

UNIVERSITY OF MEDICINE AND PHARMACY CRAIOVA
DOCTORAL SCHOOL



DOCTORAL THESSIS

**CLINICAL, HISTOLOGICAL AND IMMUNOHISTOCHEMICAL
UPDATES ON CHRONIC RHINOSINUSITIS**

ABSTRACT

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Keywords: chronic rhinosinusitis, nasal polyps, nasal endoscopy, immunohistochemistry, cytokeratin, lymphoplasmacytic infiltrate.

I.IMPORTANCE OF THE PATHOLOGY. MOTIVATION OF THE STUDY.

Chronic rhinosinusitis is the most common cause of morbidity, with a prevalence of 14, 2% (29,2 million people) in the adult population of the United States of America, and therefore more frequent than arthritis (12,47%) and hypertension (11,44%), according to data released by the National Center for Health Statistics in 2004. The economic effect of chronic rhinosinusitis is huge ; in the US , the direct cost of treatment of this condition was approximated at \$ 70 billion in 1996 and indirect costs related to loss of working days at \$ 70 million (Baraniuk , 1996). This condition affects the quality of life of patients more than heart failure (Gliklich , 1995).

In Europe , there are no statistics related to the huge costs of chronic rhinosinusitis but according Stammberger 's opinion , the situation can not be much different.

The causes of persistent inflammation sinus mucosa causing its subsequent irreversible histological changes are not fully understood.

My study addresses a a topic of great interest, attempting thereby also aligning and integrating Romanian research in large European scientific research .

The project is structured around the idea to deciphering the mechanisms that determine the persistence of chronic inflammation of sinonasal mucosa, accompanied by an eosinophilic infiltration , which can be exacerbate by the presence of bacterial aggression but do not give up entirely with the destruction of microbial germs. Determining factors that cause and maintain this inflammation will allow a more effective and targeted treatment, which could finally cure the patient.

This paper structured in two parts, aimed at the general part the study on the anatomy of the nose and paranasal sinuses, their physiology, chronic rhinosinusitis etiopathogenesis, clinical features and imagistic diagnosis of chronic rhinosinusitis, finally reviewing the main therapeutic methods used in its protocols treatment.

The special part includes a clinical study of cases with chronic rhinosinusitis diagnosed and treated in ENT Clinic of Craiova Emergency County Hospital, followed by histologic and immunohistochemical study of cases submitted to surgery.

Histological and immunohistochemical studies were facilitated by obtaining and completing a grant of the Romanian Academy (2007-2008), from whose research team I was part of.

I. MATERIAL AND METHODS

II.1. STUDY PURPOSE

Etiopathogenesis of chronic rhinosinusitis is not yet fully elucidated, despite numerous studies that have been conducted on this topic. According to documents published by the Academy of Allergology and Clinical Immunology in January 2005, documents that are a "state-of-art" for physicians who have connections with the subject, chronic rhinosinusitis is a "major health problem whose frequency is increasing, while increasing the frequency of allergic rhinitis, which results in huge costs for society".

My study aims to deepen an issue of great national and international importance, not so much by its severity, but by direct and indirect high costs, which chronic rhinosinusitis implies.

Through this thesis I intend to analyze patients with chronic rhinosinusitis from clinical, histological and immunohistochemical point of view, followed finally by a statistical analysis which try to highlight possible correlations between histopathological changes of nasal mucosa, changes in the distribution of populations and subpopulations of lymphocytes and nasal mucosa epithelial cells immunohistochemical pattern.

By establishing respiratory epithelium immunostaining pattern and especially by its correlation with immunohistochemical profile of lymphocyte subpopulations in the nasal mucosa, we can identify whether a specific profile of the respiratory epithelium can characterize a predisposition of some people, to develop a chronic sinus inflammation, when exposure to environmental factors involved in the etiopathogenesis of chronic rhinosinusitis.

Documentation on the current situation of national and international research in chronic rhinosinusitis- on its etiology, contributing factors of chronic sinus inflammation, new data on the pathogenesis of the disease, histopathological and immunohistochemical changes (cytokeratin, populations and subpopulations of lymphocytes) that characterizes sinus mucosa, was performed by studying the literature cited in the references.

The objectives of the study were the following:

1. Clinical and endoscopic study of patients with chronic rhinosinusitis enrolled in the study group: will be set criteria for inclusion in the study group and investigation protocol; statistical analysis of the study group.
2. Histopathological study of nasosinusal mucosa and nasal polyps.
3. Immunohistochemical study of epithelium and lymphocytes of the nasosinusal mucosa.

II.2. MATERIAL AND METHOD

The paper is structured in two studies:

1. Clinical study- examines a group of 1685 patients diagnosed with chronic rhinosinusitis and treated between 2007 and 2011 into the ENT Clinic of the Emergency County Hospital Craiova.
2. Histological and immunohistochemical study- this study was included in the grant research whose research team I was part - "Immunohistochemical pattern of epithelial cell and lymphocyte subpopulations of nasosinusal mucosa correlated with local microbiological determinations in chronic rhinosinusitis. "- conducted under the aegis of the Romanian Academy. We studied fragments of nasosinusal mucosa and nasal polyps from 106 patients who were diagnosed with chronic rhinosinusitis with or without nasal polyposis, who underwent endoscopic surgeries. The patients were

hospitalized in the ENT Clinic of Emergency County Hospital, Craiova between 2007-2008.

Patients were informed that they would be part of a research study, they has been shown a detailed plan of study and obtained their informed consent. The material on which we made the study included clinical observation paper of patients, imaging results, protocols and histopathological results of patients who underwent surgery, from which were collected fragments of mucosa and polyps that were sent to histopathology.

The patients were included in this study according to the following EPOS criteria on chronic rhinosinusitis: inflammation of the nose and paranasal sinuses longer than 12 weeks, characterised by two or more symptoms, one of which must be nasal obstruction / nasal congestion or rhinorrhea (anterior or posterior) with or without headache / facial tension and hypo / anosmia; different changes on the endoscopic examination: nasal polyps, mucopurulent discharge primarily from middle meatus, oedema of the middle meatus, ethmoid bulla hypertrophy, turbinate hypertrophy, of mucosal changes ostiomeatal and / or sinus.

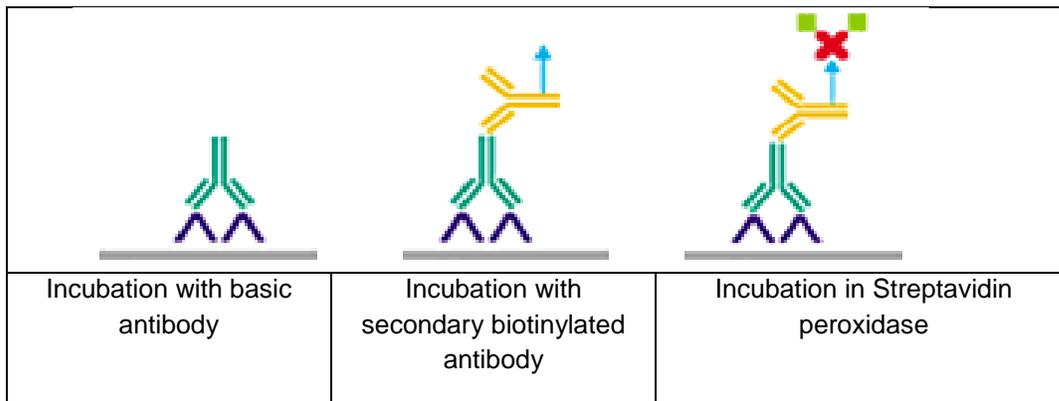
The study group was divided into two subgroups based on changes observed in nasal endoscopy:

- Pacient with chronic rhinosinusitis and nasal polyposis
- Pacient with chronic rhinosinusitis without nasal polyposis.

The study of the patients was done according to a standard protocol that included: anamnesis, general clinical examination, local clinical examination, nasosinusual endoscopy, radiological examination, histopathological and immunohistochemical examination.

For the histopathological study the tissues are initially fixed in 10% buffered formalin. Subsequently, the specimens were paraffin embedded, sectioned and Hematoxylin–Eosin stained.

The immunohistochemical method was based on soluble immunoenzymatic complexes – LSAB/HRP(Labeled Streptavidin Biotin) method. We used DAKOLSAB 2 System HRP kit (Universal DAKO Labeled Streptavidin Biotin 2 System Horseradish Peroxidase). LSAB method (with Streptavidin Biotin) is one of the ABC methods (Avidin–Biotin complex), in which Avidin substitutes Streptavidin and is based on direct conjugate of Streptavidin with enzymatic molecules.



Schematic presentation of the working procedure on LSAB method

On the immunohistochemical study, we used antibodies produced by DAKOCytomation, Denmark and ZYMED Laboratories Inc., San Francisco, whose dilution and pretreatment carried out are shown in the table below:

Antibody	Clone	Dilution	Pretreatment	Incubation time
CK 7	OV-TL	1:50	5 cicli EDTA	30 min TA
CK 20	K 20.8	1:20	5 cicli EDTA	30 min TA
CD 20	L 26	1:100	3 cicli citrat	30 min TA
CD 45.RO	UCHL1	1:100	3 cicli citrat	30 min TA
CD 4		1:50	-	30 min TA

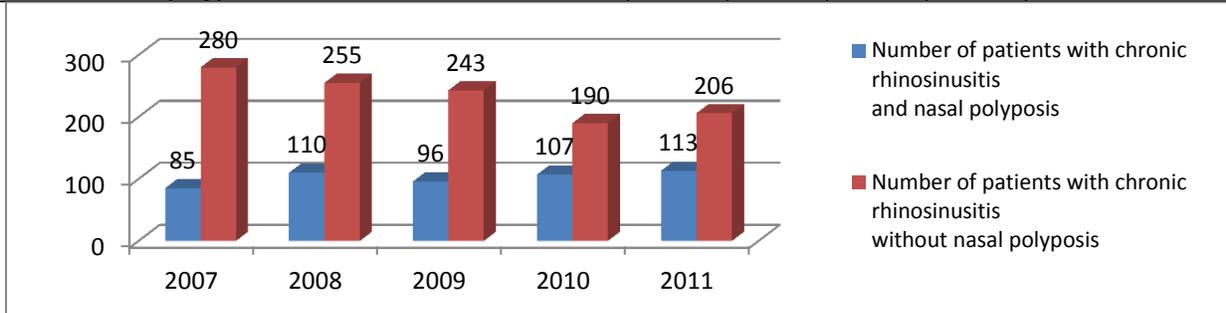
III. SUMMARY OF MAIN RESULTS

III.1. Clinical study results and discussions

1. Incidence of chronic rhinosinusitis in the period 2007- 2011, relative to the total number of hospitalizations: from the total of 17.790 patients admitted during this period in the ENT Clinic of the Emergency County Hospital Craiova were diagnosed 1685 cases of chronic rhinosinusitis, of which 1174 cases of chronic rhinosinusitis without nasal polyposis and 511 cases of chronic rhinosinusitis accompanied by nasal polyposis. Therefore chronic rhinosinusitis had an incidence of 9.4%.

2. Distribution by study years of cases of chronic rhinosinusitis with / without nasal polyposis.

Study year	2007	2008	2009	2010	2011
Number of patients with chronic rhinosinusitis and nasal polyposis	85	110	96	107	113
Number of patients with chronic rhinosinusitis without nasal polyposis	280	255	243	190	206



The analyse of the distribution of chronic rhinosinusitis cases on study years reveals a downward trend in the number of cases hospitalised with chronic rhinosinusitis without nasal polyps, which could be justified by the growing progress of the antibioterapy and the ability to policlinic treat chronic rhinosinusitis by medical treatment and when we don't obtain proper results, by endoscopic surgery requiring no hospitalization.

Instead, there is an increase in the number of cases of chronic rhinosinusitis with nasal polyposis diagnosed and operated in our clinic, which oriented me towards more detailed research of these cases. These results are in line to international studies that show frequent relapses and reinterventions in patients with chronic rhinosinusitis and nasal polyposis, despite the endoscopy progress made in recent years and numerous studies that attempt to elucidate the mechanisms involved in the etiopathogenesis of the disease.

3. Gender distribution of patients with chronic rhinosinusitis

	Chronic rhinosinusitis and nasal polyposis	Chronic rhinosinusitis without nasal polyposis
Women(no. cases)	181	667
Men(no. cases)	330	507

Gender distribution of patients with chronic rhinosinusitis shows a predominance of women with chronic rhinosinusitis without nasal polyposis which could be explained by more frequent exposure to adverse environmental conditions, for aesthetic reasons and frequent upper respiratory tract infections, most commonly through contact with young children who attend large communities (kindergartens, schools). Chronic rhinosinusitis with nasal polyposis ratio was reversed in favor of males, this proportion being found in other studies in the literature, including EPOS, being correlated with high frequency of smoking among men.

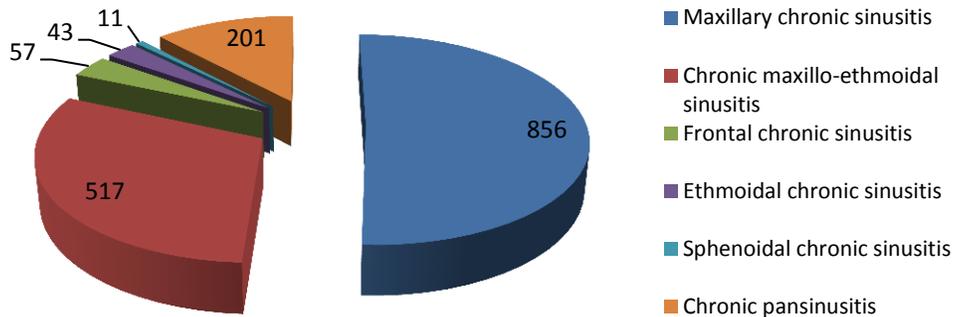
4. Distribution of cases of chronic rhinosinusitis based on the background:

	Urban	Rural
% of the no. patients with chronic rhinosinusitis and nasal polyposis	56,75%	43,24%
% of the no. patients with chronic rhinosinusitis without nasal polyposis	57,92%	42,08%

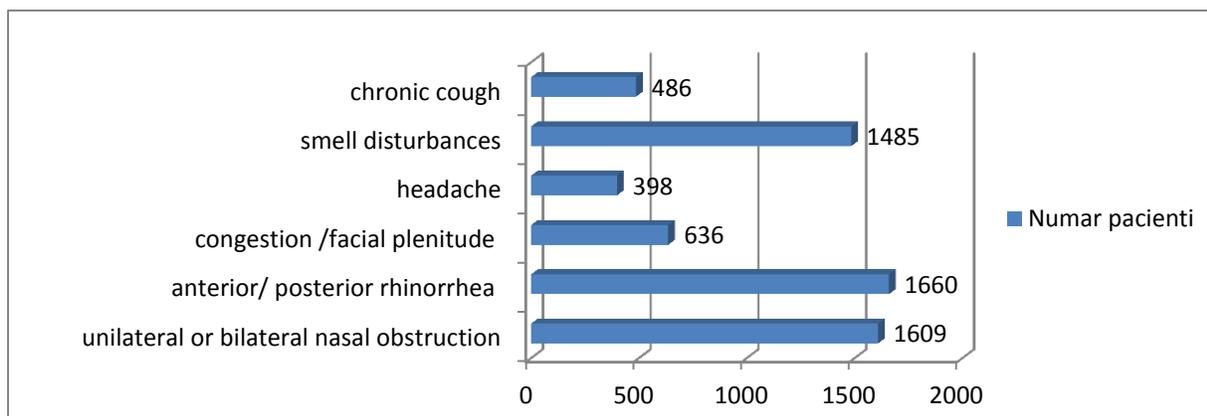
The difference in this distribution can be explained by the continuous irritation of the nasal mucosa due to exhaust gases, smoke and by the fact that crowded urban areas are a contributing factor of repeated upper respiratory tract infections by transmitting pathogens easily, favoring chronic rhinosinusitis.

5. Distribution of cases of chronic rhinosinusitis based on the affected sinus:

	Maxillary chronic sinusitis	Chronic maxillo-ethmoidal sinusitis	Frontal chronic sinusitis	Ethmoidal chronic sinusitis	Sphenoidal chronic sinusitis	Chronic pansinusitis
No. of cases	856	517	57	43	11	201
Percentage	50,70	30,68	3,38	2,55	0,65	11,92



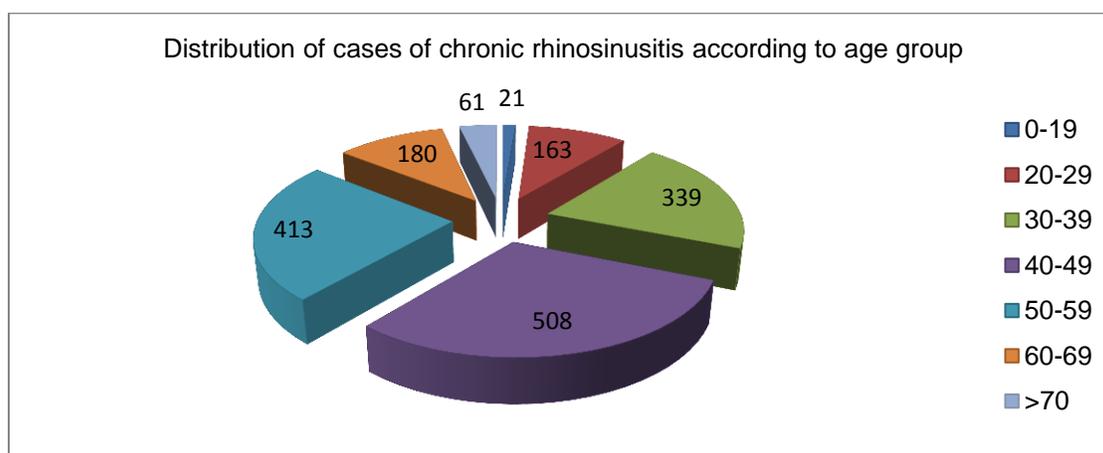
6. Distribution of cases of chronic rhinosinusitis based on symptomatology:



It is noted low frequency of chronic cough, congestion / facial fullness and headache. It is important to note that headache is not characteristic of chronic rhinosinusitis with nasal polyposis, which should be noted in preoperative patient information.

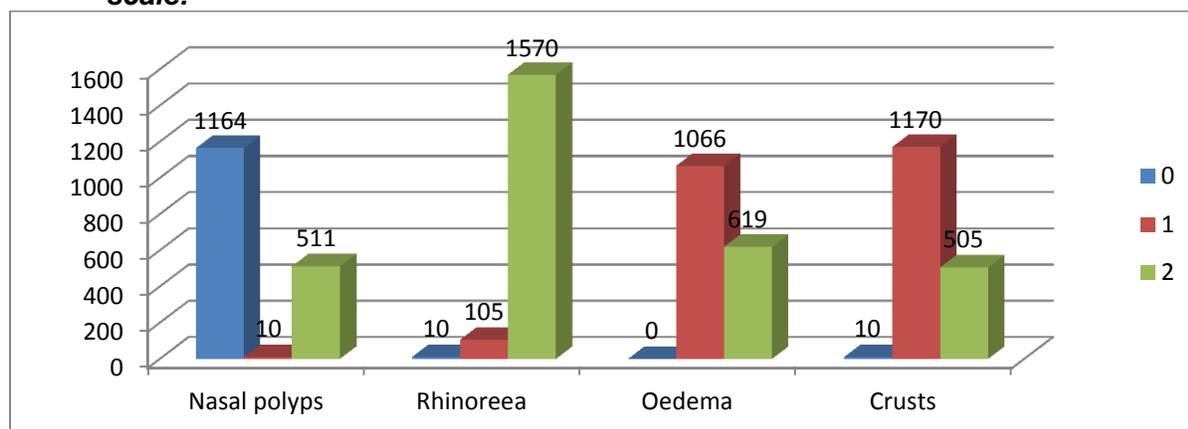
7. Distribution of cases of chronic rhinosinusitis according to age group:

Age groups (years)	0-19	20-29	30-39	40-49	50-59	60-69	>70
No. of cases	21	163	339	508	413	180	61
Percentage	1,24%	9,67%	20,11%	30,14%	24,51%	10,68%	3,62%



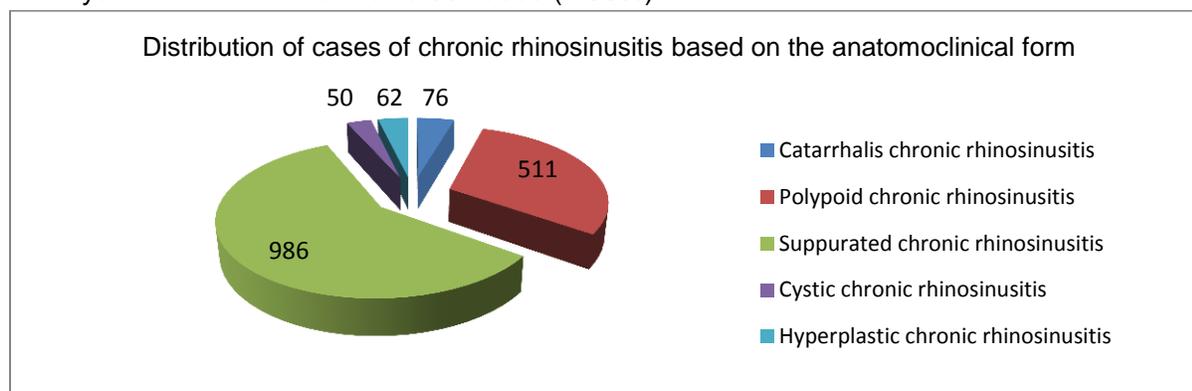
Analysing distribution by age group of patients with chronic rhinosinusitis found that chronic rhinosinusitis affects mostly adults, with a peak in the decade 40-49. This is the adult population and therefore more exposed to living and adverse working conditions, determining repeated upper respiratory tract infections and prolonged obstruction of ostiomeatal complex. The fact that the incidence of chronic sinusitis remains high at the elderly population can be explained by their decreased immunity on the background of physiological degradation and chronic pathology superimposed, which decrease body reactivity on outside attacks.

8. Distribution of cases of chronic rhinosinusitis based on the Lund-Kennedy scale:



We found that the cases of chronic rhinosinusitis with score 6 on Lund-Kennedy scale had largest share of the 1,685 cases included in the clinical study (695 cases). This distribution of cases, with predominance of the score 6 supports the idea previously held, on the high complexity of cases hospitalized. Cases with low scores were treated in the policlinic.

9. Distribution of cases of chronic rhinosinusitis based on the anatomoclinical form: revealed that the most common is suppurated chronic rhinosinusitis with 986 cases (58.51%), followed by polypoid chronic rhinosinusitis with 511 cases (30.32%), catarrhalis chronic rhinosinusitis - 76 cases (4.5%), hyperplastic chronic rhinosinusitis - 62 cases (3.67 %) and cystic chronic rhinosinusitis 50 cases (2.96%).



Suppurated chronic rhinosinusitis involves many difficulties in terms of evolution and treatment because of the many complications that can occur in its evolution, which can endanger the patient's life and can definitively invalidate him (oculo-orbital, venous or cranial complications). With one exception, all cases of suppurated chronic rhinosinusitis had a favorable evolution under well conducted antibiotic treatment, topical decongestants, corticosteroids and surgical treatment.

Polypoid chronic rhinosinusitis had a lower incidence than the suppurated rhinosinusitis. All cases of polypoid chronic rhinosinusitis were resolved favorably during hospitalisation, but we found a recurrence rate of 18%, with no significant differences related to the surgical technique used (classical or endoscopic) and despite topical corticosteroid treatment. This frequent recurrence feature of chronic rhinosinusitis with nasal polyposis, despite correct treatment led me oriented towards a more detailed study of histological and

immunohistochemical changes of nasosinusal mucosa in patients with this pathology, wishing to determine whether there are characteristic changes of epithelial profile that may explain frequent recurrence in some patients.

III.1. Results and discussion of the histological study

From histological point of view, on the nasal mucosa of the inferior turbinates, we have noticed microscopic structure of respiratory epithelium, sometimes squamous metaplasia, collagen stroma with underlayer hyaline zones, mucous glands hyperplasia, and sometimes the presence of lymphoplasmacytic or hemorrhagic infiltrates. Although there were some slight variations, inferior turbinate histopathology revealed the presence in all cases of chronic inflammatory changes. Other changes at this level consisted of the presence of vessel ectasia, some thrombosed. These changes can be due to the numerous aggressions on nasal mucosa either due to pathogens, either from exposure to environmental factors like smoking, pollution, cold, wet.

Although there were some slight variations, inferior turbinate histopathology revealed the presence in all cases of chronic inflammatory changes.

In contrast to changes noticed on the inferior turbinate, in the case of middle turbinate mucosa we observed sometimes, the presence of eosinophilic infiltrate.

The histologic study of sinus mucosa allowed us to note the following changes: a microscopic structure of respiratory epithelium, subjacent dense collagenous stroma with myxoid areas, alone or associated with hyaline zone; also noted the presence of a diffuse lymphoplasmacytic infiltrate, undelayer or periglandular, which proves chronic inflammation on sinus mucosa. I have not noticed significant differences between right sinus, respectively, at the left, on the same patient.

From the point of view of the histopathological examination, on usual color technique, nasal polyps were found to be highly variable. The 106 nasal polyps we studied were classified as follows:

- allergic polyps, with the presence of eosinophilic infiltration: 59 cases (55.6%);
- fibroinflammatory polyps: 39 cases (36.8%);
- polyps with marked hyperplasia of sero-mucous glands: eight cases (7.6%).

Microscopically, the *allergic polyps* were characterized by the presence of edematous stroma, marked hyperplasia of goblet cells, thickening of the basement membrane and an intense inflammatory leukocyte infiltrate, predominantly with eosinophils. There were also small, rare areas of squamous metaplasia of respiratory epithelium.

From microscopic point of view, *the fibro-inflammatory polyps* showed intense chronic inflammatory infiltrate (mainly lymphocytes) and a series of metaplastic changes, both at the level of coating epithelium and fiber stroma. Thus, there could be noticed areas of metaplasia of the respiratory epithelium, as well as areas of bone metaplasia of the fibrous stroma

The polyps with marked hyperplasia of the sero-mucous glands were histologically similar to the allergic polyps, presenting additional numerous sero-mucous glands.

I did not meet polyps with atypical stromal or fibrotic. In this study, the percentage of allergic polyps was not as great as in the literature, possibly due to the large-scale administration, in recent years, of topical corticosteroids (used in the treatment of acute rhinosinusitis and acute stage of chronic rhinosinusitis, not only on allergic rhinitis treatment, as in the past).

Rare presence of eosinophilic infiltration is unusual and contradicts studies made by other authors, which are finding that chronic rhinosinusitis histological marker is the presence of eosinophilic infiltration on nasal mucosa, irrespective of atopy.

III.1. Results and discussion of the immunohistochemical study

I have performed immunohistochemical analyzes for cytokeratins 7 and 20, B lymphocytes (CD20), total T lymphocytes (CD45RO) and helper lymphocytes (CD4).

Analysis of CK 7 expression

Allergic polyps expressed CK7 strongly, both at the level of respiratory type epithelium and sero-mucous stromal glands. The labeling was cytoplasmatic, expressed by intermediate and superficial cells of pseudostratified epithelium of respiratory type and glandular luminal cells. Cells of basal type did not expressed CK7. Distribution of CK7 immunolabeling was diffuse, both in respiratory and glandular epithelia.

Fibro-inflammatory polyps presented a diffuse labeling of moderate intensity of respiratory and glandular epithelia. The labeling was noticed at the level of the same cell types, similar to the case of allergic polyps, but at a lower intensity. We noticed a decrease of the intensity of CK7 expression in fibro-inflammatory polyps, comparatively with the allergic ones. Therefore, it seems that CK specific for overbasal cells decrease once the inflammatory process ev from the surface of covering epithelium. In one case, CK7 focally marked only the cells from the surface of covering epithelium. These cells reminded of “umbeliform” cells encountered in transitional epithelium. Therefore, we considered these areas as possible metaplasia areas of transitional type of respiratory covering epithelium.

Analysis of CK20 expression

Allergic polyps were negative for CK20, both at the level of respiratory pseudostratified epithelium and metaplastic squamous epithelium. Instead, we noticed a cytoplasmatic labeling of a low, focal intensity at the level of glandular epithelium.

Fibroinflammatory polyps expressed a focal labeling of low and moderate intensity for CK20 at the level of glandular epithelium. We noticed an increase in the intensity of CK20 expression in glandular epithelium of fibro-inflammatory polyps comparatively with the allergic ones, possibly in relation with the same aspect of the inflammatory process evolution.

In *polyps with hyperplasia of sero-mucous glands*, CK20 marked only the glandular epithelium, similarly to allergic polyps.

Analysis of CD45RO immunostaining

On *allergic polyps* stroma, CD 45.RO revealed T lymphocytes with a diffuse disposal. The immunostaining was continuous on membrane level.

On *fibro-inflammatory polyps*, CD 45.RO revealed T lymphocytes by a continuous immunostaining on membrane level. In addition to the diffuse T lymphocytes distribution we have noticed a underlayer and periglandular nodular distribution. Sometimes, the infiltrate with T lymphoid cells tends to the formation of lymphoid follicles. Overall T cells had their greater representation on fibro-inflammatory polyps, compared to allergic polyps.

On *polyps with marked hyperplasia of sero-mucous glands* the immunostaining was similar to the allergic polyps.

Analysis of CD4 immunostaining

Anti-CD4 monoclonal antibody has allowed detection of T helper lymphocytes by a continuous immunostaining on membrane level in these cells without being able to differentiate between subpopulations of T helper cells. The proportion of helper T cells was similar in all types of polyps analyzed.

Analysis of CD20 immunostaining

Anti-CD20 monoclonal antibody has put in evidence the presence of B lymphocytes that have been marked continuously at the membrane level. In all types of polyps, study population of lymphocytes B was much more poorly represented in comparison with the population of T lymphocytes, these cells being disposed diffuse in stroma. We noticed a slight increase in the population of lymphocytes B in fibro-inflammatory polyps compared to other types of polyps.

IV.CONCLUSIONS

1. Chronic rhinosinusitis is a disease with an etiology still unsolved, despite numerous studies over the years. According to published studies, the incidence and prevalence of this disease are increasing, coupled with increasing incidence and prevalence of allergic rhinitis.

2. In my study, the incidence of chronic rhinosinusitis was 9.4% (1685 cases) of the total of 17790 patients hospitalized during the study years (2007-2011), with a progressive annual decrease of cases of chronic rhinosinusitis without nasal polyps. We have seen an increase in cases of chronic rhinosinusitis with nasal polyps diagnosed and operated in our clinic, correlated with an increased incidence of smoking, which is involved in the etiopathogenesis of nasal polyposis.

3. Chronic rhinosinusitis predominantly affected adults, its incidence is steadily increasing, with a maximum in the decade 40-49.

4. Chronic rhinosinusitis without nasal polyps most frequently affect female (56.81%), and chronic rhinosinusitis with nasal polyps is more frequent among men, associated with greater frequency of smoking among them.

5. As regards the distribution of cases of chronic rhinosinusitis according to environment of origin, there is predominance of the disease in patients from urban -56, 75%.

6. Distribution of the percentage of cases of chronic rhinosinusitis based on the affected sinus reveals a more frequent involvement of the maxillary sinus, followed by the association of maxillary sinus and ethmoid, then frontal sinus, ethmoid sinus and last frequency sphenoid sinus.

7. The history of the patients included in the study group showed an increased frequency of the following symptoms and signs: rhinorrhea anterior and / or posterior (1660 cases), unilateral or bilateral nasal obstruction (1609 cases), hyposmia/anosmia (1485 cases). I noticed the low frequency of chronic cough (486 cases), congestion / facial fullness (636 cases) and headache (398 cases).

8. The clinical study revealed the following anatomoclinical forms of chronic rhinosinusitis: suppurated chronic rhinosinusitis- most frequent with 986 cases (58.51%), followed by polypoid chronic rhinosinusitis with 511 cases (30.32%), catarrhalis chronic rhinosinusitis- 76 cases (4.5%), hyperplastic chronic rhinosinusitis 62 (3.67%), cystic chronic rhinosinusitis 50 (2.96%).

9. Analyzing the distribution of chronic rhinosinusitis cases based on changes seen in endoscopic examination we found that most cases had six points Lund-Kennedy scoring, which means significant changes on endoscopic examination, and supports the idea that introducing into the study only hospitalized patients I studied chronic rhinosinusitis with increased level of complexity.

10. Histological study followed histopathological changes that interest nasosinusal mucosa and nasal polyps. Histological study was performed on fragments of nasosinusal mucosa and polyps collected from patients with chronic rhinosinusitis who underwent surgery in our clinic.

11. On nasal turbinate mucosa, we found histological structure of respiratory epithelium with areas of squamous metaplasia, collagen stroma with underlayer hyaline zone, mucous gland hyperplasia and sometimes the presence of hemorrhagic or lymphoplasmacytic infiltrates. Other changes at this level consisted of the presence of vessel ectasia, some thrombosed. These changes can be due to numerous assaults on the nasal mucosa from pathogens, either from exposure to environmental factors like smoking, pollution, cold, wet.

12. The histologic study of sinus mucosa allowed us to note the following changes: a microscopic structure of respiratory epithelium, subjacent dense collagenous stroma with myxoid areas, alone or associated with hyaline zone; also noted the presence of a diffuse undelayer or periglandular lymphoplasmacytic infiltrate, which proves chronic inflammation on sinus mucosa.

13. Most frequent were allergic polyps, with the presence of eosinophilic infiltration- 59 cases (55.6%), followed by fibroinflammatory polyps- 39 cases (36.8%) and polyps with marked hyperplasia of seromucous glands-8 cases (7.6%).

14. Rare presence of eosinophilic infiltration is unusual and contradicts studies made by other authors, which are finding that chronic rhinosinusitis histological marker is the presence of eosinophilic infiltration on nasal mucosa, irrespective of atopy.

16. CK 7 expression in the respiratory and glandular epithelium of the and nasal polyps decreases, while focal expression of CK 20 in the glandular epithelium increases with the inflammatory process evolution.

17. Lymphocytic inflammatory infiltrate is composed predominantly of T cells (compared with type B) in all types of polyps, with a diffuse distribution in the allergic polyps and sero-mucous gland hyperplasia polyps and nodular distribution with tendency to lymphoid follicles formation in the fibro-inflammatory polyps.

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