. *Sleep* **28**, 123–144.

Barateau, L., Jaussent, I., Lopez, R., Boutrel, B., Leu-Semenescu, S., Arnulf, I., Dauvilliers, Y. (2016) Smoking, alcohol, drug use, abuse and dependence in narcolepsy and idiopathic hypersomnia: a case-control study. *Sleep* **39**, 573–580.

Benowitz, N. L. (2008) Clinical pharmacology of nicotine: implications for understanding, preventing, and treating tobacco addiction. *Clin. Pharmacol. Ther.* **83**, 531–541.

Boutrel, B., Koob, G. F. (2004) What keeps us awake: The neuropharmacology of stimulants and wakefulness-promoting medications. *Sleep* **27**, 1181–1194.

Carlens, C., Hergens, M. P., Grunewald, J., Ekbom, A., Eklund, A., Hoglund, C. O., Askling, J. (2010) Smoking, use of moist snuff, and risk of chronic inflammatory diseases. *Am. J. Respir. Crit. Care Med.* **181**, 1217–1222.

Corrigall, W. A. (2009) Hypocretin mechanisms in nicotine addiction: evidence and speculation. *Psychopharmacology (Berl.)* **206**, 23–37.

Dauvilliers, Y., Montplaisir, J., Molinari, N., Carlander, B., Ondze, B., Besset, A., Billiard, M. (2001) Age at onset of narcolepsy in two large populations of patients in France and Quebec. *Neurology* **57**, 2029–2033.

Dauvilliers, Y., Arnulf, I., Mignot, E. (2007) Narcolepsy with cataplexy. *Lancet* **369**, 499–511.

Dimitrova, A., Fronczek, R., Van der Ploeg, J., Scammell, T., Gautam, S., Pascual-Leone, A., Lammers, G. J. (2011) Reward-seeking behavior in human narcolepsy. *J. Clin. Sleep Med*. **7**, 293–300.

Ebben, M. R., Krieger, A. C. (2012) Narcolepsy with cataplexy masked by the use of nicotine. *J. Clin. Sleep Med*. **8**, 195–196.

Engleman, H. M., Douglas, N. J. (2004) Sleep. 4: Sleepiness, cognitive function, and quality of life in obstructive sleep apnoea/hypopnoea syndrome. *Thorax* **59**, 618–622.

Fortuyn, H. A., Lappenschaar, M. A., Furer, J. W., Hodiamont, P. P., Rijnders, C. A. T., Renier, W. O., Buitelaar, J. K., Overeem, S. (2010) Anxiety and mood disorders in narcolepsy: a case-control study. *Gen. Hosp. Psychiatry* **32**, 49–56.

Hallmayer, J., Faraco, J., Lin, L., Hesselson, S., Winkelmann, J., Kawashima, M., Mayer, G., Plazz, G., Nevsimalova, S., Bourgin, P., Hong, S. C., Honda, Y., Honda, M., Högl, B., Longstreth, W. T. Jr., Montplaisir, J., Kemlink, D., Einen, M., Chen, J., Musone, S. L., Akana, M., Miyagawa, T., Duan, J., Desautels, A., Erhardt, C., Hesla, P. E., Poli, F., Frauscher, B., Jeong, J. H., Lee, S. P., Ton, T. G., Kvale, M., Kolesar, L., Dobrovolná, M., Nepom, G. T., Salomon, D., Wichmann, H. E., Rouleau, G. A., Gieger, C., Levinson, D. F., Gejman, P. V., Meitinger, T., Young, T., Peppard, P., Tokunaga, K., Kwok, P. Y., Risch, N., Mignot, E. (2009) Narcolepsy is strongly associated with T-cell receptor alpha locus. *Nat. Genet*. **41**, 708–711.

Hedstrom, K. A., Hillert, J., Olsson, T., Alfredsson, L. (2013) Smoking and multiple sclerosis susceptibility. *Eur. J. Epidemiol*. **28**, 867–887.

Hornyak, M., Feige, B., Riemann, D., Voderholzer, U. (2006) Periodic leg movements in sleep and periodic limb movement disorder: prevalence, clinical significance and treatment. *Sleep Med. Rev*. **10**, 169–177.

Iber, C., Ancoli-Israel, S., Chesson, A., Quan, S. F. (2007) *The AASM Manual for the Scoring of Sleep and Associated Events: Rules, Terminology and Technical Specifications*, 1st Ed. American Academy of Sleep Medicine, Westchester.

Jaehne, A., Loessl, A. B., Bárkai, Z., Riemann, D., Hornyak, M. (2009) Effects of nicotine on sleep during consumption, withdrawal and replacement therapy. *Sleep Med. Rev.* **13**, 363–377.

Jennum, P., Ibsen, R., Knudsen, S., Kjellberg, J. (2013) Comorbidity and mortality of narcolepsy: a controlled retro- and prospective national study. *Sleep* **36**, 835–840.

Johns, M. W. (1991) A new method for measuring daytime sleepiness: the Epworth sleepiness scale. *Sleep* **14**, 540–545.

Krahn, L. E., Martin, K. A., Silber, M. H. (2009) Narcoleptic patients' perceptions of nicotine. *J. Clin. Sleep Med*. **5**, 390.

Liblau, R. S., Vassalli, A., Seifinejad, A., Tafti, M. (2015) Hypocretin (orexin) biology and the pathophysiology of narcolepsy with cataplexy. *Lancet Neurol*. **14**, 318–328.

Mignot, E. (1998) Genetic and familial aspects of narcolepsy. *Neurology* **50**, 16–22.

Ohayon, M. M. (2013) Narcolepsy is complicated by high medical a psychiatric comorbidities: a comparison with the general population. *Sleep Med*. **14**, 488–492.

Ohayon, M. M., Priest, R. G., Zulley, J., Smirne, S., Paiva, T. (2002) Prevalence of narcolepsy symptomatology and diagnosis in the European general population. *Neurology* **58**, 1826–1833.

Palaia, V., Poli, F., Pizza, F., Antelmi, E., Franceschini, C., Moghadam, K. K., Provini, F., Pagotto, U., Montagna, P., Schenck, C. H., Mignot, E., Plazzi, G. (2011) Narcolepsy with cataplexy associated with nocturnal compulsive behaviors: a case-control study. *Sleep* **34**, 1365–1371.

Partinen, M., Kornum, B. R., Plazzi, G., Jennum, P., Julkunen, I., Vaarala, O. (2014) Narcolepsy as an autoimmune disease: the role of H1N1 infection and vaccination. *Lancet Neurol*. **13**, 600–613.

Plaza-Zabala, A., Flores, A., Martín-García, E., Saravia, R., Maldonado, R., Berrendero, F. (2013) A role for hypocretin/orexin receptor-1 in cue-induced reinstatement of nicotine-seeking behavior. *Neuropsychopharmacology* **38**, 1724–1736.

Prosise, G. L., Bonnet, M. H., Berry, R. B., Dickel, M. J. (1994) Effects of abstinence from smoking on sleep and daytime sleepiness. *Chest* **105**, 1136–1141.

Sansa, G., Iranzo, A., Santamaria, J. (2010) Obstructive sleep apnea in narcolepsy. *Sleep Med*. **11**, 93–95.

Šonka, K., Tafti, M., Billiard, M. (1993) Polysomnografické nálezy u narkoleptiků středního a vyššího věku. *Sb. Lek*. **94**, 333–344.

Sonka, K., Kemlink, D., Buskova, J., Pretl, M., Srutkova, Z., Maurovich Horvat, E., Vodicka, P., Polakova, V., Nevsimalova, S. (2010) Obesity accompanies narcolepsy with cataplexy but not narcolepsy without cataplexy. *Neuro Endocrinol. Lett*. **31**, 631–634.

Sovinová, H., Csémy, L. (2015) *The Use of Tobacco and Alcohol in the Czech Republic 2014*. National Institute of Public Health, Prague; available at: http://www.szu.cz/uploads/documents/czzp/zavislosti/TabAlkCZ_EN2014.pdf

Thannickal, T. C., Moore, R. Y., Nienhuis, R., Ramanathan, L., Gulyani, S., Aldrich, M., Cornford, M., Siegel, J. M. (2000) Reduced number of hypocretin neurons in human narcolepsy. *Neuron* **27**, 469–474.

Tizabi, Y., Overstreet, D. H., Rezvani, A. H., Louis, V. A., Clark, E. Jr., Janowsky, D. S., Kling, M. A. (1999) Antidepressant effects of nicotine in an animal model of depression. *Psychopharmacology* **142**, 193–199.

Ton, G. N. T., Longstreth, W. T. Jr., Koepsell, T. (2009) Active and passive smoking and risk of narcolepsy in people with HLA DQB1*0602: A population-based case-control study. *Neuroepidemiology* **32**, 114–121.

Vourdas, A., Shneerson, J. M., Gregory, C. A., Smith, I. E., King, M. A., Morrish, E., McKenna, P. J. (2002) Narcolepsy and psychopathology: Is there an association? *Sleep Med*. **3**, 353–360.

WHO (2008) *MPOWER*; available at: http://www.who.int/tobacco/mpower/en/

Wingerchuk, D. M. (2012) Smoking: Effects on multiple sclerosis susceptibility and disease progression. *Ther. Adv. Neurol. Disord*. **5**, 13–22.

 Prague Medical Report / Vol. 117 (2016) No. 2–3, p. 90–97

CCL5 rs2107538 Polymorphism Increased the Risk of Tuberculosis in a Sample of Iranian Population

Hamid Reza Kouhpayeh[1], Mohsen Taheri[2,3], Mana Baziboroon[1], Mohammad Naderi[1], Gholamreza Bahari[4], Mohammad Hashemi[4]

[1]Infectious Diseases and Tropical Medicine Research Center, Zahedan University of Medical Sciences, Zahedan, Iran;
[2]Genetics of Non-Communicable Disease Research Center, Zahedan University of Medical Sciences, Zahedan, Iran;
[3]Department of Genetic, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran;
[4]Department of Clinical Biochemistry, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

Received April 21, 2016; Accepted September 2, 2016.

Key words: Tuberculosis – CCL5 – CCR5 – Polymorphism

Abstract: Cysteine-cysteine chemokine ligand 5 (CCL5) with immunoregulatory and inflammatory activities has an important role in granuloma formations that activates and stimulates T-cells and macrophages. Cysteine-cysteine chemokine receptor 5 (CCR5) is a chemokine receptor, which is important for migration of immune cells to site of infection. In the present study we investigated the possible association between CCL5 –403G/A (rs2107538), CCL5 –28C/G (rs2280788) and CCR5 Δ32 polymorphisms and pulmonary tuberculosis (PTB) in an Iranian population. This case-control study was performed on 160 patients with pulmonary tuberculosis and 160 unrelated healthy subjects. The CCL5 –403G/A, CCL5 –28C/G and CCR5 Δ32 polymorphisms were genotyped by allele-specific polymerase chain reaction (AS-PCR), tetra amplification refractory mutation system polymerase chain reaction (T-ARMS PCR) and PCR, respectively. Our results showed that GA as well as GA+AA genotypes of CCL5 –403G/A (rs2107538) increased the risk of PTB in comparison with GG genotype (OR=1.70, 95% CI=1.03–2.81, P=0.038 and OR=1.64, 95% CI=1.00–2.68, P=0.049,

This study was supported by dissertation grant (MD. thesis of MB) from Zahedan University of Medical Sciences, Zahedan, Iran.

Mailing Address: Assoc. Prof. Mohsen Taheri, PhD., Department of Genetic, School of Medicine, Zahedan University of Medical Sciences, Zahedan 98167-43463, Iran; e-mail: mohsen.taheri.gene@gmail.com

http://dx.doi.org/10.14712/23362936.2016.9
© Charles University – Karolinum Press, 2016

respectively). No significant association was found between CCL5 –28C/G as well as CCR5 Δ32 polymorphism and PTB risk. In conclusion, our findings proposed that CCL5 –403G>A polymorphism may be a risk factor for susceptibility to PTB in our population. Larger sample sizes with different ethnicities are required to validate our findings.

Introduction

Tuberculosis (TB) is one of the most communicable diseases in humans caused by various strains of mycobacteria frequently *Mycobacterium tuberculosis*. According to the World Health Organization (WHO) report, most of the estimated number of TB cases occurred in Asia (55%) and Africa (30%). TB with 8 million new cases and 1.5 million deaths worldwide annually remains as a major global health problem (Millet et al., 2013). Though one third of human infected with *Mycobacterium tuberculosis*, only 10% of the infected persons develop the clinical disease (Cobat et al., 2013). Cumulative evidence designates that in addition to the environment, host genetic factors play an important role in susceptibility to TB (Bahari et al., 2012; Hashemi et al., 2013a, 2015; Naderi et al., 2014a, b).

It is well known that chemokines are essential regulators of immune system in response to *Mycobacterium tuberculosis* infectious. CCL5 is an 8 kDa protein belongs to the CC chemokines family and known as RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted). CCL5 is chemotactic for T-cells as well as macrophages and mediate the migration and activation of these cells into inflammatory sites (Alqumber et al., 2013). It is a main chemokine involved in both the acute and chronic phase of inflammation and possibly participate in pathogenesis and formation of granuloma during infection with *Mycobacterium tuberculosis* (Méndez-Samperio, 2008). Indeed, this chemokine may have a role in the inhibition of intracellular growth of *Mycobacterium tuberculosis* (Chu et al., 2007). The human *CCL5* mapped to chromosome 17 (17q11.2-q12) and consist of three exons and two introns. This gene is polymorphic and have been suggested that two polymorphisms in promoter region (–403G/A and –28C/G) effect the expression of CCL5 (Chu et al., 2007).

CCR5 or CD195 is a member of the beta chemokine receptor family. This protein is expressed by T-cells, macrophages, immature dendritic cells and granulocytes and has an important role in inflammatory response to infection (Carpenter et al., 2014). It has been reported that CCR5 is overexpressed in PTB (pulmonary tuberculosis) patients in comparison with normal subjects (Mamtani et al., 2011). The ligands of this receptor are CCL5 (RANTES), macrophage inflammatory protein (MIP-1α and MIP-1β or CCL3 and CCL4) and CCL3L1 (Han et al., 2012). The *CCR5* gene is located on short arm of chromosome 3 at the chemokine receptor gene cluster region (Mishra et al., 2012). Several polymorphisms have been recognized in *CCR5* which one of them is CCR5-delta32

(CCR5 Δ32). CCR5 Δ32 is a deletion of gene results in a non-functional receptor form of the chemokine receptor that is unable to bind CC chemokine ligands such as CCL5. Numerous studies have shown the association of *CCL5* and *CCR5* gene polymorphism and the risk of TB in various ethnic populations, but the results were controversial (Ben-Selma et al., 2011; Selvaraj et al., 2011; Carpenter et al., 2014). Therefore, the present case-control study was designed to investigate the possible association between CCL5 –403G/A (rs2107538), CCL5 –28C/G (rs2280788) and CCR5 Δ32 polymorphisms and pulmonary tuberculosis in a sample of southeast Iranian population.

Material and Methods

Patients

This case-control study was done from June 2012 to September 2013 on patients who referred to Bou-Ali Hospital (referral center for TB, Zahedan, Iran). A total of 320 subjects including 160 confirmed PTB and 160 unrelated healthy subjects with no clinical symptoms or history of TB and living in the same area as the patients with PTB (Southeast Iran) were enrolled in the study. Informed consent was taken from all subjects and the project was approved by Ethics Committee of Zahedan University of Medical Sciences. TB was diagnosed by clinical symptoms, posterior-anterior chest radiography, presence of acid-fast-bacilli (AFB) on a sputum smear, culturing *Mycobacterium tuberculosis* organisms from a specimen taken from the patient and response to antituberculosis chemotherapy as described in our previous study (Kouhpayeh et al., 2012). DNA was extracted from whole blood samples using salting out method as described previously (Hashemi et al., 2013b).

Genotyping

CCL5 –403G/A (rs2107538) as well as CCR5 Δ32 polymorphisms were genotyped using allele specific PCR (AS-PCR) and PCR method as described previously (Eskandari-Nasab et al., 2014). Briefly, primers for the CCL5 –403G/A (rs2107538) polymorphism were as follows: reverse (command): TTCTTGGGGACAACAAGGAG, forward (A allele): GGATGAGGGAAAGGCGA and forward (G allele): GGATGAGGGAAAGGCGG. The forward and reverse primers for detection of 32 bp ins/del polymorphism were 5'-TCAAAAAGAAGGTCTTCATTACACC-3' and 5'-AGCCCAGAAGAGAAAATAAACAATC-3', respectively.

For detection of CCL5 –28C/G (rs2280788) variant we designed a tetra amplification refractory mutation system polymerase chain reaction (T-ARMS PCR). We used two external primers (forward outer: 5'-AGGAGCGCAGAGGGCAGTAGCAATGA-3', reverse outer: 5'-TGAGGAGGACCCCTTCCCTGGAAGGT-3') and two internal primers (forward inner [C allele]: 5'-GGAATGAAAAATTAGAACAACAGAACCA-3' reverse inner [G allele]: 5'-TTTGCTAAAGAAATAGAAGTGGCTTACAAC-3').

PCR was performed in 25 µl reaction volumes containing 0.4 µM of each primer, 250 µM of each dNTP, 1 U Taq DNA polymerase with 2 mM $MgCl_2$, and 50 ng genomic DNA. The PCR cycling conditions was as follows: an initial denaturation step of 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, annealing at 54 °C for 30 s and extension at 72 °C for 30 s. Final extension was performed at 72 °C for 5 min. The PCR products were separated by electrophoresis in 2% agarose gels, and observed under ultraviolet light. Product sizes were 272 bp for C allele and 181 bp for G allele, whereas the product size of the two outer primers was 400 bp.

Statistical analysis

Statistical analysis was performed by SPSS software V18.0. In order to investigate potential association of the selected polymorphism with tuberculosis, the allele and genotype frequencies in patients and healthy controls were compared using Pearson's chi-squared test. Logistic regression analysis was applied to estimate odds ratio (OR) and 95% confidence intervals (CI) of genetic risk in PTB. P-values less than 0.05 were considered statistically significant.

Results

The study groups consist of 160 PTB patients (77 males and 83 females) and 160 control subjects (59 males and 101 females). Mean age of PTB patients and control groups were 48.78 ± 20.374 and 47.68 ± 15.86, respectively. No significant difference was seen between two groups regarding age and sex (P=0.592 and 0.054, respectively).

The genotype and allele frequency of CCL5 –403G/A polymorphism is shown in Table 1. The results indicated that GA as well as GA+AA genotype increased the risk of PTB in comparison with GG genotype (OR=1.70, 95% CI=1.03–2.81, P=0.038 and OR=1.64, 95% CI=1.00–2.68, P=0.049, respectively). The allele frequency of CCL5 –403G/A polymorphism was not significantly different between the groups (P=0.08).

The genotype frequency of CCL5 –28C/G polymorphism showed that all PTB patients and healthy controls had GG genotype which indicates that this variant is not polymorphic in our population.

The analysis of the PTB patients and healthy controls revealed no statistically significant difference between the groups regarding 32 bp insertion/deletion polymorphism of the CCR5 gene. In each groups 154 persons has Ins/Ins genotypes and 6 has Ins/Del (Table 2). Our finding demonstrated that the 32 bp deletion polymorphism of CCR5 is not a risk factor for PTB.

Discussion

Tuberculosis after HIV is the second leading cause of death worldwide and killing about 2 million people annually (Millet et al., 2013). Besides of environment factor

Table 1 – The genotypes and allele distribution of CCL5 –403G>A polymorphisms in pulmonary tuberculosis (PTB) patients and control groups

Polymorphism CCL5 –403G>A	Patients n (%)	Normal n (%)	OR (95% CI)	P
		Codominant		
GG	104 (65)	121 (75.6)	1.00	
GA	54 (33.8)	36 (22.5)	1.70 (1.03–2.81)	0.038
AA	2 (1.2)	3 (1.9)	0.82 (0.13–5.05)	0.827
		Dominant		
GG	104 (65)	121 (75.6)	1.00	
GA+AA	56 (35)	39 (24.4)	1.64 (1.00–2.68)	0.049
		Recessive		
GG+GA	158 (98.8)	157 (98.1)	1.00	0.369
AA	2 (1.2)	3 (1.9)	1.43 (1.04–2.58)	
		Alleles		
G	262 (81.9)	278 (86.9)		
A	58 (18.1)	42 (13.1)		

OR – odds ratio (adjusted for sex and age); CI – confidence interval

Table 2 – The genotypes and allele distribution of CCR5 Δ32 polymorphisms in pulmonary tuberculosis (PTB) patients and control groups

Polymorphism CCR5 Δ32	Patients n (%)	Normal n (%)	OR (95% CI)	P
		Codominant		
WW	154 (96.25)	154 (96.25)	1.00	
WD	6 (3.7)	6 (3.7)	1.05 (0.33–3.40)	0.92
DD	0 (0)	0 (0)	–	
		Alleles		
W	308 (96.25)	308 (96.25)		
D	12 (3.7)	12 (3.7)		

OR – odds ratio (adjusted for sex and age); CI – confidence interval

and virulence of pathogen, the differences in host immune genes polymorphisms has been proposed to play a key role in determining TB susceptibility (Möller and Hoal, 2010). In the present study, we investigated the possible association between CCL5 –403G/A (rs2107538), CCL5 –28C/G (rs2280788) and CCR5 Δ32 gene polymorphisms and susceptibility to PTB in a sample of Iranian population.

To the best of our knowledge this is the first genetic association study regarding the relationship between polymorphism in CCL5 and CCR5 genes and PTB in a sample of Iranian population. Our results showed that GA (in the codominant model) as well as GA+AA (in the dominant model) genotypes of CCL5 rs2107538 were associated with increased risk of PTB. The findings of this study indicated no significant association between CCL5 rs2280788 variant as well as CCR5 Δ32 polymorphism and PTB risk. In contrast to our finding Mhmoud et al. (2013) showed significant differences in allele distribution of CCL5 –28C/G (rs2280788) in TB patients compared with healthy controls. They found that G allele was more frequently in the patient population. However, they didn't detect any association between CCL5 rs2107538 –403G/A polymorphism and TB. The CCL5 –28G probably increased promoter activity and CCL5 expression. In agreements to our study, Mishra et al. (2012) showed that the frequency of AA genotype and A allele in CCL5 –403G/A polymorphism were significantly higher in cases than controls, thus they supposed that this polymorphism may be associated with susceptibility to TB. Also they found that CCL5 –28G/A was not associated with resistance or susceptibility to TB. Chu et al. (2007) showed that the distribution of CCL5 –28C/G and –403G/A polymorphisms was not associated with TB susceptibility in Hong Kong Chinese population. Selvaraj et al. (2011) revealed that allele and genotype frequencies of CCL5 –403G/A and –28C/G polymorphisms were not different between PTB patients and healthy individuals. Whereas, Ben-Selma et al. (2011) findings indicated an association of the CCL5 –28C/G and –403G/A polymorphisms with susceptibility to TB infection in Tunisian populations. They found that –28G/C polymorphism was significantly associated with PTB susceptibility. They reported that –28CC genotype decreased PTB risk while –403A allele increased the risk of PTB development. Sánchez-Castañón et al. (2009) identified that the frequency of the CCL5 –403G/A and –28C/G promoter polymorphisms were significantly different between PTB patients and control subjects. They observed that –403G/G and –28C/C genotypes as well as –403G allele and –28C allele were significantly more frequent in control subjects. Carpenter et al. (2014) didn't find any association between CCR5 polymorphism and susceptibility to clinically active TB. Mishra et al. (2012) have found no significant association between CCR5 polymorphism with either resistance or susceptibility to TB in Sahariya tribe of north central India population. Mamtani et al. (2011) have found an association between CCR5 gene polymorphisms and risk of TB.

During infection by *Mycobacterium tuberculosis* some chemokines including MCP-1 and CCL5 recruiting T-cells and macrophages to sites of TB infection and have important role in the formation of granuloma which enclose and control distribution of TB (Selvaraj et al., 2011). CCL5 concentration increase in PTB patients and decreased during period of recovery which indicate that play a key role in the immune response against TB infection. CCL5 function at a

precise concentration and production of CCL5 over or under this concentration accompanied with dysfunction of this chemokine (Chu et al., 2007). It is plausible that –403G/A and –28C/G polymorphisms affect the expression of CCL5 and might be associated with development of TB. Also polymorphisms in CCR5 cause alteration in binding of this receptor to its ligands such as CCL5 (Mamtani et al., 2011).

In summary, our study showed that CCL5 –403G/A (rs2107538) polymorphism might be a candidate gene, which determines the susceptibility to PTB in our population. Whereas CCL5 –28C/G (rs2280788) and CCR5 Δ32 polymorphisms may not be major genetic factors for susceptibility to PTB. Further investigations with different ethnicities and larger sample sizes are needed to validate our findings.

Acknowledgements: The authors thank to the patients and healthy subjects who willingly participated in the study.

References

Alqumber, M. A., Mandal, R. K., Haque, S., Panda, A. K., Akhter, N., Ali, A. (2013) A genetic association study of CCL5 –28 C>G (rs2280788) polymorphism with risk of tuberculosis: a meta-analysis. *PLoS One* **8(12)**, e83422.

Bahari, G., Hashemi, M., Taheri, M., Naderi, M., Eskandari-Nasab, E., Atabaki, M. (2012) Association of IRGM polymorphisms and susceptibility to pulmonary tuberculosis in Zahedan, Southeast Iran. *ScientificWorldJournal* **2012**, 950801.

Ben-Selma, W., Harizi, H., Bougmiza, I., Ben Kahla, I., Letaief, M., Boukadida, J. (2011) Polymorphisms in the RANTES gene increase susceptibility to active tuberculosis in Tunisia. *DNA Cell Biol.* **30(10)**, 789–800.

Carpenter, D., Taype, C., Goulding, J., Levin, M., Eley, B., Anderson, S., Shaw, M. A., Armour, J. A. (2014) CCL3L1 copy number, CCR5 genotype and susceptibility to tuberculosis. *BMC Med. Genet.* **15**, 5.

Chu, S. F., Tam, C. M., Wong, H. S., Kam, K. M., Lau, Y. L., Chiang, A. K. (2007) Association between RANTES functional polymorphisms and tuberculosis in Hong Kong Chinese. *Genes Immun.* **8(6)**, 475–479.

Cobat, A., Orlova, M., Barrera, L. F., Schurr, E. (2013) Host genomics and control of tuberculosis infection. *Public Health Genomics* **16(1–2)**, 44–49.

Eskandari-Nasab, E., Hashemi, M., Ebrahimi, M., Amininia, S., Bahari, G., Mashhadi, M. A., Taheri, M. (2014) Evaluation of CCL5 –403 G>A and CCR5 Δ32 gene polymorphisms in patients with breast cancer. *Cancer Biomark.* **14(5)**, 343–351.

Han, S. W., Sa, K. H., Kim, S. I., Lee, S. I., Park, Y. W., Lee, S. S., Yoo, W. H., Soe, J. S., Nam, E. J., Lee, J., Park, J. Y., Kang, Y. M. (2012) CCR5 gene polymorphism is a genetic risk factor for radiographic severity of rheumatoid arthritis. *Tissue Antigens* **80(5)**, 416–423.

Hashemi, M., Eskandari-Nasab, E., Moazeni-Roodi, A., Naderi, M., Sharifi-Mood, B., Taheri, M. (2013a) Association of CTSZ rs34069356 and MC3R rs6127698 gene polymorphisms with pulmonary tuberculosis. *Int. J. Tuberc. Lung Dis.* **17(9)**, 1224–1228.

Hashemi, M., Hanafi Bojd, H., Eskandari-Nasab, E., Bahari, A., Hashemzehi, N. A., Shafieipour, S., Narouie, B., Taheri, M., Ghavami, S. (2013b) Association of adiponectin rs1501299 and rs266729 gene polymorphisms with nonalcoholic fatty liver disease. *Hepat. Mon.* **13(5)**, e9527.

Hashemi, M., Sharifi-Mood, B., Rasouli, A., Amininia, S., Naderi, M., Taheri, M. (2015) Macrophage migration

inhibitory factor –173 G/C polymorphism is associated with an increased risk of pulmonary tuberculosis in Zahedan, Southeast Iran. *EXCLI J*. **14**, 117–122.

Kouhpayeh, H. R., Hashemi, M., Hashemi, S. A., Moazeni-Roodi, A., Naderi, M., Sharifi-Mood, B., Taheri, M., Mohammadi, M., Ghavami, S. (2012) R620W functional polymorphism of protein tyrosine phosphatase non-receptor type 22 is not associated with pulmonary tuberculosis in Zahedan, southeast Iran. *Genet. Mol. Res*. **11(2)**, 1075–1081.

Mamtani, M., Mummidi, S., Ramsuran, V., Pham, M. H., Maldonado, R., Begum, K., Valera, M. S., Sanchez, R., Castiblanco, J., Kulkarni, H., Ndung'u, T., He, W., Anaya, J. M., Ahuja, S. K. (2011) Influence of variations in CCL3L1 and CCR5 on tuberculosis in a northwestern Colombian population. *J. Infect. Dis*. **203(11)**, 1590–1594.

Méndez-Samperio, P. (2008) Expression and regulation of chemokines in mycobacterial infection. *J. Infect*. **57(5)**, 374–384.

Mhmoud, N., Fahal, A., van de Sande, W. J. (2013) Association of IL-10 and CCL5 single nucleotide polymorphisms with tuberculosis in the Sudanese population. *Trop. Med. Int. Health* **18(9)**, 1119–1127.

Millet, J. P., Moreno, A., Fina, L., del Bano, L., Orcau, A., de Olalla, P. G., Cayla, J. A. (2013) Factors that influence current tuberculosis epidemiology. *Eur. Spine J*. **22**, 539–548 (Suppl. 4).

Mishra, G., Poojary, S. S., Raj, P., Tiwari, P. K. (2012) Genetic polymorphisms of CCL2, CCL5, CCR2 and CCR5 genes in Sahariya tribe of North Central India: an association study with pulmonary tuberculosis. *Infect. Genet. Evol*. **12(5)**, 1120–1127.

Möller, M., Hoal, E. G. (2010) Current findings, challenges and novel approaches in human genetic susceptibility to tuberculosis. *Tuberculosis (Edinb.)* **90(2)**, 71–83.

Naderi, M., Hashemi, M., Pourmontaseri, Z., Eskandari-Nasab, E., Bahari, G., Taheri, M. (2014a) TIRAP rs8177374 gene polymorphism increased the risk of pulmonary tuberculosis in Zahedan, southeast Iran. *Asian Pac. J. Trop. Med*. **7(6)**, 451–455.

Naderi, M., Hashemi, M., Taheri, M., Pesarakli, H., Eskandari-Nasab, E., Bahari, G. (2014b) CD209 promoter –336 A/G (rs4804803) polymorphism is associated with susceptibility to pulmonary tuberculosis in Zahedan, southeast Iran. *J. Microbiol. Immunol. Infect*. **47(3)**, 171–175.

Sánchez-Castañón, M., Baquero, I. C., Sánchez-Velasco, P., Fariñas, M. C., Ausín, F., Leyva-Cobián, F., Ocejo-Vinyals, J. G. (2009) Polymorphisms in CCL5 promoter are associated with pulmonary tuberculosis in northern Spain. *Int. J. Tuberc. Lung Dis*. **13(4)**, 480–485.

Selvaraj, P., Alagarasu, K., Singh, B., Afsal, K. (2011) CCL5 (RANTES) gene polymorphisms in pulmonary tuberculosis patients of south India. *Int. J. Immunogenet*. **38(5)**, 397–402.

 Prague Medical Report / Vol. 117 (2016) No. 2–3, p. 98–107

The Response of C_{19} Δ^5-steroids to ACTH Stimulation and Hypoglycemia in Insulin Tolerance Test for Adrenal Insufficiency

Michaela Dušková[1], Lucie Kolátorová Sosvorová[1], Martin Hill[1], Kateřina Šimůnková[1,2], Hana Jandíková[1], Hana Pospíšilová[1], Monika Šrámková[1], Mikuláš Kosák[2], Michal Kršek[2], Václav Hána[2], Luboslav Stárka[1]

[1]Institute of Endocrinology, Prague, Czech Republic;
[2]3rd Department of Medicine – Department of Endocrinology and Metabolism, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic

Received April 7, 2016; Accepted September 2, 2016.

Key words: Dehydroepiandrosterone – ACTH test – Insulin tolerance test – Adrenal insufficiency – 7-hydroxy-DHEA – 16-hydroxy-DHEA

Abstract: Studies on the time course of ACTH- or insulin-induced hypoglycemia stimulating adrenal androgens are usually limited to dehydroepiandrosterone and/or its sulphate. Our data on dehydroepiandrosterone (DHEA) and its hydroxylated metabolites clearly show that measurements of DHEA and its sulphate (DHEAS) are valuable markers of the integrity of the HPA (hypothalamus-pituitary-adrenal) axis. Assessments of HPA function should rely on measurements of baseline and/or stimulated serum cortisol concentrations, and C_{19} Δ^5-steroids may provide additional information. The art of stimulation of 7- and 16-hydroxylated metabolites of DHEA can help our understanding of the formation sequence of these compounds.

This study was supported by grant no. NT 11277-6 IGA of the Ministry of Health of the Czech Republic.

Mailing Address: Prof. RNDr. Luboslav Stárka, MD., DSc., Institute of Endocrinology, Národní 8, 116 94 Prague 1, Czech Republic; e-mail: lstarka@endo.cz

http://dx.doi.org/10.14712/23362936.2016.10
© Charles University – Karolinum Press, 2016

Introduction

The adrenal gland is a key provider of androgens in women (Short, 1960). The adrenal androgens dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), androstenedione, 5-andostenediol and 11β-hydroxy-androstenedione exert only little androgenic activity; however, they provide a pool of circulating precursors for more active androgens or steroids, with a wide spectrum of important physiological effects. The role of DHEA and DHEAS as neurosteroids has been intensively studied (Baulieu and Robel, 1998; Morfin and Stárka, 2001; Labrie, 2010; Li and Bigelow, 2010; Hill et al., 2015; Stárka et al., 2015). Its 7-hydroxylated metabolites modulate our immunity (Pélissier et al., 2004, 2006; Stickney et al., 2011) and take part in the control of local cortisol levels through competition in the 11β-hydroxysteroid dehydrogenase system (Hennebert et al., 2007; Sedláčková et al., 2012). The 16-hydroxylated derivatives may play some role in hormonal homeostasis (Hampl and Stárka, 2000). In spite of the importance of C_{19} Δ^5-steroids, their responsiveness to corticotrophin or insulin-induced hypoglycemia has not been well studied (Rege et al., 2013). In the present study, we quantified ten C_{19} Δ^5-steroids plasma samples from women before and after ACTH stimulation, and compared the results with stimulation of the hypothalamus-pituitary-adrenal (HPA) system by insulin-induced hypoglycemia. Dynamic testing of adrenal function is a standard procedure for the diagnosis of adrenal insufficiency, and we tried to find differences in the ACTH stimulation test in terms of C_{19} Δ^5-steroids between healthy women and patients with adrenal insufficiency.

Material and Methods

Our study involved six premenopausal females (with mean/median 43.4/42 age (SD ±6.2) years, and mean/median 25.4/24.6 BMI (body mass index) (SD ±3.9) kg/m^2) with primary adrenal insufficiency verified by clinical symptoms and biochemical markers. The control group consisted of seven healthy BMI and age matched women. The women used no medications and had no history of using corticosteroids. All signed informed consent before initiating the study. The study was approved by the Ethical Commission of the Institute of Endocrinology.

All patients underwent the 250 μg "high dose" ACTH test. The control group was given two tests: the 250 μg "high dose" ACTH test and the insulin tolerance test (ITT). The minimum time between tests was one week. All tests were performed after an overnight fast, and started in the morning between 7 and 9 a.m. Synacthen and insulin were administered through a cannula inserted into the cubital vein, 15 minutes after insertion of the cannula.

Dynamic testing

Details on the dynamic testing have been presented elsewhere (Šimůnková et al., 2015; Dušková et al., 2016). The tests are described in brief as follows:

High dose ACTH stimulation (HDST): The contents of 1 ampule 250 μg/1 ml Synacthen (tetracosactide 250 μg, Novartis Pharma GmbH, Nuernberg, Germany) was given intravenously after first blood sample drawn (time = 0), and then at 30, 60, and 90 minutes.

Insulin-induced hypoglycemia stimulation – ITT: 0.1 IU per 1 kg Actrapid insulin was given intravenously. During the test, blood glucose was regularly checked with a glucometer (Accu-Chek Perform), and blood pressure and pulse rate were measured every five minutes during the first hour and every ten minutes thereafter. There was a decrease in blood glucose below 2.2 mmol/l in all of the tests, and all controls had a spontaneous blood glucose response during the first hour followed by normalization. Blood samples were taken prior to the administration of insulin at time = 0, and then after 20, 30, 40, 60, 90, and 120 minutes. The ITT was only carried out in the control group since it is unpleasant and may introduce risks in patients with adrenal insufficiency.

Analytical measurements

Steroid hormones measured by the GC/MS method

The levels of C_{19} Δ^5-steroids and of additional 27 unconjugated steroids and their polar conjugates were measured in cubital vein blood using our original GC/MS method (Hill et al., 2010). In brief, free steroids were extracted from plasma by diethyl-ether; steroid conjugates were then hydrolyzed and extracted. The resulting residues were derivatized by methoxyamine hydrochloride and analysed by GC/MS as described below.

Steroids were purchased from Steraloids (Newport, RI, USA), Sylon B from Supelco (Bellefonte, PA, USA), methoxylamine hydrochloride from Sigma (St. Louis, MO, USA) and solvents from Merck (Darmstadt, Germany).

Instruments

Measurements of steroid levels were performed on a GCMS-QP2010 Plus system by Shimadzu (Kyoto, Japan) consisting of a gas chromatograph equipped with automatic flow control, an AOC-20s autosampler, and a single quadrupole detector with an adjustable electron voltage of 10–195 V. A capillary column with a medium polarity RESTEK Rxi phase (diameter 0.25 mm, length 15 m, film thickness 0.1 μm) was used for analyses. Electron impact ionization with electron voltage fixed at 70 V and emission current set to 160 μA was used. The temperatures of the injection port, ion source and interface were maintained at 220 °C, 300 °C, and 310 °C, respectively. Analyses were carried out in the splitless mode with a constant linear velocity of the carrier gas (He), which was maintained at 60 cm/s. The septum purge flow was set at 3 ml/min. The samples were injected using the high pressure mode (200 kPa), which was maintained for 1 min. The detector voltage was set to 1.4 kV.

Statistical analysis

Data were transformed by Box-Cox transformation before further processing due to non-Gaussian distribution and non-constant variance (heteroscedasticity) in all variables. Repeated-measures analysis of variance (ANOVA) was used for monitoring levels of steroids during the tests. The ANOVA model was followed by least significant difference (LSD) multiple comparisons. The statistical software Statgraphics Centurion, version XVI from Statpoint Inc. (Warrenton, VA, USA) was used for data transformations and ANOVA analyses.

Results

Basal plasma levels of C_{19} Δ^5-steroids and their levels at 60 min after ACTH stimulation are given in Table 1, and for insulin-induced hypoglycemia in Table 2. The trends of the steroid levels after the stimulation of adrenal secretion by ACTH and by insulin-induced hypoglycemia are shown in Figures 1–5.

Table 1 – Baselines and values at 60th min of ACTH test for patients and controls

Steroid	ACTH test					
	Controls		change fold	Patients		change fold
	0 min	60th min		0 min	60th min	
dehydroepiandro-sterone	5.46 (4.22, 6.97)	19.2 (16.5, 22.4)	3.52*	4.81 (3.58, 6.37)	7.42 (5.91, 9.24)	1.54
conjugated DHEA	986 (893, 1080)	1020 (927, 1120)	1.30	441 (356, 537)	330 (271, 397)	0.75
7α-hydroxy-DHEA	0.53 (0.42, 0.66)	0.94 (0.75, 1.18)	1.78*	0.29 (0.20, 0.42)	0.40 (0.27, 0.57)	1.38
7β-hydroxy-DHEA	0.29 (0.24, 0.35)	0.31 (0.26, 0.38)	1.10	0.21 (0.15, 0.30)	0.18 (0.12, 0.25)	0.64
16α-hydroxy-DHEA	0.02 (0.02, 0.04)	0.03 (0.02, 0.04)	1.21	0.07 (0.04, 0.12)	0.04 (0.03, 0.07)	0.65
conjugated 16α-hydroxy-DHEA	2.95 (2.15, 3.88)	3.01 (2.2, 3.9)	1.10	0.57 (0.07, 1.35)	1.71 (0.95, 2.69)	3.70
5-androsten-3β, 17β-diol	0.62 (0.46, 0.89)	1.00 (0.77, 1.33)	1.60*	0.34 (0.24, 0.51)	0.25 (0.19, 0.32)	0.72
conjugated androstenediol	235 (214, 258)	219 (198, 241)	0.93	101 (81, 124)	69.70 (56.1, 85)	0.69
5-androstene-3β, 7α,17β-triol	0.11 (0.087, 0.133)	0.17 (0.13, 0.21)	1.55*	0.04 (0.03, 0.06)	0.05 (0.03, 0.06)	1.11
5-androstene-3β, 7β,17β-triol	0.08 (0.067, 0.105)	0.12 (0.09, 0.15)	1.40	0.06 (0.04, 0.08)	0.04 (0.031, 0.061)	0.72

Median and quartiles are given; *significance at $p<0.05$

Table 2 – Baselines and values at 60th min of ITT test for controls

Steroid	ITT test		
	Controls		change fold
	0 min	60th min	
dehydroepiandrosterone	6.69 (5.34, 8.41)	15.80 (12.6, 20)	2.36*
conjugated DHEA	1070 (899, 1260)	1090 (921, 1300)	1.20
7α-hydroxy-DHEA	0.43 (0.39, 0.48)	0.63 (0.557, 0.715)	1.46*
7β-hydroxy-DHEA	0.27 (0.226, 0.321)	0.33 (0.28, 0.39)	1.22
16α-hydroxy-DHEA	0.03 (0.013, 0.065)	0.02 (0.013, 0.039)	0.79
conjugated 16α-hydroxy-DHEA	2.12 (1.67, 2.63)	2.10 (1.66, 2.62)	0.99
5-androsten-3β,17β-diol	0.68 (0.57, 0.82)	0.85 (0.747, 0.97)	1.25*
conjugated 5-androstenediol	245 (236, 318)	269 (230, 312)	1.12
5-androsten-3β,7α,17β-triol	0.16 (0.088, 0.380)	0.08 (0.060, 0.117)	0.52
5-androsten-3β,7β,17β-triol	0.06 (0.041, 0.101)	0.07 (0.055, 0.098)	1.16

Median and quartiles are given; *significance at p<0.05

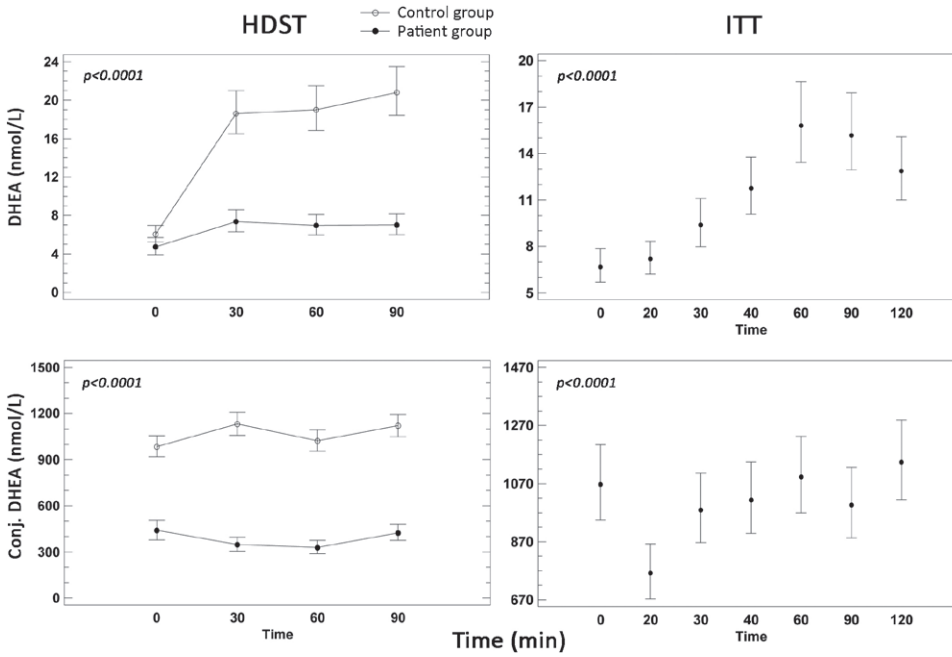

Figure 1 – Plasma dehydroepiandrosterone (DHEA) and conjugated DHEA in high dose ACTH stimulation test (HDST) for adrenal insufficient women and HDST and insulin-induced hypoglycemia stimulation test (ITT) for controls.

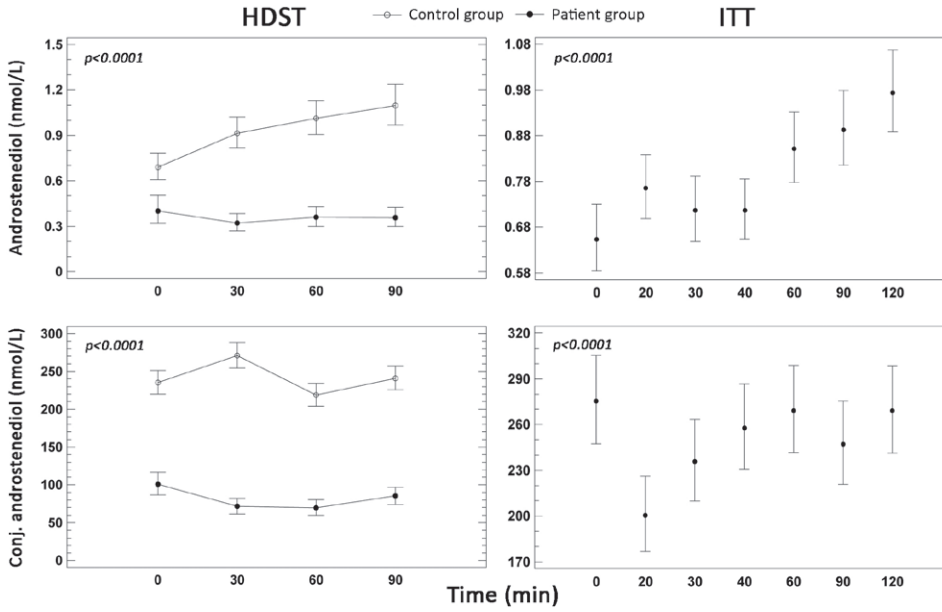

Figure 2 – Plasma 5-androstene-3β,17β-diol (androstenediol) and conjugated androstenediol in high dose ACTH stimulation test (HDST) for adrenal insufficient women and HDST and insulin-induced hypoglycemia stimulation test (ITT) for controls.

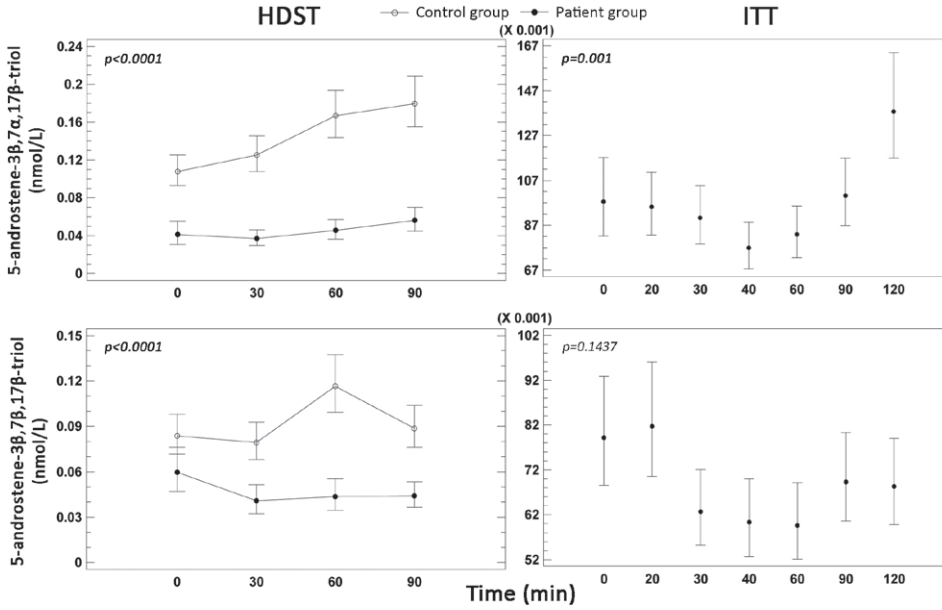

Figure 3 – Plasma 5-androstene-3β,7α,17β-triol and 5-andostene-3β,7β,17β-triol in high dose ACTH stimulation test (HDST) for adrenal insufficient women and HDST and insulin-induced hypoglycemia stimulation test (ITT) for controls.

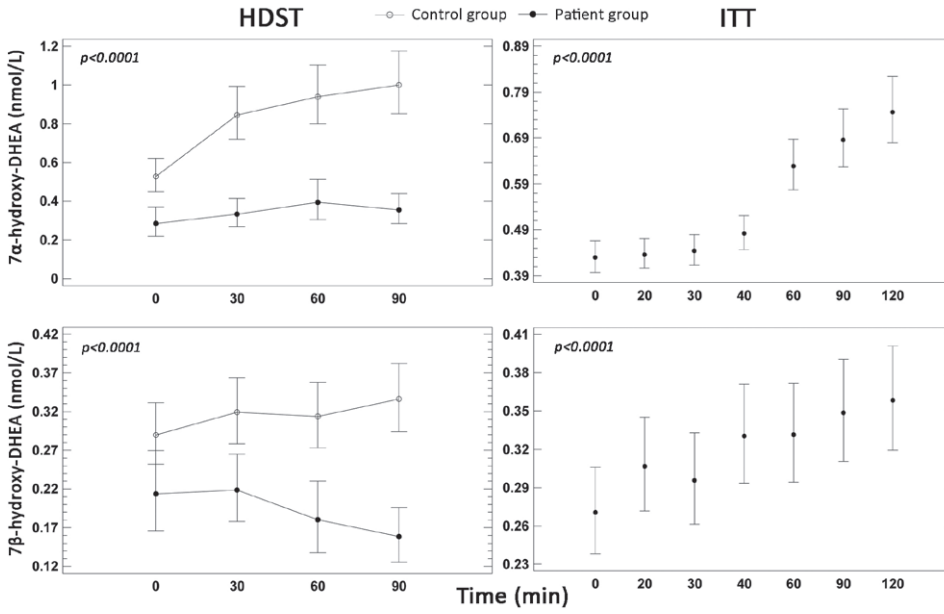

Figure 4 – Plasma 7α-hydroxy- and 7β-hydroxy-dehydroepiandrosterone in high dose ACTH stimulation test (HDST) for adrenal insufficient women and HDST and insulin-induced hypoglycemia stimulation test (ITT) for controls.

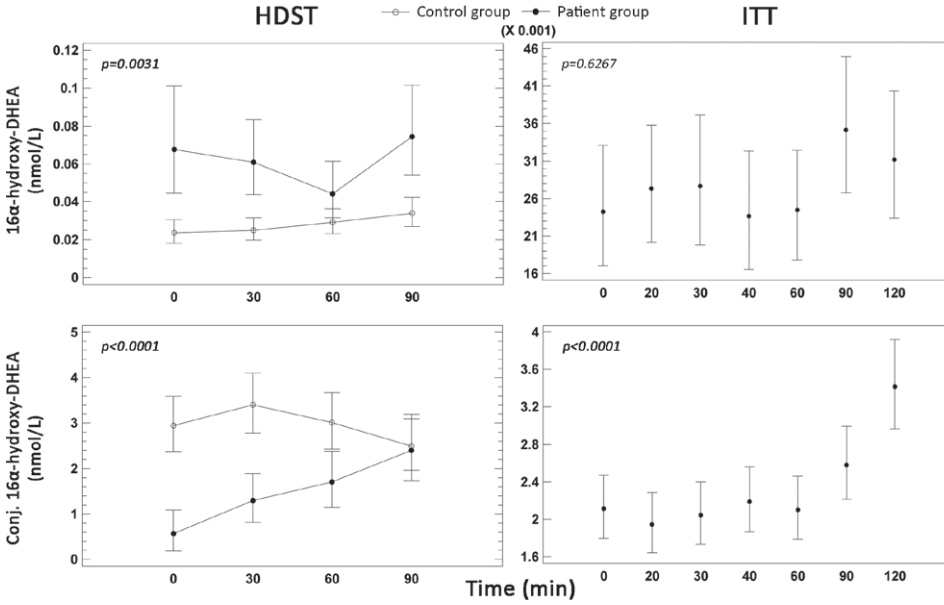

Figure 5 – Plasma 16α-hydroxy-DHEA and conjugated 16α-hydroxy-DHEA in high dose ACTH stimulation test (HDST) for adrenal insufficient women and HDST and insulin-induced hypoglycemia stimulation test (ITT) for controls.

1) For all steroids, both free and conjugated, levels during the ACTH tests in patients did not show significant increases, in contrast to the levels in healthy controls.
2) Except for free DHEA and 7β-hydroxylated derivatives of DHEA, even the starting levels of the steroids significantly differed between the patients with adrenal insufficiency and healthy controls.
3) In contrast to free steroids, changes of the levels of their conjugates during the ACTH test were insignificant.
4) During the ITT, the increase of steroid conjugates was also either insignificant, or in some rare cases, as for conjugated 16α-hydroxy-DHEA, the increase was delayed.
5) All free steroids (with the exception of 16α-hydroxy-DHEA) increased after both stimuli; however, the increase during the ITT had a latent time of about 20–40 min.
6) The differences between the baseline and peak concentration at 60 min of C_{19} Δ^5-steroids in healthy controls was higher during the ACTH test than during the ITT.

Discussion

Dynamic testing of adrenal function is a standard procedure for the diagnosis of proper adrenal function. Testing with various doses of ACTH-24 or with insulin-induced hypoglycemia (insulin tolerance test – ITT) is commonly used, and the concentration of circulating cortisol, either in plasma or in saliva, generally serves as the marker measured. In establishing the diagnosis of adrenal insufficiency, several authors have recommended measurements of baseline serum cortisol and DHEAS levels (Abdu et al., 1999; Al-Aridi et al., 2011). Our results indicated that it may be useful to additionally follow the stimulation of C_{19} Δ^5-steroids during the course of stimulation by either ACTH or ITT. Increases of DHEA or DHEAS after stimulation are observed in plasma but not in saliva (Dušková et al., 2016), due to the insufficient ultrafiltration of these compounds to saliva.

Our present data also show that the levels of C_{19} Δ^5-steroids are reliable markers of adrenal insufficiency. Even the basal levels of conjugated DHEA, free or conjugated 5-androstenediol, free 7α-hydroxy- and 16α-hydroxymetabolites of DHEA differed significantly between healthy subjects and patients with impaired adrenal function. In contrast to healthy individuals, there was no increase of C_{19} Δ^5-steroids during the ACTH test in the group of patients with adrenal insufficiency.

The lack of an increase of conjugated C_{19} Δ^5-steroids both during the ACTH and ITT tests before 60 min is in agreement with other findings of the postponed increase of conjugated DHEA (Sayyed Kassem et al., 2012), and with the observation that there is a stimulation of DHEA but no response in DHEAS production by ACTH in normal adrenocortical cell suspensions (Fehér et al., 1985). This is compatible with the differences in the half-times of DHEA (2–3 h) and

DHEAS (20 h) (Kroboth et al., 1999) and with the concept that the biosynthesis of DHEA is under the control of ACTH, while other factors may contribute to the regulation of the sulphate pathway of DHEA secretion under normal conditions.

The effective and rapid stimulation of adrenal C_{19} Δ^5-steroids by ACTH is an argument against the hypothesis of the presence of a special unknown hypothalamic or pituitary adrenal androgen-stimulating hormone (Parker and Odell, 1979; Odell and Parker, 1984–1985), a hypothesis that until now has neither been confirmed nor refuted.

Whereas changes of DHEA or DHEAS levels in the course of dynamic testing have been reported in many studies (Griffing et al., 1985; Abdu et al., 1999; Al-Aridi et al., 2011; Sayyed Kassem et al., 2012), the response of hydroxylated derivatives of DHEA had not yet been studied. 7α-hydroxymetabolites are rapidly stimulated by both ACTH and ITT in coordination with DHEA levels, while an increase of 7β-hydroxymetabolites is either delayed or absent. This is in agreement the concept that 7α-hydroxylation is the primary reaction and that 7β-epimers are a later product of either direct isomerization or conversion through a 7-oxo-derivative.

A quite exceptional reaction to stimulation was observed for 16α-hydroxy-DHEA. We found that the levels of free 16α-hydroxy-DHEA did not change significantly during either the ACTH or ITT tests; however, conjugated 16α-hydroxy-DHEA increased during the ACTH test in the group of adrenal insufficient women as well as in controls during the ITT.

In conclusion, our data clearly show that DHEA and DHEAS measurements are valuable markers of the integrity of the HPA axis. Assessments of HPA function should rely on the measurement of baseline and/or ACTH-stimulated serum cortisol concentrations, and C_{19} Δ^5-steroids may provide additional information. The art of stimulation of hydroxylated metabolites of DHEA can help improve our understanding of the sequence of the formation of these compounds.

References

Abdu, T. A., Elhadd, T. A., Neary, R., Clayton, R. N. (1999) Comparison of the low dose short synacthen test (1 microg), the conventional dose short synacthen test (250 microg), and the insulin tolerance test for assessment of the hypothalamo-pituitary-adrenal axis in patients with pituitary disease. *J. Clin. Endocrinol. Metab.* **84(3)**, 838–843.

Al-Aridi, R., Abdelmannan, D., Arafah, B. M. (2011) Biochemical diagnosis of adrenal insufficiency: the added value of dehydroepiandrosterone sulfate measurements. *Endocr. Pract.* **17(2)**, 261–270.

Baulieu, E. E., Robel, P. (1998) Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) as neuroactive neurosteroids. *Proc. Natl. Acad. Sci. U. S. A.* **95(8)**, 4089–4091.

Dušková, M., Šimůnková, K., Vítků, J., Sosvorová, L., Jandíková, H., Pospíšilová, H., Šrámková, M., Kosák, M., Kršek, M., Hána, V., Žánová, M., Springer, D., Stárka, L. (2016) A comparison of salivary steroid levels during diagnostic tests for adrenal insufficiency. *Prague Med. Rep*. **117(1)**, 18–33.

Fehér, T., Szalay, K. S., Szilágyi, G. (1985) Effect of ACTH and prolactin on dehydroepiandrosterone, its sulfate ester and cortisol production by normal and tumorous human adrenocortical cells. *J. Steroid Biochem.* **23(2)**, 153–157.

Griffing, G. T., Allen, J., Pratt, H., Melby, J. C. (1985) Discordance of plasma DHEA-S, DHEA, and cortisol responses with various ACTH regimens. *Metabolism* **34(7)**, 631–636.

Hampl, R., Stárka, L. (2000) Minireview: 16alfa-hydroxylated metabolites of dehydroepiandrosterone and their biological significance. *Endocr. Regul.* **34(3)**, 161–163.

Hennebert, O., Chalbot, S., Alran, S., Morfin, R. (2007) Dehydroepiandrosterone 7alpha-hydroxylation in human tissues: possible interference with type 1 11beta-hydroxysteroid dehydrogenase-mediated processes. *J. Steroid Biochem. Mol. Biol.* **104(3–5)**, 326–333.

Hill, M., Pařízek, A., Cibula, D., Kancheva, R., Jirásek, J. E., Jirkovská, M., Velíková, M., Kubátová, J., Klímková, M., Pašková, A., Žižka, Z., Kancheva, L., Kazihnitková, H., Zamrazilová, L., Stárka, L. (2010) Steroid metabolome in fetal and maternal body fluids in human late pregnancy. *J. Steroid Biochem. Mol. Biol.* **122(4)**, 114–132.

Hill, M., Dušková, M., Stárka, L. (2015) Dehydroepiandrosterone, its metabolites and ion channels. *J. Steroid Biochem. Mol. Biol.* **145**, 293–314.

Kroboth, P. D., Salek, F. S., Pittenger, A. L., Fabian, T. J., Frye, R. F. (1999) DHEA and DHEA-S: a review. *J. Clin. Pharmacol.* **39**, 327–348.

Labrie, F. (2010) DHEA, important source of sex steroids in men and even more in women. *Prog. Brain Res.* **182**, 97–148.

Li, A., Bigelow, J. C. (2010) The 7-hydroxylation of dehydroepiandrosterone in rat brain. *Steroids* **75(6)**, 404–410.

Morfin, R., Stárka, L. (2001) Neurosteroid 7-hydroxylation products in the brain. *Int. Rev. Neurobiol.* **46**, 79–95.

Odell, W. D., Parker, L. N. (1984–1985) Control of adrenal androgen production. *Endocr. Res.* **10(3–4)**, 617–630.

Parker, L. N., Odell, W. D. (1979) Evidence for existence of cortical androgen-stimulating hormone. *Am. J. Physiol.* **236(6)**, E616–E620.

Pélissier, M. A., Trap, C., Malewiak, M. I., Morfin, R. (2004) Antioxidant effects of dehydroepiandrosterone and 7alpha-hydroxy-dehydroepiandrosterone in the rat colon, intestine and liver. *Steroids* **69(2)**, 137–144.

Pélissier, M. A., Muller, C., Hill, M., Morfin, R. (2006) Protection against dextran sodium sulfate-induced colitis by dehydroepiandrosterone and 7alpha-hydroxy-dehydroepiandrosterone in the rat. *Steroids* **71(3)**, 240–248.

Rege, J., Nakamura, Y., Satoh, F., Morimoto, R., Kennedy, M. R., Layman, L. C., Honma, S., Sasano, H., Rainey, W. E. (2013) Liquid chromatography-tandem mass spectrometry analysis of human adrenal vein 19-carbon steroids before and after ACTH stimulation. *J. Clin. Endocrinol. Metab.* **98(3)**, 1182–1188.

Sayyed Kassem, L., El Sibai, K., Chaiban, J., Abdelmannan, D., Arafah, B. M. (2012) Measurements of serum DHEA and DHEA sulphate levels improve the accuracy of the low-dose cosyntropin test in the diagnosis of central adrenal insufficiency. *J. Clin. Endocrinol. Metab.* **97(10)**, 3655–3662.

Sedláčková, B., Dušátková, L., Zamrazilová, H., Matucha, P., Bičíková, M., Stárka, L. (2012) 7-oxygenated derivatives of dehydroepiandrosterone and obesity. *Prague Med. Rep.* **113(2)**, 147–155.

Short, R. V. (1960) The secretion of sex hormones by the adrenal gland. *Biochem. Soc. Symp.* **18**, 59–84.

Šimůnková, K., Dušková, M., Kosák, M., Kršek, M., Hána, V., Hill, M., Jandíková, H., Pospíšilová, H., Šrámková, M., Bifulco, E., Stárka, L. (2015) Response of cortisol metabolites in the insulin tolerance test and Synacthen tests. *Physiol. Res.* **64**, S237–S246 (Suppl. 2).

Stárka, L., Dušková, M., Hill, M. (2015) Dehydroepiandrosterone as a neurosteroid. *J. Steroid Biochem. Mol. Biol.* **145**, 254–260.

Stickney, D. R., Ahlem, C. N., Morgan, E., Reading, C. L., Onizuka, N., Frincke, J. M. (2011) Phase I and Phase II clinical trials of androst-5-ene-3β,7β,17β-triol. *Am. J. Transl. Res.* **3(3)**, 275–283.

 Prague Medical Report / Vol. 117 (2016) No. 2–3, p. 108–116

The Effect of Infliximab on Intestinal Anastomosis Healing in Rats

Oktay Karaköse[1], Hüseyin Eken[1], Ali Naki Ulusoy[1], Hüseyin Koray Topgül[1], Mehmet Bilgin[1], Saim Savaş Yürüker[1], Mustafa Yavuz Gülbahar[2]
[1]Department of General Surgery, Faculty of Medicine, Ondokuz Mayis University, Samsun, Turkey;
[2]Department of Pathology, Faculty of Veterinary Medicine, Ondokuz Mayis University, Samsun, Turkey

Received April 5, 2016; Accepted September 2, 2016.

Key words: Infliximab – Anastomosis – Tumor necrosis factor-α – Wound healing

Abstract: Intestinal anastomosis healing is a complex physiological process in which many local and systemic factors play a role. One of the significant cytokines in this process is TNF-α. Infliximab is a chimeric monoclonal antibody which binds to TNF-α with high affinity. Although this agent is used in ulcerative colitis and Crohn's disease, intestinal surgery may be required in these patients. In this study it was aimed to determine whether or not there was any negative effect of preoperative single dose infliximab treatment on intestinal anastomosis healing. Two groups of 10 rats were formed. One of these groups was administered with a single dose of infliximab 8 mg/kg as a 20-minute intravenous infusion from the femoral vein. Four days after the infusion, a full layer incision was made to the colon and anastomosis was applied to all the rats. At 7 days after anastomosis, the subjects were sacrificed. The anastomosis segment was removed and the bursting pressure was measured. Tissue samples were taken from this segment for hydroxyproline concentration and histopathological examination. A blood sample was taken to measure TNF-α values. No statistically significant difference was determined between the groups in terms of bursting pressure, tissue hydroxyproline concentration or histopathological scoring. A single dose of 8 mg/kg infliximab administered 4 days preoperatively was not found to have any negative effect on intestinal anastomosis healing in rats.

Mailing Address: Hüseyin Eken, MD., Department of General Surgery, Faculty of Medicine, Ondokuz Mayis University, Samsun, Turkey;
e-mail: huseyineken80@hotmail.com

http://dx.doi.org/10.14712/23362936.2016.11
© Charles University – Karolinum Press, 2016

Introduction

Many local and systemic factors play a role in intestinal anastomosis healing. Important factors in the extracellular matrix are immune cells which regulate collagen fibres, fibroblasts and wound resistance in the early postoperative healing process. In the normal inflammatory response, monocytes become macrophages by migrating into the wound. The first mediator expressed by macrophages is TNF-α. TNF-α is a cytokine of polypeptide structure expressed by activated macrophages and monocytes stimulated by endotoxins, immune complexes, toxins and other inflammation mediators (Mathews, 1981; Comert et al., 2000; Isik et al., 2015a). By increasing collagenase production, collagen synthesis is inhibited. Fibroblast proliferation, IL-6 and PG E2 production increases (Dayer et al., 1985; Regan et al., 1993). TNF-α is one of the important chemical inflammatory mediators in wound healing (Bettinger et al., 1994).

Infliximab is a chimeric monoclonal antibody in the IgG_1 subtype which binds with high affinity to both soluble and transmembranous forms of TNF-α (Suzuki et al., 2001). *In vivo*, infliximab forms stable complexes with human TNF-α and this process is in parallel with bioactivity loss in TNF-α (Suzuki et al., 2001; Nanda et al., 2013). Infliximab is used in the treatment of patients with inflammatory intestinal diseases such as Crohn's disease and ulcerative colitis. However, as it is insufficient in the treatment of the majority of these patients, surgery is still required. While some studies have reported an increase in the risk of anastomosis leakage and other postoperative complications in patients treated with preoperative infliximab (Selvasekar et al., 2007; Mor et al., 2008), others have found no difference (Colombel et al., 2004; Marchal et al., 2004; Kasparek et al., 2012). The differences between the results of these studies may be due to differences in study groups and study plans.

In this study, it was aimed to determine whether or not there was any harmful effect of preoperative single dose infliximab treatment on intestinal anastomosis healing in rats.

Material and Methods

Approval for the study was granted by the Animal Ethics Committee of Ondokuz Mayis University Faculty of Medicine. The study was conducted at the same university in the Surgical Sciences Research Laboratory. A total of 20 adult male Wistar albino rats were used, each weighing mean 224 g (range 191–310 g). Throughout the study, the rats were housed under standard conditions (room temperature of 20–24 °C with 50–60% humidity) and were fed standard food and water. All the rats were fasted for 12 hours before the surgical intervention. Ketamine-HCl at a dose of 30–50 mg/kg administered intraperitoneally was used as anaesthesia and the surgery was performed under sterile conditions.

The rats were separated into 2 groups of 10, with only 1 group given a single dose of infliximab of 8 mg/kg administered from the femoral vein as a 20-minute

intravenous infusion (Remicade 100 mg, Shering-Plough (Brinny) Co. Innishannon, County Cork, Republic of Ireland). Prior to the infliximab administration, approximately 1 cc blood was taken from the tail vein of all the rats for TNF-α value assay. In all the rats a full layer incision was made to the colon and anastomosis was applied. This procedure was applied 4 days after the administration of infliximab. At 7 days after anastomosis, the subjects were sacrificed. The anastomosis segment was removed and the bursting pressure was measured. Tissue samples were taken from this segment for hydroxyproline concentration and histopathological examination. A blood sample was taken to measure TNF-α values.

The groups were formed as:

Group 1: a full layer incision was made to the descending colon and anastomosis was applied (n=10).

Group 2: 4 days after the administration of 8 mg/kg infliximab as a 20-minute intravenous infusion, a full layer incision was made to the descending colon and anastomosis was applied (n=10).

At 7 days after anastomosis, all the rats were sacrificed.

Descending colon anastomosis

Following ketamine-HCl anaesthesia, the abdomen was entered with a midline incision. With sharp dissection in the segment 3 cm proximal of the peritoneal reflection, a full layer incision was made. Anastomosis was made one by one using 5/0 prolene suture material. Four days prior to the surgical intervention, the rats in Group 2 were administered 8 mg/kg infliximab from the femoral vein as a 20-minute intravenous infusion. The parameters related to blood biochemistry and anastomosis healing were examined 7 days after anastomosis in both groups.

Bursting pressure measurement

A colon segment 4 cm in length, was resected leaving the anastomosis line in the centre. In the bursting pressure measurement, a volumetric infusion pump, a mercury manometer and saline stained with methylene blue were used. One end of the intestine was connected to the plastic tube coming from the mercury manometer 1.5 cm from the proximal of the anastomosis and the other end to the plastic tube coming from the volumetric infusion pump. The colon segment attached between the plastic tubes was filled with saline. The intestinal lumen was filled with blue-stained saline at 2.5 ml/min with the volumetric infusion pump. The rise of the mercury manometer was monitored. The pressure at the moment of bursting was accepted with the methylene blue staining of each sample.

After measuring the bursting pressure, the colon segments were washed with saline and the lumen were opened longitudinally. For standardisation of the measurement of hydroxyproline, samples were taken as 2.2 ± 0.5 mg and frozen at –70 °C. Samples were also taken from the anastomosis line for histopathological examination.

Hydroxyproline concentration measurement
The hydroxyproline content (as an indicator of the amount of collagen) was measured in the intestine sample which included the anastomosis line and had been stored at –70 °C using the method described by Jamall et al. (1981). The results were stated as μg hydroxyproline/mg tissue.

Histopathological examination
The segment containing the whole anastomosis line was fixed in 10% formaldehyde then embedded in paraffin. Slices 5 microns in thickness were taken and stained with haematoxylin-eosin and Masson's Trichrome to determine early collagen content and the wound healing score was determined according to the Greenhalgh method (Greenhalgh et al., 1990). In each anastomosis section, the inflammatory cells, neutrophil infiltration, neovascularisation, fibroblastic activity and amount of collagen band were determined. Wound healing was evaluated by a pathologist according to the presence of these cells and scoring was made of values from 1 to 5. When there were no inflammatory cells, fibroblastic activity was evident, new collagen had formed and neovascularisation was complete, healing was accepted as very good and the reverse situation was evaluated as very poor. For statistical comparison wound healing scores were accepted as: 1 = very poor, 2 = poor, 3 = fair, 4 = good, 5 = very good.

Statistical analysis
The data obtained from measurements of hydroxyproline, TNF-α and bursting pressure were evaluated for conformity to normal distribution with the Kolmogorov-Smirnov and Shapiro-Wilk tests. As all data showed normal distribution, the Variance Analysis Post-Hoc Tukey HSD test was used in the comparisons between the groups. The anastomosis healing scores were evaluated with the Kruskal-Wallis test as they were not continuous data and did not conform to normal distribution. Data were stated as mean ± standard deviation (SD) for continuous variables and as median (minimum–maximum) for non-continuous variables. A p-value of <0.0125 was accepted as statistically significant.

Results

TNF-α was not determined in the blood samples taken at the beginning of the study.

No statistically significant difference was determined between the groups in the bursting pressure and hydroxyproline values. A statistically significant difference was determined between the groups in the mean TNF-α concentration on the 7th day of anastomosis healing ($p<0.001$) (Table 1).

In the histopathological examination of the slices stained with haematoxylin-eosin, in Group 1 (not administered infliximab), there was an appearance of widespread mononuclear cell infiltration, evident fibroblastic activity, increased

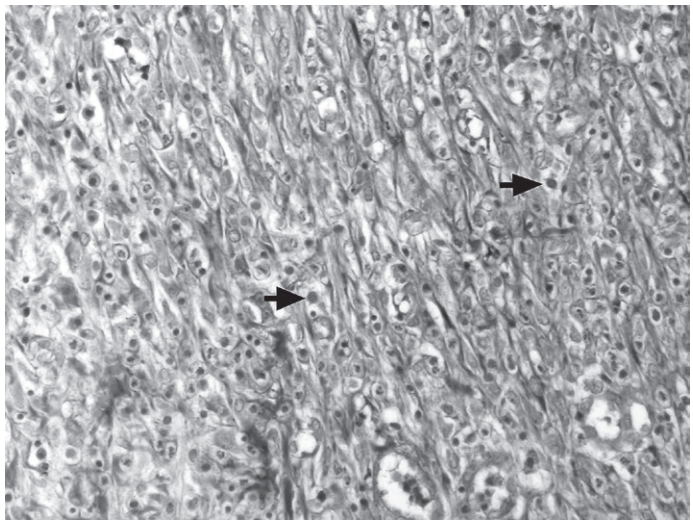

Figure 1 – Histopathology image of Group 1 (not administered infliximab). Widespread mononuclear cell infiltration, evident fibroblastic activity and significant new collagen synthesis. The arrows indicate mononuclear cells.

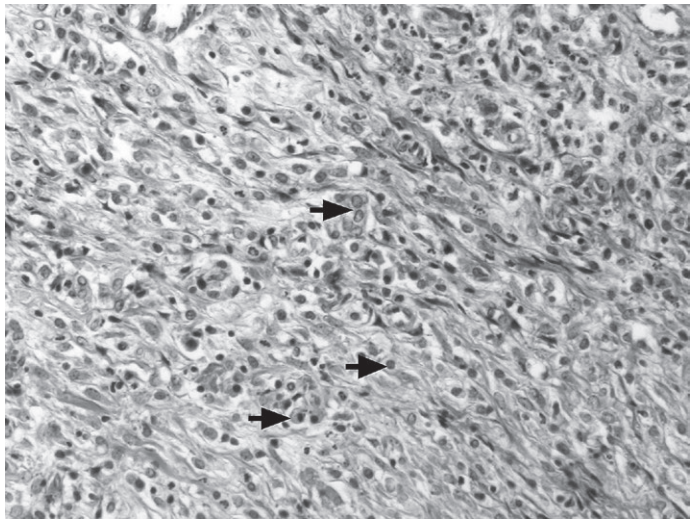

Figure 2 – Histopathology image of Group 2 (administered infliximab). Widespread mononuclear cell infiltration, new vascular formation, evident fibroblastic activity and collagen synthesis with finer fibres compared to Group 1.

Table 1 – The mean values of the parameters examined and the p-values obtained in the comparisons between the groups

	Bursting pressure (cm Hg)	Tissue hydroxyproline concentration (μg/mg tissue)	TNF-α concentration (pg/ml)	Histopathological scoring (min–max)
Group 1	10.50 ± 1.58	0.53 ± 0.06	11.98 ± 1.31	3 (3–4)
Group 2	12.20 ± 2.44	0.60 ± 0.09	5.90 ± 2.64	3 (2–4)
P-value	0.081	0.032	<0.001*	0.418

*p<0.0125

neovascularisation and evident new collagen synthesis (Figure 1). In Group 2 (administered with infliximab), mononuclear cell infiltration was more widespread than in Group 1, there was new vascular formation, evident fibroblast activity and collagen synthesis was determined with an appearance of thinner fibres compared to Group 1 (Figure 2). However, there was no statistically significant difference in the evaluation of the scoring between the groups (Table 1).

Discussion

TNF-α has a regulating function in the inflammatory phase of wound healing (Bettinger et al., 1994). The migration of neutrophils and other phagocyte cells to the wound area increases the phagocytosis capacity of neutrophils and superoxide anion structure (Mathews, 1981; Comert et al., 2000; Isik et al., 2015a). By increasing the expression of collagenase enzyme, the total collagen amount is reduced. In addition, the production of interleukine-1 and prostaglandin-E are increased in fibroblasts and vascular endothelium (Dayer et al., 1985; Regan et al., 1993). In chronic wound fluids, it has been shown that the increased TNF-α level reduced with time in the healing process (Streit e al., 2006).

Wound healing is a complex physiological process dependent on multiple intrinsic and extrinsic factors which are events such as inflammation, collagen synthesis and angiogenesis. This process can be impaired with the use of pharmacological agents such as corticosteroids, antineoplastic drugs and anti-inflammatory drugs (Papaconstantinou et al., 2014). While some studies have shown the anti-TNF agent, infliximab, to have a negative effect on wound healing (Selvasekar et al., 2007; Mor et al., 2008), others have reached the conclusion that healing is not affected (Colombel et al., 2004; Marchal et al., 2004; Kasparek et al., 2012). Despite the use of this agent in ulcerative colitis and Crohn's disease, intestinal surgery may be necessary in these patients. In this study it was aimed to determine whether or not there was any negative effect of preoperative single dose infliximab treatment on intestinal anastomosis healing in rats.

In a study conducted on rabbits by Frostberg et al. (2014), a single dose of infliximab administered one week preoperatively was found not to have a negative effect on bursting pressure. Papaconstantinou et al. (2014) determined that perioperative administration of infliximab on rats was not the reason for changes in anastomosis bursting pressure and when the postoperative septic status was examined, no difference was seen in comparison with the group not administered infliximab. Inflammation was seen to be lower in the infliximab group and in the protein expression analysis of the anastomosis region, TGFb1 and collagen V which play a significant role in colon anastomosis were found to be higher in the infliximab group. The authors concluded that preoperatively administered infliximab did not have a negative effect on intestinal anastomosis. In a rabbit study by Jensen et al. (2015), repeated high doses of infliximab were found to significantly reduce the tensile strength of anastomosis.

In the current study, no difference was found between the groups in respect of anastomosis bursting pressure, hydroxyproline concentration and anastomosis healing scores when the effects of infliximab treatment were compared on intestinal anastomosis. In the histopathological examination, although there were fewer inflammatory cells and finer collagen fibres in the infliximab group, there was no statistically significant difference. That there was no negative effect on anastomosis healing can be considered to be probably due to the single dose of 8 mg/kg administered 4 days preoperatively. However, for more significant clarification on this subject, further studies are required, primarily at the molecular level because TNF inhibition in the healing process indirectly causes an increase in some molecules. How the effect could occur in total should be examined in detail.

There were some limitations to this study, primarily that while infliximab treatment is given to patients with inflammatory intestinal disease, healthy rat intestine was used in this study. In addition, the number of subjects in the current study was extremely limited and the agent which is used on humans was used on rats. Another limitation was the preoperative dose amount and that it was a single dose. Infliximab is used at the dosage of 5 mg/kg in Crohn's disease, and ranging from 3 to 10 mg/kg in rheumatoid arthritis (Siddiqui and Scott, 2005). In an experimental study by Oruc et al. (2004), infliximab treatment of oedematous and necrotising pancreatitis created in rats was determined to have resulted in a significant histopathological regression in oedematous pancreatitis, suppression of neutrophil infiltration and a lessening of complications. Papaconstantinou et al. (2014) administered 5 doses of 5 mg/kg subcutaneously at 3-day intervals and applied surgery 3 days after the final dose. Frostberg et al. (2014) used a single dose of 10 mg/kg IV one week preoperatively in rabbits. In the current study, the dose of 8 mg/kg IV, which was effective in pancreatitis, was selected.

In the current study, when the mean TNF-α concentration of the 7th day of anastomosis healing was examined, there was seen to be a statistically significant difference between Groups 1 and 2 ($p<0.001$). This result showed the effectiveness of infliximab.

In the definition of the final status of the wound healing, histopathological examination was a subjective method, especially useful in monitoring the infiltration of specific cell types to the wound. To show the effectiveness of wound healing at various clinic conditions more studies must be done (Kesici et al., 2007; Isik et al., 2014, 2015b). In the current study, although finer fibres of new collagen were seen and more widespread mononuclear cell infiltration in Group 2 in the histopathological examination, there was no statistically significant difference compared to Group 1.

Conclusion

In the current study, no negative effect was determined on intestinal anastomosis healing in rats from a single dose of 8 mg/kg infliximab administered 4 days

preoperatively. However, both wound healing and the use of systemic anti-TNF-α is a complex subject in which several mediators have a role. For full clarification, there is a need for further studies, particularly at the molecular level.

References

Bettinger, D. A., Pellicane, J. V., Tarry, W. C., Yager, D. R., Diegelmann, R. F., Lee, R., Cohen, I. K., DeMaria, E. J. (1994) The role of inflammatory cytokines in wound healing: accelerated healing in endotoxin-resistant mice. *J. Trauma* **36**, 810–813.

Colombel, J. F., Loftus, E. V. Jr., Tremaine, W. J., Pemberton, J. H., Wolff, B. G., Young-Fadok, T., Harmsen, W. S., Schleck, C. D., Sandborn, W. J. (2004) Early postoperative complications are not increased in patients with Crohn's disease treated perioperatively with infliximab or immunosuppressive therapy. *Am. J. Gastroenterol.* **99**, 878–883.

Comert, M., Taneri, F., Tekin, E., Ersoy, E., Oktemer, S., Onuk, E., Düzgün, E., Ayoğlu, F. (2000) The effect of pentoxifylline on the healing of intestinal anastomosis in rats with experimental obstructive jaundice. *Surg. Today* **30**, 896–902.

Dayer, J. M., Beutler, B., Cerami, A. (1985) Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and fibroblasts. *J. Exp. Med.* **162**, 2163–2168.

Frostberg, E., Ström, P., Gerke, O., Qvist, N. (2014) Infliximab's influence on anastomotic strength and degree of inflammation in intestinal surgery in a rabbit model. *BMC Surg*. **14**, 23.

Greenhalgh, D. G., Sprugel, K. H., Murray, M. J., Ross, R. (1990) PDGF and FGF stimulate wound healing in the genetically diabetic mouse. *Am. J. Pathol*. **136**, 1235–1242.

Isik, A., Eryılmaz, R., Okan, I., Dasiran, F., Firat, D., Idiz, O., Sahin, M. (2014) The use of fibrin glue without surgery in the treatment of pilonidal sinus disease. *Int. J. Clin. Exp. Med*. **7**, 1047–1051.

Isik, A., Peker, K., Soyturk, M., Firat, D., Yoruker, U., Yilmaz, I. (2015a) Diagnostic evaluation and treatment of patients with rectus abdominis hematoma. *Cir. Esp*. **93**, 580–588.

Isik, A., Peker, K., Gursul, C., Sayar, I., Firat, D., Yilmaz, I., Demiryilmaz, I. (2015b) The effect of ozone and naringin on intestinal ischemia/reperfusion injury in an experimental model. *Int. J. Surg*. **21**, 38–44.

Jamall, I. S., Finelli, V. N., Que Hee, S. S. (1981) A simple method determine nanogram levels of 4-hydroxyproline in biological tissues. *Anal. Biochem*. **112**, 70–75.

Jensen, J. S., Petersen, N. B., Biagini, M., Bollen, P., Qvist, N. (2015) Infliximab treatment reduces tensile strength in intestinal anastomosis. *J. Surg. Res*. **193**, 145–152.

Kasparek, M. S., Bruckmeier, A., Beigel, F., Muller, M. H., Brand, S., Mansmann, U., Jauch, K. W., Ochsenkuhn, T., Kreis, M. E. (2012) Infliximab does not affect postoperative complication rates in Crohn's patients undergoing abdominal surgery. *Inflamm. Bowel Dis*. **18**, 1207–1213.

Kesici, H., Ulusoy, A. N., Topgül, K., Paşaoğlu, H., Bayraktar, N., Şenyürek, G., Karaköse, O. (2007) The effect of taurolidine on healing of colonic anastomosis in rats with obstructive jaundice. *Ulus. Cerrahi Derg*. **23**, 1–9.

Marchal, L., D'Haens, G., Van Assche, G., Vermeire, S., Noman, M., Ferrante, M., Hiele, M., Bueno De Mesquita, M., D'Hoore, A., Penninckx, F., Rutgeerts, P. (2004) The risk of post-operative complications associated with infliximab therapy for Crohn's disease: a controlled cohort study. *Aliment. Pharmacol. Ther*. **19**, 749–754.

Mathews, N. (1981) Production of an anti-tumour cytotoxin by human monocytes. *Immunology* **44**, 135–142.

Mor, I. J., Vogel, J. D., da Luz, M. A., Shen, B., Hammel, J., Remzi, F. H. (2008) Infliximab in ulcerative colitis is associated with an increased risk of postoperative complications after restorative proctocolectomy. *Dis. Colon Rectum* **51**, 1202–1207.

Nanda, K. S., Cheifetz, A. S., Moss, A. C. (2013) Impact of antibodies to infliximab on clinical outcomes and serum infliximab levels in patients with inflammatory bowel disease (IBD): a meta-analysis. *Am. J. Gastroenterol*. **108**, 40–47.

Oruc, N., Ozutemiz, A. O., Yukselen, V., Nart, D., Celik, H. A., Yuce, G., Batur, Y. (2004) Infliximab: A new therapeutic agent in acute pancreatitis? *Pancreas* **28**, e1–e8.

Papaconstantinou, I., Zeglinas, C., Gazouli, M., Nastos, K., Yiallourou, A., Lykoudis, P., Evangelou, K., Papalois, A., Papaioannou, M., Vlachogiannakos, J., Tzathas, C. (2014) Effect of infliximab on the healing of intestinal anastomosis. An experimental study in rats. *Int. J. Surg*. **12**, 969–975.

Regan, M. C., Kirk, S. S., Hurson, M., Sodeyama, M., Wasserkrug, H. L., Barbul, A. (1993) Tumor necrosis factor-alpha inhibits *in vivo* collagen synthesis. *Surgery* **113**, 173–177.

Selvasekar, C. R., Cima, R. R., Larson, D. W., Dozois, E. J., Harrington, J. R., Harmsen, W. S., Loftus, E. V. Jr., Sandborn, W. J., Wolff, B. G., Pemberton, J. H. (2007) Effect of infliximab on short-term complications in patients undergoing operation for chronic ulcerative colitis. *J. Am. Coll. Surg*. **204**, 956–962.

Siddiqui, M. A. A., Scott, L. J. (2005) Infliximab: A review of its use in Crohn's disease and rheumatoid arthritis. *Drugs* **65**, 2179–2208.

Streit, M., Beleznay, Z., Braathen, L. R. (2006) Topical application of the tumour necrosis factor-alpha antibody infliximab improves healing of chronic wounds. *Int. Wound J*. **3**, 171–179.

Suzuki, S., Kurachi, K., Yokoi, Y., Tsuchiya, Y., Okamoto, K., Okumura, T., Konno, H., Baba, S., Nakamura, S. (2001) Intrahepatic cholangiojejunostomy for unresectable malignant biliary tumors with obstructive jaundice. *J. Hepatobiliary Pancreat. Surg*. **8**, 124–129.

Thoracic Outlet Syndrome: A Significant Family Genetic Phenotypic Presentation

David Janák[1,3], Karel Novotný[1], Miloslav Roček[2], Vilém Rohn[1]
[1]Department of Cardiovascular Surgery, Second Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czech Republic;
[2]Department of Radiology, Second Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czech Republic;
[3]Institute of Physiology, First Faculty of Medicine, Charles University, Prague, Czech Republic

Received April 1, 2016; Accepted September 2, 2016.

Key words: Thoracic outlet syndrome – Genes – Mutations

Abstract: We report on a very rare case of diagnosis and successful surgical treatment of three young family members with a four-fold presentation of thoracic outlet syndrome. In the relevant family case, we are considering and discussing the population incidence, a possible HOX genes disorder, and a significant phenotypic presentation.

Mailing Address: David Janák, MD., Department of Cardiovascular Surgery, Second Faculty of Medicine, Charles University and University Hospital Motol, V Úvalu 84, 150 06 Prague 5, Czech Republic; e-mail: janakdavid@seznam.cz

http://dx.doi.org/10.14712/23362936.2016.12
© Charles University – Karolinum Press, 2016

Introduction

Thoracic outlet syndrome (TOS) is a clinical syndrome characterised by a series of symptoms brought about by the compression of the neurovascular bundle by bony or/and muscular obstacles in the area of the superior aperture. In 1927 Adson and Coffey first described the compression of the subclavian artery using the anterior scalene muscle with arterial symptomatology. The term "thoracic outlet" was first introduced by Peet et al. in 1956. Galen and Vesalius were the first to describe the presence of a cervical and abnormal first rib (Borchardt, 1901). In 1861 Coote performed the first cervical rib resection, which was described in the literature. The term TOS is non-specific. The clinical presentation can vary from light symptoms, to even vein thrombosis and upper extremity muscle atrophy. By the character of the compression and a compressed structure, we can specify TOS and divide it into arterial (ATOS), venous (VTOS), and neurogenic (NTOS). The symptoms of ATOS include ischemia of the fingers, limb claudication, pallor, coldness, and paraesthesia. Pain, paraesthesia and weakness in the hand, arm and shoulder, plus neck pain and occipital headaches, are the classical symptoms of NTOS. VTOS involves arm swelling, finger paraesthesia, plus cyanosis. TOS is often misdiagnosed and significantly underdiagnosed in the population. Heredital TOS is not exactly defined in the literature. A significant part of patients is diagnosed between the ages of 20 and 50 (Köknel Talu, 2005). Teenagers are rarely diagnosed with TOS and pediatric patients are very rarely diagnosed (Arthur et al., 2008; Rigberg and Gelabert, 2009). The incidence of TOS in the population is, at least, 1–2%. The incidence of NTOS in the population is, roughly, 94–97%, whereas the incidence of ATOS is, roughly, 1% (Jusufovic et al., 2012). Cervical rib anomalies in the population are extremely rare, and the frequency of detection of this anomaly in the general population constitutes approx. 1–2% (Walden et al., 2013). Approximately 70% of all cases are in women. Most cases of cervical rib are demonstrated as NTOS. The incidence of bone abnormalities resulting in TOS is estimated to be 30%. The most frequent bone anomalies leading to TOS included cervical ribs (70%), clavicular anomalies (20%), and isolated first rib aberrations (10%) (Weber and Criado, 2014). Cervical ribs can develop with a unilateral or bilateral distribution. The frequent occurrence of cervical ribs is described in the Klippel-Trenaunay syndrome (Glass et al., 2002), which is associated with other vascular pathologies. Rib anomalies are the most frequent causes of the compression of cervical plexus. The personal history of a significant part of NTOS patients includes a neck trauma or a car accident. VTOS can be presented in the case of excessive unilateral load of arms. Symptoms of ATOS usually develop spontaneously, and in most cases it is associated with a cervical rib and with isolated first rib aberrations. A golden diagnostic standard is a clinical examination, provocative tests (Adson's, Wright's test), X-ray scanning, ultrasound, and magnetic resonance imaging.

Case report

We admitted two female patients to our Department of Cardiovascular Surgery with incidentally diagnosed TOS for resection of the first rib; a 14-years-old female patient, and her 35-years-old aunt on the mother's side; both patients had manifested TOS of the right upper extremity. The 37-years-old mother of the 14-years-old female patient and, at the same time, the sister of the 35-years-old female patient had transaxillary first rib resection six years ago, due to a manifested VTOS on the right side, at another hospital. The mother had manifested VTOS in the form of Paget-Schroetter syndrome, with repeated thromboses of the subclavian vein; a stent was implanted into the subclavian vein after the first rib resection.

Case 1

The 14-years-old female patient suffers with asthma, atopic dermatitis, S/P adenoidectomy at the age of two, with polyvalent allergy a year ago due to manifested VTOS after the first rib resection on the left side with a good effect; now admitted due to oedema of the right upper extremity, receives a Dabigatran therapy, a ultrasound examination carried out with maximum provocative

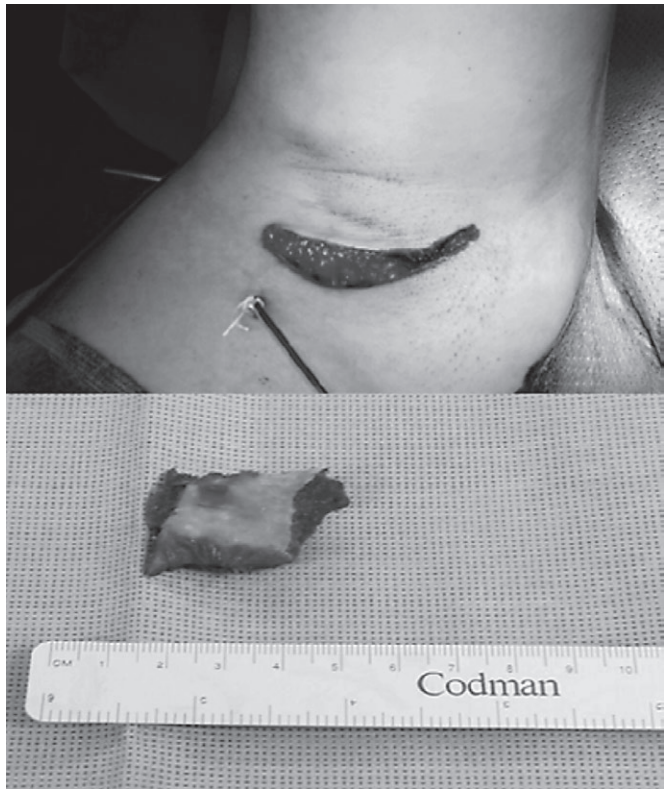

Figure 1 – The 14-years-old female patient – surgical wound – the resected first rib.

manoeuvres (limb elevation while she was breathing in), with alterations of Doppler signal on the subclavian artery, approximately 40% of artery compression, and flow alterations in the subclavian vein with roughly 50% of vein flow. Transaxillary first rib resection was performed on the patient (Figure 1). The procedure was without any complications. Following the operation, small apical pneumothorax was in regression. The patient was discharged from the hospital for home treatment on the sixth post-operative day. After the 21 days the post-operative ultrasound examination revealed no flow alterations in the subclavian artery and vein, the surgical wound was healed; the patient remained without oedema of the right upper extremity and without clinical problems.

Case 2

The 35-years-old female patient suffers with asthma, thyroid gland hypofunction, with polyvalent allergy, now with oedema of the right upper extremity mainly during the elevation of the extremity, and with finger paresthesia. The patient was examined with an X-ray photograph which shows a significant lateral fragment of vertebra C7 the character of which is even an incipient cervical rib on the right; according to the control ultrasound examination with maximum augmentation

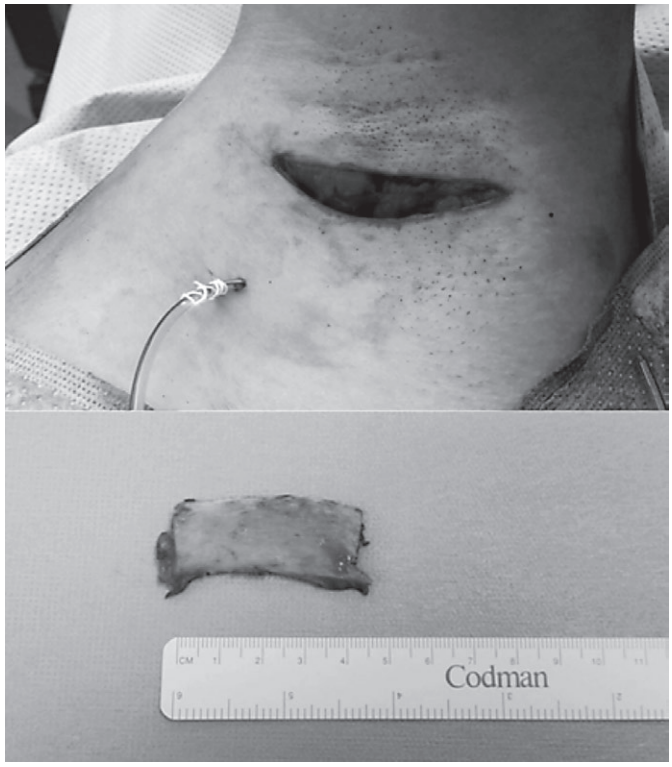

Figure 2 – The 35-years-old female patient – surgical wound – the resected first rib.

manoeuvres (limb elevation while she was breathing in), significant flow alterations in the subclavian vein colliding with the abnormal first rib, without any alteration of flow in the subclavian artery. Transaxillary first rib resection was performed on the patient (Figure 2). The procedure was without any complications. The patient was discharged from hospital for home treatment on the sixth post-operative day. After the 21 days the post-operative ultrasound examination with provocative manoeuvres revealed no flow alterations in the subclavian vein and artery, the surgical wound was healed; the patient's condition were clinically improved, without subjective problems.

Discussion

We presented a very rare case of four-fold TOS syndromes manifested in three members of the same family. We have performed transaxillary first rib resections with optimum clinical results. In addition to standard clinical examinations using elevation and traction tests, also ultrasound and standard imaging methods of anteroposterior X-ray photographs and CT scans were used to diagnose TOS. Based on the studies dealing with this diagnosis, there is very frequent underreporting and an incorrect and poor detection of the presence of bone anomalies including the cervical rib and structural anomalies of the ribs resulting in the clinical symptoms of TOS (Viertel et al., 2012). Transaxillary or supraclavicular decompression can be used for vascular bundles or for the removal of bone anomalies. We selected the universal transaxillary approach in which we are experienced in our Department of Cardiovascular Surgery and which is normally applied. In our department, we have operated on patients with TOS syndrome for more than 30 years. We operate on a full range of patients with different symptomatology. We have not yet encountered such a strong family-related presentation of TOS syndrome. In a detailed clinical examination, no significantly genetically determined syndrome was identified in this family. Female patients were without other obvious abnormalities. No specific abnormality associated with the known and also molecularly genetically detectable mutation of HOX genes, was identified in the patients. No molecular genetic techniques have been developed yet for undefined nonspecific mutations of HOX genes. As a result, at the moment it is impossible to objectify these mutations in detail. The occurrence of this pathology fully falls within the category of polygenic inheritance, but with a very strong polygenically determined phenotypic penetrance. Following available anatomical-embryological studies, the axial skeleton, to which the paraxial mesoderm gives rise, is determined by the so-called HOX genes (Kmita and Duboule, 2003; Mallo et al., 2009; Wellik, 2009). These genes present an evolutionary conserved group. This group of genes is extremely important, among other things, for the differentiation and structuring of the vertebral column, and today 39 HOX genes are described for the development of the vertebral column, which are divided into four different clusters. The four clusters described as HOXA, HOXB, HOXC and HOXD, are

located on four different chromosomes 7p14, 17q21, 12q13 and 2q31, respectively, and contain 9–11 genes (Quinonez and Innis, 2014). Up to now, 10 mutations of HOX genes were described with a clear mechanism of inheritance, expressivity and mechanism of pathogenesis, and phenotypic manifestation (e.g. HOX1A mutation reported as Bosley-Salih-Alorainy syndrome, HOXA13 as Guttmacher syndrome, HOXC13 ectodermal dysplasia 9, HOXD13 syndactyly type 5). Non-specified mutations in HOX genes are described, too. They are associated with anomalies and abnormalities of the vertebral column, the quantity and anomalies of ribs, and include the occurrence of a cervical rib (Kmita and Duboule, 2003). It is described that aberrations in HOX gene expression have a role in tumour suppression and in oncogenesis (Merks et al., 2005; Shah and Sukumar, 2010). The literature emphasizes, that for complete phenotypic penetrance (complete homeotic transformation), an alteration of expression in several homologous or paralogous HOX genes, is required (Deschamps and van Nes, 2005; Bots et al., 2011). Mutagenic agents of HOX genes leading to the occurrence of bone abnormalities represented mainly by cervical ribs and structural abnormalities of costal arch, which can be represented by TOS syndrome, are not yet known, and they require further analysis. However, within the population incidence their acceleration and higher line phenotypic presentation in the relevant family line, is possible. In the case reported by us concerning the family presence of TOS, we can take into account the acceleration of agents leading to alterations of HOX gene expression. However, in the relevant family, potential risk factors are not known. It is advisable to examine the family members of patients with TOS syndrome caused by bone abnormalities in relation to suspected cases of clinical TOS symptomatology, and also to bear in mind the possible association between family history of TOS and incidence. In addition, for in patients with bone abnormalities with clinical TOS presentation, it is necessary to consider possible higher cancer incidence, mainly in patients with higher genetic family burden, and to bear in mind this potential risk. The aim of our report is to demonstrate HOX gene mutations associated with the occurrence of TOS syndrome, which have not been genetically specified yet. The literature available in fact does not describe this correlation. Many variations of mutations in this area can be represented by a wide phenotype diversity. It is necessary to take into consideration the relevant mutations of HOX genes, and in certain cases, also their strong family transmission between individuals; a significant clinical presentation, and the possibility of the prevalence of other cancer types with increasing age.

Conclusion

Future studies concerning the frequency of cervical ribs and structural bone anomalies of the first cervical rib presented with clinical TOS syndrome will have to address the relevant issue as an indicator of potentially higher medical risks for the relevant affected population, and its possible inheritance.

References

Adson, A. W., Coffey, J. (1927) Cervical rib: A method of anterior approach for relief of symptoms by division of the scalenus anticus. *Ann. Surg*. **85**, 839–857.

Arthur, L. G., Teich, A., Hogan, A., Caniano, A. D., Smead, W. (2008) Pediatric thoracic outlet syndrome: a disorder with serious vascular complications. *J. Pediatr. Surg*. **43**, 1089–1094.

Borchardt, M. (1901) Symptomatologie und Therapie der Halsrippen. *Berl. Klin. Wochenschr*. **38**, 1265.

Bots, J., Wijnaendts, L. C. D., Delen, S., Van Dongen, S., Heikinheimo, K., Galis, F. (2011) Analysis of cervical ribs in a series of human fetuses. *J. Anat*. **219**, 403–409.

Coote, H. (1861) Pressure on the axillary vessels and nerves by an exostosis from a cervical rib; interference with the circulation of the arm; removal of the rib and exostosis, recovery. *Med. Times Gaz*. **2**, 108.

Deschamps, J., van Nes, J. (2005) Developmental regulation of the Hox genes during axial morphogenesis in the mouse. *Development* **132**, 2931–2942.

Glass, R. B., Norton, K. I., Mitre, S. A., Kang, E. (2002) Pediatric ribs: a spectrum of abnormalities. *Radiographics* **22**, 87–104.

Jusufovic, M., Sandset, E. C., Popperud, T. H., Soldberg, S., Ringstad, G., Kerty, E. (2012) An unusual case of the syndrome of cervical rib with subclavian artery thrombosis and cerebellar and cerebral infarctions. *BMC Neurol*. **12**, 48.

Kmita, M., Duboule, D. (2003) Organizing axes in time and space; 25 years of colinear tinkering. *Science* **301**, 331–333.

Köknel Talu, G. (2005) Thoracic outlet syndrome. *Agri* **17**, 5–9.

Mallo, M., Vinagre, T., Carapuco, C. (2009) The road to the vertebral formula. *Int. J. Dev. Biol*. **53**, 1469–1481.

Merks, J. H., Smets, A. M., Van Rijn, R. R., Kobes, J., Caron, H. N., Maas, M., Hennekam, R. C. (2005) Prevalence of rib anomalies in normal Caucasian children and childhood cancer patients. *Eur. J. Med. Genet*. **48**, 113–129.

Peet, R. M., Hendriksen, J. D., Anderson, T. P., Martin, G. M. (1956) Thoracic-outlet syndrome: evaluation of a therapeutic exercise program. *Proc. Staff Meet. Mayo Clin*. **31**, 281–287.

Quinonez, S. C., Innis, J. W. (2014) Human HOX gene disorders. *Mol. Genet. Metab*. **111**, 4–15.

Rigberg, D. A., Gelabert, H. (2009) The management of thoracic outlet syndrome in teenaged patients. *Ann. Vasc. Surg*. **23**, 335–340.

Shah, N., Sukumar, S. (2010) The Hox genes and thein roles in oncogenesis. *Nat. Rev. Cancer* **10**, 361–371.

Viertel, V. G., Intrapiromkul, J., Maluf, F., Patel, N. V., Zheng, W., Alluwaimi, F., Walden, M. J., Belzberg, A., Yousem, D. M. (2012) Cervical ribs: a common variant overlooked in CT imaging. *AJNR Am. J. Neuroradiol*. **33**, 2191–2194.

Walden, M. J., Adin, M. E., Visagan, R., Viertel, V. G., Intrapiromkul, J., Maluf, F., Patel, N. V., Alluwaimi, F., Lin, D., Yousem, D. M. (2013) Cervical ribs: identification on MRI and clinical relevance. *Clin. Imaging* **37**, 938–941.

Weber, A. E., Criado, E. (2014) Relevance of bone anomalies in patients with thoracic outlet syndrome. *Ann. Vasc. Surg*. **28**, 924–932.

Wellik, D. M. (2009) Hox genes and vertebrate axial pattern. *Curr. Top. Dev. Biol*. **88**, 257–278.

 Prague Medical Report / Vol. 117 (2016) No. 2–3, p. 124–128

IgG4-related Diseases – A Rare Polycystic Form of Ormond's Disease

Miroslav Průcha[1], Petr Czinner[2], Petra Prokopová[3]
[1]Department of Clinical Biochemistry, Haematology and Immunology, Na Homolce Hospital, Prague, Czech Republic;
[2]Department of Vascular Surgery, Na Homolce Hospital, Prague, Czech Republic;
[3]Department of Pathology, Na Homolce Hospital, Prague, Czech Republic

Received May 18, 2016; Accepted September 2, 2016.

Key words: Ormond's disease – Retroperitoneal fibrosis – Polycystic form

Abstract: Currently, Ormond's disease is classified among IgG4-associated diseases. Its clinical manifestation varies and is characterized by the presence of fibrous retroperitoneal tissue that often affects the ureters or abdominal aorta and iliac arteries. We present a unique case of the polycystic form of Ormond's disease, imitating tumour in the retroperitoneal space. At the time of diagnosis, the disease was not metabolically active and did not require immunosuppressive therapy. The polycystic mass was removed surgically. There has been no exacerbation of the disease during the last 12 months.

Mailing Address: Assoc. Prof. Miroslav Průcha, MD., PhD., Department of Clinical Biochemistry, Haematology and Immunology, Na Homolce Hospital, Roentgenova 2, 150 30 Prague 5, Czech Republic; Phone: +420 257 273 051; e-mail: miroslav.prucha@homolka.cz

http://dx.doi.org/10.14712/23362936.2016.13
© Charles University – Karolinum Press, 2016

Introduction

IgG4-related diseases comprise a relatively newly defined group of diseases characterized by IgG4 hypergammaglobulinaemia with the presence of IgG4-producing plasma cells in affected organs with fibrotic or sclerotizing changes (Kamisawa et al., 2015). A disease associated with IgG4 may affect virtually any organ – salivary glands, periorbital tissue, kidneys, lungs, meninges, aorta, prostate, pericardium or skin. Histopathological findings are uniform, characterized by a major lymphoplasmocytic infiltrate and the presence of IgG4-producing plasma cells, irrespective of the affected site. Ormond's disease (idiopathic retroperitoneal fibrosis) is an example of a disease associated with IgG4. It is characterized by chronic periaortitis and retroperitoneal fibrosis. The inflammatory process affects the infrarenal part of the abdominal aorta and the iliac arteries and is characterized by the presence of infiltrates encasing the ureters and inferior vena cava.

Case report

A 46-year-old male patient presented to the Department of Urology, Na Homolce Hospital, for backache and fatigue. Clinical and ultrasound assessments and a CT (computed tomography) examination were performed, which revealed an obstructed ureter on the left-hand side. The CT scan showed a mass of 7×5×7 cm that was obstructing the left ureter and iliac arteries. The patient was referred for a surgical procedure to be performed in the Department of Vascular Surgery. Upon admission, the following data were collected. Family history: his mother died at the age of 68 following Grawitz tumour surgery; his father died at the age of 70 from a laryngeal tumour. The patient had been treated for hypertension. Health state: heart rate 97 beats/min, BP (blood pressure) 150/100 mm Hg, respiratory rate 16/min, temperature 36.5 °C, BMI (body mass index) 31.0, eupnoeic, oriented; no jaundice, cyanosis or anaemia; adequate hydration and skin turgor. Head was painless; ears and nose showed no pathological finding. Thyroid gland was not enlarged; cervical veins had an adequate filling; carotid arteries had a symmetric beat, with no murmur. The patient had a regular heartbeat, with two distinct sounds; the heart was not enlarged on percussion; breathing was alveolar, with no additional phenomena. The abdomen was painless on palpation; a resistant mass was palpable in the left mesogastrium; liver and spleen were not enlarged; tapotement was bilaterally negative. Extremities showed no signs of inflammation; pulse on peripheral arteries on both the upper and lower extremities was well palpable all the way into the periphery; joints showed no signs of inflammation and had a regular mobility. A preoperative assessment was performed that did not reveal any contraindications for the surgical treatment. Laboratory findings: serum minerals – Na, K, Cl, urea, creatinine, glycaemia, AST, ALT, cholesterol, triglycerides, all with no pathology, C-reactive protein 9 mg/l, Hb 113 g/l, erythrocytes 3.74×10^{12}/l, haematocrit 0.34 l/l, leukocytes 9.6×10^{9}/l, thrombocytes 636×10^{9}/l, lues, HbSAg, HIV negative, FW 28/52, cardiac and lung X-ray showed no

pathological finding. An 8 cm large tumour mass affixed to the retroperitoneum, well vascularized on the surface, was removed during the vascular surgical procedure. The ureter was encased into the tumour wall. The lower part of the tumour had close contact with the fascia of the psoas muscle and was affixed to the anterior vertebral fascia. The inferior mesenteric artery was encased by the anterior part of the tumour. Similarly, the tumour was growing behind the aorta, deviating it vertebrolaterally to the right. The tumour involved the inferior vena cava. A complete removal of the tumour was performed. The following histopathological examination showed. Macroscopic finding: a partially emptied cystic cavity (70×60×55 mm) with serous contents and a 1–10 mm thick wall made of homogenous grey tissue. Microscopic finding (Figure 1): in the examined excisions, the cavity wall is composed of hyalinised scarred tissue with a large number of blurred round-cell plasmocytic infiltrates which infiltrate the adjacent adipose tissue on the periphery; its remnants are also found in the scarred tissue of the wall. The cavity has a character of a pseudocyst with no clear epithelial lining. The scarred tissue comprises also some small neural rami and arteries. The mass is assessed as a tumour-like pseudocyst variation of interstitial retroperitoneal fibrosis (Ormond's disease). No tumour was detected. The finding is consistent with an idiopathic retroperitoneal fibrosis (Ormond's disease). IgG4-positive plasmocytes were detected, 30 IgG4+ cells/HPF. The subsequent

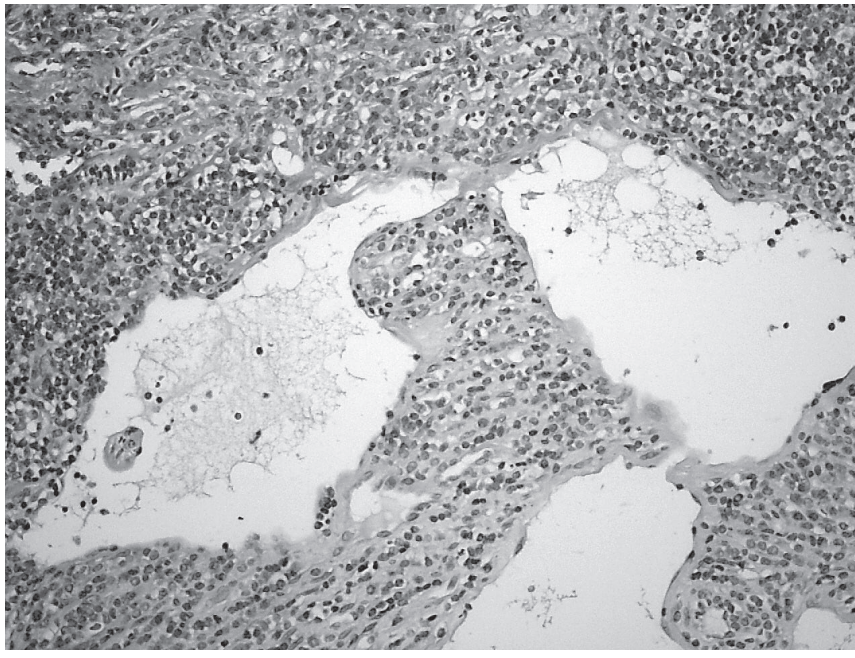

Figure 1 – Tissue with cysts.

PET/CT scan revealed only a slightly increased glucose metabolism and soft tissue lying paravertebrally and posteriorly to the aortic bifurcation, which may present a minor residual mass of active Ormond's disease. The immunology findings of the patient: IgA, IgG, IgM, C3 and C4 part of the complement with no pathological findings, ANA negative, anti-ds DNA negative, ANCA negative. IgG 12.1 G/l, IgG4 0.34 G/l. Since both laboratory findings and imaging methods showed no inflammatory activity, no immunosuppressive therapy was indicated. A cystic form of Ormond's disease was confirmed in the patient, which was not active at the time of diagnosis. The patient has been regularly followed up at the Department of Clinical Immunology. No activity of the disease has been seen in the last 12 months of follow-up.

Discussion

Diseases associated with IgG4 are a newly defined group of diseases, some of which have been known previously (Stone et al., 2015). They are defined by an increased serum IgG4 concentration and the finding of plasma cells in inflammatory infiltrates producing these antibodies. Their clinical presentation varies greatly – the disease may affect completely different organs and systems. The pathogenetic role of IgG4 has not been established as yet. Our case report revealed an interesting clinical form of Ormond's disease. We did not find the described case of the cystic form of retroperitoneal fibrosis confirmed histopathologically in the literature, only one case of a patient with recurrent retroperitoneal fibrosis (RPF), in whom a pseudocyst in the periaortic fibrotic mantle was diagnosed without histopathological confirmation (Jansen et al., 2010). The absence of inflammatory activity at the time of surgery is another interesting feature. It confirms our experience that there are patients in whom the disease is not clinically active (Průcha et al., 2016). For practical reasons, it is important to mention that the disease responds well to systemic corticosteroid therapy. Immunosuppressive therapy is the treatment of choice and has no alternative (Scheel and Feeley, 2013). Patients with a hollow renal system or abdominal aorta are treated by both urologist and vascular surgeon. Systemic corticosteroid monotherapy, however, leads more often to exacerbation of the disease; therefore, combined immunosuppression is the preferred method of treatment, if it is possible.

Conclusion

Diseases associated with IgG4 are a relatively rare group of diseases, often with unpronounced clinical symptomatology, affecting a great variety of organs and systems. Ormond's disease is one example of an IgG4-associated disease. We diagnosed a rare case of a polycystic form of Ormond's disease. Its diagnosis and treatment require a multidisciplinary approach that will be of greater value to the patient.

References

Jansen, I., Hendriksz, T. R., Van Bommel, E. F. (2010) Pseudocyst formation in retroperitoneal fibrosis relapse. *Br. J. Radiol.* **83**, e111–e113.

Kamisawa, T., Zen, Y., Pillai, S., Stone, J. H. (2015) IgG4-related disease. *Lancet* **385**, 1460–1471.

Průcha, M., Kolombo, I., Štádler, P. (2016) Combination of steroids and azathioprine in the treatment of Ormond's disease – a single centre retrospective analysis. *Prague Med. Rep.* **117**, 34–41.

Scheel, P. J. Jr., Feeley, N. (2013) Retroperitoneal fibrosis. *Rheum. Dis. Clin. North Am.* **39**, 365–368.

Stone, J. H., Brito-Zerón, P., Bosch, X., Ramos-Casals, M. (2015) Diagnostic approach to the complexity of IgG4-related disease. *Mayo Clin. Proc.* **90**, 927–939.

Bone Metabolism of the Patient with a Malignant Melanoma during the Entry Examination and the Check-up of Whole-body Bone Scintigraphy

Jaroslav Weissensteiner[1], Eva Babušíková[2]
[1]Department of Nuclear Medicine, Hospital Poprad, Poprad, Slovakia;
[2]Department of Medical Biochemistry, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia

Received May 24, 2016; Accepted September 2, 2016.

Key words: Malignant melanoma – Bone scintigraphy – Osteocalcin – Beta-carboxyterminal cross-linked telopeptide of type I collagen – Human epididymis protein 4

Abstract: Malignant melanoma is a malignancy located predominantly in the skin and the incidence of melanoma increases. We compared the markers of bone metabolism – osteocalcin (OC), beta-carboxyterminal cross-linked telopeptide of type I collagen (β-CrossLaps, β-CTx) and tumour marker – human epididymis protein 4 (HE4) in the serum with finding during the entry examination and the check-up of whole-body bone scintigraphy of the patient with a malignant melanoma. Serum concentrations of OC, β-CTx, HE4 were determined in 1 patient (female, age 64 years) with malignant melanoma and correlated with the presence of equivocal bone metastases detected by whole-body bone scintigraphy (the entry examination and check-up after 6 months). Concentrations of bone metabolism markers decreased during six months and we observed progress in bone metastases. The change of the markers levels during the entry examination and the check-up of the whole-body bone scintigraphy with equivocal finding of bone metastases could be a sign of a possible initiating progression of malignant melanoma despite a clinically negative finding that does not prove the progression of the disease.

Mailing Address: Assoc. Prof. Eva Babušíková, MSc., PhD., Department of Medical Biochemistry, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Malá Hora 4D, 036 01 Martin, Slovakia; e-mail: babusikova@jfmed.uniba.sk

http://dx.doi.org/10.14712/23362936.2016.14
© Charles University – Karolinum Press, 2016

Introduction

Malignant melanoma, a neoplasm of melanocytes or a neoplasm of the cells that develop from melanocytes is the most dangerous type of skin cancer (Dummer et al., 2012). The etiology of malignant melanoma is combination of sun exposure (particularly UVB radiation), genetic and environmental influences (Koh et al., 1993). Metastases develop in regional lymph nodes, as satellite or in-transit lesions or in distant organs (Zbytek et al., 2008). The lung is most commonly involved followed by brain, liver, bone marrow, and intestine (Deepali et al., 2007). Bone marrow metastases of melanoma are in 5–7% of patients with disseminated disease but in up 45% when an autopsy-staging procedure is performing (Serrier and Lesesve, 2013). Typical bone metastases are osteolytic with medullary origin. Lesion growth caused erosion and destruction, pathologic fractures and soft-tissue involvement. Atypical skeletal metastases exhibit as a mixed osteolytic-osteoblastic pattern or hardly ever as a completely osteoblastic (Potepan et al., 1994; Brountzos et al., 2001). Whole-body bone scintigraphy is commonly used for detection of bone metastases in cancer patients and it is more sensitive than X-rays. It is nuclear imaging procedure, offering of total body examination, low cost, and high sensitivity (Brenner et al., 2012). The recording is performed 2 to 4 hours after intravenous application of radioactive tracer (Technecium-99m methylene disphosphonate, Tc-99m MDP) with a double head gamma camera as a whole-body scan in anterior and posterior projection (Kane et al., 2013; Ghosh, 2014). Osteocalcin (OC) is the most important noncollagen protein in bone matrix, accounts for approximately 1% of the total protein in human bone and OC is produced by osteoblasts and is widely accepted as a marker of bone osteoblastic activity. Elevated levels of osteocalcin indicate metabolic bone disease including osteoporosis, osteomalatia, hyperparathyroidism, renal osteodystrophy, thyrotoxicosis, fractures and bone metastasis (Kardamakis et al., 2009). Osteoclasts secrete during bone resorption a mixture of acid and neutral proteases that degrade the collagen fibrils into molecular fragments including carboxyterminal cross-linked telopeptide of type I collagen (CTX). As bone ages, the alpha form of aspartic acid present in CTX converts to the beta form. Beta form is released into the bloodstream and serves as a specific marker for the degradation of nature type I collagen. Beta-carboxyterminal cross-linked telopeptide of type I collagen (β-CTx) levels are increased by bone resorption and are associated with osteoporosis, osteopenia, Paget's disease, hyperparathyroidism and hyperthyroidism (Biver, 2012). Human epididymis protein 4 (HE4), also known as WFDC2 (WAP four – disulphide core domain 2), is a secretory protein detectable in human serum and was originally identified in human epididymis (Li et al., 2013; O'Neal et al., 2013). This protein is a useful tumour marker for ovarian cancer and endometrial cancer. Recent studies have suggested that HE4 is overexpressed also in other malignant tissues and in a number of tumour cell lines including melanoma cell line (Ross et al., 2000; Garber et al., 2001; Ryu et al., 2002; Escudero et al., 2011; Iwahori et al., 2012; Karlsen et

al., 2014). Human epididymis protein 4 may be a novel, useful tumour marker and the increased concentration of HE4 may be a marker for metastasis and a negative prognostic marker for patients after chemotherapy.

Case report

A 64-year-old patient (woman, Caucasian) was examined at the Department of Dermatology with a suspected malignant melanoma of skin (the diameter of 6 mm) on the lateral side of the right arm and a malignant melanoma of skin (the size of 12×6 mm) on the medial side of the right arm. Lymph node on the neck and in the axillae were not palpable. The lymph nodes were not affected during the sonographic examination of the axillae and the neck; struma nodosa bilateral present, gallstones present during the sonography of stomach, steatosis of liver, hepatopathy. Myopathic, reciprocally dilated heart and multiplied bronchovascular marking on an X-ray picture of the chest. She fought off common childhood disease, embolisation into lungs. She undergoes a long-term treatment of arterial hypertension and she is being observed for the struma within a replacement therapy. The value of protein S-100 was 0.093 (standard: <0.105). The total extirpation of malignant melanoma with a 10mm-protective edge recommended by the Melanoma Committee. The intervention was realized in local anaesthesia. The histological examination of dermal lesion situated on the lateral side of the right arm discovered a regressively changed malignant melanoma and a melanocyte lesion (nevus) with epithelioid features and atypical junction proliferation from the dermal lesion on the medial side of the right arm. The proliferation activity of the cells was examined in the immune-chemical way Ki-67 and a higher share of cells expressing Ki-67 was detected in the middle and upper layers of the lesion, less in the base. The wounds were healed with no complications after a surgical intervention. During the examination by computed tomography (CT) – without deposit changes of the metastases character on brain and thoracic and abdominal organs. Older hypodense deposits of the post-ischemic lesions nature and moderate cerebral atrophy were present on the brain in the front part and in the area of basal ganglia on the left. There was also a finding of hypodense deposit in the right lobe of the thyroid gland and a calcified wall of the gallbladder. The entry examination of the whole-body bone scintigraphy was realized 1 month after the operation. The recording was acquired 2 hours after intravenous application of 600 MBq of radioactive tracer Tc-99m – MDP as a whole-body scan of the skeleton in anterior and posterior projection with scanning speed of 10 centimetres per minute. There was the finding of equivocal bone metastases in the bones of the proximal part of the right forearm. Before the examination itself we realized the blood collection, we acquired the serum from a peripheral vein that was consequently stored in sterile plastic test tubes at –50 °C. A check-up was realized 6 months after the entry examination of the whole-body bone scintigraphy with a finding of new equivocal bone metastases in the area of Th7 vertebra and a

moderate regression of the finding in the bones of the proximal part of the right forearm was detected. The blood was again collected and serum stored. Then the patient was observed at the Department of Oncology and Dermatology. The scars after the surgical intervention are unchanged with no palpable resistance. The serum acquired from entry examination and the check-up was used at the workplace of Clinical Biochemistry for the determination of osteocalcin, beta-carboxyterminal cross-linked telopeptide of type I collagen and human epididymis protein 4 in the serum by electrochemiluminescent immunoanalysis – ECLIA method (Elecsys, Roche Diagnostics, at the Department of Clinical Biochemistry, University Hospital in Martin, Comenius University in Bratislava, Slovakia) (Hasanbegovic et al., 2015). Reference values: OC women: 15–46 ng/ml, β-CTx women: 0.33–0.78 ng/ml, HE4: ≤140 pmol/l.

Results

The concentrations of bone metabolism markers in the serum of the patient with a malignant melanoma were: OC: 25.22 ng/ml, β-CTx: 0.513 ng/ml and the concentration of HE4 was 55.33 pmol/l, after the first entry examination of the whole-body bone scintigraphy, and the concentrations were after the second check-up: OC: 17.99 ng/ml, β-CTx: 0.321 ng/ml, HE4: 67.76 pmol/l (Table 1).

We observed the reduction of the concentration of the markers of bone metabolism of OC and β-CTx and the moderate increase of the HE4 concentration comparing the concentrations of OC, β-CTx and HE4 in the serum during the entry examination and the check-up of the whole-body bone scintigraphy with equivocal finding of bone metastases.

Table 1 – The concentrations of osteocalcin (OC), beta-carboxyterminal cross-linked telopeptide of type I collagen (β-CrossLaps, β-CTx) and human epididymis protein 4 (HE4) in the serum during the entry examination and the check-up scintigraphic examination of the patient with the malignant melanoma

Examination	Bone scintigraphy	OC (ng/ml)	β-CTx (ng/ml)	HE4 (pmol/l)
Entry examination	Equivocal metastases in the bones of the forearm	25.22	0.513	55.33
Check-up after 6 months	Equivocal metastases in the area of the Th7 vertebra, moderate regression in the bones of the forearm	17.99	0.321	67.76

Th – thoracic vertebra; referential values: OC women: 15–46 ng/ml, β-CTx women: 0.33–0.78 ng/ml, HE4: ≤140 pmol/l

Conclusion

We observed increased bone metastasis by whole-body bone scintigraphy in patient with melanoma after one month after total extirpation of lesion and bone metastasis progressed after next six months. The changes of the bone metabolism markers levels were observed during six months as well and could be a sign of a possible initiating progression of malignant melanoma, either in a local or distant form of bone metastases and the metastases in the structures of soft tissues and organs despite a clinically negative finding that does not prove the progression of the disease. It would be appropriate to examine the set containing a higher number of patients with a malignant melanoma in order to evaluate the finding more precisely as well as to observe them during a longer period and correlate the given parameters in the serum with the finding during the whole-body bone scintigraphy. There is no available information about the determination and observation of these parameters in the serum of the patients with a malignant melanoma in the literature. Des Grottes et al. (2001) observed the increased concentration of osteocalcin in patients with melanoma and Escudero et al. (2011) observed the increased concentration of HE4 in the case of the group of 9 patients with a malignant melanoma.

References

Biver, E. (2012) Use of bone turnover markers in clinical practice. *Curr. Opin. Endocrinol. Diabetes Obes.* **19**, 468–473.

Brenner, A. I., Koshy, J., Morey, J., Lin, C., DiPoce, J. (2012) The bone scan. *Semin. Nucl. Med.* **42**, 11–26.

Brountzos, E., Panagiotou, I., Bafaloukos, D., Kelekis, D. (2001) Bone metastases from malignant melanoma: a retrospective review and analysis of 28 cases. *Radiol. Oncol.* **35**, 209–214.

Deepali, J., Tejindar, S., Naresh, K., Mradul, K. D. (2007) Metastatic malignant melanoma in bone marrow with occult primary site – a case report with review of literature. *Diagn. Pathol.* **2**, 38.

des Grottes, J. M., Dumon, J. C., Body, J. J. (2001) Hypercalcaemia of melanoma: incidence, pathogenesis and therapy with bisphosphonates. *Melanoma Res.* **11**, 477–482.

Dummer, R., Hauschild, A., Guggenheim, M., Keilholz, U., Pentheroudakis, G. (2012) Cutaneous melanoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **23**, 86–91 (Suppl. 7).

Escudero, J. M., Auge, J. M., Filella, X., Torne, A., Pahisa, J., Molina, R. (2011) Comparison of serum human epididymis protein 4 with cancer antigen 125 as a tumor marker in patients with malignant and nonmalignant disease. *Clin. Chem.* **57**, 1534–1544.

Garber, M. E., Troyanskaya, O. G., Schluens, K., Petersen, S., Thaesler, Z., Pacyna-Gengelbach, M., van de Rijn, M., Rosen, G. D., Perou, C. M., Whyte, R. I., Altman, R. B., Brown, P. O., Botstein, D., Petersen, I. (2001) Diversity of gene expression in adenocarcinoma of the lung. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 13784–13789.

Ghosh, P. (2014) The role of SPECT/CT in skeletal malignancies. *Semin. Musculoskelet. Radiol.* **18**, 175–193.

Hasanbegovic, L., Alicelebic, S., Sljivo, N. (2015) Comparison of specific ovarian tumor markers by Elecsys Analyzer 2010. *Acta Inform. Med.* **23**, 86–89.

Iwahori, K., Suzuki, H., Kishi, Y., Fujii, Y., Uehara, R., Okamoto, N., Kobayashi, M., Hirashima, T., Kawase, I., Naka, T. (2012) Serum HE4 as a diagnostic and prognostic marker for lung cancer. *Tumour Biol.* **33**, 1141–1149.

Kane, T., Kulshrestha, R., Notghi, A., Elias, M. (2013) Clinical utility (applications) of SPECT/CT. In: *Practical*

SPECT/CT in Nuclear Medicine. Jones, D. W., Hogg, P., Seeram, E., Editors, pp. 165–226, Springer-Verlag, London.

Kardamakis, D., Vassiliou, V., Chow, E. (2009) *Bone Metastases. A Translational and Clinical Approach.* Springer Science + Business Media B. V., Dordrecht.

Karlsen, N. S., Karlsen, M. A., Hogdall, C. K., Hogdall, E. V. (2014) HE4 tissue expression and serum HE4 levels in healthy individuals and patients with benign or malignant tumors: a systematic review. *Cancer Epidemiol. Biomarkers Prev.* **23**, 2285–2295.

Koh, H. K., Sinks, T. H., Geller, A. C., Miller, D. R., Lew, R. A. (1993) Etiology of melanoma. *Cancer Treat. Res*. **65**, 1–28.

Li, J., Chen, H., Mariani, A., Chen, D., Klatt, E., Podratz, K., Drapkin, R., Broaddus, R., Dowdy, S., Jiang, S. W. (2013) HE4 (WFDC2) promotes tumor growth in endometrial cancer cell lines. *Int. J. Mol. Sci.* **14**, 6026–6043.

O'Neal, R. L., Nam, K. T., LaFleur, B. J., Barlow, B., Nozaki, K., Lee, H. J., Kim, W. H., Yang, H. K., Shi, C., Maitra, A., Montgomery, E., Washington, M. K., El Rifai, W., Drapkin, R. I., Goldering, J. R. (2013) Human epididymis protein 4 is up-regulated in gastric and pancreatic adenocarcinomas. *Hum. Pathol.* **44**, 734–742.

Potepan, P., Spagnoli, I., Danesini, G. M., Laffranchi, A., Gadda, D., Mascheroni, L., Guzzon, A. (1994) The radiodiagnosis of bone metastases from melanoma. *Radiol. Med.* **87**, 741–746.

Ross, D. T., Scherf, U., Eisen, M. B., Perou, C. M., Rees, C., Spellman, P., Iyer, V., Jeffrey, S. S., Van de Rijn, M., Waltharn, M., Pergamenschikov, A., Lee, J. C., Lashkari, D., Shalon, D., Myers, T. G., Weinstein, J. N., Botstein, D., Brown, P. O. (2000) Systematic variation in gene expression patterns in human cancer cell lines. *Nat. Genet.* **24**, 227–235.

Ryu, B., Jones, J., Blades, N. J., Parmigiani, G., Hollingsworth, M. A., Hruban, R. H., Kern, S. E. (2002) Relationships and differentially expressed genes among pancreatic cancers examined by large-scale serial analysis of gene expression. *Cancer Res.* **62**, 819–826.

Serrier, C., Lesesve, J. F. (2013) Metastatic malignant melanoma in the bone marrow. *Blood* **121**, 721.

Zbytek, B., Carlson, J. A., Granese, J., Ross, J., Mihm, M. C., Slominski, A. (2008) Current concepts of metastasis in melanoma. *Expert Rev. Dermatol.* **3**, 569–585.

Instructions to Authors

Prague Medical Report is an English multidisciplinary biomedical journal published quarterly by the First Faculty of Medicine of the Charles University. Prague Medical Report (Prague Med Rep) is indexed and abstracted by Index-medicus, MEDLINE, PubMed, and Scopus.

Articles issued in the journal

a) Primary scientific studies on the medical topics (not exceeding 30 pages in standardized A4 format – i.e. 30 lines and 60–65 characters per line – including tables, graphs or illustrations)
b) Short communications
c) Case reports
d) Reviews
e) Lectures or discourses of great interest
f) Information about activities of the First Faculty of Medicine and other associated medical or biological organizations

Layout of the manuscript

a) Title of the study (brief and concise, without abbreviations)
b) Information about the author(s) in the following form:
   - first name and surname of the author(s) (without scientific titles)
   - institution(s) represented by the author(s)
   - full corresponding (mailing) author's reference address (including first name, surname and scientific titles, postal code, phone/fax number and e-mail)
c) Abstract (maximum 250 words)
d) Key words (4–6 terms)
e) Running title (reduced title of the article that will appear at the footer (page break), not more than 50 typewritten characters including spaces)
f) Introduction
   - The use of abbreviations should be restricted to SI symbols and those recommended by the IUPAC-IUB. Abbreviations should be defined in brackets on first appearance in the text. Standard units of measurements and chemical symbols of elements may be used without definition.
g) Material and Methods
h) Results
i) Discussion

j) Conclusion
k) References
   - All the sources of relevant information for the study should be cited in the text (citations such as "personal communication" or "confidential data" are not accepted).
   - It is not permitted to cite any abstract in the References list.
   - References should be listed alphabetically at the end of the paper and typed double-spaced on separate pages. First and last page numbers must be given. Journal names should be abbreviated according to the Chemical Abstract Service Source Index. All co-authors should be listed in each reference (et al. cannot be used).
   - Examples of the style to be used are:
     Yokoyama, K., Gachelin, G. (1991) An Abnormal signal transduction pathway in CD4–CD8– double-negative lymph node cells of MRL *lpr/lpr* mice. *Eur. J. Immunol.* **21**, 2987–2992.
     Loyd, D., Poole, R. K., Edwards, S. W. (1992) *The Cell Division Cycle. Temporal Organization and Control of Cellular Growth and Reproduction.* Academic Press, London.
     Teich, N. (1984) Taxonomy of retroviruses. In: *RNA Tumor Viruses,* eds. Weiss, R., Teich, N., Varmus, H., Coffin, J., pp. 25–207, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
     References in the text should be cited as follows: two authors, Smith and Brown (1984) or (Smith and Brown, 1984); three or more authors, Smith et al. (1984) or (Smith et al., 1984). Reference to papers by the same author(s) in the same year should be distinguished in the text and in the reference list by lower-case letters, e.g. 1980a, or 1980a, b.
l) tables, figures, illustrations, graphs, diagrams, photographs, etc. (incl. legends)

Technical instructions

a) Manuscripts (in UK English only) must be delivered in the electronic form via Online Manuscript Submission and Tracking system (http://www.praguemedicalreport.org/). In case of problems, contact the Prague Medical Report Office (medical.report@lf1.cuni.cz). The online submission has to include the complete version of the article in PDF format, separately the manuscript as a MS Word file and a cover letter. The detailed version of the Instructions to Authors can be found at: http://www.praguemedicalreport.org/download/instructions_to_authors.pdf.
b) Text should be written in MS WORD only. We accept only documents that have been spell-checked with UK English as a default language.
c) Please, write your text in Times New Roman script, size 12, and line spacing 1.5.
d) Text should be justified to the left, with no paragraph indent (use Enter key only); do not centre any headings or subheadings.

e) Document must be paginated-numbered beginning with the title page.
f) Tables and graphs should represent extra files, and must be paginated too.
g) Edit tables in the following way: Make a plain text, indent by Tab (arrow key) all the data belonging to a line and finish the line by Enter key. For all the notes in table, use letter x, not *.
h) Make your graphs only in black-and-white. Deliver them in electronic form in TIFF or JPG format only.
i) Deliver illustrations and pictures (in black-and-white) in TIFF or JPG format only. The coloured print is possible and paid after agreement with the Prague Medical Report Office.
j) Mark all the pictures with numbers; corresponding legend(s) should be delivered in an extra file. Mark the position of every picture (photo) in the manuscript by the corresponding number, keep the order 1, 2, 3...

Authors' Declaration

The corresponding (or first author) of the manuscript must print, fill and sign by his/her own hand the Authors Declaration and fax it (or send by post) to the Prague Medical Report Office. Manuscript without this Declaration cannot be published. The Authors' Declaration can be found by visiting our web pages: http://pmr.lf1.cuni.cz or web pages of Prague Medical Report Online Manuscript Submission and Tracking system: http://www.praguemedicalreport.org/.

Editorial procedure

Each manuscript is evaluated by the editorial board and by a standard referee (at least two expert reviews are required). After the assessment the author is informed about the result. In the case the referee requires major revision of the manuscript, it will be sent back to the author to make the changes. The final version of the manuscript undergoes language revision and together with other manuscripts, it is processed for printing.

Concurrently, proofs are electronically sent (in PDF format) to the corresponding (mailing) author. Author is to make the proofs in PDF paper copy and deliver it back to the editorial office by fax or as a scanned file by e-mail. Everything should be done in the required time. Only corrections of serious errors, grammatical mistakes and misprints can be accepted. More extensive changes of the manuscript, inscriptions or overwriting cannot be accepted and will be disregarded. Proofs that are not delivered back in time cannot be accepted.

Editorial Office
Prague Medical Report
Kateřinská 32, 121 08 Prague 2, Czech Republic
e-mail: medical.report@lf1.cuni.cz
Phone: +420 224 964 570. Fax: +420 224 964 574

Prague 2017 ONCO
oncology cooperation

3rd announcement

„8th Prague Interdisciplinary Colloquium in Oncology“

Joint action against the cancer

25th - 27th of January 2017
Clarion Congress Hotel Prague ****
Freyova 33, Prague 9, Czech Republic

Themes for the Colloquim PragueONCO 2017:

- Post ASCO GI 2017
- Pancreatic cancer
- Oncogynecology
- Breast cancer
- Lung cancer
- Gastrointestinal cancer
- Genitourinary cancer
- Tumors of head and neck
- Supportive oncological treatment

If you are interested in presenting a lecture (or you would like to nominate someone as a speaker) during the Colloquium, please apply online untill 30th of November, 2016 through the colloquium online system **www.PragueONCO.cz.** Use the opportunity to present your work to a large audience and to interact more deeply with those who are interested to learn more. Authors of submitted lectures will have been notified about this fact before 31st of December, 2016

This year's colloquium will also feature parallel sessions addressed to **nurses** and focused on **palliative care.**

During the Colloquium special attention will be created for the poster session. The organisers would like to encourage authors to prepare their posters, the topics are not strictly limited on the above mentioned themes, you can choose the subject of your interest.Passive and active participation in the medical section (including foreign participants) will be evaluated by an appropriate number of credits valid for EU countries.

Contact:
We Make Media, s. r. o. / tel.: +420 775 476 475
e-mail: info@wemakemedia.eu / **www.PragueONCO.com**

we make media

Prague Medical REPORT

(Sborník lékařský)

Published by the First Faculty of Medicine, Charles University, Karolinum Press,
Ovocný trh 3, 116 36 Praha 1 – Staré Město, Czech Republic, www.karolinum.cz

Editorial Office: Prague Medical Report, Kateřinská 32, 121 08 Prague 2, Czech Republic,
Phone: +420 224 964 570, Fax: +420 224 964 574,
e-mail: medical.report@lf1.cuni.cz
Editor in Chief: Kateřina Jandová, MD., PhD.
Editor: Assoc. Prof. Jan Šváb, MD., PhD.
Foreign Language Editor: Prof. Jaroslav Pokorný, MD., DSc.
Executive Editors: Mgr. Jiří Frühauf, Mgr. Lucie Šulcová
Editorial Board: Prof. Jan Betka, MD., DSc.; Zdeněk Kostrouch, MD., PhD.;
Prof. Jiří Mazánek, MD., DSc.; Prof. Emanuel Nečas, MD., DSc.;
Prof. František Perlík, MD., DSc.; Prof. Karel Smetana, MD., DSc.; Prof. Karel
Šonka, MD., DSc.; Assoc. Prof. Jan Tošovský, MD., PhD.; Prof. Jiří Zeman, MD., DSc.

Published as quarterly journal. Typeset and printed by Karolinum Press.
Annual subscription (4 issues) EUR 60,–. Single copy EUR 20,–.
Distribution: A.L.L. Production, Ve Žlíbku 1800/77 – Hala A7, 139 00 Praha 9, Czech Republic,
tel.: +420 840 306 090, e-mail: predplatne@predplatne.cz, www.predplatne.cz

ISSN 1214-6994 (Print)
ISSN 2336-2936 (Online)

Reg. No. MK ČR E 796