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Group A beta Haemolytic Streptococcus Infection in Children in Latvia

Summary

PhD Thesis

Earning Doctor of Medicine Degree in Children's Infectious Diseases

Riga, 2010

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PhD Thesis has been done in the Department of Pediatrics, Riga Stradins University

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PhD Thesis are available in the library of the Riga Stradins University.

Secretary of Promotional Council:
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1. ESF National Programme "Support for Implementation of Doctoral Programmes and Postdoctoral Research in Medical Sciences", Contract Nr. 2004/0005/VPD1/ESF/PIAA/ 04/NP/3.2.3.1./ 0001/0004/0066.
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5. Ministry of Education and Science, Development of Scientific Activities in 2007.Branch of Science: Medicine Research direction and subdirection: Study of factors causing mortality and disability in children, children's infectious diseases, pediatric surgery, children's rheumatology, immunogenetics, embryology.

   Project: *Study of factors causing children's mortality and invalidity related with development of new undertakings of diagnostics, treatment and prevention in order to improve children's health in Latvia.*
6. State Research Programmes- SRP 7 *"Reduction of children's mortality by improving early diagnostics, treatment results and prophylaxis of life-threatening diseases in Latvia, employing methods of modern molecular biology, cytometry and immunogenetics"*, within the project in 2008-2009.

Content

1. Abbreviations

GAS - Group A beta hemolytic streptococcus

RF - rheumatic fever

PSGN - poststreptococcal glomerulonephritis

PANDAS - pediatric autoimmune neuropsychiatric disorder associated with streptococci

RHD - rheumatic heart disease

CCUH - Children Clinical University Hospital

ICD-10 – International Classification of Diseases (10th edition)

ASLO - antistreptolysin O

antiDNaseB - antideoxyribonuclease-B

RADT - rapid antigen detection test

MLS_B - macrolide-lincosamide-streptogramin B

PCR – polymerase chain reaction

CDC - Centers for Disease Control and Prevention

DNA – deoxyribonucleic acid

HLA - *human leukocyte antigen* system

MVR - mitral valve regurgitation

AVR - aortal valve regurgitation

MVL – multi valvular lesion (MVR + AVR)

SAM - The State Agency of Medicines

RSU – Riga Stradins University

LU – University of Latvia

2. Importance of the Problem.

Group A beta hemolytic streptococcus (GAS) is essential and frequently encountered human pathogen all over the world. GAS causes diseases of broad spectrum - from uncomplicated pharyngitis and pioderma to invasive, life-threatening infections, and to such severe complications as rheumatic fever (RF) and poststreptococcal glomerulonephritis (PSGN).

Connection of both the pace of inflammation process caused by GAS and untreated acute streptococcal pharyngitis, with such nonpurulent complications as acute rheumatic fever, poststreptococcal glomerulonephritis and PANDAS *(pediatric autoimmune neuropsychiatric disorder associated with streptococci),* is an important stimulus to understand and control this infection. It has been proved that accurate diagnosis, effective and adequate antibacterial treatment helps to solve this problem in cost-effective and inexpensive way.

Notwithstanding the fact that diseases caused by Group A beta hemolytic streptococcus, are known already since 19th century and much research work has been done upon them, several problems remain unsolved:

- despite improvement of socio-economic circumstances, environmental factors and environmental sanitation level in highly industrialized countries, pharyngitis of GAS etymology is still as frequent there as in developing countries. In Latvia the GAS prevalence in etiology of acute pharyngitis in children has not been studied until now, also incidence of Group A streptococcus carriers has not been found out. Not solved stays early diagnostics of GABHS acute pharyngitis, the early diagnostics being very important in finding correct treatment and protecting children from late complications (RF, PSGN). In developing countries laboratory diagnostics is not available, whereas in industrialized countries hyperdiagnostics may appear, because a positive GAS culture could be present also in Group A carriers, when antibacterial therapy should not be appointed.

- the rise of incidence of acute rheumatic fever focal outbreaks has been observed in the USA in 1980ies, but in Latvia in 1990ies. Also the increase of frequency of invasive infection, points to the existence of other factors, like interaction between host and pathogen, which are very significant in causality and pathogenesis of the disease. Acute rheumatic fever can develop as a consequence of GAS infection, but it is known, that certain HLA class II alleles, genotypes and

haplotypes are associated with the risk or protection to develop rheumatic fever (RF), as a result of which a patient may develop rheumatic heart disease (RHD), and these associations are unambiguous, if analyzed in clinically homogeneous patient groups. Such predisposition has not been studied in Latvia before.

- In the beginning of the 21 st century GAS is unique in that sense that as of to date there has not been found such GAS clinical isolate which would produce *in vitro* resistance to penicillin, perhaps the cheapest and most affordable antimicrobial agent, nevertheless determination of antimicrobial sensitivity is important in providing rational antimicrobial therapy, when penicillin cannot be an optional preparation. Testing of isolates is required not only for the need to choose appropriate therapy, but also to monitor and possibly control the spread of antimicrobial-drugs-resistant microorganisms in public places and hospitals. Acute pharyngitis is one of the most frequent reasons of irrational antibacterial therapy, because doctors willingly appoint antimicrobial drugs for children with exudative pharyngitis, and abandon them in cases of catharal inflammation, which points to the strategy of inappropriate diagnostics and treatment of patients with pharyngitis symptoms, and in the result of which, during last ten years more and more studies show convincing data on the rise of GAS antibacterial resistance to macrolides. Previously in Latvia no studies have been done on GAS antibacterial resistance. Such necessity only increases with the growing of multidrug-resistance of microorganisms.

3. Objective of the Work

The goal of the present work is to study Group A beta hemolytic streptococcal infections - their most common clinical manifestations in children and predisposition to severe nonpurulent complications, as well as the agent's molecular biological description in Latvia.

4. Terms of Reference

1. To prepare a systematic literature review on the issues of the study.
2. To do research on GAS caused acute pharyngitis in children by determining its prevalence at the Children Clinical University Hospital (CCUH) Emergency department, and determining peculiarities of clinical-laboratory diagnostics, that could serve as predictive factors of early diagnostics
3. To research the obtained children's Group A beta hemolytic streptococcal initial molecular description, and determine in the isolated GAS cultures, the presence of antibiotics resistance genes (*ermA, ermB* un *mefA*) and their association with certain *emm* types.
4. To research the peculiarities of macroorganism to develop RF and subsequent RHD in Latvia by determining HLA class II risk and protective alleles and HLA class II of DR and DQ genotype and haplotype distribution in clinically homogeneous RF patient groups.

5. Questions of the Study

1. What is the most common GAS clinical form in children in hospitals in Latvia, and is its prevalence similar to such in other European countries?
2. Do there exist any predictive clinical symptoms, that would allow to diagnose early the acute GAS caused pharyngitis?
3. Are indicators of GAS genotypical antibacterial resistance and their association with certain *emm* types similar to such in other European countries?
4. Which HLA class II DR and DQ alleles have an essential role in the development of rheumatic fever and rheumatic heart disease, and is this association stronger, if analyzed in clinically homogeneous patient groups?

6. Scientific Novelty of the Study

1. Defined is the most frequent GAS infection caused clinical form in children in hospital.
2. Defined is the prevalence of GAS caused acute pharyngitis in children with acute pharyngitis symptoms in The Children's Clinical University Hospital; not done before.
3. Defined, that GAS caused pharyngitis does not have any predictive clinical symptoms, that would allow to diagnose in advance the pharyngitis caused by streptococcus.
4. Defined are the indicators of GAS genotypical antibacterial resistance in Latvia. High antimicrobial resistance to macrolide group was found (78%) were resistant to erythromycin and clindamycin; prevailingly associated with *emm 89.0* type.
5. For the first time in Latvia, rheumatic fever and rheumatic heart disease risk and protective haplotypes and genotypes, controlled by HLA class II DR and DQ alleles, were studied and defined, and analyzed in clinically homogeneous groups.

7. Practical Value of the Study

General data of the study on GAS infection's clinical forms in children, acute pharyngitis prevalence, its clinical symptoms and signs, as well as genotypic antimicrobal resistance of isolated GAS cultures, were used to create practical, evidence-based guidelines for students, residents and doctors for them to make accurate diagnosis and optimal therapy of GAS caused diseases in children in Latvia. The expected results of the introduction of these guidelines will improve not only detection of clinical symptoms and signs of GAS infection and prevention of early purulent and late complications (RF, PSGN), but also reduce irrational antimicrobal therapy and its induced side-effects.

8. Structure and Extent of the Work

The PhD Thesis is written in the Latvian language, consists of 13 chapters: Introduction, Importance of the Problem, Goal of the Work, Terms of Reference, Questions of the Study, Novelty of the Study, Literary Description, Materials and Methods, Results, Discussion, Conclusions, References and Annex. Ph Thesis consists of 156 pages, including 26 tables, 11 pictures and 3 figures. Reading list comprises 238 references.

9. Approbation of the Work

Approbation of the PhD Thesis *Group A ß Hemolytic Streptococcal Infection in Children* took place in extended session of RSU Paediatrics Department and Department of Infectology, on 15th March, 2010.

10. Publications on the Theme

There are 28 publications regarding the present PhD Thesis, 3 of which published in internationally quoted medical research editions (all registered in PubMed database), 2 in Latvian scientific editions, 12 thesis of the study are published in international scientific congresses and 9 theses in domestic scientific congresses and conferences.

11. Materials and Methods

11.1. Structure of the Study

Research work Group A ß Haemolytic Streptococcal Infection in Children in Latvia has been started in 2002 and is made up of 4 sections: I, II, III, and IV.

Section I - Structure of clinical forms of GAS caused infections in children at Children's Clinical University Hospital (CCUH) in Riga.

Section II - Prevalence of GAS caused acute pharyngitis in children at CCUH Emergency Department and peculiarities of clinical-laboratory diagnostics.

Section III - Molecular-biological analysis of GAS isolates obtained during the research time, and defining of antimicrobal resistance genes.

Section IV - Research of genetic associations in children with rheumatic fever (RF) carrying HLA Class II alleles, in Latvia.

In the setting of PhD thesis the systematic review was done. We searched several bibliographic databases: *The Cochrane Library* (www.cochrane.org), *The Centre for Evidence-Based Medicine* (www.cebm.net), *The NHS Centre for Reviews and Dissemination* (www.york.ac.uk/inst/crd) un *PubMed Clinical Queries: Find Systematic Reviews* (www.ncbi.nlm.nih.gov/entrez/query/static/clinical.shtml). Keyword search was used and reviewed all abstracts and articles (12593 for Group A Streptococcus from 1980, 3955 for streptococcal pharyngitis from 1932, 11009 for macrolide resistance from 1953 and 160 for HLA and rheumatic fever from 1975) and included most relevant studies for detailed full text review. Each article was analyzed to determine the sample characteristics, study settings, measurement strategy. We exclude duplicate publications or multiple articles reporting identical data over the same time period on the same population.

11.2. Design and Description of the Study Sections

11.2.1. Section I - *Structure of clinical forms of GAS caused infections in children at Children's Clinical University Hospital (CCUH) in Riga.*

In Section I, retrospectively and making use of 160 medical case records, analysis was made at CCUH Emergency department and in-patient departments within years 2004-2006 from the data collected by CCUH Microbiological laboratory, on the connection of obtained GAS isolates with the infection's clinical form, with the aim to define the most frequent clinical form of GAS infection in children.
Simultaneously CCUH Medical Statistics data of last 5 years (2004-2008) were analyzed in relationship with The International Classification of Diseases, 10th edition (ICD-10) diagnosis codes, which are attributed to verified and presumptive GAS infection.

11.2.2. Section II - *Prevalence of GAS caused acute pharyngitis in children at CCUH Emergency Department and peculiarities of clinical-laboratory diagnostics.*

Three hundred forty children seeking care at the state children's hospital – CCUH, Emergency department for acute respiratory infection, were included in the prospective observational study during 2002.–2006.

Inclusion criteria:

- male and female children from 24 months to 12 years;
- any child with at least one of the following symptoms: pharyngeal erythema, cough, cold, sore throat.

Exclusion criteria:

- Documented antibiotic use during last three days;
- Documented use of intramuscular benzathine penicillin G during last 28 days;
- Presence of ear discharge or impetigo at the time of examination;
- History of previous rheumatic fever or rheumatic heart disease;
- History of allergy to amoxicillin or penicillin;
- Presence of any other infection requiring antibiotics;
- Presence of any other known severe illness requiring hospitalization; EXCEPT: malnutrition or tuberculosis;
- Previous enrollment in the study;
- Physician's diagnosis of wheezing, bronchitis, or pneumonia;
- Patient unable to return for follow up visit;
- Parent's or guardian's consent not available.

After enrollment each child had information on identification, demographic variables, medical history, physical examination, laboratory investigations, and information on treatment and follow up visits recorded. A throat culture was taken and a serum sample (5 ml) for antistreptolysin O (ASLO) and antideoxyribonuclease (antiDNaseB) was obtained both at initial and follow up visits after 21 -32 days. These data were collected uniformly of every child. At the time of the enrollment rapid Biostar Strep A OIA MAX GAS antigen detection tests (RADT) were performed.

Criteria for episode of acute GAS pharyngitis and GAS carrier status was defined.

Criteria for episode of acute GAS pharyngitis

- Positive GAS antigen detection test and
- Positive throat GAS culture and
- As a serological response at least double elevation in antistreptolysin O (ASLO) and/or antiDNase B titers in pair seras.

Criteria for GAS carrier status

- Positive GAS antigen detection test and
- Positive throat GAS culture and
- **there were no** at least double elevation in antistreptolysin O (ASLO) and/or antiDNase B titers in pair seras **or** elevated (above normal) ASLO and/or antiDNase B titers already at first visit and not doubled at follow up visit.

The data on culture positivity and symptoms were analyzed by regression techniques to formulate a clinical prediction instrument for clinicians to use in the diagnosis of streptococcal pharyngitis in children in the absence of throat cultures or other diagnostic tests.

11.2.3. Section III - *Molecular-biological analysis of GAS isolates obtained during the research time, and defining of antimicrobal resistance genes.*

Ninety-six randomly chosen of 200 previosly in Section I obtained GAS isolates from non-sterile sites such as throat swabs, and pus sample from two different patient subgroups, were studied in Section III in the period 2005-2006.

Antibiotic resistance was determined by disc susceptibility tests according to CLSI standards. The presence of *ermA, ermB* and *mefA* was established using amplification of streptococcal DNA with specific primers.

PCR mixture and conditions were made according to CDC guidance and *emm* type and subtype assignments were determined as described in the Centers for Disease Control and Prevention website (http://www.cdc.gov/ncidod/biotech/strep/doc.htm).

11.2.4. Section IV - *Research of genetic associations in children with rheumatic fever (RF) carrying HLA Class II alleles, in Latvia.*

In the study Section IV were included 70 white children in Latvia under the age of 18 who had RF during the period from year 1984 - 2004. Patient consent was obtained. The RF diagnosis was confirmed according to the Jones criteria. Eight RF patients had chorea minor. As a result of RF, 47 patients (67.1%) had developed RHD. Cardial valve damages were diagnosed by echocardiography (ECG) and/or heart catheterisation. RHD patients were further split into groups with mitral valve regurgitation (MVR), aortal valve regurgitation (AVR) and MVR + AVR or MVL. Data of healthy individuals (n=100) were obtained from the Databank of Laboratory

of Clinical Immunology and Immunogenetics, Riga Stradins university, Latvia. The above individuals were free of autoimmune disease and had no family history of RF. In both groups (RF patients and healthy individuals) HLA Class II alleles were determined by polymerase chain reaction (PCR).

11.3. Organization of Research

The GAS isolates, mentioned in Sections I and II, were obtained and proved with standard methods in the Microbiological laboratory of Children's Clinical University Hospital (CCUH), (Head of Laboratory Ņ. Pugačova). Throat posterior pharyngeal wall culture swab for obtaining GAS, was performed prospectively in the manner described in Section II, and as a routine examination of patients with respiratory complaints at Emergency Department or hospital ward of CCUH.

Section III, previously in CCUH Microbiological laboratory obtained, GAS isolates were tested on the presence of antibiotic resistance genes *(erm, tet)* and on polymorphism of M protein gene *(emm)* at the Department of Molecular biology and genetics of Stradiņš Clinical Univeristy Hospital, Central Laboratory (Head of Department Dr.biol. E. Miklašēvičs)

Section II, Serological diagnostics of patients' blood serum was performed in the CCUH Clinical biochemical laboratory (Head of laboratory D. Grāvele) and in E. Gulbis Laboratory (Person in charge of the present study: dr. Didzis Gavars).

Section IV, Immunogenetical tests were performed in the Immunogenetic and Immunology Interdepartment Laboratory of Riga Stradiņš Clinical University hospital (Head of Laboratory J. Eglīte). For control group, data of healthy children (n=100) were used from database of the Clinical Immunogenetic and Immunology Interdepartment Laboratory (former Latvian Institute of Immunology, Head of Institute A. Sočņevs).

Statistical analysis of the data was performed in the Department of Physics (Head Prof. U. Teibe); consultant of the present study: RSU Lecturer Oskars Rasnačs.

Patient selection and survey for prospective and retrospective analysis were performed at the Clinic of Paediatric Diseases, Children's Clinical University Hospital in Riga (Head of Clinic Prof. D. Gardovska), in Sections II and IV with the consent of children's parents or guardians as patients' case records (N. Kaufmane, Head of Department of Medical Statistics and Information Technologies, CCUH) and the

Anamnesis part of WHO Multicentre Research *The GRASP (Group A Streptococcus pyogenes study)*. Study Protocol of Section II was approved by Institutional Review Board of *John Hopkins University* within the framework of the multicentre research. It was submitted for review and approval to the WHO Institutional Review Board and to Ethics Committees of all participating countries, including Latvia.

Each child involved in the study (Section II) received therapy according the present domestic guidelines for treating streptococcal pharyngitis

Documentation and information of the study was handled and kept confidentially and in accordance with the Latvian Law.

11.4. Data analysis and statistics

In Section I and III to analyse patients' and GAS isolates data descriptive statistics were used.

In Section II for entering, storing and processing data in compliance with the approved standard computerized methods of biological research, a specially tailored data storing and analyzing system EPI INFO 2000 was used; for processing statistical data, the established Mann-Whitney and Chi –Square tests were used, which are common in biological and medical research statistical data processing. Indicators of head tendency were evaluated: the mean, median and moda, as well as dispersion indicators: standard deviation, mean standard error and interquartiles. Differences in values were estimated with credibility $p<0.05$. As for symptoms, if they statistically credibly differed ($p<0.05$), odds ratio (OR) was evaluated

In Section IV the HLA-DRB1, DQA1 and DQB1 allele frequencies in patients and control subjects were compared. Typing of all 3 loci was performed on all patients and control subjects. Allele and haplotype frequencies of HLA class II were determined by the method of gene counting tests. The difference in predisposing and protective effects of RHD were measured using the odds ratio method: OR = ad/bc or OR = (2a+1)(2d+1)/(2d+1)(2c+1) when b or c = 0. The statistical significance was examined by Fisher's exact test in RHD and the subgroup of RHD. Allele frequencies (AF) were calculated using the following formula: AF (%) = the sum of the allele/2ñ 100 when n is the sum of the total number of individuals analyzed. Haplotype frequecies (HF) were determined by the method of gene counting the following formula HF (%) = sum of given haplotype/2nx100. P-value was calculated using EPI INFO software version 06 with 95 % confidence intervals, Mantel – Hanzszel and Fisher exact correction for small numbers

12. Results

12.1. Section I - *Structure of clinical forms of GAS caused infections in children at Children's Clinical University Hospital in Riga.*

In Section I, retrospectively and making use of 160 medical case records, analysis was made at CCUH Outpatient Emergency department and in-patient departments within years 2004-2006 from the data collected by CCUH Microbiological laboratory, on the connection of obtained GAS isolates with the infection's clinical form.

In 2004 there were 55 isolates, in 2005 – 32 and in 2006 – 73 GAS isolates obtained from throat swabs, identically the number of case records. GAS strains from other body fluids were not isolated .

Analyzing association of isolated strains with clinical presentations of disease:

- upper respiratory infection cases – acute rhinopharyngitis, pharyngitis, pharyngotonsillitis, paratonsillar abscess - 137 (85.6%) clinical cases and identically isolates;
- scarlet fever – 13 (8.3%) clinical cases and isolates;
- rheumatic diseases – 6 (3.7%) clinical cases and isolates, including 1 RF case;
- vulvitis – 1 (0.6%) clinical case and isolate;
- varicella – 1(0.6%) clinical case and isolate;
- glomerulonephritis - 1(0.6%) clinical case and isolate and
- celiac disease- 1(0.6%) clinical case and isolate.

Simultaneously CCUH Medical Statistics data of last 5 years (2004-2008) were analyzed in relation with The International Classification of Diseases, 10th edition (ICD-10) diagnosis codes, which are attributed to verified and presumptive GAS infection. It was found that the most common GAS infection clinical form in children had been acute phatyngitis and acute tonsillitis.

Therefore in the next Section of the study it was chosen to analyze acute GAS pharyngitis in children in CCUH due to its frequency and possible severe complications.

12.2. Section II - *Prevalence of GAS caused acute pharyngitis in children at CCUH Emergency Department and peculiarities of clinical-laboratory diagnostics.*

Three hundred forty children seeking care at the state children hospital – CCUH, Emergency department, for acute respiratory infection were included in the prospective observational study during 2002.–2006

From 340 children having complaints of sore throat, 27.6% (n=94) had positive RADT and 22% (n=75) of those - positive GAS culture (Table 1). Serological response, which is at least double elevation in antistreptolysin O (ASLO) and/or antiDNase B titers in pair seras, had 11,5% (n=39) children.

Such who had just positive RADT were 1.8% (n=6), in its turn such, who had both – positive RADT and positive GAS throat culture - 5.9% (n=20) (Table 1).

Acute GAS pharyngitis patients

By study definition acute pharyngitis patients, e.g., - positive RADT, positive throat GAS culture and a serological response at least double elevation in antistreptolysin O (ASLO) and/or antiDNase B titers in pair seras, were 6.5% (n=22) (Table 1).

Pharyngitis patients with positive RADT, positive throat GAS culture and double elevation of antistreptolysin O (ASLO) in follow up visit were 2.6% (n=9) (Table 1).

Such who had positive RADT, positive throat GAS culture, and double elevation of antiDNaseB in follow up visit were 1.5% (n=5) (Table 1).

Summing up both above mentioned groups of patients, as acute GAS pharyngitis patients were considered 10.6% (n=36) and prevalence of acute GAS pharyngitis is 0,106±0,1.

Table 1.

Distribution of patients(n=) by laboratory findings

Laboratory findings		GAS **carriers** (n=)	**Acute** GAS pharyngitis (n=)
positive RADT		**6**	-
positive RADT + positive GAS culture		**20**	-
positive RADT + serological response - 2×↑ ASLO and/or antiDNaseB **initial visit**		6	-
positive RADT + serological response - 2×↑ ASLO and/or antiDNaseB **follow up visit**		-	3
positive RADT + positive GAS culture + serological response **initial visit**	2×↑ ASLO **and** antiDNaseB	17	-
	2×↑ ASLO		
	2×↑ antiDNaseB		
positive RADT + positive GAS culture + serological response **follow up visit**	2×↑ ASLO **and** antiDNaseB	-	**22**
	2×↑ ASLO	-	**9**
	2×↑ antiDNaseB	-	**5**
TOTAL		**26** (49)	**36** (39)

Pink colour and bold numbers – defined by study criteria

Ligt yellow colour and grey numbers– study variations

GAS carriers

As GAS carriers were defined those patients who had positive RADT, positive throat GAS culture, but there was no at least double elevation in antistreptolysin O (ASLO) and/or antiDNase B titers in pair seras or elevated (above normal) ASLO and/or antiDNase B titers already at first visit and not doubled at follow up visit. Such patients in the study group were 5.9% (n=20). To the previously mentioned patients we included also those who had just positive RADT - 1.8% (n=6). Summing up both above mentioned groups of patients, as GAS carriers were considered 7.6% (n=26) and therefore GAS carrier prevalence is 0,07 ±0,098.

Not all patients with positive culture swab have true GAS infection. According to the published data, 50% of these patients are GAS carriers, who do not have an increased risk to develop RF. Analyzing patients with positive culture from the throat posterior pharyngeal wall (n=75), 37 patients were defined as GAS carriers, in percentage 49%. It follows, that half of all the patients with positive culture swab from the throat posterior pharyngeal wall, in reality were only GAS carriers with clinical picture of acute pharyngitis of another etiology.

GAS carrying incidence compared to the patients with acute pharyngitis picture, testifies that approximately 1/4 of school-age children with culture positive pharyngitis are not really infected, but are only GAS carriers whose pharyngitis clinic at that moment is of acute non-streptococcal etiology.

Pharyngitis or "sore throat" is a very frequent affection throughout the world, especially among the children. It is estimated that every child at least once a year has some acute pharyngitis episode. GAS pharyngitis risk group are children from the age of 5 to 15, younger children on their turn, have more often pyodermia of streptococcal etiology. The incidence of pharyngitis of streptococcal etiology in the world is calculated from 3 000 to 6 000 per 100 000 children a year, though the actual incidence is difficult to assess, because not all countries of the world require pharyngitis of streptococcal etiology to report epidemiologically as a case.

In about 10-30% episodes of acute pharyngitis, the agent is GAS, in all other cases most often virus infection acts as etiological agent. In the present study, GAS as an etiological factor of acute pharyngitis was found only in 10.6% of cases, which conforms to the data of international sources.

Clinical symptoms of GAS pharyngitis (Table 2)

After enrollment each child had physical examination, aiming to find signs or symptoms as predictive in diagnostics of acute GAS pharyngitis.

In compliance with the approved standard computerized methods of biological research, the established Mann-Whitney and Chi –Square tests, two different patient groups were compared: acute pharyngitis patients and GAS carriers. There was statistical difference found in the following symptoms: hoarseness, nose congestion, difficulties to swallow, abdominal pain and tonsillar enlargement I and III degree ($p<0,05$).

However, it should be noted that these symptoms, from the clinical point of view, are present also in other diseases, as well as in acute pharyngitis of other etiology, that is why their expedience as predictive clinical symptoms in diagnostics of acute GAS pharyngitis, is questionable.

One of the most common diagnostic criteria in cases of acute streptococcal pharyngitis in doctors' opinion is fur on the tonsils or posterior pharyngeal wall. Very often it becomes the criteria for prescribing antibacterial therapy. In the present study, exudative pharyngitis in children with acute Streptococcal infection was found in 54% ($n=21$). So it may be concluded, that this symptom is not a safe diagnostic criteria of acute Streptococcal pharyngitis, and cannot be used as the only indicator for prescribing antimicrobial therapy. Furthermore, in cases of virus-caused acute pharyngitis, fur on the tonsils or posterior pharyngeal wall can be present, but in the therapy of these pharyngitis, antimicrobial preparations are absolutely not indicated.

Table 2.

Frequency of Clinical Symptoms in Acute GAS Pharyngitis Patients and GAS Carriers

Symptoms	p value	Acute pharyngitis patients (n=)	%	GAS carriers (n=)	%
Hoarseness	**p<0.05**	**21**	**54%**	**14**	**28,5%**
Fever	p>0,05	33	85%	34	69%
Chills	p>0,05	28	72%	26	53%
Running nose	p>0,05	12	31%	11	22%
Nose congestion	**p<0.05**	**24**	**61,5%**	**18**	**37%**
Sore throat		32	82%	30	61%
Difficult to swallow	**p<0.05**	**28**	**72%**	**20**	**41%**
Vomitting	p>0,05	11	28%	8	16%
Earache	p>0,05	4	10%	4	8%
Abdominal pain	**p<0.05**	**16**	**41%**	**10**	**20%**
↓ activity level	p>0,05	32	82%	31	63%
Bad sleep	p>0,05	25	64%	21	43%
Cough	p>0,05	10	26%	8	16%
Pharyngeal erytema	p>0,05	35	90%	37	75,5%
Tonsillar erytema	p>0,05	36	92%	34	69%
Erytema posterior	p>0,05	32	82%	31	63%
Faucial erytema	p>0,05	29	74%		29
Tonsillar enlargement I	**p<0.05**	**2**	**5%**	**11**	**22%**
Tonsillar enlargement II	p>0,05	17	43,5%	16	33%
Tonsillar enlargement III	**p<0.05**	**16**	**41%**	**8**	**16%**
Tonsillar enlargement IV	p>0,05	2	5%	3	6%
Exudate	p>0,05	21	54%	21	43%
Tender cervical lymphnodes	p>0,05	29	74%	25	51%
Scratched nostrils	p>0,05	3	8%	4	8%
Pharyngeal exudate	p>0,05	1	2,5%	5	10%
Petechias of palate	p>0,05	13	33%	8	16%
Strawberry tongue	p>0,05	12	31%	12	24%

Analyzing frequency of clinical symptoms in comperable study groups, acute GAS pharyngitis patients most often had pharyngeal (90%, n=35) and/or tonsillar erytema (92%, n=36), as well as fever (85%, n=33), lowered activity level, sore throat and erytema posterior (82%, n=32). The most often found symptoms in GAS carriers group were the same as in acute GAS pharyngitis group – the most often was pharyngeal erytema (75,5%, n=37), equally often was found fever and tonsillar

erytema (69%, n=34), as well as lowered activity level and erytema posterior (63%, n=31).

Less frequently found symptoms of acute GAS pharyngitis patients were pharyngeal exudate (2,5%, n=1), tonsillar enlargement I and IV (5%, n=2), scratched nostrils (8%, n=3) and earache (10%, n=4). The least found symptoms in GAS carriers group were tonsillar enlargement IV (6%, n=3),), scratched nostrils and earache (8%, n=4), also pharyngeal exudate (10%, n=5).

Such symptoms which are evaluated routinely in association with presumable GAS pharyngitis, in GAS pharyngitis patient group were found in following frequency: tonsillar exudate (54%, n=21), difficulties to swallow (72%, n=28), tenderness of cervical lymphnodes (74%, n=29), fever (85%, n=33) and strawberry tongue (31%, n=12).

Comparably in GAS carriers group: tonsillar exudate (43%, n=21), difficulties to swallow (41%, n=20), tenderness of cervical lymphnodes (51%, n=25), fever (69%, n=34) and strawberry tongue (24%, n=12).

Data from the prospective, observational cohort study published in 2008 where signs and symptoms of children presenting with GAS pharyngitis in several countries among them also in Latvia were compared, showed that clinical presentation of pharyngitis may differ by country or region. It is therefore crucial that clinicians continually evaluate local presentation of signs and symptoms associated with GAS infection in the specific region to reach an accurate clinical diagnosis.

12.3. Section III - *Molecular-biological analysis of GAS isolates obtained during the research time, and defining of antimicrobal resistance genes.*

A total of 96 GAS isolates from throat swabs of both inpatients and outpatients with sore throats were used to detect antimicrobial resistance. Antimicrobial susceptibility tests revealed that all the strains tested were sensitive to vancomycin, linezolid, penicillin and ceftriaxone. Simultaneously, high levels of resistance to macrolides were evident; 78% of the isolates were resistant to clindamycin and erythromycin. There was no statistical difference in resistance to macrolides between the two subgroups (Table 3), and no significant change in the yearly or seasonal incidence of resistance was observed.

Molecular studies demonstrated that the majority of strains harboured *ermA* (n=27), *ermB* (n=23) or both genes (n=24). *mefA* was detected only in one strain.

Table 3. **Macrolide resistance in patients**

	Patients (n)	CC	E	*ermA*	*ermB*	*mefA*
Outpatients	32	26	8	17	13	1
Inpatients	64	52	14	39	35	0
Total	96	78	23	56	48	1

Treating GAS-induced infections with antibiotics has several different aims, including the prevention of acute rheumatic fever and acute glomerulonephritis, to achieve fast resolution of the symptoms and clinical signs of acute illness, to prevent suppurative complications and to eradicate GAS in order to reduce transmission.

A number of oral and intravenous antibiotics are effective in the treatment of group A streptococcal infections. Penicillin resistance has not been a significant problem in the treatment of GAS; no clinical isolate of group A *Streptococcus* has been reported to be resistant to penicillin. However, increased macrolide usage following the introduction of the second-generation macrolides has been directly associated with the relatively high increase in resistance to these agents.

Macrolides have been the class of antibiotics most widely prescribed by general practitioners in Latvia for the treatment of upper respiratory tract infections during the last 5 years.

By the mid 2000s, consumption of conventional macrolides had declined in favour of newer macrolides such as clarithromycin (Figure 1) or azithromycin (Figure 2), which were prescribed by general physicians or specialists. With the exception of clarythromycin, consumption of conventional macrolides in hospitals during 2006 decreased in favour of newer macrolides. The number of packs of clarithromycin sold in drug stores in 2002 totalled 20,921; this approximately tripled to 54,981 packs in 2006. Sales of azythromycin reached 11,106 in 2002 but by 2006, 22,193 packages were sold.

Figure 1. **Consumption of Claritomycin in Drug Stores and Hospitals in Latvia from 2002 to 2006. (SAM)**

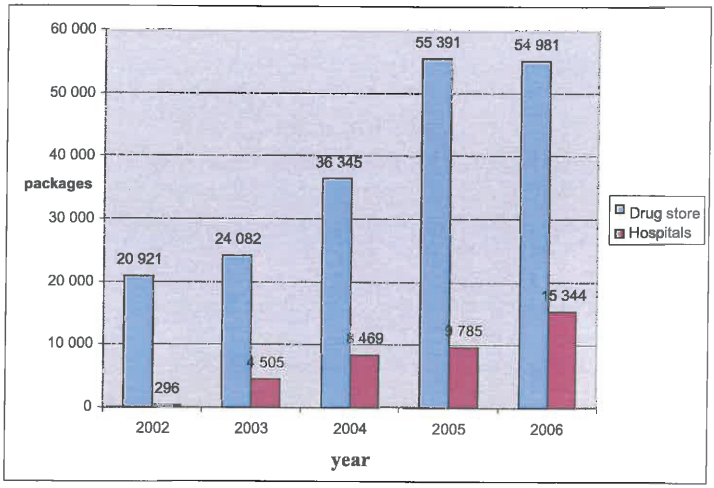


Figure 2. **Consumption of Azitromycin in Drug Stores and Hospitals in Latvia from 2002 to 2006. (SAM)**

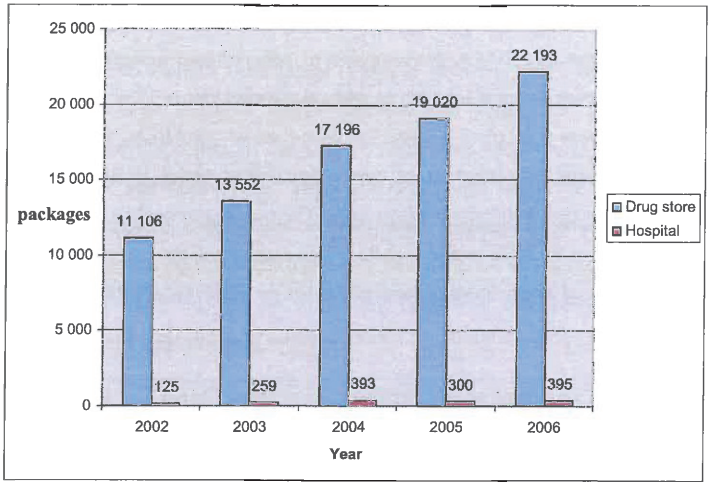
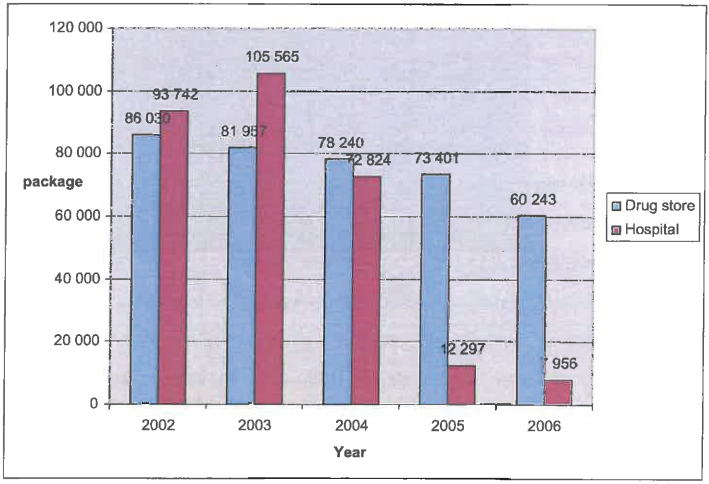


Figure 3. **Consumption of Eritromycin in Drug Stores and Hospitals in Latvia from 2002 to 2006. (SAM)**



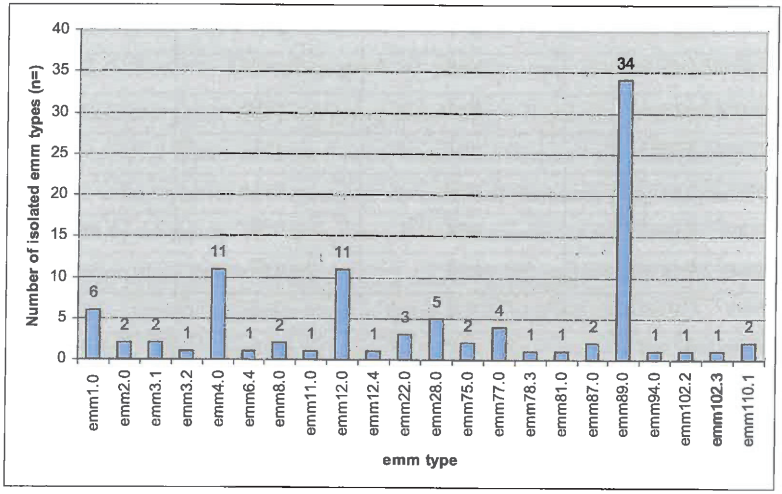
Consumption of erythromycin decreased among both outpatients and inpatients (Figure 3).

Better knowledge of established guidelines for antimicrobial treatment of upper respiratory tract infections by general practitioners and primary care pediatricians is needed in Latvia.

GAS *emm* type distribution

In total, 22 different *emm* types were identified among 96 non-invasive isolates. (Picture 1; Table 4). The most prevalent *emm* types were *89.0* (34/96; 35%), as follows *4.0* and *12.0* (11/96; 11%), which comes next *1.0* (6/96; 6%) and *28.0* (5/96; 5%). The rest of isolated *emm* types is shown in Picture 1.

Picture 1. **GAS *emm* type distribution (n=).**



emm 89.0 type was found prevailingly associated with antimicromial resistance to eritromycin (n=25) and clinamycin (n=31), all *emm4.0* (n=11) showed resistance to both MLS_B (macrolide-lincosamide-streptogramin B) antibiotics. The rest of GAS isolates *emm* types in association with antimicrobial resistance shown in Table 4.

During *emm* typing two different isolates were found, where the difference appeared from the type *emm 1.0*. Data on different isolates were sent to *CDC*, and received confirmation for new subtype *emm 1.51* isolation in Latvia.

Table 4.

GAS isolates *emm* types in association with antimicrobial resistance

***emm* type (number of isolates)**	**Resistant isolates to**		
	clindamycin and erythromycin	**erythromycin**	**clindamycin**
emm1.0 (n=6)	n=3	n=3	n=3
emm2.0 (n=2)	n=0	n=2	n=0
emm3.1 (n=2)	n=2	n=2	n=2
emm3.2 (n=1)	n=0	n=0	n=0
emm4.0 (n=11)	n=11	n=11	n=11
emm6.4 (n=1)	n=0	n=1	n=0
emm8.0 (n=2)	n=1	n=1	n=2
emm11.0 (n=1)	n=0	n=0	n=0
emm12.0 (n=11)	n=5	n=9	n=6
emm12.4 (n=1)	n=1	n=1	n=1
emm22.0 (n=3)	n=0	n=1	n=1
emm28.0 (n=5)	n=2	n=2	n=2
emm75.0 (n=2)	n=2	n=2	n=2
emm77.0 (n=4)	n=3	n=3	n=3
emm78.3 (n=1)	n=0	n=0	n=1
emm81.0 (n=1)	n=0	n=0	n=0
emm87.0 (n=2)	n=2	n=2	n=2
emm89.0 (n=34)	n=25	n=25	n=31
emm94.0 (n=1)	n=1	n=1	n=1
emm102.2 (n=1)	n=1	n=1	n=1
emm102.3 (n=1)	n=1	n=1	n=1
emm110.1 (n=2)	n=1	n=2	n=1

emm 89.0 type in studies more often had been found in association with invasive streptococcal diseases, which is different from the recent study – it had been isolated from acute pharyngitis, e.g. non-invasive, cases. Similar to previously published data, it had been suggested that the high rate of macrolide resistance was caused by *emm 89.0* type, harbouring *erm*(B) gene.

Taking into account GAS resistance data in the world and data of the research made in Latvia, it is important to remember, that still today, penicillin as an optional preparation should be prescribed in treatment of streptococcal acute pharyngitis. Adequate penicillin therapy prevents from developing of rheumatic fever later, even if the treatment is started 9 days after the onset of acute streptococcal pharyngitis; it shortens also the clinical course, reduces the risk of transmission, as well as decreases the risk to develop purulent complications. For these reasons giving antimicrobial therapy in cases of acute pharyngitis is not urgent, and doctor may make a decision on how to start the treatment after awaiting and receiving all laboratory results that he or she thinks necessary to have.

12.4. Section IV - *Research of genetic associations in children with rheumatic fever (RF) carrying HLA Class II alleles, in Latvia.*

According to data from the Latvian Rheumatic Disease Patient Registry, more than 1500 children suffer from rheumatic diseases, and rheumatic fever (RF) is in the third place by frequency. In Latvia, since 1991 the number of RF cases in children under the age of 18 has increased, reaching a 7.5/100 000 incidence in 1998. During the last three years the incidence has been stable at 0.03/100 000 children.

In study Section IV were included 70 white children – 48 boys (68.5%) and 22 girls (31.4%) – in Latvia under the age of 18 who had RF. In the age group younger than 7 years there were 23 individuals (32.8%), and over 7 years – 47 individuals (67.1%). The RF diagnosis was confirmed according to Jones criteria. Eight RF patients had *chorea minor*. As a result of RF, 47 patients (67.1%) had developed RHD. RHD patients were further split into groups with mitral valve regurgitation (MVR, n= 24; 34.3%), aortal valve regurgitation (AVR, n=3; 4.3%) and MVR + AVR or MVL (n= 20; 28.6%). Only 23 of the patients (32.8%) had fully recovered by the age of 18. RF set-back was recorded for 15% of the patients because they had not received prolonged penicillin treatment.

RF is an autoimmune sequel of group A streptococcal infections and one of the leading causes of morbidity and mortality in many parts of the world. The disease is often preceded by rheumatic fever episodes that may, in susceptible individuals, progress to a chronic valvular disease. The relatively low attack rate of RF (0.3 – 3%)

after untreated streptococcal tonsillopharyngitis suggests the involvement of the host genetic factors within the susceptibility to RF with consequential progression to rheumatic heart disease (RHD). The basis of autoimmune processes that contribute to the development of RHD are T-cell molecular mimicry between streptococcal and heart proteins. RHD is initiated by certain serotypes connected with group A streptococcus M protein.

Several studies have suggested that genetic susceptibility to RF and RHD is linked to HLA class II alleles. However, there has been an apparent discrepancy as to the nature of susceptibility and/or protective alleles. Several years ago it could have been associated with different types of laboratory methods, but this may be partly because of ethnic differences within the distribution of HLA alleles and the contribution of other genes that may have displayed amongst different populations. Genetic associations are more likely to be detected in clinically homogeneous groups of patients, and thus it is important to separate carditis patients from patients with different RF sequel.

Frequency of DRB1*, DQB1* Alleles in RF Patients and Control Subjects

In RF patients HLA Class II DRB1*07 (OR=4.18, $p<0.01$), DQB1*0302 (OR=3.13, $p<0.0002$), and DQB1* 0401-2 (OR=4.33, $p<0.0001$) alleles were found more frequently compared to the control group, while the DRB1*06 (OR=0.18, $p<0.0023$), DQB1*0602-8 (OR=0.4, $p<0.0127$), and DQB1*0501 (OR=0.26, $p<0.0027$) alleles were less frequent

Frequency of DRB1 and DQB1 Alleles in RF, RHD, and *chorea minor* Patients Compared to Control Subjects

In the homogeneous patient groups, DRB1*07 had the highest odds ratio in all RF groups: patient group with no acquired valvular heart disease developing after RF carditis (OR=7.35, $p<0.001$), MVR patients (OR=4.45, $p<0.03$), MVL patients (OR=5.44, $p<0.01$), and chorea minor patients (OR= 11.31, $p< 0.002$). The least frequent allele was DRB1*06 (OR=0.18, $p<0.0023$). The DQB1 allele frequencies differed for RHD patients with MVR and MVL: DQB1*0401-2 (OR=8.20, $p<0.001$) was more common in MVR patients and DQB1*0302 (OR= 4.18, $p< 0.001$) in MVL

patients. In chorea minor patients a high frequency of DQB1*0401-2 (OR=6.36, p<0.005) was found.

Protective HLA Class II alleles are as important as the risk alleles. Alleles that can be designated as protective include DRB1*03/*06, DQB1*0201-2/*0201-2 and DQB1*0303/*0602-8. Higher protection against RF and RHD is ensured by the allele genotype DRB1*06 – DQB1*0602-8.

DRB1* and DQB1* Genotypes Associated with RF patients

The strongest associations between DRB1* alleles and RF patients were for DRB1*01/07 (OR= 8.57, p<0.05), DRB1*15/07 (OR= 2.86, p<0.02), DRB1*04/05 (OR=3.57, p<0.04), and DRB1*05/07 (OR=2.86, p<0.02). The DRB1*03/06 allele (OR= 0.48, p< 0.01) had the lowest OR.

Common alleles associated with RF are: DQB1*0301/0302 (OR=5.44, p< 0.05), DQB1*0302/0303 (OR=4.43, p<0.02), DQB1*0302/0601 (OR=2.91, p<0.01), and DQB1*0302/0602-8 (OR=2.19, p<0.01). The lowest allele frequencies in RF patients were observed for DQB1*0201-2/0201-2 (OR= 0.28, p<0.001) and DQB1*0303-0602-8 (OR=0.22, p<0.01).

Distribution of HLA DQA1 alleles and genotypes in RF patients

In RF patients HLA class II DQA1* 0401 (OR=3.31, p<0.01) is found more frequently than in control group, while the DQA1*0102 (OR=0.34, p<0.001) is less frequent. In the RF homogeneous patient groups DQA1*0401 has the highest OR, also in the MVL group, as well as DQA1*0501 (OR=3.25, p<0.03) and DQA1*0301 (OR=3.45, p<0.02) appears in MVL group patients. In chorea minor patients credibly often found DQA1*0201 (OR=3.33, p<0.05)

The DQA1*0102 allele was absent in all RF patients, whereas its frequency was 9% in control subjects (p<0.001), but showed no significant protective effect in the homogeneous patient groups. Significant HLA DQA1 protective genotypes were not found, though DQA1 genotypes *0103/*0201 (OR=7.62, p< 0.03) and *0301/*0501 (OR=2.61, p<0.009) were found more frequently.

Distribution of HLA DRB1/DQA1 genotypes

The most frequently found genotypes in RF patients are *07/*0201 (OR=2.01, p<0.06) and *01/*0501 (OR=3.18, p<0.005), which are preserved significantly also often in MVL group (OR=5.69, p<0.001). The genotype *07/*0201 has often been found in chorea minor patient (OR=3.72, p<0.04), but DRB1*04/DQA1*0401 found in RF patients without RHD (OR=11.1, p<0.004).

Distribution of HLA DQA1/DQB1 genotypes

Both in RF patients and in homogeneous patient groups the least frequent genotypes are *0102/*0602-8 and *0501/*0201-2. The genotype DQA1*0501 with DQB1 risk allele *0301 often found in RF patients (OR=2.10, p<0.01), in MVL group – (OR=3.35, p<0.001) and also in patients without RHD (OR=2.58, p<0.03).

The genotype *0301/*0402 has been significantly found in RF patient group and in chorea minor patient group, but there is no validity in RHD groups.

This cannot be considered as a statement. It is only a supposition. In order to develop the supposition into statement it is necessary to continue the experiments by increasing the group under the study.

Distribution of DRB1-DQA1-DQB1 haplotype

The haplotype *07-*0201-*0302 (OR=21.94, p<0.001) frequently found in RF and homogeneous patient groups – MVL (OR=26.0, p<0.001) and patients without RHD (OR=35.1, p<0.002). In its turn haplotypes *04-*0401-*0301 (OR=16.6, p<0.003) and *04-*0301-*0401-2 (OR=78.0, p<0.0001) are frequent amongst patients with chorea minor.

Protective alleles DQA1*0102 and DQB1*0602-8 in haplotype DRB1*15 showed no significant protective effect in RF patients.

It is significant that the patients were grouped into clinically homogeneous groups as opposed to the total RF patient group, as they differed in DQ allele frequencies.

The severity of RF is likely associated with the DRB1*07 allele and development of certain RHD may be dependent on specific DQ alleles.

Results of the present study support our hypothesis and indicate that certain HLA class II alleles, genotypes and haplotypes are associated with risk/protection from RHD and that these associations are more evident in patients among clinically

homogeneous groups. Also ethnical differences should be taken in account in spite of the division in homogeneous groups, also presuming that in the last 5 years all studies have been performed with the PCR-SSP method.

Our study provides further information on the genetic predisposition for RF hypothesis and on the protective immune responses in RHD. Further insight into the molecular mechanisms of the disease will be a useful tool for predicting the clinical outcome in rheumatic fever patients and thus potentially offer new means and approaches to treatment and prophylaxis, inter alia the invention of potential vaccine.

13. Conclusions

1. The most frequent clinical form of GAS caused infection in children is acute pharyngitis, which confirms one of the study questions. The prevalence of GAS caused acute pharyngitis in children is 10.6±0,1 % (n=36) and is similar to such in other European countries.

2. 49% (n=37) of study population children who had GAS obtained from the throat posterior wall culture, and 52% (n=49) of study population children, who had positive RADT, were noticed carrying GAS with acute pharyngitis clinical picture of some other unspecified etiology.

3. Predictive clinical symptoms, that would allow to diagnose early the acute GAS caused pharyngitis, were not found.

4. GAS strains used in the study, have a very high macrolide resistance -78%, which exceeds the mentioned figures in study's promoted question, and from now on great attention should be paid to a deliberative strategy of antibiotics use. Association of antimicrobial resistance with certain GAS *emm* types, is similar to that in other European countries. During the course of the study a new GAS *emm 1.51* subtype was discovered, which is now approved by *CDC (Centers for Disease Control and Prevention)*.

5. Results of the present study indicate that certain HLA class II alleles, genotypes and haplotypes are associated with risk/protection from RHD and that these associations are more evident in patients among clinically homogeneous groups. It is a useful tool for predicting the clinical outcome in rheumatic fever patients and thus potentially offer new means and approaches to treatment and prophylaxis, inter alia the invention of the potential vaccine.

14. Publications on the Study Theme

1. Stanevicha V, Eglite J, Sochnevs A, Gardovska D, Zavadska D, Shantere R. HLA class II associations with rheumatic heart disease among clinically homogeneous patients in children in Latvia. *Arthritis Res Ther* 2003; 5:R340-R346.
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5. Zavadska D, Drukaļska L, Pugačova N, Bērziņa D, Gardovska D, Miklaševičs E. Macrolide resistance of group A beta haemolytic Streptococcus isolated from outpatient children in Latvia. *APMIS* 118: 366–370.

15. Conference Theses on the Study Theme

1. Staņēviča V, Eglīte J, Zavadska D, Sočņevs A, Šantere R, Gardovska D. HLA class II associations with rheumatic heart disease among clinically homogeneous patients in children in Latvia. *J Annals of Rheumatic diseases*, 2002, Vol 61, suppl.1, p.83.
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