Q1 Age and gender associated changes in immunoglobulin Q2 subclass levels specific to *S. pneumoniae*, serotype 1

Sanja Knežević[a], Dejana Kosanović[a], Luka Dragačević[a], Irena Živković[a], Vesna Ilić[b], Ljiljana Hajduković[c], Olivera Savić[d], Rajna Minić[a,*], ~~minicrajna@gmail.com~~, rajna.minic@imi.bg.ac.rs

[a]Institute of Virology, Vaccines and Sera, Torlak, Vojvode Stepe 458, 11000 Belgrade, Serbia

[b]Institute for Medical Research, National Institute of Republic of Serbia, University of Belgrade, Dr. Subotića 4, POB 39, 11129 Belgrade 102, Serbia

[c]Institute for Application of Nuclear Energy, University of Belgrade, Banatska 31b, 11080 Beograd, Serbia

[d]Institute for Blood Transfusion of Serbia, Svetog Save 39, Belgrade, Serbia

*Corresponding author.

Abstract

Q3 Q4 *S. pneumoniae* is an important human pathogen which has a polysaccharide capsule with virulent properties. This work aims to estimate the titres of *S. pneumoniae* specific IgG and IgA isotypes, with respect to age and sex.

An in-house whole bacterial cell ELISA was used for the determination of relative levels and endpoint titres of IgG subclasses and IgA1 subclass specific for *S. pneumoniae* serogroup 1, and to quantify specific IgG1 and IgG2 levels.

Significantly lower anti-pneumococcus IgG1 titres were found in older individuals, which was more pronounced in men. Lower IgG2 titres were detected in men over 50 years of age, in comparison to women under 50 years of age. The levels of IgG3 and IgG4 did not differ between different sex and age groups. Lower IgA1 levels were detected in male individuals in both age groups in comparison to females under 50 years of age. The levels of IgG1 showed a moderate correlation with IgG4 in younger individuals of both sexes (r = 0.61 in men and 0.63 in women) which was not noted in the older age group.

We highlight the deficiency in humoral immunity in older people, especially male and suggest immunization of this population with pneumococcal vaccines.

Keywords:

Ageing, Anti-bacterial antibodies, Anti-pneumococcus antibodies, Sex characteristics

Abbreviations

No keyword abbreviations are available

1 Introduction

During the famous 1918 influenza pandemic, bacterial pneumonia, caused predominantly by pneumococci and streptococci was a major cause of death [1]. *Streptococcus pneumoniae* (Klein, 1884), the pneumococcus, is an important human pathogen which has a polysaccharide capsule with virulent properties. The capsule enables pneumococcal survival during infection by sheltering it from phagocytosis. It is widely studied, and this has led to the

identification of a large number of serotypes (more than 95) with more capsular types theoretically existing in nature [2] .

Streptococcus pneumoniae is responsible for causing invasive pneumococcal disease (IPD): pneumonia, otitis media, bacteraemia, and meningitis [3]. Today, in a number of COVID-19 patients pneumococcal pneumonia co-infections, super-infections and IPDs have been found [4,5].

Humoral immunity or antibody-mediated immunity interacts with pathogens at mucosal sights and in the circulation. Human immunoglobulin G (IgG) is the most abundant class of antibodies in the peripheral blood, and the most important one for adaptive immunity. It is classified into four subclasses, IgG1, IgG2, IgG3 and IgG4, with IgG1 being the most abundant and IgG4 the least [6]. The antibodies belonging to IgG1 subclass mostly act against protein antigens and IgG2 against polysaccharide antigens [7] including antigens expressed on encapsulated bacteria. While the deficiency in IgG1 subclass usually displays as total IgG deficiency and probably represents a form of common variable immune deficiency, compensation for deficiency in IgG2 antibodies may be found in IgG1 and IgG3 antibodies [6,7]. IgG1 antibodies against polysaccharide antigens have been noted in *Haemophilus influenzae* b infection [8] and a shift to IgG1 and IgG3 response was observed against protein-conjugated glycans after pneumococcal vaccination [7].

Immunoglobulin A is mostly regarded as mucosal antibody class, as it is massively secreted on mucosal surfaces. It is also the second most abundant antibody class in the circulation [9]. There are two IgA isotypes in the circulation, of which IgA1 is more abundant.

An effective (i.e. protective) immune response to *Streptococcus pneumoniae* is antibody mediated [10] but the role of different antibody isotypes in this protection is not entirely known. The aim of this research was to dissect the reactivity of different IgG subclasses and of IgA1 from peripheral blood of healthy people who have not been vaccinated against pneumococci, towards whole pneumococcus cells, in order to evaluate age and sex differences. For this purpose, we have used an in-house whole cell ELISA to measure all antibodies binding to this microorganism.

2 Materials and methods

2.1 *Streptococcus pneumoniae* cells plate coating procedure

In this study *Streptococcus pneumoniae* ATCC® 6301, belonging to serogroup 1 was used. It was propagated in hemo-aerobic culturing medium (HEMO-AE, Torlak, Serbia) at 37 ºC, 5 % CO_2.

The procedure for coating MaxiSorp ELISA plates (Nunc, ThermoFisher Scientific, Denmark) with microorganisms was carried out as previously described [11].

2.2 Anti-bacterial ELISA

The procedure was done, with minor modifications, as previously described [11]. Namely, the washing step was modified and included washing 3 times with PBS with 0,05% 0.05 % Tween 20 (TPBS) and once with PBS. For preliminary analysis of bacteria-specific IgG, the sera were diluted 400 x for IgG1 and IgG2, while for the analysis of IgG3, IgG4 and IgA1 subclasses - 200 x dilutions were used. The following monoclonal antibodies were used: anti-human IgG1 clone HP6019, anti-human IgG2 clone HP-6014, anti-human IgG3 clone HP-6050 (all from Sigma-Aldrich) and anti-human IgG4 clone JDC-14 (Biolegend) all produced in mouse and coupled to biotin and anti-human IgA1 alkaline phosphatase, clone B3506B4 (Abcam). Inhibitory ELISA was performed by mixing appropriate amounts of purified IgG of a particular subclass isolated from patients with IgG monoclonal gammopathies, with the diluted secondary antibody, details are given in Supplement 1.

2.3 Study subjects

The exclusion criteria were the presence of a severe infection, immunization with pneumococcal vaccines, use of probiotics and antibiotics one month before sample collection, recent surgical intervention, or existence of chronic diseases. In this study sera of five groups of people were analysed: 1) both sexes, median age 23 years (IQR: 22-24)22–24) (10 men and 12 women), for pooled sample in quantification studies; 2) W<50W< 50 – women median age 32 years (IQR: 18-46) (n = 24);18–46) (n = 24); 3) W>50W> 50 – women median age 67.5 years (IQR: 57-78) (n=24);57–78) (n = 24); 4) M<50M< 50 – men median age 38.5 (IQR: 25-52) (n = 24) 25–52) (n = 24) and 5) M>50M> 50 men median age 68 (IQR: 58-78) (n=24).58–78) (n = 24). Statistical significance of differences in the age between men and women of same age groups was not found (women versus men in < 50 years of age groups, p = 0.09; p = 0.09; and women versus men in > 50 years of age groups, p=0.332).p = 0.332). All the experiments were done in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Ethics Committee of The Institute of Virology, Vaccines and Sera, Torlak, Serbia.

2.4 Statistical analysis

For group comparison, endpoint titres were determined, and results are expressed as the geo mean ~~± 95%~~± 95 % confidence interval, Kruskal–Wallis one-way analysis of variance was used with Dunn's Multiple Comparison test; ~~p < 0.05~~ $p < 0.05$ was considered statistically significant. For statistical analysis, including IC50 calculation GraphPad Software was used.

3 Results

3.1 Analysis of a steady-state IgG and IgA response to *S. pneumoniae*

For the evaluation of antibacterial antibody response against *S. pneumoniae* in ELISA with whole bacterial cells ~~pooled serum sample from~~sera from 24 young adults were~~was~~ used. As can be seen in Fig. 1. in this system, all of human IgG subclasses and IgA1 subclass bound *S. pneumoniae*, and the binding was not restricted to IgG2.

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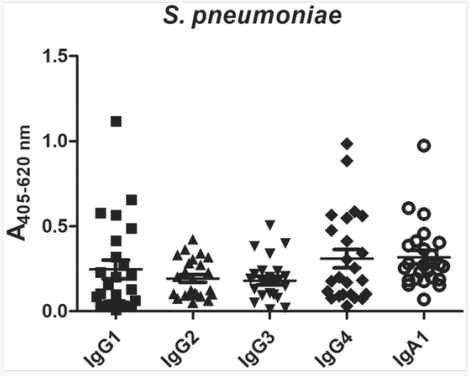
alt-text: Fig. 1

Fig. 1



Levels of different antibody subclasses (IgG1, IgG2, IgG3, IgG4 and IgA1) in human serum of healthy young adults at steady state towards *S. pneumoniae*.

3.2 Quantification of specific anti-*S. pneumoniae* serotype 1 antibody subclasses

Results of inhibitory ELISA shoved that monoclonal IgG1 (Supplement, Fig. S1A) gave mean IC50 value 45 ng/ml (95 % CI 7–276 ng/ml). The quantification of IgG2 (Supplement, Fig. S1B) gave mean IC50 value 5.7 μg/ml (95 % CI 3.5–9.3 μg/ml). The quantification of the IgG3 and IgG4 antibody subclasses specific to *S. pneumoniae* was not performed, due to the low abundance of IgG3 and IgG4.

Due to wide confidence intervals obtained for IC50 analysis end-point titration was used for the assessment of antibody reactivity in different sex and age groups.

No differences in specific antibody titres were detected between sera from young adults (male and female) used in a pooled form for quantification experiments and the ~~<50~~ ≤ 50 age groups of both sexes.

The results of endpoint titres for four different groups of people for IgG1 are shown in Fig. 2A, B, C, D. Statistical analysis, Kruskal–Wallis with Dunn's Multiple Comparison test, of the obtained data revealed significantly lower levels of IgG1 anti-pneumococcal antibodies in older individuals of both sexes in comparison to ~~<50~~ ≤ 50 age group females (Fig. 2E). Of note is that the levels obtained for females ~~>50~~ ≥ 50 almost equal the levels obtained for males ~~<50.~~ ≤ 50.

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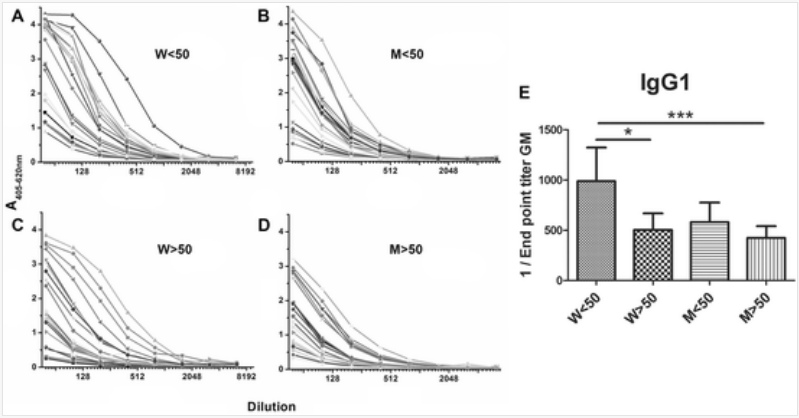
Fig. 2



~~Endpoint titres of serum IgG1 from different human age groups towards~~Endpoint titres of serum IgG1 from different human age groups towards ~~*S. pneumoniae*~~ *S. pneumoniae*, serotype 1~~. A) W~~. A) W< 50, B) M< 50, C) W> 50, D) M> 50. E) Endpoint titres were compared with Kruskal–Wallis one-way analysis of variance test with Dunn's Multiple Comparison test; $p < 0.05$ was considered statistically significant. 1 / Endpoint titres GM - geometric means with 95 % confidence intervals are shown. W – women, M – men.

Determination of endpoint titres of *S. pneumoniae* specific IgG2 revealed relatively uniform titres of this IgG subclass with lower confidence intervals, Fig. 3A, B, C, D. Significantly lower titres of IgG2 were detected in males over fifty years of age in comparison to females under fifty years of age, while there were no statistically significant differences between the other tested groups, Fig. 3E.

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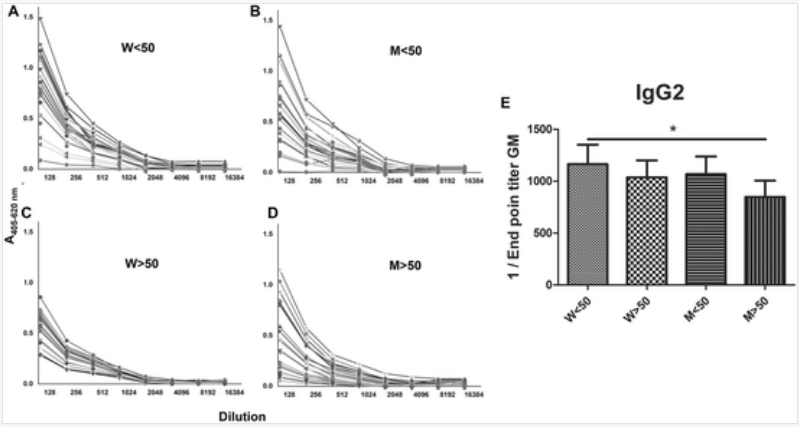
Fig. 3



~~Endpoint titres of serum IgG2 from different human age groups towards~~Endpoint titres of serum IgG2 from different human age groups towards ~~*S. pneumoniae*~~ *S. pneumoniae*, serotype 1~~. A) W~~. A) W< 50, B) M< 50, C) W> 50, D) M> 50. E) Endpoint titres were compared with Kruskal–Wallis one-way analysis of variance test with Dunn's Multiple Comparison test; $p < 0.05$ was considered statistically significant. 1 / Endpoint titres GM - geometric means with 95 % confidence intervals are shown. W – women, M – men.

The endpoint titres of IgG3 for different age groups are shown in Fig. 4A, B, C, D. No significant differences in IgG3 titres were obtained between the groups, Fig. 4E.

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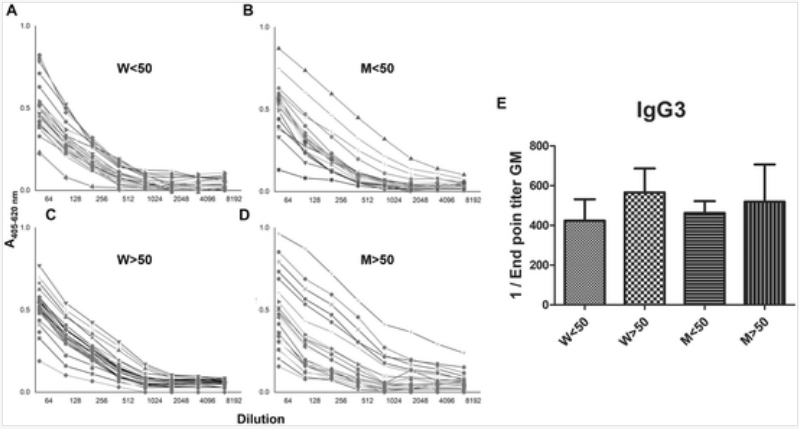
Fig. 4



~~Endpoint titres of serum IgG3 from different human age groups towards~~Endpoint titres of serum IgG3 from different human age groups towards ~~*S. pneumoniae*~~ *S. pneumoniae*, serotype 1~~. A) W~~. A) W< 50, B) M< 50, C) W> 50, D) M> 50. E) Endpoint titres were compared with Kruskal–Wallis one-way analysis of variance test with Dunn's Multiple Comparison test; $p < 0.05$ was considered statistically significant. 1 / Endpoint titres GM - geometric means with 95 % confidence intervals are shown. W – women, M – men.

The results of endpoint titters for IgG4 are shown in Fig. 5A, B, C, D. Widest confidence intervals for the geometric means were obtained for this antibody subclass. Statistical analysis, Kruskal–Wallis with Dunn's Multiple Comparison test of the obtained data revealed no significant differences between the groups, Fig. 5E.

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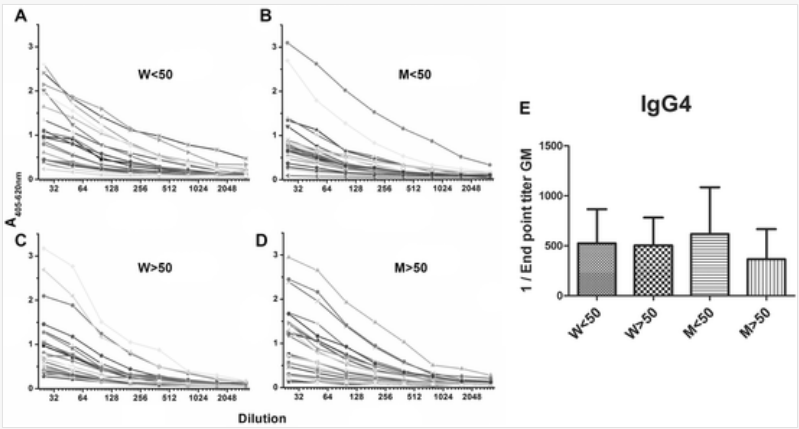
Fig. 5



~~Endpoint titres of serum IgG4 from different human age groups towards~~Endpoint titres of serum IgG4 from different human age groups towards ~~*S. pneumoniae*~~ *S. pneumoniae*, serotype 1~~. A) W~~. A) W< 50, B) M< 50, C) W> 50, D) M> 50. E) Endpoint titres were compared with Kruskal–Wallis one-way analysis of variance test with Dunn's Multiple Comparison test; $p < 0.05$ was considered statistically significant. 1 / Endpoint titres GM - geometric means are shown. W – women, M – men.

The results of endpoint titres for four different groups of people for IgA1 are shown in Fig. 6A, B, C, D. In case of IgA1, lower levels of anti-pneumococcus IgA1 was detected only in males of both sexes compared to females ~~<50,~~≤ 50, Fig. 6E.

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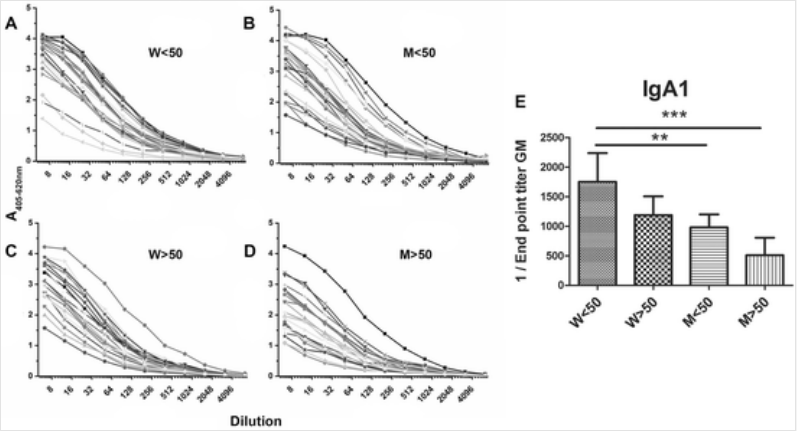
Fig. 6



~~Endpoint titres of serum IgA1 from different human age groups towards~~Endpoint titres of serum IgA1 from different human age groups towards ~~*S. pneumoniae*~~ *S. pneumoniae*, serotype 1~~. A) W~~. A) W< 50, B) M< 50, C) W> 50, D) M> 50. E) Endpoint titres were compared with Kruskal–Wallis one-way analysis of variance test with Dunn's Multiple Comparison test; $p < 0.05$ was considered statistically significant. 1 / Endpoint titres GM - geometric means are shown. W – women, M – men.

In order to see if there is mutual relationship between different isotypes of interest to us, we have performed Pearson correlation. The levels of IgG1 showed moderate of correlation with IgG4 in both sexes below the age of 50 ~~(r=0.61~~(r = 0.61 in men and 0.63 in women) this was not noted in the older age group, Table 1. In the older age groups a moderate correlation of IgG1 with IgA1 was obtained ~~(r=0.57~~(r = 0.57 in men and 0.49 in women), Table 1.

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Table 1

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Pearson correlation coefficients, showing correlation between different immunoglobulin isotypes, specific to *S. pneumoniae*, serotype 1 within the four tested age groups. Women under the age of 50 - ~~W<50;~~W< 50; men under the age of 50 - ~~M<50;~~M< 50; women over the age of 50 – ~~W>50;~~W> 50; men under the age of 50 – ~~M>50.~~M> 50.

~~Study group~~Study group		~~IgA1~~IgA1	~~IgG4~~IgG4
~~W<50~~ W< 50	IgG1	0.36	0.63 *
	IgA1		0.01
~~M<50~~ M< 50	IgG1	0.67 *	0.61 *
	IgA1		0.22
~~W>50~~ W> 50	IgG1	0.49 *	0.01
	IgA1		0.01
~~M>50~~ M> 50	IgG1	0.57 *	0.25
	IgA1		0.15

4 Discussion

According to literature records, measurements of the specific anti-pneumococcal antibody levels have been performed in various ways [12], mostly to separate pre-existing (natural) from antigen-induced antibodies, as antibacterial antibodies can be both natural and induced upon infection or colonization. Apart from capsule polysaccharides, cell wall antigens also bind antibodies. Therefore, clinical studies used to evaluate the influence of immunizations with anti-pneumococcal conjugate vaccines have used methods to eliminate antibodies to cell wall polysaccharides

(pneumococcal C polysaccharide - CPS) [12]. These polysaccharides are species specific and common to different strains [13].

Phosphocholine is a major component of the CPS and both children and adults have natural anti-CPS antibodies in the serum [14]. Moreover, patients with pneumococcal pneumonia acquire anti-CPS antibodies in response to infection [15]. As was highlighted by Koskela [12], although the value of anti-CPS antibodies in humans is unknown, the anti-phosphocholine antibodies have been able to protect against invasive encapsulated pneumococci in studies done in mice [16,17].

In this work we have used whole *S. pneumoniae* cells, meaning that both capsular polysaccharides and CPS were included. In this experimental setting, with inhibitory ELISA, we have obtained less than 0.1 % of total IgG1 binding to *S. pneumoniae* serogroup 1 in healthy adults under 50 years of age, while for IgG2 there was 14,4 % of *S. pneumoniae* ATCC 6301-specific IgG2 in healthy adults in this age group.

S. pneumoniae serotype 1, used in this study, has a high likelihood of causing invasive disease, or has a relatively high rate of invasion when adjusted for its rate of colonization [18]. It has zwitterionic polysaccharide capsule, which contains both positive and negative charge [19]. This type of polysaccharide is associated with T cell stimulation [20]. Although protein antigens from bacteria most often induce specific T helper cell which modulates B cell response this is not so common for polysaccharide antigens.

The question of which antibody subclass/subclasses give protection towards pneumococci has still not been answered entirely and measurement of anti-pneumococcus antibodies level are usually done for total IgG. The importance of the IgG1 subclass was also noted by Anttila et al. [10], where both the levels of IgG1 and IgG2 correlated significantly with opsonophagocytic activity. The appearance of IgG4 specific for *S. pneumoniae* is particularly interesting. It is in accordance with literature data findings of IgG4 forming following upon repeated or long-term exposure to the antigen [7]. In immunotherapy, relief of symptoms correlates with IgG4 induction. Switching to IgG4 might be modulated by IL-10, linking this subclass with down regulation of immune responses or tolerance induction [21]. Whether IgG4 in pneumococcal infections is associated with relief of symptoms and/or development of tolerance remains to be explained. What is also interesting is that in older individuals there was no decline in the titres of specific IgG3 and IgG4 antibodies.

We consider that the most important result of this study is the finding of lower anti-pneumococcal IgG1 in individuals over the age of 50, especially males (Fig. 2A), which is accompanied with a reduction in specific IgG2 antibodies in this population.

The clinical relevance of the detected reduction in IgA1 is unknown, especially as pneumococcus produces a specific IgA1 cleaving protease, but higher levels of specific IgA1 could imply higher protection, especially as IgA1 comprises over ~~90%~~ 90 % of the antibody in the upper and lower respiratory tract where these infections begin [22].

With the ageing process happens a decline in the functioning of the immune system [23]. This phenomenon includes increased secretion of pro-inflammatory cytokines, such as TNF, IL-6, and IL-1β [24], but also a reduction in lymphopoiesis and increased proportions of memory and memory-like lymphocytes, which is all associated with impaired immune function. [25,26] In the specific case analyzed here, which is the titer of anti-pneumococcus antibodies, the secretion of anti-pneumococcus antibodies is generally lower in the older age groups, implying that the higher proportion of memory B lymphocytes, which is a consequence of lower number of naïve lymphocytes, did not maintain stable antibody levels.

Until relatively recently, it has been assumed that ageing influences the immune system equally in males and females [27], but more recent literature data, including the data presented here, as well as data from the recent Covid-19 pandemic situation, highlights gender differences in the ageing of the immune system.

Gender differences in the immune response are driven by hormonal action; hence oestrogen influences many different components of the immune system. As it comes to the influence on antibody producing cells, oestrogen was shown to influence the development, survival, activation, and cytokine production in B cells [28], which is evidenced here by differences in anti-pneumococcus antibodies between premenopausal and postmenopausal women. Similarly, testosterone levels decrease with ageing, as 20% of men aged over 60 have total testosterone levels below the normal range and the figure rises to 50% in those aged over 80 [29]. But testosterone exerts mainly immunosuppressive properties [30,31], and the decline in anti-pneumococcus antibody titres obtained here is not easily explained by the decline in testosterone levels although it may explain gender differences between the same age groups of different sexes.

The drawback of the study is the lack of functional tests, but the results presented here corroborate the results of Antilla et al. [10]. Another potential drawback of this study was that the colonization status of study subjects was not assessed, and colonization rates are not equal in the tested populations, in fact, colonization is rarely detected in older adults, despite high rates of pneumococcal disease [32]. But according to the study performed by Adler et al. colonization does not confer significant serotype-specific immune boosting four weeks after colonization [33].

In conclusion, significantly lower anti-pneumococcus IgG1, IgG2 and IgA1 titres in men were observed in comparison to women, which was especially pronounced in men ~~>50.~~ > 50, implying lower protection and deficiency in humoral immunity. This is in accordance with results of a recent meta-analysis finding male sex to be a risk factor in Covid-19 pandemic [34]. During the Covid-19 pandemics, critically ill patients had the highest percentage of bacterial coinfection ~~(34.5%)~~ (34.5 %) compared to patients in the moderately ill and severely ill groups ~~(3.9%~~ (3.9 % and ~~8.3%,~~ 8.3 %, respectively) [35–37].

Thus, immunization with conjugate pneumococcal vaccines or careful infection management is needed in older individuals, especially male.

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Author contributions

Authors' Contributions Rajna Minic and Dejana Kosanović made substantial contributions to the conception and design of the study. Material preparation and data collection was done by Sanja Knežević, Luka Dragacevic, Vesna Ilić, Ljiljana Hajduković and Olivera Savić. Data analysis and interpretation was done by all authors. The first draft of the manuscript was written by Rajna Minic and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The authors have no conflict of interest to declare.

Appendix A Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2022.101834.

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The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

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