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CHANGES IN SEVERAL INFLAMMATORY AND OXIDATION MARKERS AFTER OVALBUMIN-SENSITIZATION IN A GUINEA PIG MODEL OF ALLERGIC ASTHMA – A PILOT STUDY

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Abstract

Ovalbumin (OVA)-sensitization is a common way to evoke changes similar to changes in allergic asthma in humans. Activated cells produce various pro-inflammatory and vasoactive substances including reactive oxygen species. The goal of this pilot study was to evaluate mobilization of leukocytes into the lungs and oxidation processes induced by OVA-sensitization in a guinea-pig model of allergic asthma. Guinea-pigs were divided into OVA-sensitized and naïve animals. After sacrificing animals, blood samples were taken and total and differential leukocyte counts were calculated, and eosinophil cationic protein (ECP) and total antioxidant status (TAS) in the plasma were determined. Left lungs were saline-lavaged and total number of cells and differential leukocyte count in the bronchoalveolar lavage fluid (BAL) were calculated. Right lung tissue was homogenized, ECP, TAS and products of lipid and protein oxidation (thiobarbituric acid-reactive substances and lysine-lipoperoxidation end-products) were determined in the lung homogenate. OVA-sensitization increased a total number of cells and percentages of eosinophils and neutrophils and slightly increased ECP in the blood and in the BAL fluid. In addition, increased lipid and protein oxidation in the lung homogenate, and decreased TAS in the plasma was found in OVA-sensitized compared to naïve animals. In conclusion, OVA-sensitization increased mobilization of leukocytes into the lungs and elevated production of reactive oxygen species (ROS), accompanied by a decrease in plasma TAS.

Key words: allergic asthma, ovalbumin-sensitization, oxidation stress, reactive oxygen species, guinea pig

INTRODUCTION

Allergic asthma is a respiratory disease with a complex pathophysiology. It is characterized by airway hyperresponsiveness, increased irritability of sensory nerves in the airways and enhanced mucus secretion, as well as by acute and chronic inflammation, edema and airway remodeling (1). Activation of eosinophils, neutrophils, T-lymphocytes and mast cells is associated with increased production of reactive oxygen species (ROS) and reduced antioxidant status, as it has been described (2, 3). ROS, such as superoxide anion, hydrogen peroxide, hydroxyl radicals etc., may directly damage proteins, lipids and DNA, forming products that can be used as biomarkers of oxidative stress. In addition, oxidative stress through the activation of mitogen-activated protein kinase (MAPK) signaling as well as by increase of nuclear factor erythroid-2 related factors (NRF)-1 and -2, and nuclear factor- κ B (NF- κ B) promotes inflammation (4) and modulates airway responses (5). Even, higher ROS may contribute to the induction of allergic asthma (4, 6).

Participation of various pathomechanisms results in a heterogeneity of clinical expressions of bronchial asthma and finally relates to the different response to administered therapy (1). Therefore, understanding the pathophysiology of the disease may be useful not only for developing and testing novel therapeutic strategies, but the precisely addressed therapeutic intervention may finally reduce also the proportion of patients not responding to the therapy.

Whereas the mobilization and activation of the cells in allergic inflammation and overproduction of ROS are the fundamental factors in the pathophysiology of the allergic

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asthma, the goal of this pilot study was to evaluate the mobilization of cells into the lungs and to determine the severity of oxidation processes induced by OVA-sensitization in a guinea-pig model of allergic asthma.

METHODS

The study protocol was approved by a local Ethics Committee at Jessenius Faculty of Medicine, Comenius University in Martin. In total, twelve healthy male guinea pigs (250-350 g) were used in the study, divided into two groups. There were no differences between the groups in age or body weight. Animals were kept in an animal house and had food and water ad libitum.

In six animals, model of allergic asthma was induced with ovalbumin allergen (Albumin from chicken egg white; Sigma-Aldrich, Germany). Other six animals served as non-sensitized (naïve) controls. Sensitization of animals with ovalbumin was performed during 14 days (7). Ovalbumin in a 1% concentration (10 mg of ovalbumin in 1 ml) was administered on the 1st day of sensitization intraperitoneally (0.5 ml) and subcutaneously (0.5 ml), on the 3rd day intraperitoneally (1 ml). Ovalbumin challenge by the inhalation (3 min) was performed on the 14th and 21st day using an Aerosol Jet Nebulizer (HSE, Germany). Saline was given to the control (naïve) animals at the same dose and regimen as in OVA-sensitized ones. Animals were killed 30 min. after the second ovalbumin challenge.

After sacrificing the animal, sample of blood was taken and centrifuged at 3000 rpm for 20 min. Blood plasma was stored at -70 °C till biochemical analyses were made.

Left lungs were lavaged with saline (2 x 10 ml/kg b.w.). Total number of cells in BAL was evaluated microscopically and expressed per 1 µl of BAL fluid. BAL fluid was then centrifuged at 1500 rpm for 10 min. In the sediment, differential leukocyte count was evaluated microscopically after staining by Pappenheim, as described previously (8).

Right lung tissue was homogenized, ECP, TAS and markers of lipid and protein oxidation (TBARS and lysine-LPO end-products) were determined (see below).

Lung tissue was washed and homogenized in 50 mmol of phosphate buffer (pH 7.4) and 1 mmol butylated hydroxytoluene in a ratio 1:5 using a homogenizer (Potter, B. Braun Melsungen A.G., Germany) at a temperature 0-4 °C. Protein assay was performed by a method of Lowry et al. (9). For dilution of the homogenate, 1% sodium dodecyl sulfate was used. Concentration was calculated using a bovine serum albumine as a standard.

Fluorescence measurements were performed in a solution containing 50 µg of membrane protein per ml, 10 mmol/l HEPES, 100 mmol/l KCl, pH 7.0 at 25 °C using spectrofluorimeter (RF-540, Shimadzu, Japan). Fluorescence emission spectra (380-440 nm, slit width 5 nm) of dityrosine, a product of tyrosine oxidation, were measured at excitation wavelength 325 nm (slit width 5 nm) (10). Emission spectra (from 425 to 480 nm, slit width 5 nm) of lysine conjugates with lipid peroxidation (LPO) end-products were recovered at excitation of 365 nm (5 nm slit width). Excitation spectra (from 325 to 380 nm, 5 nm slit width) were measured at 440 nm (5 nm slit width) (11). Fluorescence intensity was expressed in arbitrary units (A.U.). Thiobarbituric acid-reactive substances (TBARS) in the lung homogenate were determined from the absorbance at 532 nm and expressed in nmol/mg protein (12).

Concentrations of eosinophil cationic protein (ECP) in the lung homogenate and in the blood plasma were evaluated by enzyme-linked immunosorbent assay (ELISA) using ECP kit (Diagnostics Development, Sweden). Quantification of total antioxidant status (TAS) in the lung homogenate and in the plasma was carried out using ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline sulphonate]) radical formation kinetics (Randox TAS kit, Randox Laboratories Ltd., UK) and were expressed in mmol/l.

Statistics: Statistical analysis was made by Systat for Windows. Because of relatively small number of animals in the groups and non-parametric distribution of some data, Kruskal-Wallis test for between-group comparisons was used. A $P < 0.05$ was considered statistically significant.

RESULTS

Cells in the BAL fluid and in the arterial blood

OVA-sensitization elevated a total number of cells in the BAL fluid compared to naïve controls ($69.1\pm6.6 \times 10^3/\mu\text{l}$ in OVA-sensitized group vs. $9.0\pm1.5 \times 10^3/\mu\text{l}$ in controls; $P<0.01$). In addition, OVA-sensitization increased percentages of both eosinophils ($P<0.01$; **Fig. 1**) and neutrophils ($P<0.05$; **Fig. 2**).

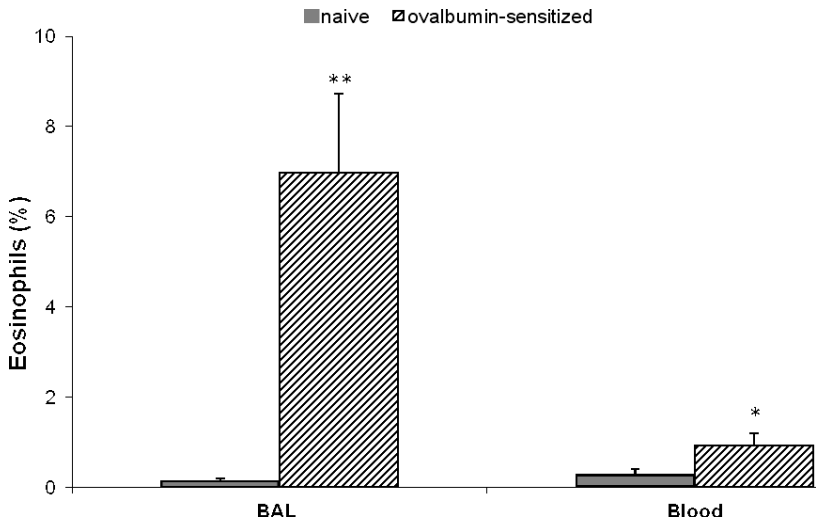


Fig. 1 Eosinophils (%) in the BAL fluid and in the blood. For differences between OVA-sensibilized group vs. naïve animals: ** $P<0.01$, * $P<0.05$

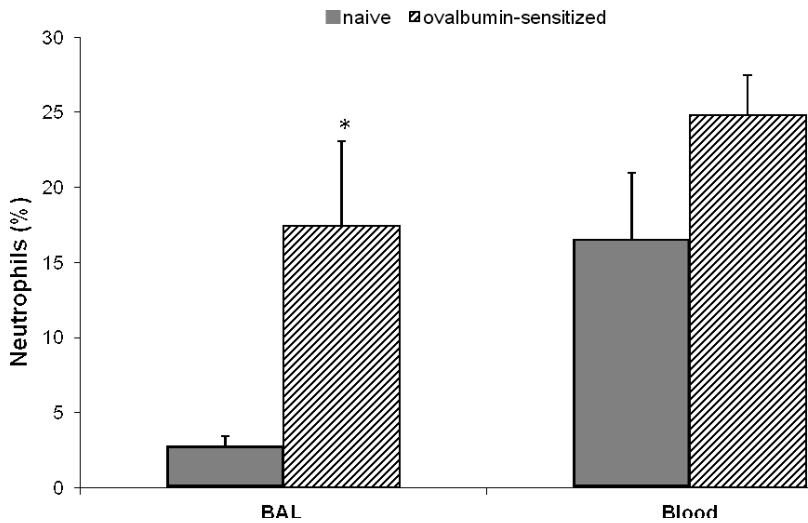


Fig. 2 Neutrophils (%) in the BAL fluid and in the blood. For differences between OVA-sensibilized group vs. naïve animals: * $P<0.05$

Total number of leukocytes in the blood increased after OVA-sensitization compared to controls ($4.1 \pm 0.5 \times 10^9/l$ in OVA-sensitized group vs. $2.5 \pm 0.3 \times 10^9/l$ in controls; $P < 0.05$). Similarly, OVA-sensitization increased percentage of eosinophils ($P < 0.05$; Fig. 1) and showed a trend to elevate also a percentage of neutrophils ($P > 0.05$; Fig. 2).

Markers of inflammation and oxidation

OVA-sensitization slightly increased levels of eosinophil cationic protein (ECP) in the lung homogenate ($1.41 \pm 0.27 \mu\text{g/l}$ in OVA-sensitized group vs. $0.85 \pm 0.15 \text{ g/ml}$ in controls) and in the plasma ($2.10 \pm 0.29 \mu\text{g/l}$ in OVA-sensitized group vs. $1.21 \pm 0.36 \mu\text{g/l}$ in controls), however, these differences were not significant (both $P > 0.05$).

In OVA-sensitized animals, higher concentrations of thiobarbituric acid-reactive substances (TBARS) and lysine-LPO end-products, markers of lipid and protein oxidation, were observed in the lung homogenate compared to non-sensitized controls (both $P < 0.01$; Fig. 3A and 3B).

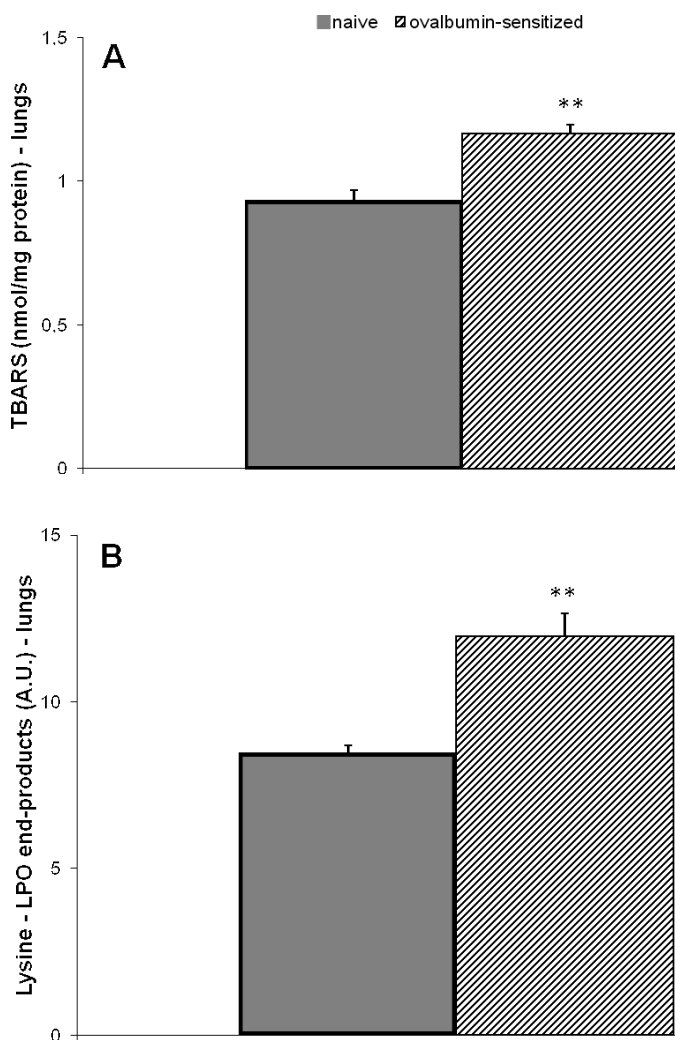


Fig. 3. Thiobarbituric acid-reactive substances (TBARS) (A) and lysine-lipoperoxidation (LPO) end-products (B) in the lung homogenate. For differences between OVA-sensitized group vs. naïve controls: ** $P < 0.01$

Inversely to increased oxidation stress, total antioxidant status (TAS) decreased in the blood plasma ($P<0.05$) and a trend to decrease TAS was observed also in the lung homogenate ($P>0.05$; Fig. 4).

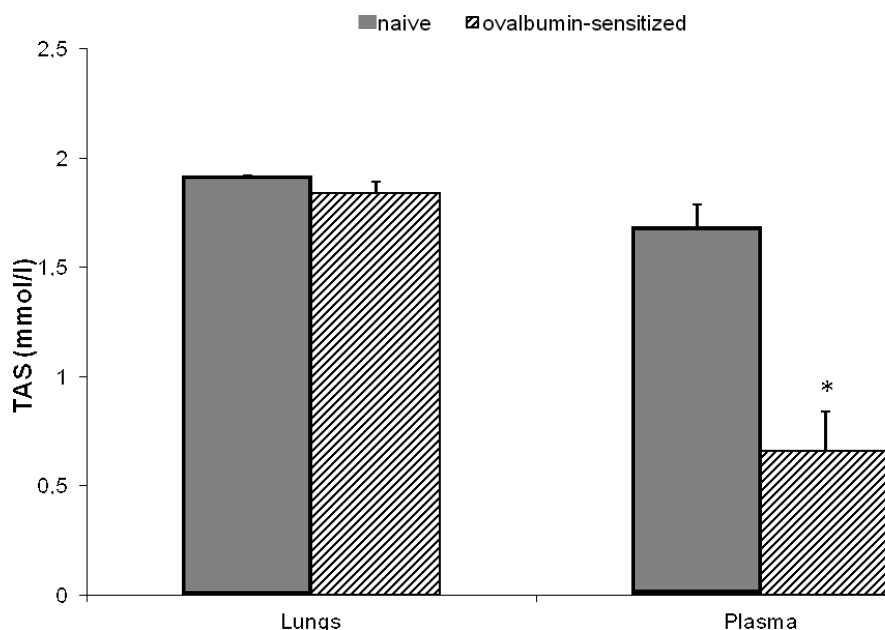


Fig. 4 Total antioxidant status (TAS) in the lung homogenate and in the blood plasma. For differences between OVA-sensitized group vs. naïve controls: * $P<0.05$.

DISCUSSION

Migration and activation of the inflammatory cells and abundant production of ROS are the fundamental factors in the pathophysiology of allergic asthma. Thus, the goal of this pilot study was to evaluate the mobilization of cells into the lungs and to determine the severity of oxidation processes induced by OVA-sensitization. In a guinea-pig model, OVA-sensitization elevated numbers of total leukocytes and percentages of eosinophils and neutrophils in the blood and increased total number of cells and percentages of eosinophils and neutrophils in the BAL fluid. Enhanced activation of cells, particularly of eosinophils, was confirmed by slightly increased levels of eosinophil cationic protein (ECP) in the lung and plasma. In addition, OVA-sensitization increased generation of free radicals and forming by-products of lipid and protein peroxidation, associated with reduced total antioxidant status (TAS) in the lungs and in the blood plasma.

OVA-sensitization is a generally accepted method of preparation of an experimental model of airway inflammation and hyperreactivity, resembling the changes in bronchial asthma (3, 5, 7). Several species of rodents may be used for this purpose, however, a guinea pig model has been well established.

Ovalbumin-sensitization in guinea pigs caused an increase in total circulating leukocytes with a particular increase in eosinophils and neutrophils compared to non-sensitized controls. This may be attributed to demarginalization and release of the mentioned types of leukocytes from the stores in the spleen and other organs. Anyway, we cannot exclude also

the production *de novo* in the bone marrow, as the OVA-sensitization is a long-term process. An increase in circulating leukocytes goes hand-in-hand with an increase in a number of cells in the BAL fluid. Similarly to blood, a particular increase was found for percentages of eosinophils and neutrophils. It is known that these two types of phagocytosing microcytes are able within a very short time to migrate from the circulation into the tissue. The process of their activation is associated with an increased production of a variety of bioactive substances (1, 13). Eosinophils are a rich source of granule basic proteins, but they generate prostacyclin, cysteinyl leukotrienes, reactive oxygen species, growth factors and a wide range of cytokines and chemokines, as well. Despite eosinophils produce lower amounts of cytokines and free radicals than the other leukocytes, they play an essential role in the immune response, airway hyperreactivity, airway remodelling and mucus production in allergic asthma (1, 13). On the other hand, neutrophils are potent producers of reactive oxygen species, however, their role as the secondary effector cells in a „classical“ allergic asthma of Th2-type, particularly mild-to-moderate asthma, is questionable (1). Nevertheless, neutrophilic inflammation was noted in patients with severe asthma and during acute exacerbations, as well as in patients treated with glucocorticoids (1, 14). Finding increased neutrophils in an absence of eosinophils suggests that the disease becomes more aggressive and chronic, representing non-eosinophilic asthma of Th1-type (1). More prominent increase in the eosinophils in our study is, definitively, in accordance with characteristics of the used model of allergic asthma.

To evaluate activation of eosinophils, ECP concentrations were determined in the lungs as well as in the blood plasma. Despite we found a slight increase in both lungs and plasma, the differences to the controls were not significant. This finding may be related to small number of animals included in the groups. In addition, we may speculate that although ECP increases in allergic asthma in humans (15), it might be not enough sensitive indicator of the eosinophil activation in this animal model of asthma and any other marker may be more appropriate, e.g. eotaxin etc.

Nevertheless, activation of eosinophils and neutrophils was satisfactory confirmed by finding increased concentrations of by-products of lipid and protein oxidation. A product of lipoperoxidation, malonyldialdehyde, may react with free sulfhydryl groups of cysteine or amino groups of lysine and generate complexes reacting with thiobarbituric acid, creating thiobarbituric acid-reactive substances (TBARS). In this study, higher concentrations of TBARS and lysine-LPO products have proven an overproduction of free radicals after ovalbumin-sensitization in guinea-pigs. Similarly, intensive oxidation stress characterized by increased TBARS was found in asthmatic children compared with healthy controls (16).

Inversely to enhanced generation of free radicals, a total antioxidant status (TAS) representing a cumulative action of all antioxidant systems in the plasma or lung tissue, decreased after OVA-sensitization. Similarly to our results, lower plasma total antioxidant capacity negatively correlated with oxidation markers were found also in patients with asthma, particularly in acute exacerbations (17). In addition, finding of reduced TAS levels in plasma suggests significant inflammatory and oxidation changes resulting from OVA-sensitization also on the systemic level.

In conclusion, OVA-sensitization in guinea pigs increased a number of circulating leukocytes in blood, particularly the percentages of eosinophils and neutrophils. Migration of these cells into the lungs was associated with a slight increase in eosinophil cationic protein, and significant overproduction of free radicals expressed by elevated levels of by-products of lipid and protein oxidation, and reduced total antioxidant status in the plasma.

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ACID-BASE CHANGES IN YOUNG MEN AFTER ORAL INTAKE OF ETHANOL

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Abstract

The authors studied a group of healthy men of an average age 24.4 ± 2.1 yrs ($x \pm \text{SEM}$) before the oral intake of ethanol and 1 and 2 hours after its ingestion. The volunteers, $n = 10$, of an average weight $79.1 \text{ kg} \pm 2.7 \text{ kg}$ ($\pm \text{SEM}$) consumed within one minute $0.42 \text{ g ethanol/kg}$ of individual body weight diluted in water (ratio 1:3). Concentrations of alcohol in venous blood were determined using Widmark method. One hour after the peroral intake the ethanol concentration reached $0.39 \pm 0.02 \text{ g/kg}$, 2 hours after peroral intake it was $0.33 \pm 0.03 \text{ g/kg}$.

Parameters of acid-base balance (ABB) from venous blood samples were monitored in probands. Compared with baseline values, the pH of blood did not change significantly. The values of pCO_2 in blood were significantly decreased, even the values of actual HCO_3^- and standard HCO_3^- . The decrease of base excess continued 2 hours after intake of ethanol. There were significant variations in ABB (paired t - test), however in tolerable range. The values of glycaemia, AST, ALT, GGT, and AMS before ethanol intake and 1 and 2 hours after intake of ethanol were in the reference range and they did not change significantly.

The authors find important to document even "small" intake of alcohol which may be the cause of ABB shift to a pathological range. The doctors should always keep this in mind when doing the diagnostic statement. The changes of ABB due to alcohol consumption must be expected, especially if they are associated with other pathological conditions affecting the ABB.

Key words: ethanol, venous blood, healthy young persons, acid-base balance, hypocapnia, glycaemia, AST, ALT, GMT, AMS

INTRODUCTION

Ethanol and its metabolic products affect homeostasis of the organism. Only a very small amount of consumed alcohol is excreted unchanged by the kidneys, lungs and skin. A high percentage (90 - 98%) is degraded in the liver. Ethanol metabolism in the body is closely linked to its oxidation: first, the influence of a cytosolic enzyme alcohol dehydrogenase to acetaldehyde (only a small percentage by microsomal alcohol-oxidizing system, MEOS and catalase) and then there is the action of liver aldehyd dehydrogenase that creates acetic acid. Acetaldehyde is highly reactive substance that inhibits the catabolism of acetate in the Krebs cycle; it activates collagen synthesis and stimulates the formation of free oxygen radicals with subsequent lipoperoxidation (16). Acetaldehyde has the ability to bind to the nucleic acids and proteins in particular. Measuring its bound protein amount can be used as a marker for alcoholism, similarly as it is in pursuit of glycosylated hemoglobin in diabetics (4, 18). Acetaldehyde in connection with the free amino acids, especially lysine amino residues can form neoantigenes, which are then directed against the body's own proteins, especially against hepatocytes (16). The final metabolic products of ethanol removal in the body are carbon dioxide and water.

In the way of alcohol dehydrogenase, that forms NADH and acetaldehyde is the main way of degradation of ethanol in non-drinkers, who drank alcohol only occasionally (15). With rising incomes of alcohol after several weeks ethanol exposure does not increase the amount

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of enzyme alcohol dehydrogenase. Supporting role in oxidation happening assumes inducible MEOS office system (known in the literature also as cytochrome P450-CYP2E1-11, 14), which can increase their activity 2-3 times, but this is associated with greater demands for oxygen. This may gradually lead to the development of centrilobular necrosis and alcoholic liver cirrhosis (17). In the pathogenesis of alcoholic hepatitis is involved also endotoxin transfer from G-bacteria through the impaired intestinal wall into the circulation and binding to Kupffer cell receptor, which subsequently released mediator TNF-alpha which induces other inflammatory cytokines (11).

Ethanol may cause fluctuations in acid-base balance, which is strongly reflected particularly in cases of intoxication with higher doses, unfortunately, these cases currently often occur not only in adults, but also in children, adolescents and young subjects (7,8). Alcohol intake increases concentration of NADH and the ratio of NADH/NAD. Higher concentrations of NADH inhibit the performance of Krebs cycle and β -oxidation of fatty acids in mitochondria, increase the lactate/pyruvate ratio and β -hydroxybutyrate/acetoacetate leading to the acidosis with formation of ketone, the inhibition of glycolysis and gluconeogenesis. In the renal tubules is increased resorption of uric acid, which may provoke the hyperuricemia and the gouty attack. Alcohol increases the concentration of catecholamines and through their effect also depletes glycogen reserves in the liver (15).

Monitoring the impact of peroral, less precisely quantified dose of ethanol on the parameters of acidosis and their following evaluation in relation to levels of ethanol found in the blood at defined periods of time, can provide a picture of early changes in acid-base balance (ABB) and correction of the compensatory mechanisms of the organism.

Not least is not specifically estimated whether and to what extent, small doses of alcohol affect the acid-base. More precise knowledge of the situation - a possible shift in the acid-base parameters - would help to reveal whether doctors should consider in the diagnostic statement after blood collection to investigate ABB, reflect that pathological values can also be caused by the summation of certain disease plus a small but already significant "ethanol background" when e.g. a history of the patient did not, sometimes intentionally, ingested of alcohol recently.

Therefore, the authors decided to assess the effect of peroral small doses of ethanol on acid-base balance in healthy persons in relation to the monitoring of blood ethanol concentrations. Evaluation of the selected parameters of circulation after peroral intake of alcohol has been the subject of our previous study (1).

SUBJECTS AND METHODS

Authors examined a group of healthy men of average age of 24.4 ± 2.1 years ($x \pm \text{SEM}$) before peroral ethanol intake and after its ingestion of 1 and 2 hours. Probands ($n= 10$, of average weight $79.1 \text{ kg} \pm 2.7 \text{ kg}$) consumed within 1 minute 0.42 g of ethanol/kg body weight diluted with water in a ratio of 1:3. Alcohol concentrations in venous blood were studied before and after 1 and 2 hours after its intake by Widmark method (after disinfection of the sampling point with solution of hydrogen peroxide). In parallel in the same situations were assessed also parameters of ABB, before and after 1 and 2 hours after drinking alcohol, in three samples of capillary blood from a finger pad (after friction, before taking the blood in order to improving arterialization). In all samples of venous blood were also determined: glycaemia, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GMT) and total amylase (AMS). Investigated men after breakfast (2 rolls and tea or a glass of fruit juice, between 6-7 a.m.) passed the examination between 9.30 to 12.30 pm. During the examination they were sitting. During blood taking, they were lying for a short time.

Informed consent was obtained from all subjects under investigation. The results were processed by mathematical and statistical procedures, by calculating the average values and $\pm \text{SEM}$, using paired t-test.

RESULTS

Figure 1 shows the average concentration of ethanol in venous blood before and after 1 and 2 hours after ingestion of ethanol. Mean value of blood ethanol was 0.10 ± 0.03 g/kg, after 1 hour 0.39 ± 0.02 g/kg and after 2 hours 0.33 ± 0.03 g/kg.

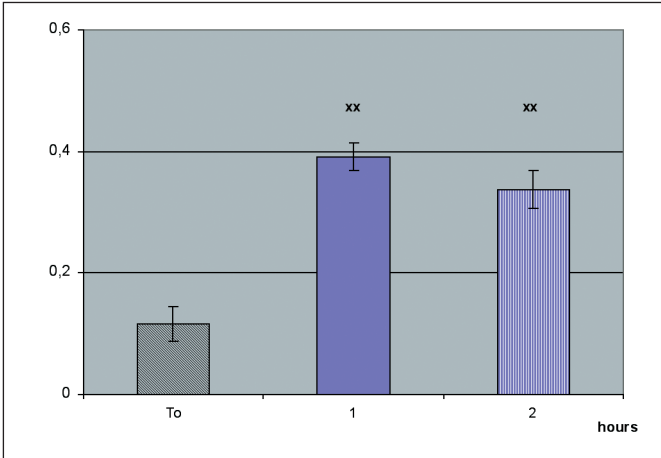


Fig.1 Average level of ethanol in venous blood before the ethanol ingestion, 1 hour after ingestion and 2 hours after ingestion. xx p < 0.01 compared with To (To = initial time).

Fig. 2, 3 and 4 present ABB parameters before ingestion of ethanol and 1 and 2 hours after the ingestion. Figure 2a presents values of pH in the blood before and 1 and 2 hours after the ingestion of ethanol. We found that 1 hour after ingestion of ethanol the pH was 7.42. After 2 hours the pH in blood was 7.41 (NS).

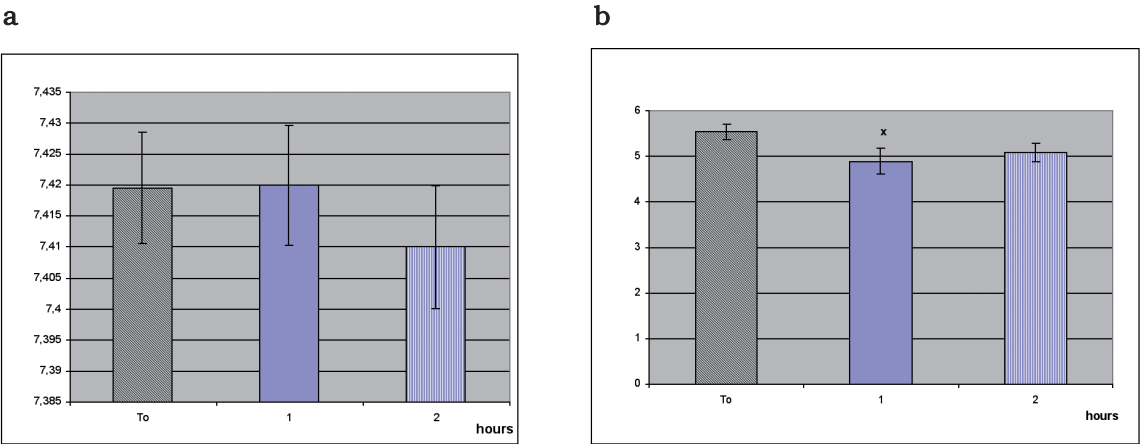


Fig. 2 a) The average pH of blood before the ethanol ingestion, 1 hour after ingestion and 2 hours after ingestion. **Fig. 2 b)** Average concentration of pCO₂ before the ethanol ingestion, 1 hour after ingestion and 2 hours after ingestion. x p < 0.05 compared with To (To = initial value).

Figure 2b is the illustration of the $p\text{CO}_2$ values in blood before the ingestion of ethanol (5.51 kPa) and 1 and 2 hours after ingestion. We found that 1 hour after ingestion of alcohol the $p\text{CO}_2$ was 4.89 kPa, therefore it significantly decreased by 0.62 kPa ($p < 0.05$). After 2 hours, the $p\text{CO}_2$ was 5.22 kPa (NS to initial value).

Figure 3a is the comparison of the mean values of the **base excesses** in the blood before the ingestion of ethanol (+1.53 mmol.l⁻¹) with values after 1 and 2 hours after ingestion. Base excess 1 hour after ingestion of ethanol significantly decreased to negative value -0.31 mmol.l⁻¹, it means by 1.84 mmol.l⁻¹ ($p < 0.05$). The decline continued even during 2nd hour, when the BE was -0.48 mmol.l⁻¹; difference from initial value was significant ($p < 0.05$) by 2.01 mmol.l⁻¹. Since the normal range is taken as 0 ± 3.0 mmol.l⁻¹, the situation cannot be evaluated as a base deficiency (10).

Figure 3b presents the mean values of the **buffer base**, which was after 1 hour decreased by 1.36 mmol.l⁻¹ (from 50.7 mmol.l⁻¹ to 49.32 mmol.l⁻¹) and after 2 hours by 8.16 mmol.l⁻¹ (from 50.7 mmol.l⁻¹ to 42.54 mmol.l⁻¹). This decrease after 2 hours to the initial level was significant ($p < 0.01$).

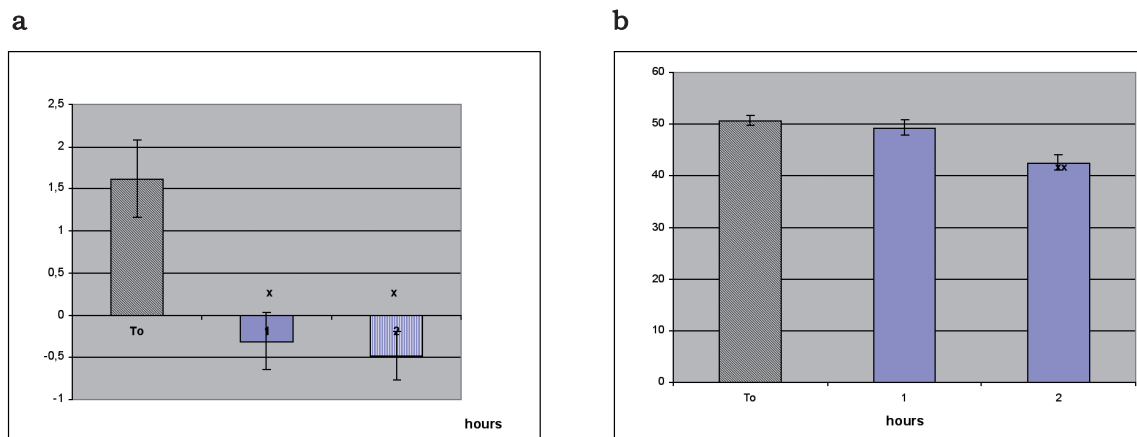


Fig. 3 a) The average value of excesses of bases in the blood before the ethanol ingestion, 1 hour after ingestion and 2 hours after ingestion. x $p < 0.05$ compared with initial value;

Fig. 3 b) The average value of buffer base prior to ingestion of ethanol, 1 hour after ingestion and 2 hours after ingestion. xx $p < 0.01$ compared with initial value.

Figure 4a shows the values of **standard HCO_3^-** , which fell after one hour by 1.58 mmol.l⁻¹ (from 25.79 mmol.l⁻¹ to 24.21 mmol.l⁻¹; $p < 0.05$). After 2 hours the decline persisted (24.0 mmol.l⁻¹).

In the Fig. 4b are values of **actual bicarbonate**. This parameter was decreased significantly after 1 hour by 2.74 mmol.l⁻¹ (from 26.32 mmol.l⁻¹ to 23.58 mmol.l⁻¹ - $p < 0.01$). The decrease was found even after 2 hours, 23.79 mmol.l⁻¹, however it was statistically non-significant ($p = 0.05$).

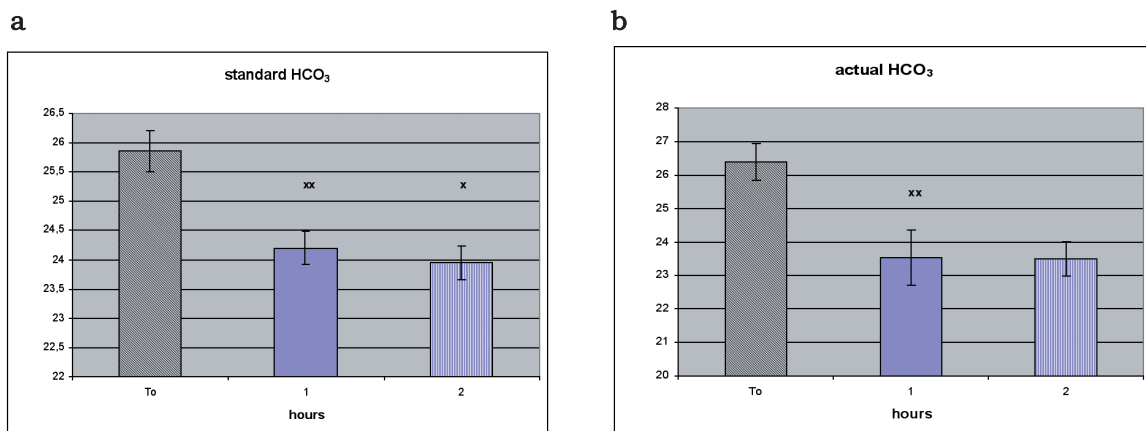


Fig. 4 a) Concentration of standard HCO_3^- in the blood before the ethanol ingestion, 1 hour after ingestion and 2 hours after ingestion. x $p < 0.05$; xx $p < 0.001$ compared with To;

Fig. 4 b) Concentration of actual bicarbonate in the blood before the ethanol ingestion, 1 hour after ingestion and 2 hours after ingestion; xx $p < 0.001$ compared with To (To = initial value).

Before the ingestion of ethanol was the average glycaemia 4.89 ± 0.22 mmol/l, AST averages 0.23 ± 0.02 $\mu\text{kat/l}$, ALT averages 0.38 ± 0.04 $\mu\text{kat/l}$, GMT averages 0.21 ± 0.04 $\mu\text{kat/l}$, AMS averages 2.92 ± 0.42 $\mu\text{kat/l}$. All parameters were within the reference range (10). The shift of these values after ingestion of ethanol was not different from the initial values.

DISCUSSION

Alcohol metabolism is also influenced by various factors included genetic and racial ones (2, 14, 15 and others). Alcohol dehydrogenase and aldehyd dehydrogenase are enzymes that are present in several racial and genetic variations. Caucasian population breaks down alcohol faster than the Indians and Eskimos. In Japanese, Koreans and Thais is genetically determined an increased reactivity to small amounts of ethanol because they have different enzyme background for oxidation of ethanol. In our study group all probands were Caucasians.

We decided for Widmark method to determine concentrations of ethanol in blood, which is very laborious, but still accurate, most common in people who are alive (14). This method enables a more accurate determination of ethanol in blood in comparison with the determination using breath analyzers. Only 20.8 % of the results of testing 610 cases from 11 workplaces in the Czech Republic and Slovakia using a respiratory analyzer tests correspond to the detected real alcohol level in blood (5).

In our group the input value of ethanol in blood was very slightly elevated. This can be explained by an early cup of juice or some confectionery products. Small quantities of alcohol are sometimes included in mouthwashes and certain toothpastes. Interestingly, the tolerability limits of blood alcohol concentration for drivers in EU are not yet uniform. Permissible concentration of alcohol in blood is usually 0.5 g.l^{-1} ; in the Czech republic, in Romania and Hungary 0.0 g.l^{-1} ; in Slovakia, Poland, Norway and Sweden 0.2 g.l^{-1} , in Great Britain, Ireland and Malta 0.8 g.l^{-1} .

The amount of taken alcohol in young men in our group increased the concentration of ethanol in blood for a long time. The difference between the values of ethanol after 1 and 2 hours was not significant. We performed our testing on healthy probands. They were not exposed to physical exertion during the observation to avoid a possible effect of exercise on

degradation of alcohol. We excluded disease states and drug effects that could slow down the degradation of ethanol in blood.

Hypoglycemia slows down absorption from stomach because it accelerates gastric emptying. Vice versa, hyperglycemia would slow down gastric emptying and thus accelerate the absorption from stomach (3, 14, 19). Blood glucose concentration during our 2-hour observation did not change significantly.

In general, after ingestion of ethanol, the peak of its blood concentration is reached within 30-40 minutes. There is a subsequent fall which can be prolonged by e.g. slow oxidation in liver under absence of alcoholdehydrogenase, aldehyddehydrogenase, in chronic hepatitis of various genesis, during fasting or malnourishment, in chronic alcoholics or when MEOS activity is exhausted, etc. We monitored blood glucose, transaminases and amylase to try to exclude also the eventual hidden abnormalities in internal environment of probands to make sure that it did not affect the degradation of alcohol and ABB parameters. Straka et al. (2011) point out that small amounts of ingested alcohol can be eliminated from body even for 3 hours. The alcoholemic curve has a so called Grehan plateau (14). These protracted changes in acid-base balance could be an example of this phenomenon in our probands.

Food especially slows down the absorption of ethanol in stomach and small intestine. However, we did not expect a slower resorption of ethanol in our probands because they had had a light breakfast two hours before. We followed up peripheral acral circulation by using skin thermometer and digital reography on upper extremities. The probands were for two hours monitored in semihorizontal position and had no physical activity. That is why we cannot exclude whether this could not have delay the peak values of blood ethanol (6, 14). Although the arterial blood pH did not change significantly, our observation points out small but significant changes of some other values of ABB affected by ethanol. The results vary in the distribution of normal values.

A relatively small intake of alcohol had an effect on base excess, possibly through an increase in lactate at the expense of pyruvate, as is the case with physical exertion. We have not yet benefited from the possibility of monitoring the lactate. Tendency to hypocapnia during one hour monitoring of $p\text{CO}_2$ was not clinically associated with signs of pronounced hyperventilation. The probands were in a cheerful mood and partially logorrhic, which could obscure a mild hyperventilation syndrome. We did not do measurement of ventilatory functions of lung. The aim of our work was not a closer monitoring of mental function after a single small dose of ethanol. In current literature the impact of ethanol on central and peripheral nervous system and other organs is often discussed (2, 9, 12, 13, 14).

In outpatient clinical practice we sometimes meet patients who recently drunk a small amount of alcohol. Similarly, especially in traumatology, accidents often arise due to alcohol consumption. Low blood values of alcohol can be present in a patient who is admitted to a clinic for various unexpected acute conditions. The ABB values are assessed for essential diagnostics or its dynamics. In such cases it is not common to detect blood values of alcohol. The authors find important to document even "small" intake of alcohol which may be the cause of ABB shift to a pathological range. The doctors should always keep this in mind when doing the diagnostic statement.

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GENETIC DETERMINATION AND IMMUNOPATHOGENESIS OF TYPE 1 DIABETES MELLITUS IN HUMANS

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Abstract

Type 1 diabetes comprises autoimmune-mediated and idiopathic beta-cell destruction of the pancreatic islets of Langerhans[†] resulting to absolute insulin deficiency. Susceptibility to T1D is influenced by both genetic and environmental factors. It is generally believed that environmental agents, such as viral infections, dietary factors in early infancy or climatic influences, trigger disease development in genetically susceptible individuals. Many candidate regions for diabetes genes have been reported, the insulin, glutamic acid 65, insuloma associated antigen 2, and zinc transporter ZnT8 genes being among the most important. The destruction of b cells is mediated mainly by cellular mechanisms; antibodies seem to reflect the ongoing autoimmune process only, not being directly involved in tissue damage. They, however, appear prior to the onset of insulin deficiency what makes us to profit from in prevention of the disease.

Keywords: type 1 diabetes mellitus, autoantigens, autoantibodies, regulatory T cells, HLA alleles.

INTRODUCTION

Diabetes mellitus is a group of metabolic disorders characterised by chronic hyperglycemia, with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, action, or both. Diabetes mellitus (DM) is a leading cause of blindness, amputations and end-stage renal disease, and is a major factor contributing to cardiovascular diseases and premature death. In 1979, the first general institutionalised classification of DM was accepted from ADA (American Diabetes Association), EASD (European Association for the Study of Diabetes) and NIH (National Institutes of Health). The insulin-dependent DM and non-insulin-dependent DM forms of diabetes mellitus became accepted. Later, the classification was updated. The four categories were proposed: type 1, type 2 (with a major component of insulin resistance, defined as non-insulin dependent diabetes mellitus), other types of diabetes (e.g. the maturity-onset diabetes of youth) and gestational diabetes (1) (Table 1).

Type 1 diabetes mellitus (T1D) is a condition caused by an autoimmune response against insulin-secreting b-cells of the pancreas resulting in their destruction. This autoimmune destruction is irreversible and the disease incurable. If new pancreas or islets are transplanted they too are destroyed, unless heavy immunosuppression is applied. T1D is usually characterised by acute onset and dependence on exogenous insulin for survival. In children, beta cell destructions is almost complete, in adults there remains a residual cell function for some years; this form was denominated "latent autoimmune diabetes" (LADA) (2,3,4).

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Table 1 Diabetes mellitus classification
(ADA 2010, modified)

I. Type 1 diabetes
A, Immune mediated B, Idiopathic
II. Type 2 diabetes
III. Other specific types A. Genetic defects of beta cell function 1. – 6. MODY1 – MODY 6 7. Mitochondrial DNA 8. Others B. Genetic defects in insulin action C. Diseases of the exocrine pancreas D. Endocrinopathies E. Drug or chemical-induced F. Infections G. Uncommon forms of immune-mediated diabetes 1. “Stiff man” syndrome 2. Anti/insulin receptor antibodies H. Other genetic syndromes
IV. Gestational diabetes mellitus MODY – maturity onset diabetes of youth

GENETICS

Type diabetes mellitus is a genetically determined disease and many genes or genetic regions were found to be associated with its induction (for reviews see 5,6,7, www.t1dbase.org). Several genome-wide linkage studies have been conducted to identify candidate regions that may contain unidentified susceptibility genes. About 50 candidate regions for diabetes genes have been reported in linkage studies of affected sib pairs. Most of the known or suspected susceptibility loci have been designated IDDM, e.g. IDDM1 refers to genes mapping to the HLA region at 6p21, IDDM2 to the insulin region at 11p15, etc. (8,9) (Table 2).

HLA genes account for approximately 40% of the genetic risk for the T1D development. The major determinant of genetic susceptibility to autoimmune diseases resides in the HLA class II region. Individuals with the highest risk for type 1A diabetes express both predisposing haplotypes: DQA1*05:01-DQB1*02:01 (DQ2), which is almost always inherited with DRB1*03:01 (DR3) and DQA1*03:01-DQB1*03:02 (DQ8), inherited with DRB1*04:01 or DRB1*04:02 (DR4) (9,10,11,12). These individuals have been referred to as DR3/DR4, DQ2/DQ8 heterozygotes. The genotype, DQ2/DQ8 (DR3/DR4), is commonly observed in type 1 diabetics. Individuals who carry this high risk haplotypic combination have ~ 5% absolute risk of T1D, however within affected families, this genotype shows ~ 20% risk; approximately 40% of diabetic children possess this genotype compared to 2% of children in the healthy population (7,10,13). Observations of transmission frequencies of particular haplotypes have helped to illustrate the importance of certain haplotypes in contributing to diabetes susceptibility. For example, analysis of the Human Biological Data Interchange (HBDI) family collection has revealed that DQA1*05:01-DQB*02:01 and DQA1*03:01-DQB1*03:02 are transmitted to more than 80% of diabetic children (9).

HLA-alleles have also been associated with protection from type 1A diabetes, esp. the haplotype DQA1*01:02/DQB1*06:02/DRB1*15:01 (DQ6, DR15) confers strong protection. Evidence suggests that such protection may be mostly encoded by the DQB1*06:02 allele;

Table 2 T1D susceptibility loci

Locus	Region	Locus	Region
IDDM1 (<i>HLA</i>)	6p21	IDDM11	14q24.3-14q31
IDDM2	11p15	IDDM12 (<i>CTLA-4</i>)	2q31-q33
IDDM3	15q26	IDDM13	2q34-q35
IDDM4	11q13	IDDM15	6q21
IDDM5	6q25	IDDM16 (<i>IgH</i>)	14q32
IDDM6	18q12-q21	IDDM17	10q25
IDDM7	2q31-33	IDDM18 (<i>IL-12p40</i>)	5q33
IDDM8	6q27		
IDDM9	3q21-q25	PTPN22	1p13
IDDM10	10p11-q11	?	7q25
		?	16q22-q24
		?	1q42
		?	8q22-q24

CTLA-4 – cytotoxic T lymphocyte antigen 4, HLA – major histocompatibility complex of man, IDDM – insulin dependent diabetes mellitus, IgH – heavy chain of immunoglobulins, IL – interleukin, INS – insulin, PTPN – protein tyrosine phosphatase non-receptor type 22

even in individuals heterozygous for one of the major HLA T1D-associated genes. Further, the rare T1D subject who does carry DQB1*06:02 tends to develop disease at a later age, even in subjects who are positive for T1D autoantibodies (7,14). To summarise, as some HLA haplotypes are associated with high, moderate, low risk and even “protection”, their identification is useful in disease prediction (Table 3).

There are also some reports on associations between the last group of class II HLA alleles and T1D. HLA-DPB1*0101, -DPB1*0301, and -DPB1*0202, respectively, were reported to be positively and HLA-DPB1*0402 negatively associated with (15,16,17).

The major role of HLA-associated genetic susceptibility is related to their central role in recessive (central) tolerance induction, antigen presentation and activation of T helper cell mediated immune response. High probability for loss of tolerance to specific islet peptides appear to depend upon the manner in which specific peptides bind to class II-presenting molecules and are recognised by T cell receptors. Poor peptide binding due to only partially filling the MHC groove is probably responsible for escape of autoreactive T cells from the thymus. In the periphery, an abundance of particular self-antigens, such as insulin in the islets, or modification of the antigen, should allow for sufficient presentation of odd binding epitopes to autoreactive T cells (18).

The second well established T1D susceptibility locus was mapped to the chromosome 11p15.5, to the **insulin** promoter region (IDDM2). The region is typical by its variable num-

Table 3 Spectrum of diabetes risk HLA haplotypes

High risk haplotypes			
DR3	DRB1*03:01	DQA1*05:01	DQA1*05:01
DR4	DRB1*04:01	DQA1*03:01	DQA1*03:01
	DRB1*04:02	DQA1*03:01	DQA1*03:01
	DRB1*04:05	DQA1*03:01	DQA1*03:01
Moderate risk haplotypes			
DR1	DRB1*01	DQA1*01:01	DQB1*05:01
DR8	DRB1*08:01	DQA1*04:01	DQB1*04:02
DR9	DRB1*09:01	DQA1*03:01	DQB1*03:03
<i>Protective haplotypes</i>			
Strong protection			
DR2	DRB1*15:01	DQA1*01:02	DQB1*06:02
DR6	DRB1*14:01	DQA1*01:01	DQB1*05:03
DR7	DRB1*07:01	DQA1*02:01	DQB1*03:03
Moderate protection			
<i>DR5</i>	<i>DRB1*11:01</i>	<i>DQA1*05:01</i>	<i>DQB1*03:01</i>
Weak protection			
<i>DR4</i>	<i>DRB1*04:01</i>	<i>DQA1*03:01</i>	<i>DQB1*03:01</i>
	<i>DRB1*04:03</i>	<i>DQA1*03:01</i>	<i>DQB1*03:02</i>
<i>DR7</i>	<i>DRB1*07:01</i>	<i>DQA1*02:01</i>	<i>DQB1*02:01</i>

ber of tandem repeat (VNTR) polymorphism. There are three classes of the VNTR polymorphism: VNTR I contains 26–63 repeating units (5'- - - ACAGGGGTGGTGGGG - - - 3'), VNTR II 80 units, and VNTR III 140-210 units, respectively. Homozygous individuals with short VNTR elements develop T1D more likely than those with VNTR III; VNTRII is associated with resistance to the disease induction. The VNTR III genotype is associated with high expression of thymic proinsulin in thymic medullary epithelial cells, suggesting a correlation between antigen level and negative selection of high-avidity autoreactive T cells, consistent with a prominent role for recessive (central) tolerance in protection from insulin autoimmunity (6,14). Recently, it has also been demonstrated that these individuals harbour a higher frequency of insulin-specific T cells by tetramer analysis when compared to individuals with the protective insulin VNTR element (19).

The next T1D susceptibility genetic region, IDDM12, is located on the long arm of chromosome 2 (2q33) and contains the gene coding for **CTLA-4** (*cytotoxic T lymphocyte antigen-4*). CTLA-4 plays an important role in regulation of the immune response, esp. in suppressive activities of FOXP3⁺CD4⁺CD25⁺ T regulatory cells. The genetic analyses and correlation

of CTLA-4 gene haplotypes with messenger RNA levels suggest that it is a reduction in the amount of soluble CTLA-4 that is responsible for the increased susceptibility to T1D in T cells (20), including nTreg cells (21).

PTPN 22 (*Protein tyrosine phosphatase non-receptor type 22*; chromosome 1p13) is the fourth well established human DM 1A susceptibility gene. It encodes a lymphoid protein tyrosine phosphatase playing an important role in negative control of T cell activation and development (22,23). In particular, it is critically involved in signaling of cytokines, such as IL-2 and IL-15. The T1D-associated variant of PTPN2 leads to diminished phosphorylation of STAT5 in T cells, interfering with cytokine signaling, raising the possibility that this variant may interfere with nTreg cell survival, which is highly dependent on IL-2 activity (24).

Interleukin 2 (IL-2) has paradoxical functions in T cells homeostasis, acting as a potent T cell growth factor during the initiation of immune responses and having a crucial function in the termination of T cell responses and maintenance of self-tolerance. The latter function has been proposed to be due to a requirement for IL-2 signaling for the development and function of natural regulatory T cells. IL-2 could therefore take part in T1D pathogenesis. Really, it was reported that the region containing CD25 (IL-2RA; a chain of IL-2 receptor) on chromosome 10p14 was associated with the T1D (25). It is of interest that human linked T1D susceptibility segregates with 30%–50% decrease in the expression of CD25 pre-mRNA and surface protein on activated-memory T cells and, to a lesser extent, nTreg cells. Moreover, lower expression of CD25 in memory T cells from individuals with IL2RA susceptibility genotypes is associated with lower IL-2 secretion, which might synergise with lower expression of CD25 on nTreg cells to impair their function (26,27). Because IL-2R signaling plays a key role in nTreg cell biology (28) and both the murine IL-2 and human IL-2RA associations to T1D correlate with reduced expression of IL-2 and IL2RA, respectively, they may both operate by impairing nTreg cell development or function. Really, a recent study supports it as it found that defects in functional IL-2 signaling had an effect of FOXP3 expression (24). Thus, CD25 defects in patients with T1D may be mapping to a similar pathway as that, which leads to T1D in subjects with IPEX¹.

More recently, the **TLR7-TLR8** region of chromosome Xp22.2 has been mapped in T1D (7, www.t1dbase.org). These genes encode intracellular receptors for viral RNAs and for DNA and RNA from apoptotic cells (29). This finding suggests that a variety of viral infections could, via increased type 1 interferon levels, enhance susceptibility to autoimmune b cell destruction and T1D, provided that susceptibility alleles at other loci are present. Seasonal differences in viral infections, combined with other seasonal effects such as reduced vitamin D levels in more northern countries during the winter months (30), could help to explain the well-established seasonality of T1D diagnosis itself.

A role of viruses in T1D development is further supported by the report of its association with IF1H1 (interferon induced with helicase C domain 1) gene (31). IF1H1 codes for the pattern recognition receptor **MDA5** (melanoma differentiation associated gene 5). It recognises certain types of double stranded RNAs, which are commonly produced during the replication of some RNA viruses. MDA5 is triggered also by enteroviruses, known as possible environmental triggers or accelerators of islet autoimmunity leading to type 1 diabetes (32).

The association of **enteroviruses** (including entero-, echo- and coxsackie viruses) with T1D has attracted attention when reported that enterovirus infections had been diagnosed in 51% of cases and 28% of controls in the six months before the development of islet antibodies (33,34). It was suggested that these viruses could stimulate a b-cell autoimmune response. Sequence similarity exists between the Coxsackie viral protein 2 (amino acid residues 249–279) and GAD65. This amino acid sequence homology is highly conserved in

¹ IPEX is an immunodeficiency syndrome caused by mutations in FOXP3 gene (Xp11.23-q13.3.), which results to a breakdown of natural regulatory T cells (nTregs) development. It manifests itself by various autoimmune disorders, among them also with T1D.

Coxsackie B4 isolates, and the relevant peptide has been found to bind to HLA-DR3 but not HLA-DR4 (9,35). It was thought that these viruses induced activation and proliferation of T cells specific for the viral epitope that mimics a protein unique to b cells (phenomenon of molecular mimicry) causing cytotoxic T cells to respond to the b cells autoantigens. However, further studies have proved that enteroviruses could be potentially causal at DM 1A onset, but not at the initiation of islet autoimmunity (36,37). The work of Winkler et al. (2011) further confirms this opinion (31). They, by examining individual islet autoantibodies, found no association with the development of IAAs or GADAs, which appear early in the disease process, and borderline association with IA-2As (31). It seems therefore that the development of T1D autoantibodies is strongly influenced by the HLA class II genotype, whereas progression to diabetes is rather connected to infections. So initiation of autoimmunity and progression to disease might be mediated by different mechanisms, first by failure of tolerance induction and second by molecular mimicry.

There are also some genes that confer resistance to T1D development. Except already mentioned HLA-DQ6, there is a form of IL-7R alpha gene (38) and a delta 32 variant of CCR5 (39) that were reported to be associated with protection, from T1D.

Genes not associated with T1D can also inform on its immunopathogenesis. In Crohn's disease, psoriasis, and other diseases a set of susceptibility loci encoding candidate genes with known functions in the T_H17 pathway have been reported (40,41). Using T1D database (www.t1dbase.org) and the current SNP association results, however, there is no evidence that these " T_H17 genes" are associated with T1D. Hence, there is no support for a primary etiological role for this T cells subset in T1D. Also other genetic regions, such as 4p15.5, 6q22.32, 7p15.2, 7p12.1, 10q23.31, 14q24.1, 16q23.1, 19q13.32, 20p11, 22q12.2, and Xq28 are known not to contain T1D functional candidate genes (7).

There are reports on other associations and one can expect that the genetic T1D map will be completely filled up in the near future.

AUTOIMMUNE MECHANISMS IN DESTRUCTION OF BETA CELLS OF PANCREAS

Type 1 diabetes mellitus results from progressive loss of pancreatic islet mass through autoimmunity targeted at diverse molecules that are expressed in the pancreatic β cells. This chronic destructive process involves both cellular and humoral components of immunity. Once autoimmunity appears (evidenced by islet autoantibodies and progressive loss of insulin secretion) decades can elapse before the appearance of hyperglycaemia (9,42).

The two distinctive features of type 1A diabetes are the infiltration of pancreatic islets by macrophages and lymphocytes and the presence of autoantibodies to various b cells antigens in the plasma (43,44). Islet inflammation or insulitis is the hallmark lesion of T1D. Mononuclear cells, initially macrophages and dendritic cells accumulate first around the islets. Subsequently, B and T cells (both helper and cytotoxic) arrive and form a periinsulitis. Insulitis occurs when macrophages and activated T cells invade the islets of Langerhans[†] in the pancreas and attack the insulin-secreting β cells. Insulitis occurs during the pre-diabetic phase ultimately leading to the complete depletion of β cells (35,45).

By far, T cells have been the one immune cell population that has consistently been shown to be critical for disease pathogenesis (46). T cells are known to infiltrate the islets in both rodent models of disease (NOD and BB) and constitute a substantial fraction of infiltration observed in humans (47). Removal or the complete absence of mature T cells in the two rodent models prevents diabetes (48,49). The pathogenic process is largely believed to be dependent upon the presence of autoreactive $CD8^+$ and $CD4^+$ T cell subsets. It is thought that $CD8^+$ T cells may be more important during the early phases of pathogenesis, whereas $CD4^+$ T cells are required throughout (42,48,50).

Infiltration of autoreactive T cells into the islets is essential for the development of diabetes. Studies performed in NOD mice have revealed that the influx of T cells into the pan-

creas is associated by *de novo* formation of lymphoid follicles what is a prototypic feature of a chronic progressive inflammation. The experimental results show that the interaction between membrane lymphotoxin (LTA1 β 2) of T cells and its receptor (LT β R) in membranes of stromal cells is essential for the development and maintenance of this lymphoid microenvironment. Lymphotoxin knockout mice and wild-type mice treated with LT β receptor-immunoglobulin fusion protein (LT β R-Ig) reverse insulinitis and prevent the formation of lymphoid follicles, even when insulinitis is well established (51). This effect is likely caused by a failure to induce lymphoid tissue chemokines and adhesion molecules (52). In humans, LTA gene is polymorphic. From this point of view is interesting that LTA*1 allele is in a linkage disequilibrium with HLA-A1, -B8, -DR3, the haplotype predisposing its carriers to T1D development (53).

The autoimmune process is initiated by activation of autoreactive T cells that escaped thymic negative selection and populated the peripheral lymphoid organs. Unknown insults trigger cell autoantigens shedding into the milieu. Antigen-presenting cells (APCs, i.e. dendritic cells, macrophages, and B cells) engulf them and start to migrate to the pancreatic lymph nodes (LNs). During their migration they process autoantigens and their split products, peptides, bind to groove of HLA molecules. When in LNs, they prime naive autoreactive CD4⁺ and CD8⁺ T cells. CD8⁺ T cells recognise peptide-HLA class I (pHLA) complexes in membranes of APCs and in the presence IL-2 and other cytokines, produced by autoreactive CD4⁺ T helper cells, become activated. They expand, differentiate into mature cytotoxic T cells, and migrate via the bloodstream into pancreatic islets, where they cause cell damage (18).

The resolution of severe insulinitis by LT β R-Ig treatment (51) suggests that migration of inflammatory cells into the islets is a dynamic process in which these cells may constantly move in and out of the target tissue what results in continuous activation of autoreactive T cells by previously unrecognised autoantigens (epitope spreading) and tissue damage.

Many individuals with immunological markers of pre-clinical T1D do not progress to clinical disease. A possibility exists that such individuals may develop a successful regulatory immune response. However, a number of contradictory observations are reported in this field. Regulatory T cell numbers have been reported to be decreased or normal in T1D (54,55,56), and functional assays have similarly described low, slightly decreased, or normal regulatory activity (57,58,59). Much of these controversial findings results probably from methodological differences in various laboratories.

Macrophages by their production of reactive oxygen species and nitric oxide may have a destructive effect too. Activated macrophages produce also proinflammatory cytokines, esp. TNF and IL-1 (60), which are directly cytotoxic to β cells (61). Moreover, β cells themselves may be a source of IL-1 too, particularly in response to glucose, suggesting a destructive cycle in which hyperglycemia induces expression of the inflammatory mediators that results in immune activation and a further β cell destruction (62).

Type I interferons (IFNs) secreted during early viral infection act in a paracrine manner to lower the permissiveness of neighbouring and distant cells to viral infection. During certain viral infections, early innate production of IFN- γ may also contribute to the transition to an antiviral state. However, the IFN response not only lowers cell permissiveness to viral (e.g. Coxsackie viruses B4) infection, but also contributes to the escape from NK cell-dependent killing. These data raise the possibility that cell responses elicited by interferons preserve self-tolerance of NK cells to pancreatic cells during a viral infection. Defective cell antiviral defences could therefore augment cellular damage and the release of otherwise sequestered cell antigens, providing a rich pool of epitopes to prime self-reactive lymphocytes and initiating autoimmunity (63).

iNKT cells are a population of T cells that share some characteristics with natural killer (NK) cells. The key features characteristic of iNKT cells include heavily biased T-cell receptor gene usage (Va24J α Q and V β 11, so called Va24*i*-NKT cells), CD1d restriction and high levels of cytokine production, particularly IL-4 and IFN- γ . Because of this property, NKT

cells participate in regulation of the immune response; they can skew it into either T_H1 or T_H2 direction and so substantially contribute to the development of autoimmune processes. Several reports on iNKT cell analysis in type 1 diabetic patients have been published and there is no consensus since some authors described a decreased frequency and function of iNKT cells in these patients but it has not been confirmed by other investigators (64,65). Despite this complexity of iNKT cell analysis in humans, it has been extensively shown that manipulations of iNKT cells prevent and even cure T1D in various mouse models (66).

Although T cells are the main players in the T1D field, B cells contribute to its pathogenesis too. NOD mice deficient in B cells, but displaying normal numbers of T cells, are prevented from developing disease (67). Treatment of both man (68) and NOD mice displaying the human CD20 molecule (69) with a B cell-specific anti-CD20 antibody alters progression to diabetes, again demonstrating the importance of B cells. Since B cells cannot transfer neither insulinitis nor diabetes, they have been considered to fulfil a non-destructive, function. The role of B cells by way of their function as antigen-presenting cells in T1D etiology is now widely accepted (68). Nevertheless, autoantibodies can be detected in the plasma in 80% of patients; their appearance reflects the progression of autoimmunity to β cells, their number is positively correlated with the evolution rate of autoimmune destruction. The presence of autoantibodies to just one of the four antigen groups alone is associated with only a marginal increase in risk. However, T1D risk is markedly increased when islet autoantibodies to two or more of the antigen groups are found in a child. In young, first-degree relatives the 5-year risk of diabetes is of the order < 25%, 25–50%, and > 50% if they have autoantibodies to one, two, and three islet autoantigens, respectively. In particular, the presence of antibodies to IA-2 (or ICA512 or IA-2b or PHOGGRIN) is associated with highest risk (14,42,70) (Table 4).

Table 4 Type 1 diabetes risk

Risk categories			
Low	Intermediate	High	Very high
Single islet antibodies with low affinity	Single islet antibodies with high affinity	Two or three islet antibodies	Four islet antibodies; higher titers
Older age	Young age	Young age	Young age
Non-susceptible HLA genotype	Susceptible HLA genotype	Susceptible HLA genotype	Susceptible HLA genotype
			Impaired glucose tolerance

Moreover, the earlier autoantibodies appear, the faster the rate of progression to diabetes (71). Longitudinal studies of children born with high genetic risk of T1D showed that circulating autoantibodies to islet antigens can be seen as early as 6 months. A large proportion of childhood cases has seroconverted by the age of 3 years, and almost all cases by the age of 7 years, even although median age-at-diagnosis is approximately 12 years (4).

There is, however, no evidence that autoantibodies play a direct role in the pathogenesis of T1 D. Evidence against autoantibodies contributing directly to β -cell damage comes from studies of infants born to mothers with type 1 diabetes: anti-islet autoantibodies of mothers are transferred to the infants transplacentally, yet these infants do not develop disease. Moreover, about 10% of patients have no detectable antibodies at diagnosis (9).

Pancreatic β cell components that are recognised by autoantibodies include mainly insulin or proinsulin, GAD65, IA-2, and ZnT8. **Insulin** was the first autoantigen identified in T1D

and is still the only β cell-specific. Insulin is derived from the enzymatic removal of a connecting peptide (C-peptide) from proinsulin within islet secretory granules and C-peptide is secreted along with insulin. In general, autoantibodies react to mature insulin, whereas specific proinsulin autoantibodies have been difficult to demonstrate. Antibodies against insulin (IAA) are ones of the earliest clinical markers of prediabetes. They can be detected in the sera of some 60% of newly diagnosed patients prior to insulin therapy. The presence of IAA is inversely correlated with age of diabetes onset, with almost 100% of newly diagnosed children less than 5 years of age expressing IAA, compared to less than 20% of those diagnosed after 15 years of age. Moreover, the levels of IAA are associated with the rate of the autoimmune destruction of the β cells making their detection an important aspect of diagnosis and prevention (42).

Glutamic acid decarboxylase of M_r 65,000 (GAD65) is an enzyme that converts glutamate to γ -amino butyric acid (GABA). It is expressed in human β cells (but also by α , and δ cells of the islets) and represents the major target autoantigen in type 1 diabetes. Antibodies to GAD65 can be detected in the sera of 75% of new-onset T1D patients. When GAD65 antibodies coexist with additional diabetes specific antibodies, they indicate a high risk; only GAD65-positive patients are slow progressors. LADA patients present frequently anti-GAD65 antibodies only (72).

60-75% of new-onset T1D patients have autoantibodies against the islet cell antigen 512 (ICA512 or IA-2), compared to only 2% of healthy controls. ICA512 is a tyrosine phosphatase-related molecule (it has no enzymatic activity) expressed in the pancreas and the brain. The molecule is produced in two alternative splice variants, with the variant usually termed IA2b (phogrin). Autoantibodies usually react with both variants, although about 10% of sera of new-onset patients reacts with one or the other (9). The presence of antibodies to IA-2 is associated with highest risk (14).

Zinc transporter ZnT8 was reported as a new T1D specific autoantigen recently. The ZnT8 molecule is within the membrane of islet β cell secretory granules and transports zinc where it forms a complex with insulin. Autoantibodies were detected in 60-80% of new-onset T1D compared with < 2% of controls. Individuals followed from birth to T1D showed ZnT8 antibodies as early as two years of age and generally emerged later than GADA and IAA. The combined measurement of ZnT8A, GADA, IA-2, and IAA raised autoimmunity detection rates to 98% at disease onset, a level that approaches that needed to detect a possible development of the disorder in a general children's population (73).

The above-mentioned autoantibodies appear in a sequential manner rather than simultaneously, with insulin and GAD65 usually appearing first. This appearance reflects the progression of humoral autoimmunity to β cells. Longitudinal studies of children born with high genetic risk of T1D showed that circulating autoantibodies to islet antigens can be seen as early as 6 months. A large proportion of childhood cases has seroconverted by the age of 3 years, and almost all cases by the age of 7 years, even although median age-at-diagnosis is approximately 12 years (7). The earlier autoantibodies appear, the faster the rate of progression to diabetes (71).

Childhood diabetes is characterised by early appearance of autoantibodies. Observations show that a second peak incidence period appears around puberty. The characteristics of the islet autoantibody profiles seen in "late" autoantibody-positive children are heterogeneous, and the typical profile is different compared to that seen in the 1 to 2 year olds. Autoantibodies to single antigen groups, typically, insulin or GAD65, without spreading to other islet proteins, is common. Lower-affinity IAAs or GAD65 antibodies are also more common, as are antibodies directed against atypical epitopes. Whereas early autoantibody development is strongly linked to T1D-associated HLA class II genotypes, the distribution of HLA class II genotypes in children who develop islet autoantibodies late is less dominated by HLA-DRB1*04; DQB1*03:02 genotypes. This trend toward less HLA dominance and more heterogeneous antibody profiles is also pronounced in older individuals developing autoantibodies who lack a family history of T1D (14) (Table 5). One interpretation of the differences

Table 5 Comparisons of neonate and puberty autoimmunity

Neonate autoimmunity	Puberty autoimmunity
Insulin/proinsulin reactive	Single antibodies to insulin or GAD65
DR3/DQ2 or DR4/DQ8 more common	Lower DR3/DQ2 or DR4/DQ8 prevalence
Early and frequent autoantibodies to GAD65, IA-2, and ZnT8	Less IA-2 and ZnT8 autoantibodies
High affinity IgG1 autoantibodies	Presence of low affinity autoantibodies

associated with age of islet autoantibody appearance is that aetiology and immunisation are different, i.e. events that lead to an insulin-dominant spreading autoimmunity at the age of 1 year are different from those that lead to a GAD65-restricted autoimmunity at the age of 11 years or later. An intriguing possibility is that immune response thresholds for islet autoimmunity during childhood are programmed during foetal life.

CONCLUSION

It is now clear that T1D is caused by a complex autoimmune process that is triggered by ill-defined environmental factors in permissive genetic backgrounds. A constellation of gene variants scattered throughout the genome, generally operating via relatively small changes in expression and/or function, provides the basis for series of immune events that involve virtually every cell type of the immune system and eventually culminate in near complete loss of β cells. Understanding this complexity is essential in the quest for a cure. Arrival of sophisticated technologies, techniques, refinement of statistical methods will surely bring new knowledge and will bring us to this goal.

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COEXISTING DEPRESSION AND ANXIETY: CLASSIFICATION AND TREATMENT

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Abstract

Despite of the fact, that comorbidity of depression and anxiety is a frequent condition in clinical practice, current psychiatric classification systems (according to WHO-ICD 10 and according to APA-DSM IV-TR) are not taking this reality into account sufficiently. The concept of anxious depression is very important for clinical practice. Recommended guidelines and algorithms of treatment based on evidence based medicine (EBM), established mainly on randomized controlled trials are designed separately for depression and separately for anxious disorders. This presents very often a significant complication in clinical practice. The aim of this article was to bring the concept of anxious depression to closer attention with highlighting of possible therapeutic approaches.

Key words: depression, anxiety, classification, treatment

INTRODUCTION

Depression is a common mental disorder that affects about 121 million people worldwide, it is the leading cause of disability as measured by YLDs (years lived with disability) and is already the 2nd cause of DALYs (Disability Adjusted Life Years) in the age category 15-44 years for both sexes combined. Depression, at its worst, is associated with the loss of about 850,000 lives every year (1).

The prevalence of depression in Europe is estimated in 10.05% for women and 6.61% for men (2).

The definition of depression varies according to the classification system. According to the DSM-IV the illness defined as depressive disorders include major depressive disorder, dysthymia and depressive disorders not otherwise specified (3). And according to the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) is part of mood (affective) disorders in which is included bipolar affective disorder, current episode of depression; depressive episode, recurrent depressive disorder, cyclothymia, dysthymia and unspecified mood disorders (4).

Illness currently defined in DSM-IV as anxiety disorders includes panic disorder, obsessive compulsive disorder, posttraumatic stress disorder (PTSD), generalized anxiety disorder, social phobia or social anxiety disorder and agoraphobia (3). The ICD-10 classifies as phobic anxiety disorders: agoraphobia, social phobias, specific (isolated) phobias, other phobic disorders and unspecified. Other anxiety disorders in the ICD-10 includes: panic disorder, generalized anxiety disorder, and mixed anxiety and depressive disorder, other mixed anxiety disorders, other specified anxiety disorders and unspecified; obsessive-compulsive disorder, reaction to severe stress and adjustment disorders. General anxiety disorder is one of the most frequent (up to 10%) of all mental disorders seen in primary care and they are high utilizers of all health care resources (5).

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COMORBIDITY

Comorbidity of depression with other psychiatric disorders is the rule, not the exception, in clinical practice. Often patients with depression have a comorbid anxiety disorder, and the converse is true as well (6). Studies of adults suggest that both disorders often share common risk factors and that similar interventions may be effective (7).

Closely related to anxiety and depression is also somatization and somatoform disorders, where characteristics are multiple physical complaints that are not caused by or cannot fully be explained by a known nonpsychiatric medical condition. The physical complaints include pain in different areas and symptoms other than pain involving gastrointestinal tract, sexual/reproductive system, respiratory system, dermatologic and neurologic system. Stekel refers to it also in terms of organ speech of the mind, meaning the organic expression of mental processes. It is as if the organs of the body were translating into a psychopathological language the mental troubles of the individual (8).

At least a third of patients with somatoform disorders have comorbid anxiety or depressive disorders, whereas depression and anxiety co-occur with one another up to 50% of the time (9).

PREVALENCE

Several studies focused on comorbidity of depression and anxiety were made.

According to a study by Aartijan et al. (7), high levels of comorbidity were found among major depressive disorder and anxiety disorders (47.5% of those with major depressive disorder had comorbid anxiety disorders; 26.1% of those with anxiety disorders had comorbid major depressive disorder). In a comparison of specific anxiety disorders, comorbid major depressive disorder was more common in panic disorder and Obsessive-Compulsive Disorder - OCD (50.0% and 44.4%, respectively) than in generalized anxiety disorder and social phobia (30.3% and 25.0%, respectively) (7).

Another study shows that 75% of depressed patients had comorbid anxiety, somatization or both; 57% of the patients with anxiety had comorbid depression, somatization; or both and 54% of the patients with somatization had comorbid depression, anxiety or both (9).

Although it has been repeatedly confirmed that concurrent occurrence of depressive and anxious symptomatology is often, the concept of anxious depression is not sufficiently incorporated into the classification and appropriate therapeutic recommendations of this comorbidity are lacking.

Additionally, the probability that depression will be comorbid with well defined anxiety syndromes is nearly twice as high as probability that depression will be comorbid with alcohol dependence (10).

IMPORTANCE

Each of these disorders is associated with substantial functional impairment, increased disability days and elevated health care costs, but the contribution of the commonalities of depression, anxiety and somatization to functional impairment substantially exceeded the contribution of their independent parts (10).

Individuals who suffer from both anxiety and depression appear to experience a more chronic course and more impairment of social and occupational functioning than individuals with either anxiety or depression alone (10).

Comorbidity of depression and anxiety should be considered for several reasons:

a) *Bigger severity*: The group with anxiety disorders plus major depressive disorder may represent those with a more severe disorder (7).

b) *Worse outcome*: If not treated properly, patients with comorbid anxiety and depression have a worse outcome than patients with either illness alone. Thus, the most effective treatment requires attention to symptoms of both anxiety and depression (6).

c) *Withdrawal from treatment and decreased response to treatment*: Several studies have found that greater severity of anxiety symptoms is associated with an increased risk of withdrawal from treatment, a decreased response to acute antidepressant treatment, and a longer time to both response and remission (11).

d) *Risk of recurrence*: High pretreatment levels of anxiety symptoms increase not only the risk of non-response in acute treatment of late-life depression but also the risk of recurrence of the disorder in the first 2 years after response to treatment (11).

e) *Higher number of symptoms*: In addition, patients with chronic medical illness and comorbid depression or anxiety compared to those with chronic medical illness alone report significantly higher numbers of medical symptoms (12).

Syndrome overlap explanation:

1. Depression, anxiety and somatization are defined by partially overlapping diagnostic criteria (3), which increase the likelihood of co-occurrence.

2. One syndrome might act as risk factor for the development of the other syndromes. For example, it has been shown that anxiety is a risk factor for the development of depression (13).

3. Depression, anxiety and somatization might emerge from shared psychosocial and biological diatheses. In fact, there is evidence to suggest that many of the phenomena of the somatoform disorders are caused by clustering of psychiatric syndromes or their incomplete or atypical manifestations and a low sensation threshold (14).

CLASSIFICATION

The first category of the newer classification system, which is currently used in DSM-IV as well, is a diagnosis of major depressive disorder in addition to a diagnosis of panic disorder, social phobia, generalized anxiety disorder, obsessive-compulsive disorder, or post-traumatic stress disorder.

The second category is a diagnosis of mixed anxiety-depressive disorder, which can only be made if the criteria for any other anxiety or mood disorder are not currently met (10). This diagnosis reflects the current, usually long lasting presence of mild depressive and mild anxiety symptomatology. Etiologically, it is neurotic, stress-induced impairment and thus, the diagnosis cannot be used for major depression-depressive episode with anxiety.

All or- nothing type of classification of mental disorders is a rather arbitrary process, which may hinder achieving better understanding of anxiety and depression (7).

The large syndrome overlap suggests that there may be a broader underlying construct consisting of depressive, anxiety, and somatic symptoms, that is, one disorder with three related types of symptoms rather than three distinct disorders. Indeed, some authors argue for a broader underlying disorder, labeled general neurotic syndrome or negative affect syndrome (9).

TREATMENT

Therapeutic approaches to comorbid depression and anxiety include pharmacologic and psychosocial options.

The pharmacologic choices include benzodiazepines, azapirones (non-benzodiazepine anxiolytics e.g. buspirone), antidepressants or combined treatment. Antidepressants are a safer alternative to benzodiazepines in patients who might become dependent on the latter agents (7).

It is generally believed that all antidepressant medications are approximately equally effective for the treatment of depression. However, these medications are not equally effective in the treatment of anxiety disorders. For example, the serotonin reuptake inhibitors are more effective than tricyclic antidepressants in the treatment of OCD, and monoamine oxidase inhibitors may be more effective than tricyclic antidepressants in treating social phobia.

Several antidepressant medications are indicated for the treatment of certain anxiety disorders, whereas other antidepressants have not been consistently shown to also be effective in treating anxiety disorders. Certain medications have acquired a reputation of being more or less anxiogenic or anxiolytic than others and pharmaceutical companies have developed promotional campaigns suggesting that some medications are particularly well suited for treating depressed patients with anxious features. While knowledge of the presence of an anxiety disorder in a depressed patient might influence the choice of medication prescribed, there are, in fact, few data to support suggestions that depressed patients with anxious features respond differently to the range of antidepressant medications (16).

Determination of the pattern of the depression (e.g. endogenous or melancholic, atypical, nonendogenous, agitated, retarded) can have important clinical implications. For example, atypical depression, which is characterized by hypersomnia / hyperphagia, weight gain, reversed diurnal variation (worse in the evening), reactive mood, and heightened interpersonal sensitivity, is preferentially responsive to the monoamine oxidase inhibitors (MAOIs), and possibly selective serotonin reuptake inhibitors (SSRIs), that to the tricyclic antidepressants (TCAs).

It is important to note though, that using of the MAOIs may have possible limitations resulting from their known adverse effects. This fact is predominantly referred to their older generation of nonselective and irreversible IMAOs (e.g. fenelzine, izokarboxazide), where diet free of tyramine was necessary and hypertension crisis occurred together with their hepatotoxic potential.

The newer generation of selective IMAOs from the end of the 1980-ties: reversible type A (moclobemide) and irreversible type B (selegiline), are considered as a safer alternative (15).

In evaluation of anxiety symptoms, many of the same considerations regarding depression apply, such as a review of other causes for anxiety symptoms. Determination of the presence of a specific anxiety disorder is important and can have implications for the choice of treatment also (19). Even when subdiagnostic anxiety symptoms exist with major depression, targeting the coexisting anxiety syndrome may be important.

Coexisting major depression and panic disorder: Panic disorder is characterized as an episodic paroxysmal anxiety. The efficacy of the available SSRIs (citalopram, escitalopram, fluoxetine, paroxetine, sertraline and fluvoxamine) in the acute treatment and long term maintenance of patients with major depression has been documented in several research trials (19). The SSRIs have also been used successfully in the treatment of uncomplicated panic disorder and are increasingly used as the first-line treatment for panic disorder (20). It is plausible, therefore, that these agents may be useful in the treatment of panic disorder with comorbid depression. The use of these agents is favoured especially in cases in which other antidepressants are not well tolerated (17). Worth mentioning is surely also group of SNRI agents (e.g. venlafaxine), which shows promising results in the treatment of coexisting major depression and panic disorder.

Coexisting major depression and social phobia: Social phobia is a disorder characterized by fear and avoidance of situations in which an individual may be scrutinized by other and fears that he or she will be embarrassed of humiliated (3). Social phobia is the third most common lifetime mental disorder in the general U.S. population (13.1%), following major depression (17.1%) and alcohol dependence (14.1%) (21). Major depression is frequently associated with social phobia, and conversely, social phobia is often complicated by major depression. From a differential diagnostic perspective, it is important to discriminate social phobia from the social withdrawal associated with depressive illness (18).

Treatment of the patients with social phobia is particularly important because of the significant impairment in functioning, social isolation, and depression that accompany it (22).

Based on the information currently available, patients with comorbid major depression and social phobia should probably receive antidepressant treatment. For patients suffering from comorbid atypical depression, MAOIs may be the optimal treatment. The SSRIs have been established as effective for patients with major depression (22), and, like the MAOIs, also appear to be more effective than the TCAs for patients with atypical depression (23). There is also growing evidence that SSRIs are beneficial in the treatment of social phobia. Thus, these agents may also have a therapeutic role in patients with coexisting major depression and social phobia. Because of the inconvenience associated with MAOI treatment, a trial with 1 or more SSRIs is a reasonable first treatment for major depression coexisting with social phobia. If the SSRIs are ineffective or not tolerated, after a washout of 2 weeks (or 5 weeks for fluoxetine), an MAOI such as phenelzine or tranylcypromine may be used. Addition of a high/potency benzodiazepines may be considered, if necessary (17).

Coexisting major depression and obsessive-compulsive disorder: A relatively small group of patients presenting with major depression also have OCD, but there is a strong association of OCD with depression. Rasmussen et al. (24), observed that 80% of a 44 OCD patient sample had a lifetime history of major depression; 30% met criteria for major depression at the time of evaluation.

The SSRIs are effective in the treatment of both depression and OCD (25). The first systematic study of comorbid major depression and OCD was reported by Hoehn-Saric et al. (26), in that study, which compared the SSRI sertraline, the TCA desipramine, and placebo, the SSRI was superior to desipramine and placebo in alleviating both depression and OCD; (although, there is evidence that clomipramine, another agent from the TCA group, has anti-obsessional effect and should be also considered as one of the possible treatment option). Generally, SSRIs appear to be superior in alleviating concurrent depressive and OCD symptoms; other anti-obsessionals have not yet been studied in rigorous fashion (17).

Coexisting major depression and posttraumatic stress disorder: Davidson et al. (27), evaluated lifetime diagnoses of 44 World War II and Vietnam veterans and found that 59% met lifetime criteria for major depression. Despite high rates of depression in posttraumatic stress disorder (PTSD) patients, antidepressants and psychotherapeutic techniques targeted at reducing depressive symptoms were less effective in depressed patients with PTSD than in patients with depression alone (28).

Based on clinical neuroendocrine challenge response differences between patients with major depression with and without PTSD, Southwick et al. (28), suggested that depressive symptoms in PTSD may not simply be manifestation of a concurrent major depressive episode, but rather may reflect either secondary or "nontypical" depression.

Since etiology of comorbid depression accompanying PTSD may differ significantly from the primary major depression, patients with PTSD probably require different, individualized treatment interventions (17).

Coexisting major depression and generalized anxiety disorder (GAD): Sanderson et al. (28), reported that over half of a sample of approximately 100 patients presenting for treatment of major depression or for dysthymia also had anxiety disorders; approximately 20% of each group had GAD. Other study evaluated 109 patients with GAD and found that 42% had experienced at least 1 major depressive disorder during their lifetime, with the first episode typically beginning shortly after the onset of GAD (30).

Benzodiazepines have traditionally been the treatment of choice in patients with GAD. However, coexisting depression and GAD raise questions regarding monotherapy with benzodiazepines in this patient subgroup. There is some evidence in the literature suggesting that the triazolobenzodiazepine alprazolam, may have specific antidepressant properties (31).

Based on the data, it appears that the TCAs or buspirone may have useful role in the treatment of GAD with prominent depressive symptomatology, in the same time, is suggested that patients with anxious depression respond well to the SSRIs and mirtazapine, NaSSA agent, which is noradrenergic and specifically serotonergic antidepressant with dual mechanism of effect (by increasing synthesis and releasing of noradrenalin and serotonin). These agents also appear to ameliorate depression-related anxiety of GAD associated with major depression (32).

At this point, prior treatment responses, patient preference, and side effect profiles should be considered in the treatment decision process (17).

In the group of patients with anxious depression, where the intensity of symptoms is not severe and the risk of suicidal tendencies can be excluded, the combined therapy with psychopharmacs and procedures of psychosocial treatment (e.g. rehabilitation, cognitive behavioral therapy, interpersonal psychotherapy and other treatments) may be applied.

Additionally, also effect of other agents from different categories has been studied as a considerable form of pharmacologic treatment.

As effective appear: pregabalin, newer nonbenzodiazepine anxiolytic and GABA precursor; antihistaminics, e.g. hydroxyzine; buspirone; other groups of antidepressants - trazodone, with anxiolytic-sedative and sleep-inducing effect; agomelatine; SNRI (venlafaxine, duloxetine); some of the antipsychotics are proved to have anxiolytic and antidepressive effect and are often used in clinical practice in combination with AD, also those from the *first generation (basal)*, but because of adverse effects (anticholinergic, adrenergic and other) they are used rather rarely and transiently in lower doses (e.g. chlorprothixen, levopromazine). More often are used the *second generation antipsychotics* in lower doses: benzamides - sulpiride, amisulpride, others like quetiapine, aripiprazole, risperidone, ziprasidone, olanzapine. If the cardiovascular symptoms of anxiety are prominent (tachycardia, palpitations, hypertension, but also tremor, hyperhidrosis, hyperventilation) beta-blockers may be effective; it is also considered anxiolytic - antidepressive effect of some of the antiepileptics, which could be possibly used as a therapy for patients with bipolar depression with anxious features (34).

From the natural substances effect of *omega-3-fatty acids* as adjuvant therapy of anxiety and mild depression is examined, as anxiolytics the possible effect have phytopharmaceuticals, e.g. *Kava Kava*, *Galphimia glauca* (Mexican natural medicine), *Passionflower* (passiflora), St. John's wort and others (34).

CONCLUSION

The presentation of patients with mixed symptoms of anxiety and depression is common in general medicine. It is estimated as the third most common ambulatory diagnosis in primary care practice (16).

The impairment and health care utilization associated with untreated mixed anxiety-depression is significant, and recognition and treatment of these patients are important (33).

It is clear that the presence of comorbid depression and anxiety can affect the course and treatment outcome. Identification of coexisting anxiety is very important and may determine the treatment of choice. In the same time, many patients with comorbid depression and anxiety require higher doses of medication and more intensive treatment to achieve remission than those without comorbid symptomatology (19).

Anxious depression as a diagnostic category does not exist in the DSM-IV; however, more study is needed to identify whether there is a residual category of patients with major depression with problematic anxiety symptoms.

The need for controlled studies comparing different agents that specifically include patients with coexisting anxiety and mood disorders is clear. Issues of comparative efficacy, treatment resistance, length of treatment, and relapse prevention remain areas requiring further investigation (19).

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CASTLEMAN'S DISEASE MIMICKING CAROTID BODY TUMOR

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Abstract

The authors present a case of 41-year-old patient with unicentric type of Castleman's disease in the neck imitating carotid glomus tumor. This rare condition can affect any lymph-node in the body and neck localization occurs only in 14% of cases. The case when Castleman's disease simulating chemodectoma have not been described yet in the literature. The authors give details on the course of the disease, its diagnosis and treatment and the discussion focuses on the reasons why the auxiliary imaging methods have led to the clinical diagnosis of carotid glomus tumor, and why the treatment was chosen.

Keywords: Castleman's disease, carotid body tumor, chemodectoma, Castleman's disease therapy, preoperative embolization

INTRODUCTION

Castleman's disease is a rare, usually benign disease of unclear etiology which is now considered an immunopathological process. Castleman with his colleagues first published it in 1956. It was described as benign hyperplasia of mediastinal lymph-nodes, similar to thymus tissue [1]. Some authors use synonymous terminology of this disease: angio-follicular hyperplasia, angio-fibroid hamartoma, follicular lymphoreticuloma, giant cell hyperplasia, benign giant lymphoma. It could, however, lead to misinterpretation of a neoplasia or hamartoma and therefore the most commonly used name is Castleman's disease.

In 1972 Keller with co-workers have described two different histological types of the disease: hyaline-vascular (91% of cases) and plasma-cell (9% of cases) [2]. The disease can occur as an unicentric type localized in one anatomic area or a multicentric type - a systemic disorder with disseminated lymphadenopathy. The disease is unicentric in most cases of hyaline-vascular type; plasma-cell type is represented only in about 10% of cases. The multicentric variation is seen almost always in plasma-cell type [3] and clinical development of the disease often leads to malignancy, both in terms of development of malignant lymphoma, or sarcoma of dendritic reticular cells.

Although Castleman's disease could be theoretically found in any lymph-node in the body, the chest is the most common location (60%). The neck has been reported in only about 14% of cases [4]. At head and neck, Castleman's disease is preferentially located in perijugular lymph-nodes in the submandibular and parotid region [5].

On the basis of clinical status, physical examination and auxiliary imaging techniques Castleman's disease was often diagnosed as a different nosological unit (MTS cancer to lymph-nodes, lymphoma, schwannoma, a pathological process in the thyroid, hepatocellular carcinoma, tumor of adrenal glands and others) until the histopathological examination led to correct diagnosis. The cases in which Castleman's disease imitates chemodectoma, are not reported in the literature and this is the first study doing so.

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CASE REPORT

A 41-year-old patient with massive resistance on the left side of the neck is presented. Tumor was located in the middle third of the neck on the left and first diagnosed at the age of two years. In childhood the patient was operated 4 times (1974, 1976, 1981 and 1986). Histopathological findings were repeatedly ambiguous. The conclusion of the last examination in 1986 was: nonspecific lymphadenopathy. The course of the last operation (1986) as recorded in the documentation was complicated by massive bleeding requiring surgical suture of damaged wall of *a.carotis interna* (ACI) *l.sin.* and the patient required blood transfusion.

In the further course of the disease the tumor was slowly expanding. In spite of that, the proposed surgical treatment was rejected by patient's parents and after attaining full age also by the patient herself. She was sporadically followed up by otorhinolaryngologist. The size of the tumor was virtually unchanged since puberty. The last time, the excision for histopathological examination was recommended 2 years ago but it was refused by the patient. The possible zoonotic disease was excluded by infectologist.

At the age of 41 years, 3 months before admission to the Clinic of Otorhinolaryngology and Head and Neck Surgery, Jessenius Faculty of Medicine, Comenius University (JFM CU), Martin and University Hospital Martin (UHM), the patient observed a tumor growth, mainly in cranial part that was associated with a dull pain of fluctuating intensity.

At the ENT examination, in addition to increased volume of lesions, newly formed left vocal cord paralysis was detected. At ultrasound examination, a well-bounded packet of vascularized lymph-nodes 7x4 cm surrounding *a.carotis communis* (ACC) and its bifurcation was found without obturating or infiltrating blood vessels. *V.jugularis interna* (VJI) was pushed dorsolaterally.

CT scan revealed a huge vascular tumor of rough but relatively sharp edges on the left side of the neck size 5.5x6.5x12.5 cm. It ranged from jugular to the infratemporal fossa almost to the carotic canal. ACC, *a.carotis externa* (ACE) and ACI on the left side were inside in the expanded tumor. Due to pressure of the tumorous masses ACI has been thinning. VJI on the left was obturated and replaced by a widespread network of veins ventrally to the tumor. The tumor was located in 3 to 3.5 cm large node with central necrosis, that was not detected in the last CT scan two years ago (Figs. 1, 2, 3).

Due to the suspicion of a malignant transformation of the tumor, the patient was admitted to the Clinic of Otorhinolaryngology and Head and Neck Surgery JFM CU and UHM for further diagnosis and treatment.

Because of the tight relationship of tumor to major vessels angiography was indicated. It revealed hypervascularized tumor in the left part of the neck supplied by branches of ACE *l.sin.* and involving bifurcation of ACC *l.sin.*

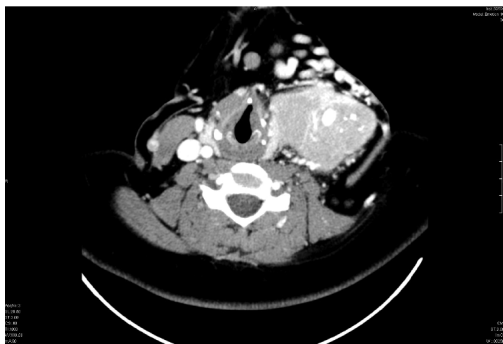


Fig. 1 CT scan axial cut

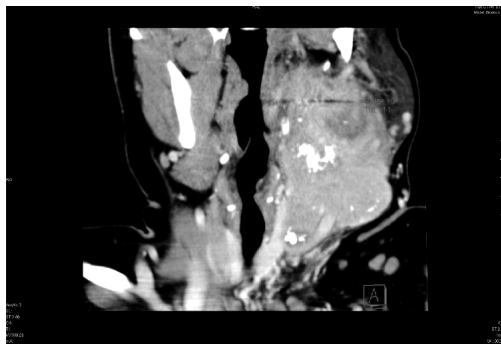


Fig. 2 CT scan frontal cut

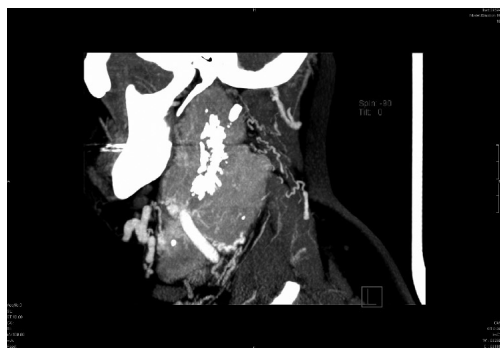


Fig. 3 CT scan sagittal cut



Fig. 4 CE-MRA frontal cut

Subsequently, Matas test of ACI l.sin. was performed. After administration of contrast medium into ACI l.dx. both *a.cerebri anterior* (ACA) and *a.cerebri media* (ACM) were filled bilaterally without delay in venous phases of vessel basin of ACA and ACM on left. After administration of contrast medium into *a.vertebralis* (AV) l.sin. vertebrobasilar venous system was filled and through the *a.communicans posterior* on the left also ACM l.sin. with no delay in venous phase. After 15 min of the occlusion test the patient was clinically without motor and phatic disorders. At the end of the test (total length 45 min) she described a visual sensation in the left eye („like a flash“), and headache that disappeared with restoration of the blood flow in ACI l.sin.

Prior to the planned surgical treatment examination of extracranial cerebral arteries by Contrast Enhanced Magnetic Resonance Angiography (CE-MRA) was completed. ACC, ACI and ACE on the right side were without significant hemodynamic changes. Left ACC was involved into the tumor in the length of 3.5 cm to the bifurcation and it was deviated laterally by about 3 cm. Left ACI was involved into the tumor in 5 cm and ACE l.sin. in 2 cm of their length. Hemodynamically the vessels were without significant changes with homogeneous contrast filling (Fig. 4).

Preliminary diagnosis based on clinical course and auxiliary imaging studies was given as tumor originating from the left carotid artery body. According to the Shamblin's classification it was type III [6] – a large tumor, pressing carotid artery and cranial nerves (in our case *n.vagus*) whose removal is difficult and often requires reconstruction of the ACI. Vascular surgeon considered the tumor as operable with high risk of serious complications and massive bleeding.

Prior to the planned surgical treatment embolization of the tumor was performed by application of the embolization substances into the branches of *a.occipitalis l.sin.* and *a.pharyngea ascendens l.sin.* The control application of contrast fluid into ACE and ACC revealed only small contrast areas in the ventrocaudal parts of the tumor on the left probably supplied from branches *a.thyroidea sup.l.sin.*

At the next day, the patient was operated in co-operation with vascular surgeons. A radical excision of the tumor sized 15x8x7 cm was performed with ACC, ACI and ACE passing through its center. VJI was fibrotized, *n.vagus* involved into the tumor, *truncus symphaticus* was unidentified, *n.hypoglossus* was intact and separated from the tumor, but in the place of its crossing with ACI the integrity of the vessel was broken. ACC and ACI on the left were therefore replaced by a graft of autologous inersposit of *v. sap. magna l.sin.* ACE on the left was ligated (Fig. 5, 6). Perioperative blood loss was 1500 ml.

A sample of the tumor tissue was examined by perioperative histopathology with conclusion of lymphoid tissue with interfollicular proliferation of uncertain biological significance. Definitive diagnosis was based on immunohistochemical processing: nonmalignant, immunopathological lymph-node proliferation from spectrum of Castleman's disease, hyaline-vascular type „stroma rich“ variant [7] (Fig. 7).

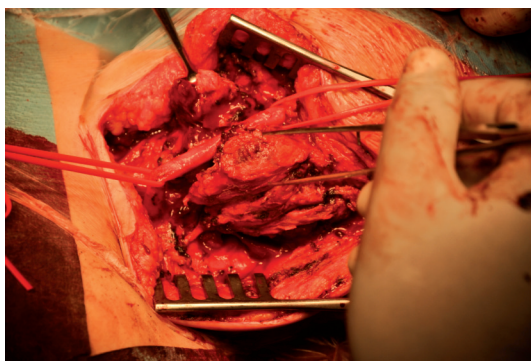


Fig. 5 ACC partially removed from tumor

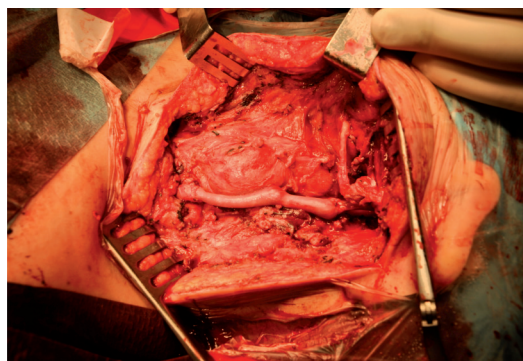


Fig. 6 Tumor removed and ACC and ACI replaced by a graft of autologous interposit

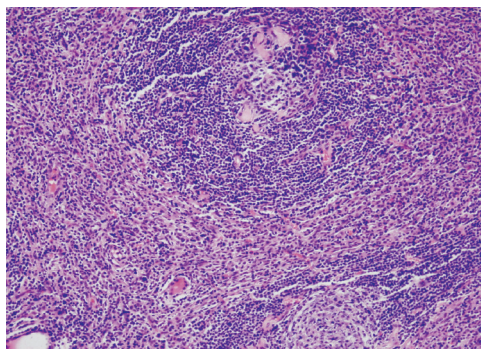


Fig. 7 Hematoxylin and eosin, 10 x magnification

DISCUSSION

When reviewing the literature on Castleman's disease it was found out that a histopathological diagnosis is more often surprising than expected result, especially in cases with extrathoracic tumor localization. After a more detailed analysis it seems that the disease most often mimicks other processes characterized by the growth of tumor mass. After entering the key words *Castleman* and *mimicking* into the database MEDLINE®/PubMed® displayed 14 results, words *Castleman/simulating* and *Castleman/masquerading* 2 publications each and words *Castleman/imitating* 1 publication. The papers identically describe the cases of patients with a clinical diagnosis different from that done after histopathological examination as Castleman's disease. According to these publications the disease mimicks tumors of pancreas, liver, lungs, retroperitoneum, ovary, nerve sheaths, or lymph-nodes, but also sequester in mediastinal space, or malabsorption syndrome [8-15]. In none of the articles studied, Castleman's disease imitated chemodectoma as in our reported patient.

The most common tumors at the point of carotid bifurcation are *carotid glomus* tumors, but they can be imitated by other expansive processes in this area. For instance, schwannomas of the cervical sympathetic fibers were preoperatively falsely considered as carotid body tumors [16, 17].

Carotid glomus tumors are causing an expansion of carotid artery bifurcation. The increased distance (called "splaying") between ACI and ACE demonstrated by supportive imaging techniques resembles lyre-like picture. Lyre sign is considered a crucial mark in the diagnosis of carotid body tumors [16] and it was present also in our patient (Fig. 4). Chemodectomas are usually easily diagnosed by conventional angiography [17]. In this case report angiography revealed hypervascularized tumor involving bifurcation of ACC l.sin that has been supplied by branches of ACE l.sin. Clinical diagnosis was based on these signs.

Among non-invasive methods ultrasonography can be used. However, in 13% of cases this technique leads to an incorrect diagnosis of carotid body tumor [18]. The examination was performed also in our patient and 7x4 cm large bounded packet of vascularized lymph-nodes involving ACC and its bifurcation was seen.

CT and MR images are useful to determine the extent of tumors as well as their position in relation to the surrounding blood vessels [17]. Both tests supported an expected diagnosis also in our case report illustrating a huge vascularized tumor involving ACC, ACI and ACE.

All above-mentioned imaging techniques are helpful in the diagnosis of extensive vascularized tumors of the neck, but not in all cases they lead to correct clinical diagnosis.

There is no consensus on optimal treatment of Castleman's disease in the literature [19]. Surgical removal is considered the most appropriate therapy for unicentric type of the disease [2, 20-22]. Unresectable unicentric variants of these tumors have been treated by various strategies: primary radiotherapy or incomplete resection [23], and chemotherapy [24]. In some cases, significant reduction in tumor mass achieved by primary radiotherapy allowed radical resection of previously unresectable tumors [19].

In the treatment of carotid body tumors the surgical resection is the method of choice [25-27]. In very large tumors their selective transarterial embolization may be applied preoperatively. This method has been for the first time successfully used by Schick and colleagues in 1980 [28]. It is aimed to block the vessels supplying the tumor without affecting the blood flow into the healthy tissue, the back-flow of the embolization agent into the carotid stream or its penetration into the distal circulation [29]. The benefit of preoperative embolization includes reduced perioperative blood loss and thus reduced need of transfusion [30, 31]. Moreover, tumor size uses to be reduced by 25% with this technique [32]. Reducing the blood supply to the tumor allows to determined the margins of resection more accurately and to reduce postoperative neurological disability [29]. The disadvantages include mainly the risk of postembolised stroke that occurs in more than 10% of cases [32-34].

Because of the size of the tumor (15x8x7 cm) and its extent (according to Shamblin type III) our patient was treated by selective preoperative tumor embolization and its subsequent surgical removal.

CONCLUSION

In the case reported here we would like to point out the possible variability of clinical manifestations and morphological picture of Castleman's disease. The disease can affect any lymph-node in the body and simulate various nosological units. Location of the neck is described only in 14% of cases. To our knowledge, there is no case of Castleman's disease imitating carotid body tumor published in the literature. Clinical examination and imaging methods in presented patient were helpful in predicting the disease, however, the histopathological analysis led to the final correct diagnosis. The treatment combined selective preoperative embolization and radical surgical resection and was chosen on the basis of the tumor size and its type.

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