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1. Pop CF, Rajka D, **Bocsan IC**, Coblisan PA, Ichim GE, Lazar A, Chereches-Panta P. Insights into self-reported food allergies in Romanian schoolchildren. Front Allergy. 2025 Jan 21;5:1472673. **IF=3,1, Q2 pag 2**

2. Feketea G, Vassilopoulou E, Andreescu O, Berghea EC, Pop RM, Sabin O, Zdrenghea M, **Bocsan IC**. Vitamin D Level and Immune Modulation in Children with Recurrent Wheezing. Children (Basel). 2024 Feb 8;11(2):219. **IF=2.1, Q2 pag 12**

3. **Bocsan IC**, Măgureanu DC, Pop RM, Levai AM, Macovei ȘO, Pătrașca IM, Chedea VS, Buzoianu AD. Antioxidant and Anti-Inflammatory Actions of Polyphenols from Red and White Grape Pomace in Ischemic Heart Diseases. Biomedicines. 2022 Sep 20;10(10):2337. **IF=4.757, Q2 pag 22**

4. Muntean IA, **Bocsan IC**, Wiest LK, Pintea I, Dobrican CT, Duca E, Ureche C, Buzoianu AD, Deleanu D. Predictive Factors for Oral Immune Modulation in Cow Milk Allergy. Nutrients. 2022 Jan 23;14(3):494. **IF=5.9 Q1 pag 51**

5. **Bocsan IC**, Muntean IA, Miron N, Pintea I, Dobrican CT, Ureche C, Buzoianu AD, Deleanu D. Are Markers of Allergic Inflammation in Grass Pollen Allergy Influenced by H1 Antihistamines? J Clin Med. 2022;11(1):113. **IF=3.9 Q2 pag 64**

6. Feketea G, Vassilopoulou E, Geropanta F, Berghea EC, **Bocsan IC.** Alternative Fish Species for Nutritional Management of Children with Fish-FPIES-A Clinical Approach. Nutrients. 2022;14(1):19. **IF=5.9, Q1 pag 73**

7. Feketea G, Vlacha V, Tsiros G, Voila P, Pop RM, **Bocsan IC,** Stanciu LA, Zdrenghea M. Vitamin D Levels in Asymptomatic Children and Adolescents with Atopy during the COVID-19 Era. J Pers Med. 2021 Jul 25;11(8):712. **IF=3.508 Q2 pag 84**

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9. Muntean IA, **Bocsan IC**, Vesa S, Miron N, Nedelea I, Buzoianu AD, Deleanu D. Could FeNO Predict Asthma in Patients with House Dust Mites Allergic Rhinitis? Medicina (Kaunas). 2020 May 14;56(5):235. **IF=2.43, Q2 pag 113**

10. **Bocsan IC,** Muntean IA, Ureche C, Pop RM, Neag MA, Sabin O, Deleanu D, Buzoianu AD. Characterization of Patients with Allergic Rhinitis to Ragweed Pollen in Two Distinct Regions of Romania. Medicina (Kaunas). 2019 Oct 24;55(11). **IF=1.205,Q3 pag 123**

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Insights into self-reported food allergies in Romanian schoolchildren

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The prevalence of food allergy (FA) varies worldwide with an increasing rate in the last decades. Data of self-reported FA have been recorded by most European countries, the US, Canada and Australia, but not Romania. The aim of this study is to analyze the prevalence and severity of FA and to assess the extent of information the medical and teaching staff in schools have on students' medical history.

Methods: A cross-sectional survey was performed in schoolchildren from Cluj-Napoca, Romania, using an online questionnaire delivered to their parents.

Results and conclusions: Seven hundred and eight individuals completed the entire questionnaire. The prevalence of self-reported FA was 8.9%, 28.6% presented food-induced angioedema and 38.1% required ER presentation. Cow milk (36.5%), egg (9.5%), strawberry (20.6%) and nuts (2.7%)were the most frequent culprit foods. The lack of an appropriate and accurate communication with the medical and teaching staff in the school suggest the requirement for further measures for parents and children educations regarding food allergy detection and management.

KEYWORDS

food allergy, schoolchildren, food-induced anaphylaxis, prevalence of food allergy, teachers

1 Introduction

Food allergy is still a major concern worldwide due to both the increasing prevalence of the disorder and its burden in the specific management. Schoolchildren, mainly those in their first decade of life, represent a vulnerable age category. Food allergy imposes a great burden on both patient and family, affecting their emotional status. The risk of a severe reaction induces anxiety in the daily life of children with FA and is often associated with significant limitations in their social interactions (1, 2).

The prevalence of food allergy (FA) varies according to many factors. Recently, Lyons SA et al. reported the prevalence of FA in schoolchildren aged 7–10 years, in a cross-sectional study performed in 8 European countries (1). The prevalence of self-reported FA varied between 13.1% and 47.5%, the lowest in Greece and the highest in Poland and Lithuania (1).

By the end of 2021, an interesting review on food allergy globally was published, focusing on incidence, diagnosis and therapy of FA in different guidelines (2). The authors pointed out the differences between different continents in the prevalence of self-reported FA and the relevance of the definition of FA (2).

Self-reported FA is an obvious cause for overestimated prevalence of FA. Nevertheless cross-sectional surveys on significant large population groups showed that self-reported FA estimated rate is extremely variable among countries and continents, varying from 5 to 6 up to 19% in some African countries (3). The confirmed FA based on oral food challenge test has a lower rates in most of the studies (3, 4). In USA survey showed that a prevalence of 7.6% probable IgE-mediated FA (38,408 parent-reported FA in a 2018 US survey) (4).

A suspicion of food-induced allergic reaction should be confirmed in order to have a positive diagnosis of food allergy. The gold standard for the diagnosis of food allergy is an oral double-blind placebo-controlled food challenge (DBPCFC) to the culprit allergen that elicits reproducible clinical symptoms (5). Because the DBPCFC may induce severe reactions, it is not used routinely in most clinical settings. Trained doctors, who are equipped to manage potential adverse reactions, including anaphylaxis (6), can underwent it only under close clinical observation. Other diagnostic tools, like skin prick tests with standardized extracts or culprit food, sIgE to whole extract or to components, where available, allow an accurate assessment of FA and they can also identify the patients that might need oral food challenge (OFCs) test. Thus, an extended analysis of the factors associated with the presence and severity of FA is necessary in order to help the physicians from schools to provide adequate care to schoolchildren and refer them to an allergy specialist.

The main aims of our study are to establish the lifetime prevalence of FA in schoolchildren in Cluj-Napoca, Romania, based on parents self-report, and to assess the level of information available to medical and teaching staff in the school about their students' medical history and their awareness of possible severe reactions. Secondary objectives of this study are to characterize the clinical features of FA, to identify possible risk factors for FA, to evaluate the correlation between FA and other allergic diseases in children and to assess the impact of it in the child's social relationships.

2 Materials and methods

This is an open cross-sectional non-randomized survey study. We conducted the study in March 2023 in four schools from Cluj-Napoca, Romania. The study protocol and the survey content were approved by the Ethics Committee of Iuliu Hatieganu University of Medicine and Pharmacy (no. AVZ62/2023).

2.1 Method

The study is based on an anonymous questionnaire delivered online to parents by the school doctor who works in the institution where the child studies. An informative letter addressed to the parents about the outcomes of the study, the anonymous and non-coercive nature of participation in the study, and the approval of the Ethics Committee were added to the questionnaire.

All primary and secondary school students were included in the study and their parents received the online questionnaire. A number of 708 parents (54.9% of referred questionnaires) completed the questionnaire entirely and data were included in the analysis.

All schoolchildren whose parents answered the questionnaire were included in the study. After analyzing the answers, the study participants were divided into 2 groups: group A, students with a history of food allergy, and group B, students with no history of food allergy. Parents reported FA based on convincing clinical history and prior diagnosis of FA established by a physician, either paediatrician or allergist.

2.2 Collected data

The information in the questionnaire refers to:

1. Demographic data: age (date of birth), gender, the child's level of education (primary school, between 0 and 4th grade, or lower secondary education, between 5th and 8th grade);
2. Data regarding food allergy: the age of onset for the first symptoms, clinical manifestations (hives, flexural eczema, angioedema, gastrointestinal manifestations), the culprit foods, dietary interventions and the history of previously required medication (H1 antihistamines, adrenaline, corticosteroids). The foods that were listed in the questionnaire were: milk or dairy products (yoghurt, cheese, butter), hen egg, peanut, tree nuts (hazelnut, walnut or other nuts), wheat or cereals, soybeans, fish and seafood, and also several types of non-priority food like citrus fruits, strawberries, kiwi, chocolate. We included an open-ended question in the list so that the parent could add other potential culprits besides the ones listed in the questionnaire.
3. Data regarding the child's medical history: previous diagnosis of allergic diseases (asthma, allergic rhinitis, allergic conjunctivitis) and their impact on their social life and relationships.
4. Possible associated risk factors (duration of breastfeeding, family history of any allergic disorders). The questionnaire contained detailed history of allergic diseases in both parents and siblings. The last part of the questionnaire referred to a diagnosis of food allergy, atopic dermatitis, urticaria, allergic rhinitis or rhino-conjunctivitis, drug allergy, or asthma in both the mother and the father, and to any other sister or brother.
5. The extent of the information the teacher or the school doctor have about the children's medical history, the current diet, the daily treatment or the need for an emergency kit that the child may require under certain circumstances.

2.3 Statistical analysis

The results were analysed using Excel, SPSS version 19 and MedCalc Statistical Software version 19.0.3. The prevalence of

self-reported food allergy was calculated as a percentage from the total number of analyzed questionnaires. The severity was reported based on the clinical data mentioned: the number of any emergency visit due to the child`s allergy to certain foods, or the need for adrenaline therapy, or prior use of self-administered adrenaline or systemic corticosteroids during an allergic episode. These were also reported as percentage from the total number of children that were included for the analysis.

We analyzed the positive predictive value, negative predictive value, the specificity and sensitivity of several allergic comorbidities, like asthma, allergic rhinitis, and/or allergic rhino-conjunctivitis, comparing the two groups, A and B. We also analyzed the positive predictive value, negative predictive value, the specificity and sensitivity of some risk factors like the duration of breastfeeding, family history of allergies by comparing the two groups, A and B.

All the answers were analyzed and if for all of the questions related to personal and family history of allergies a positive answer was counted, positivity of them was considered and defined in the statistical analysis, regardless of their number (only one or more than one positive answer to these questions).

We compared the students in the two groups, A and B, and the differences were analyzed using the Mann-Whitney test, the χ^2 test and the *t*-test. We used SPSS version 19 to analyze the correlation between different variables in group A and group B, with bivariate correlation and also student's *t*-test. The conventional thresholds of a *p* value below 0.005 for statistical significance, and the confidence interval of 95% were applied for data interpretation.

3 Results

3.1 Demographic characteristics of the study group

After sending the questionnaires, 708 were completed entirely and were available for analysis. The study group consisted of 362 female students (51.1%) and 346 male students (48.9%) between ages 6 and 15.

The 708 students were divided into two groups: group A, consisting of 63 children with self-reported food allergy, and group B, in which we included 645 children without self-reported food allergy. The overall prevalence of FA in our study group was 8.89%. Gender distribution and age distribution (children between 6 and 10 years old vs. children between 11 and 15 years old) were similar into the two subgroups. Demographic data are shown in Table 1.

Additionally to food allergies, the questionnaire included information regarding respiratory or skin allergic diseases. In group A, 10 parents reported no allergic reaction in their children, but based on the physicians evaluation of their clinical history, their children were included in the FA group, because they have positively answered to questions referring to specific foods induced clinical manifestations. In group B, 14.1% of parents reported the onset of possible clinical allergic symptoms without prior diagnosis of FA, but the analysis of them invalidate a possible FA. In most of the cases, the allergic symptoms occurred before the age of 2 (88 cases, 12.4%), while 68.5% of children had no allergies (see Table 1).

TABLE 1 Demographics of the two study groups.

	Group A with food allergy (*n* = 63)	Group B without food allergy (*n* = 645)
Males, no (%)	29 (46.03%)	312 (48.37%)
Age, mean ± SD (years)	10.69 ± 2.83	9.98 ± 2.68
Age groups, no (%)		
- 6–10 years	27 (42.85%)	352 (54.57%)
- 11–15 years	36 (57.14%)	293 (45.42%)
Onset of any allergic reaction, no (%)		
- Below 2 years of age	29 (46.03%)	59 (9.15%)
- Between 2 years and 4 years	9 (14.29%)	48 (7.44%)
- Above 5 years of age	15 (23.81%)	64 (9.92%)
- Newer	10 (15.87%)	474 (78.49%)
Nationality, no (%)		
- Romanian	44 (69.84%)	404 (62.63%)
- Hungarian	18 (28.57%)	236 (36.58%)
- Polish	0	1 (0.15%)
- Arab-Greak	0	1 (0.15%)
- Israeli	0	1 (0.15%)
- Moldavian	0	1 (0.15%)

In group A, the age of onset reported with the highest prevalence was below the age of 2 (46.0% of the children) and almost a quarter had symptoms after the age of 5. In this group, 10 parents reported no symptoms and their comment referred to skin problems during childhood that were related to any type of food. The highest prevalence was in Romanians, with 448 children, both in group A and B. Overall class distribution of the subjects illustrates the homogeneous distribution by age groups for the two levels of education: primary school (between 0 and 4th grade) and lower secondary education (between 5th and 8th grade). The prevalence of FA was 6.7% in children aged between 6 and 10, and 11.9% in children above 11 years old.

3.2 The prevalence of clinical features

In group A, 47 children presented skin rash with itchy lesions. In 38 of them (60.3%) the lesions were located at the ears, around the eyes and neck, ankle, popliteal and elbow area. A reduced number of children from group B had skin lesions (9.3%) (Table 2).

The gastrointestinal disorders related to food consumption included diarrhea, with explosive or bloody stools, and abdominal pain. In group A, 30.2% of children presented such symptoms, while in group B, only 15.0% of them. The culprit foods that induced gastrointestinal symptoms were extremely variable, including cow milk and dairy products, fish and seafood, egg, different types of berries (strawberry, raspberry) grapes, eggplant, peanut and tree nuts (hazelnut, walnut) chocolate, honey, pineapple, caramel sauce, sausages, mayonnaise, cereal, and hummus (see Figure 1). Neither of

TABLE 2 The prevalence of food allergy clinical manifestations.

Food allergy clinical manifestation	Group A, (n = 63) No., %	Group B, (n = 645) No., %	p	Sensitivity%	Specificity%	PPV[a], %	NPV[b], %
Associated atopic dermatitis	46 (73.01%)	61 (9.45%)	<0.01	74.603	82.791	29.747	97.091
Food-induced angioedema	10 (15.87%)	15 (2.32%)[c]	<0.01	28.571	97.364	51.429	93.314
Gastrointestinal disorders	18 (28.57%)	92 (14.26%)	0.006	30.159	84.961	16.379	92.568
Wheezing episods	23 (36.50%)	137 (21.24%)	0.016	33.333	79.380	13.636	92.419
Exercise-induced wheezing	7 (11.11%)	25 (3.87%)	0.016	11.111	96.189	24.138	91.415
Asthma	14 (22.22%)	54 (8.37%)	0.001	22.222	92.558	22.581	92.415
Ithcy or runny nose without a could	34 (53.86%)	167 (25.89%)	<0.01	53.968	74.884	17.347	94.336
Sneezing, itchy eyes	24 (38.09%)	105 (16.27%)	<0.01	41.270	83.876	20.000	93.599
Allergic rhinitis	29 (46.03%)	100 (15.50%)	<0.01	46.032	85.601	24.167	94.087

[a]PPV, positive predictive value.
[b]NPV, negative predictive value.
[c]Parents did not report any correlation with a specific food and tests were not performed, but the answer was positive to this question.

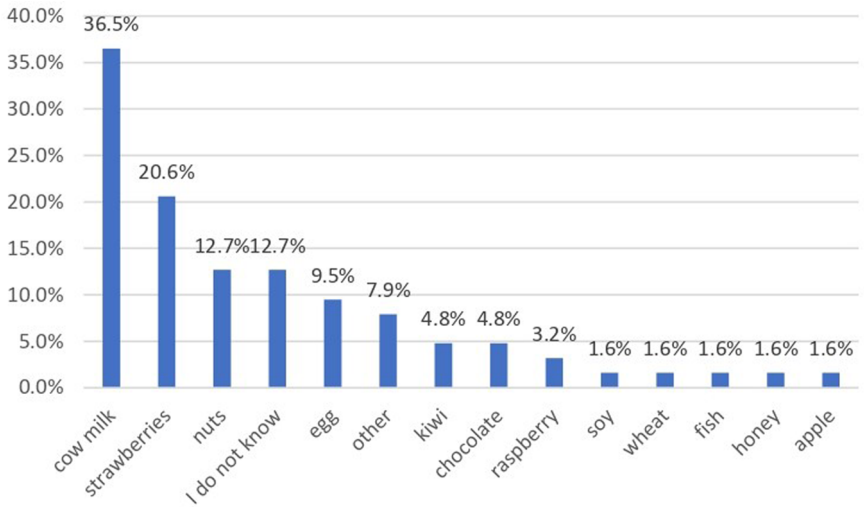

FIGURE 1
Types of food associated with allergic reactions.

these 97 children had a prior diagnosis of food allergy. The parents of 17 children from group B reported angioedema, but in the majority of the cases, the relationship with a specific food allergen was not established.

Respiratory symptoms like wheezing, itchy and/or runny nose, sneezing, itchy eyes and prior diagnosis of asthma and/or allergic rhinitis were significantly more prevalent in group A as compared to group B (Table 2).

3.3 Risk factors for FA

Potential risk factors for any type of allergic disease including food allergy were investigated in the questionnaire. Among these factors, we assessed family history of atopic diseases and the duration of breastfeeding Table 3. History of atopic conditions in the family and other risk factors

Regarding the other risk factor assessed, it was found that the absence of breastfeeding was similar in the two groups of children (Table 3). Breastfeeding during first 6 months of age was noticed in 60.8% of children from group B, as compared to 47.6% of children from group A, with a negative predictive value of 88.462%, but with low sensitivity and specificity.

3.4 Culprit foods

The questionnaire included and we analysed the main foods that might produce allergies as previously mentioned. The highest prevalence was noticed for cow milk or derivatives (yoghurt, cheese, butter) that were involved in an allergic reaction in 36.5% of cases, and strawberry in 20.6% (Figure 1). In 12.7% of cases the exact incriminated food was not established and 7.9% of parents reported different types of foods.

Spontaneous report of drug allergy was mentioned by one parent and insect-bite non-anaphylactic reaction by one parent, both children belonging to group A.

TABLE 3 History of atopic disorders in the family and other risk factors.

	Group A, (n = 63) No. of cases, %	Group B, (n = 645) No. of cases, %	p	Sensitivity, %	Specificity, %	PPV[a], %	NPV[b], %
Family history of allergic disorders							
Mother	36 (57.14%)	179 (27.75%)	<0.01	55.556	70.078	15.351	94.167
Father	34 (53.96%)	133 (20.62%)	<0.01	42.857	78.140	16.074	93.333
Siblings	18 (28.57%)	102 (15.81%)	<0.01	30.159	78.140	11.875	91.971
Duration of breastfeeding							
Absent	4 (6.35%)	34 (5.27%)	0.045	6.319	94.729	10.526	91.194
Less than 3 months	17 (26.98%)	125 (19.38%)	0.357	26.984	80.620	11.972	91.873
Between 4 and 6 months	12 (19.05%)	95 (14.73%)	0.004	19.048	85.271	11.215	91.514
Over 6 months	30 (47.62%)	392 (60.77%)	0.001	47.619	39.225	7.109	88.462

[a]PPV, positive predictive value.
[b]NPV, negative predictive value.

3.5 Severity of FA and treatment

Out of the 63 children with FA, 38.1% required presentation to the emergency department and 9.5% received epinephrine, while 34.9% received systemic (oral or intravenous) corticosteroids (Table 4). Almost all cases with FA received H1 antihistamines during the exacerbation of symptoms and after the reaction 68.3% of them had long-term exclusion diet. In group B, 5.1% of the children required emergency department presentation as reported by the parents, although they had neither consistent history nor diagnosis of FA.

3.6 Teacher and medical staff awareness regarding the medical history of the schoolchildren

In what concerns the extent of information the teacher and/or the school physician have about food allergies in their children and the seriousness of the disease (severe, required diet or necessary treatment), in our study group only 33 parents from group A (52.4%) reported that they informed school authorities about these special circumstances. In group B, a statistically significant lower number of parents (38 parents, respectively 5.6%) informed the school teachers and healthcare providers on a possible suspicion of food allergy ($p < 0.001$).

Analysing the possible restrictions that children with food allergies might have during trips, parties or any other extracurricular activities with their colleagues, we noticed that only one parent from group A (1.6%) and eight parents from group B (1.2%) reported any interference.

TABLE 4 Allergy treatment.

	Group A, (n = 63) No. of cases, %	Group B, (n = 645) No. of cases, %	p
Diet restriction	43 (68.25%)	22 (3.41%)	<0.001
H1 antihistamine drugs	59 (93.65%)	224 (34.73%)	<0.001
Systemic corticosteroids	22 (34.92%)	41 (6.35%)	<0.001
Adrenaline	6 (9.52%)	8 (1.24%)	0.103
Emergency room presentation	24 (38.10%)	33 (5.12%)	<0.001

3.7 Age distribution of FA symptoms

We divided the children with food allergy in group A into two age groups: young children aged between 6 and 10 years and children aged between 11 and 15 years, and we analysed the differences between them (Table 5).

The age distribution in primary school and lower secondary education was similar, with a slightly higher prevalence in children between ages 11 and 15 (57.1%). Male subjects showed a lower percentage than female subjects in both age groups. Based on self-reported prevalence there was no significant prevalence of allergic comorbidities (atopic dermatitis, asthma or allergic rhinitis) in older children as compared to the younger group. Food-induced angioedema was reported in 29.62% of young children and 27.78% of children above the age of 11.

4 Discussion

The present study reports clinical characteristics and results from investigations in schoolchildren from four schools in Cluj-Napoca, Romania. To our knowledge, this is the first study that assesses food allergy in Romania, mainly in the pediatric population. During the early 1990s, several epidemiological studies on allergic diseases were carried out in Romania (7). The prevalence of asthma, eczema and allergic rhinitis were assessed using a standardized study protocol, designed by the International Study on Asthma and Allergic Diseases in Children (ISAAC) (7, 8). This questionnaire did not include specific questions about food allergy, therefore the researchers did not have reliable data to compare the extent of FA in our geographical area.

A decade ago, the incidence of allergic diseases was characterized as a wave of the allergic epidemic, which mainly affected infants and preschool children (3). Based on the lack of epidemiological data between the ISAAC study and the time

TABLE 5 The two age groups in children with food allergy.

	Children aged between 6 and 10 (n = 27) No. (%)	Children aged between 11 and 15 (n = 36) No. (%)	p
Male subjects	12 (44.44%)	17 (47.22%)	0.890
Associated atopic dermatitis	18 (66.66%)	29 (80.55%)	0.307
Food-induced angioedema	8 (29.62%)	10 (27.78%)	0.480
Gastrointestinal disorders	9 (33.33%)	10 (27.78%)	0.520
Asthma, no. (%)	5 (18.51%)	9 (25.00%)	0.424
Allergic rhinitis, no. (%)	14 (51.85%)	15 (41.66%)	0.420

point in 2010, the surveys on the prevalence of FA proved the increasing trend of all allergies, including FA. In a more recent review, Spolidoro et al. commented that although the frequency of FA in Europe seems to have an increasing trend, there are still not enough revised data (9). Between 2000 and 2012, the lifetime prevalence was 5.9%, while during the past decade the prevalence increased almost three times, up to 14.9% during 2012 and 2021. Their analysis estimates that the current prevalence of any FA during a lifetime in children is 18.7%, with a point prevalence of reported FA of 14.2%. The review included 110 studies and showed that any FA prevalence was higher in Eastern and Northern Europe as compared with Southern and Western Europe. The authors emphasized that there is a very limited number of studies from Eastern Europe (9). None of these studies reflected the prevalence of FA in Romania.

We compared the data in our study, conducted in Cluj-Napoca (Romania), with current data on prior prevalence of self-reported FA in other European countries (1) and worldwide (2). The overall prevalence of food allergy in our study group was 8.89%. The prevalence in our study is lower than the previous prevalence of self-reported FA to different foods in Europe. The prevalence varied in European countries between 13.1% in Athens (Greece), 16.3% in Zurich (Switzerland), 16.7% in Reykjavik (Iceland), 17.1% in Utrecht (The Netherlands), 17.9% in Madrid (Spain), 19.7% in Sofia (Bulgaria), up to significantly higher percentages of 43.4% in Lodz (Poland) and 47.5% in Vilnius (Lithuania) (1). The percentages reported in other countries were lower, and similar to our overall prevalence, when data were addressed only to priority foods.

Over 160 foods are incriminated in food allergy (2). The present study analyzed the answers to 12 foods and the authors offered parents the opportunity to add other food allergens that triggered the reaction in their child. Among the main allergenic foods, a higher prevalence was recorded for milk or derivatives (36.5%), different types of nuts (12.7%) and hen egg (9.5%), with a very low prevalence for wheat or cereals, fish, seafood and soybeans. Out of the other foods, the most common were strawberries (20.6%), while 12.7% of parents could not report a certain food involved in the FA. In a recent survey, Messina M et al. refer to priority foods as the Big 8, as classified by the Food Allergen Labeling and Consumer Protection Act (FALCPA) (10). The origin of this classification of foods relies on the prevalence of allergic reactions to different type of foods as well as clinical evidence of severe reaction to food, including fatal anaphylactic shock. Based on data from 5 surveys in large population samples on the prevalence of self-reported FA in children, the authors noticed that the prevalence of soybean allergy is lower than the other 7 major allergen. The Japanese list includes only 7 food allergens for mandatory labeling, soy being excluded. On the other hand, sesame allergy seems to be increasing, therefore sesame is a potential candidate for the Big 8 list (10). A list of 14 major food allergens is currently being discussed in the European Union. The observed differences could be explained also by different dietary particularities. In Romania fish and seafood is not a common foods included in many diet, so the exposure to these ones is reduced. The same observation is also available for soybean.

We noticed an unexpected high prevalence of strawberry allergy in our study group. Several authors report strawberry allergy, with prevalence values between 0.3% and 9.2%, but with a very high prevalence of this allergy among severe reactions, up to 13.2% (11–14). It is relevant to emphasize that strawberry allergy can be a cross-reactions with pollen allergy. In Romania grass pollen season coincides with strawberry season and many parents consider that an acute urticarial is a consequence of food ingestion rather than a secondary reaction to pollen exposure.

The estimated prevalence of FA in children from the United States in 2005 was 6.0% for any food, 2.5% for milk, 1.3% for egg, 0.8% for peanut, and 0.1% for fish (10). In a study performed as a random-digital-telephone survey with 20,686 individuals, both children and adults, the prevalence of self-reported FA in children from the United States was 6.53% for any food, 1.94% for milk, 0.64% for egg, 1.16% for peanut, and 0.43% for fish (15). In contrast, children from Canada included in Messina M. review showed higher prevalence than those in the United Stated, with 7.14% FA for any food, 2.23% for milk, 1.77% for peanut, 1.23% for egg, but lower prevalence rates of 0.18% for fish (10, 15). These last two surveys were carried out during 2007–2010 (15) and 2008–2009 (16).

This variable prevalence is depending on the method of data collection and supplementary investigations needed to confirm the positive diagnosis of FA. In the present study, the authors analyzed only the lifetime self-reported prevalence of FA among children, referring also to the main foods analyzed also in the aforementioned research. Each type of analysis have benefits and limits. Self-reported prevalence of FA gives a rapid estimation of possible patients with FA, but for an accurate positive diagnosis, the confirmation is needed.

There are differences in the prevalence of FA in different age groups. In the review published in 2013, the authors reported a

higher prevalence in younger children, below the age of 5 or even during infancy, as compared with children above the age of 5 (3). In our study, the overall prevalence of self-reported FA in children aged between 6 and 10 was 6.67%, while in older children, above the age of 11, the prevalence of FA was reported in 11.88% of them. Some other studies also showed that in older children, mainly above the age of 14, the prevalence of FA is lower than in the younger group or when compared to the overall prevalence in children of all ages (4, 10). Previous studies showed that self reported lifetime prevalence of FA is higher in younger than in older children. This could be explained also by the fact that some allergies (e.g., milk or egg allergies) may have spontaneous resolutions after 5 years old. The present study did not included children of 5 years old because parents of schoolchildren completed the questionnaire and in Romania the minimal age for primary school is 6 years old. But the same tendency is maintained in the present research.

Regarding potential risk factors for FA, our data showed a significantly higher prevalence of family history of allergic diseases in group A, as compared to no family history in 55.81% of the children in group B. Breastfeeding for a period of less than 3 months and between 4 and 6 months was similar in both groups, as well as the absence of breastfeeding. The only significant difference was regarding the duration of breastfeeding for more than 6 months, a higher rate being reported in children from group B (60.77%) as compared to children in group A (47.62%), with a negative predictive value of 88.462%. Current recommendations for the duration of breastfeeding is a minimum of 4 months according to the World Health Organization, respectively for at least 6 months according to the EAACI (European Academy of Allergy and Clinical Immunology). The data published does not yet provide consistent evidence about the beneficial and protective role of prolonged breastfeeding (2). Recent data suggest that early exposure of infants to various food allergens could induce tolerance and improve the maturation of the mucosal immune system (2).

The authors assessed the association of other allergic diseases in our study group. Children with FA had significantly higher rates of atopic dermatitis, allergic rhinitis and allergic asthma as compared to children from group B. When the two age groups of children with FA were analysed, the prevalence of these comorbidities was similar for both children between 6 and 10 years of age and for those older than 11. In a recent analysis on 3,233 individuals, Peters et al. stratified FA in infants in different phenotypes and noticed a correlation of early onset of FA with lower pulmonary function tests after the age of 6 (17). Even if the children presented transient egg or peanut allergy, their FEV1 had lower values. Compared to the general population, food allergy, and in particular egg allergy, correlates with a significantly higher prevalence of atopic dermatitis, asthma or eosinophilic esophagitis (18). The prevalence of egg allergy was reported by Samady et al. in a complex survey on 38,408 children, 1.3% in children below the age of 5, while the overall prevalence of egg allergy in children all ages was of 0.9% (18). Additional to atopic dermatitis, "food-protein induced protein losing enteropathy" (FPIPLE) was described in children with allergic reaction to egg, cow milk and nuts (19).

The main limitation of our study is the lack of proof that FA is based on either demonstration of specific IgE and an oral food challenge test (OFC) or double-blind placebo-controlled food challenge (DBPCFC). The parents reported FA based on convincing clinical history and prior diagnosis of FA established by a physician, either paediatrician or allergist. This could lead to the overestimation of FA, with inclusion of both IgE-mediated reaction and other reactivity to food, like food intolerance, food toxicity or non-IgE mediated reactions. A minority of the parents added in their comments that they underwent either skin prick-tests and/or specific serum IgE for foods. Since we did not formulate a question on diagnostic tools, we did not report these data. In fact, in a study carried out by Lyons SA, the applied protocol had three phases: the first one analysed self-reported FA, the second phase included food-sensitized patients based on specific serum IgE, and the third investigated the patients through DBPCFC (1). The authors defined possible FA when patients with positive tests had concurrent symptoms. The prevalence of probable FA dropped to 1.9% from 5.6% children across Europe, with a match between self-reported FA and food-sensitization of about 17.2%, depending on the various foods tested. The best match was proven for lentils, apple and hazelnut (between 37% and 46%) and poorer correlation for different seeds or corn. Cow milk had a match of 8% in this study and hen egg 15.8% (1). The authors reported that very few patients in their multinational study group agreed to take part in phase III (18 of 16,935 subjects). The lack of exact data regarding real confirmed FA based on gold standard for the diagnosis is a recognised as bias in other studies. The DBPCFC involves risks that require the test to be performed in specialized centres (20, 21). The overestimation of the prevalence of FA can also be due to the increased awareness in recent years, the situations in which investigation is requested through skin tests or specific IgE being more frequent. Component-Resolved-Diagnosis for the identification of IgE is a diagnostic method recently introduced in the evaluation of allergic patients. However, its high costs limit access to this investigation (20). The great majority of studies on prevalence are based on questionnaires with self-reported FA or parent-reported FA. Even a physician-diagnosed allergy is not always reported in prevalence studies. In a large cross-sectional survey on egg allergy, 27.8% of the participants did not have physician-diagnosed allergy (18, 22).

The most recent review on the frequency of FA in Europe summarized self-reported FA in 20% of the children, the sensitization proven with skin prick tests in 6% of them and with IgE in 17%, while food challenge-verified FA was 0.8% (9). The most commonly used food challenge was OFC, as compared with DBPCFC. The authors emphasized the significance of a convincing clinical history, and the fact that the recent studies did not include any of the challenge tests in the assessment of the prevalence of FA.

The burden of FA is even greater as the number of specialists is reduced and the availability of adrenaline auto-injectors is still low. In Romania, these children are referred to either the pediatrician, or to the allergist, since in Romania there is no distinct medical specialty of pediatric allergology and immunology. Although the

most severe cases are primarily diagnosed in the Emergency Department, the diagnostic work-up, the training in self-administration of adrenaline, and the role of other therapeutic options are part of a subsequent evaluation. Adrenaline auto-injectors (AAI) are available in Romania, but they are not reimbursed by the healthcare system. In our study group, the number of children that were addressed to the Emergency Department due to an acute event was 24 (38.10%) in group A and 33 (5.12%) in group B, but only 14 children received adrenaline during acute episodes. A higher number of children received systemic steroids (34.92% from group A and 6.35% from group B) and almost all children in group A (93.65%) were treated with H1 antihistamines for acute symptoms. In a recent cross-sectional study on FA in children from the US, there were 47.7% FA-related Emergency Department visits in Hispanic individuals and 45.4% in African-American individuals (10). Other studies have reported a higher Emergency Department presentation rate of children with egg allergy, as 21.1% of the children with egg allergy report severe reactions, compared to those with allergic reactions to other foods (18, 22). The use of AAI was 20.9% in Caucasian children and even higher, up to 23.6% and 24.6% in African-American and Hispanic children. The authors analyzed the prescription of AAI and the rates were above 25% in all races (23). The strict requirement for training in order to prevent severe FA events, mainly the use of AAI, was pointed out in a recent review (24). Food-induced anaphylaxis, including fatal reactions, have demonstrated an increasing prevalence during recent decades (22, 25). This aspect leads to increasing concern for families as well as the need for greater awareness for policymakers.

In the present study, the authors assessed the incidence of severe FA, teacher and school physician awareness about the history and therapy of FA of schoolchildren, as well as the parents' training on the use of adrenaline. Only 52.38% of parents informed the teacher and the school physician on their child's history of FA. Although 38.10% of children had prior visits to the Emergency Department and 28.57% had proven food induced angioedema, the number of patients who received adrenaline during a severe episode was of only 9.52% of total cases with FA. The low percentage of parents who reported the health issues to the teaching staff and to the school physician has no reasonable explanation. The number of children with severe FA in primary school was double compared to children in lower secondary education, aged above 11, and this is an aggravating factor for the risk of accidental exposure during school time. This is an alarm for a better training of children with FA and their parents, for the awareness on the potential risks and for the crucial role of informing the teaching and medical staff in the school. Surprisingly, the authors noticed that parents with children with FA declared that their children have no issues regarding extracurricular activities with their colleagues, since only up to 1.59% of them reported interference with social activities like parties or trips.

The management of severe allergic reactions in the community is a constant topic for allergists worldwide. A decade ago, Food Allergy and Anaphylaxis Guidelines were published by the European Academy of Allergy and Clinical Immunology (EAACI) (26). The main purpose of this guideline was, on one hand, the high percentage of severe reactions induced by food, and on the other hand, the fact that these reactions usually take place in the community (kindergarten, school, restaurants, playgrounds, etc.). Parents have the responsibility to take the appropriate measures for their child when recognizing potentially risky circumstances, avoiding specific allergens and training to use emergency medication, like adrenaline auto-injectors (AAI). Food allergy and its potentially severe course is little known by teachers, who have poor knowledge about anaphylaxis and, furthermore, about the appropriate management. The fact that parents do not inform the school about the student's allergy increases the risk of severe and potentially fatal reactions. In 2020, a questionnaire-based assessment was conducted on raising awareness to allergic pupils in schools, training of school staff and parents on the correct treatment of allergies (27). The information about the number of children with severe FA was correct, but the preparedness for its management was poor. A high percentage of schools (81%) expressed the need for further training. A questionnaire-based study on the preparedness of school teachers in Greece regarding FA has recently been published (28). The results confirm the lack of knowledge in teachers and other school staff members, as well as school principals, both on the symptoms and on the use of adrenaline auto-injectors. Similar data were published in Saudi Arabia (29) and Italy (30). Artificial intelligence (AI) could be an important tool for education, addressing to both parents and teachers. Using AI the parents could learn how to use the auto-injector, while teachers may learn how to detect a characteristic symptom for allergic reactions. A recent study performed in Romania showed that AI had a good acceptance among caregivers of children (31).

5 Conclusions

Lifetime self-reported prevalence of FA was 8.89% in a cohort of schoolchildren, with lower values in those between 6 and 10 years old. Self-reported anaphylaxis was mentioned in 28.57% of children with FA. Family history of allergic diseases was correlated with a higher risk for FA. Breastfeeding was not found to be a significant protective factor to FA development. The use of AAI was reported in very few children and parents informed the teaching and medical staff in schools on their child's allergy in a very low percentage. These aspects are strong recommendation for further educational programs for children and parents of children with FA, and also for teachers and school staff. These data offer a new perspective regarding the perception of FA in a country with a relative recent major change in lifestyle that has impacted both nutritional and allergic behavior in children and young adults.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This is an open, cross-sectional non-randomized survey study. We conducted the study in March 2023 in four schools from Cluj-Napoca, Romania. Study protocol and the survey content were approved by the Ethic Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy (no. AVZ62/2023).

Author contributions

CFP: Writing – original draft, Writing – review & editing, Conceptualization, Methodology, Software. DR: Methodology, Software, Writing – original draft, Writing – review & editing. ICB: Writing – original draft, Writing – review & editing, Formal Analysis, Validation. PAC: Formal Analysis, Software, Writing – original draft, Writing – review & editing. GEI: Investigation, Writing – original draft, Writing – review & editing. AL: Methodology, Software, Writing – original draft, Writing – review & editing. PC-P: Conceptualization, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing.

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children

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Article

Vitamin D Level and Immune Modulation in Children with Recurrent Wheezing

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Abstract: Introduction and aim: A direct causal relationship between vitamin D (vit D) deficiency and recurrent wheezing has not been proven. The present study investigated the role of vit D in enhancing the risk of asthma or recurrent wheezing by modifying the intensity of the inflammatory process. Material and method: Forty children with wheezing presenting at the emergency service and sixteen healthy control subjects were included in the study. Children with wheezing were either in the first episode (20) or with recurrent wheezing (20). Children with chronic diseases, and other conditions that present with acute wheezing or that might influence the vit D level, were excluded. Blood samples were taken at presentation and 3–6 months later, to evaluate the serum levels of total IgE, vit D, IL-10 and IL-31. Statistical analysis was performed using the SPSS 25 program, with a significance level of $p < 0.05$. Results and conclusion. The vit D level was lower in patients with recurrent wheezing compared with those with a single episode and with the control group, and this increased with time. IL-10 was significantly higher in children with wheezing than in the control group, with the highest values in those with an acute episode of wheezing. IL-31 was higher in children with recurrent wheezing than in those with a first episode only at the initial point, while at the final time point it was lower. Low levels of vit D appear to be detected more frequently in recurrent wheezing than in simple wheezing. Immune modulation, as measured by Th2 status reflected by IL-10 and IL-31 levels, appears to depend on the wheezing phenotype and on the general health status.

Keywords: wheezing; recurrent wheezing; vitamin D; IL-10; IL-31; immune modulation

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1. Introduction

Childhood respiratory diseases induced by viruses can be defined in many terms depending on the dominant manifestation, such as acute bronchiolitis, viral lower respiratory tract infection (LRTA), acute viral bronchitis, viral pneumonia, recurrent/transient/nonspecific and/or virally induced wheezing and the exacerbation of asthma [1]. Recurrent wheezing

in infants and young children is one of the most common conditions for which parents seek medical attention, and it constitutes a global health problem [2].

In infants and preschool children, wheezing is a frequent clinical symptom of diseases of the respiratory system; it is characterized by a distinctive musical sound in the expiratory phase, and sometimes also in the inspiratory phase, with an increase in respiratory frequency [3]. Wheezing is a multifactorial symptom, and recurrent wheezing in preschool children is largely due to recurrent respiratory tract infection (RTI) [4]. Recurrent wheezing is defined as three or more episodes of this condition in the previous year [5,6] Children who have episodes of wheezing only when they have viral RTIs and have no symptoms between episodes are characterized as having episodic viral wheezing [2,7].

Observational studies have shown that the frequency of asthma increases with latitude [8], as does the prevalence of low levels of vitamin D (vit D) in childhood [9]. Epidemiological studies have indicated that low serum levels of vit D are associated with a higher risk of respiratory infections in children [10]. Experimental and clinical studies document positive effects of vit D on the immune system, and on the response to the treatment of asthma [11]. Several studies have reported that vit D deficiency may lead to an increased frequency of wheezing episodes [12,13], and the need for more medication. Vit D sufficiency, insufficiency and deficiency are defined as serum levels of 25(OH)D of >30 ng/mL, 20–30 ng/mL and <20 ng/mL, respectively, [>75 nmol/L, 50–75 nmol/L and <50 nmol/L, respectively] [14]. Supplementation with vit D may protect children against viral infections and decrease the number and/or severity of recurrent wheezing episodes [15,16]. Studies in the Northeastern US have shown that increased maternal vit D intake during pregnancy, from either dietary sources or supplements, may lower the risk of wheezing in early childhood [17,18]. However, a recent updated Cochrane review found no evidence to support the administration of vit D supplementation to reduce the risk of exacerbations in mild and moderate asthma [19]. Most reported associations between vit D levels and infectious disease outcomes, including respiratory tract infections, were not statistically significant [20].

The immune modulation observed during infection and inflammation takes a variety of forms. Interleukin 10 (IL-10), a cytokine with anti-inflammatory properties closely related to T regulatory (T reg) cells, plays an important role in Th1–Th2 balance, limiting the immune response to pathogens, and in this manner preventing local damage [21,22]. IL-31 is a relative novel cytokine produced by Th2 cells involved in the pathogenesis of bronchial inflammation [23]. It seems that IL-31 might have a dual effect on Th2-type inflammation depending on disease stage [24]. An increased IL-31 could lead to a Th2-dominant inflammation in the early stage, but with late anti-inflammatory action in asthma [25] It is also a potential biomarker for the phenotypic classification of viral bronchiolitis, depending on the type of virus [26].

2. Hypothesis and Aims of the Study

To date, a direct causal relationship between vit D deficiency and recurrent wheezing has not been proven. Most relevant studies report that deficiency or insufficient levels of vit D predispose to wheezing episodes and exacerbations of asthma [27]. Infections may result as a consequence of low vit D levels, due to a decrease in its synthesis, which could be a result of the reduced exposure to sunlight of children who spend more time indoors [28]. It can also be speculated that the low level of vit D in these children is due to excessive consumption of vit D in the immune processes that occur during asthma exacerbations or wheezing episodes, but this remains to be proven. The present study aimed to analyze the level of vit D and some Th2 inflammatory parameters in children with recurrent wheezing. The main purpose is an exploration of whether vit D deficiency may influence the severity of the inflammatory process and may correlate with wheezing severity in two groups of children with different wheezing phenotypes. After a period of 4–6 months without further infections, vit D and inflammatory parameters levels should return to normal, or there should be no significant difference between the studied groups.

3. Methods

3.1. Study Population

This was a cross-sectional, analytical study of children who presented consecutively at the emergency service of the regional Hospital of Amaliada, Greece, between September 2019 and February 2020, with wheezing as the main complaint. The study evaluated, over time, vit D levels in children with multiple episodes of wheezing compared with those who had a single episode and with healthy children. The children with wheezing were assigned to two groups: group 1 consisted of 20 patients who presented with a first episode of wheezing, and group 2 of 20 patients with recurrent wheezing, defined as more than 3 episodes of wheezing within the last 3 months. A control group was recruited of 16 healthy children who attended the Department of Pediatrics for regular check-ups and mandatory vaccinations in the same time period (during the winter), and had no history of chronic disease, lower respiratory tract disease or wheezing. The entire study population lived in the same region and reported no other episode of wheezing between the initial and final time point, and no differences were recorded in religious and alimentary practices affecting dietary vit D intake. The exclusion criteria were chronic diseases, musculoskeletal and congenital diseases, genetic syndromes, malabsorption disorders and prematurity; children with bronchiectasis, gastroesophageal reflux, swallowing disorders, foreign body aspiration, primary and secondary immunodeficiencies, malignancy and neurological diseases were also excluded. In addition, children with a severity of wheezing that required hospitalization were not included in this study. In order to reduce the influence of other factors that could be associated with wheezing [29], children were also excluded that had taken antibiotics for some reason in the first 2 months of life, and vit D supplements in the last 3 months, those with obesity, i.e., body mass index (BMI) >28, and those whose parents smoke or who had clinical signs of allergy.

The study was approved by the Scientific and Ethics Council of the Amaliada Hospital Unit, General Hospital of Ilia, Greece, and by the Ethics Commission of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj Napoca, Romania. Before recruitment, the parents provided their written informed consent for participation of their children in the study.

3.2. Study Design and Data Collection

The data recorded for each child included age, sex, type of birth, type of nutrition in the first 3 months of life (breastfeeding, artificial milk formula or mixed feeding) and clinical data related to the current acute illness: duration and amplitude of fever, and details of signs and symptoms characteristic of an RTI (i.e., cough, chest pain, shortness of breath, myalgia, headache, sore throat, rhinorrhea, diarrhea, nausea, vomiting). The respiratory rate, heart rate and oxygen saturation at presentation were also recorded.

Venous blood samples were collected from each child with wheezing at presentation (basal) and at 3–6 months after the wheezing episode (final evaluation) and the following parameters were determined: blood cell count, erythrocyte sedimentation rate (ESR), serum levels of C-reactive protein (CRP), calcium, alkaline phosphatase, vit D [25-hydroxycholecalciferol, 25(OH)D], IL-10 and IL-31, total immunoglobulin E (IgE) and liver function tests (SGOT, SGPT). Blood samples were taken from the children in the control group at recruitment and after 3–6 months, at the time of routine testing. The biochemical measurements and hemogram were performed using a Unicel DxH 600 Coulter Cellular Analysis System by Beckman Coulter, Krefeld, Germany, for the blood count and a Siemens The Dimension® RxL Max® Integrated Chemistry System, Munich, Germany, for the other determinations. A part of each sample after centrifugation at 3500 rpm for 15 min was stored at −80 °C, until the determination of the serum levels of 25(OH)D, IL-10 and IL-31 and total IgE.

Total IgE was determined by the electrochemiluminescence technique, using a Cobas e-411 analyzer. Vit D (25(OH)D), IL-31 and Il-10 were determined by ELISA technique. The following determination kits were used: 25(OH)D Total ELISA Kit (DIAsource ImmunoAs-

says, Louvain-la-Neuve–Belgium), Human Quantikinine IL-10 (R&D system) and Human Il-31 Duoset ELISA (R&D System, Minneapolis, MN, USA). The samples and standard dilutions were assayed according to the manufacturer's instructions.

3.3. Statistical Analysis

Statistical analysis was conducted using the SPSS 25 program. The distribution of continuous variables was checked using the Kolmogorov–Smirnov normality test and they were characterized as mean and standard deviation (±SD) or median and 25–75th percentiles. Nominal variables were expressed as number and percentage. Quantitative variables were compared using the non-parametric Mann–Whitney and Wilcoxon tests. Nominal variables were compared using the Chi-square test or Fisher's Test. The level of statistical significance was set at $p < 0.05$.

4. Results

The demographic data of the children included in the study are presented in Table 1. Eleven girls (55%) and nine boys (45%), with a median age of 3.37 (1.5–5) years presented with the complaint of wheezing with no previous episode (group 1). Seven girls (35%) and thirteen boys (65%) with a median age of 3.12 (1.5–5) years, who presented with wheezing, had a history of more than three recurrent episodes of wheezing in the last 3 months (group 2). In the healthy control group (group 3), eight were girls (50%) and eight were boys (50%), with a median age of 10.59 (9–14) years. No gender distribution difference was found between the groups ($p > 0.05$) (Table 1). The control group had a higher median age than the patient groups.

Table 1. Demographic data of children with wheezing (group 1), recurrent wheezing (group 2) and healthy children (group 3—control).

	Group 1—Wheezing N = 20	**Group 2—Recurrent Wheezing N = 20**	**Group 3—Control N = 16**	***p***
Sex No (%)				
Male	9 (45%)	13 (65%)	8 (50%)	
Female	11 (55%)	7 (35%)	8 (50%)	$p > 0.05$
Age (Median/percentiles)	3.37 (1.5–5)	3.12 (1.5–5)	10.59 (9–14)	$p < 0.001$

Years of age are presented as median and (25th–75th) percentiles.

The laboratory data of the children in the study are presented in Table 2.

Between the initial and the final measurements, the median level of vit D decreased in group 1 (single episode of wheezing) and in the control group, while in children with recurrent wheezing, it was very low initially, but increased, reaching a level similar to the other two groups by the final measurement 3–6 months later (Table 2, Figure 1).

Between the two groups of patients with wheezing, IL-31 was higher in group 2, the children with recurrent wheezing, at the initial time point, but lower at the final time point.

IL-10 was lower in children with recurrent wheezing than in group 1, both at initial presentation and at the end of the study.

At the initial evaluation, MPV, ESR and levels of CRP, IL-10 and vit D showed significant differences between the studied groups, in as shown in Table 3. Also, IL-31 was lower in the control group vs. for children with wheezing, but the differences were not statistically significant. Vitamin D level was positively correlated with IL-31 median values in children with acute wheezing (R = 0.499, $p = 0.025$), but not in those with recurrent wheezing ($p > 0.05$).

Table 2. Laboratory data of children with wheezing (group 1), recurrent wheezing (group 2) and healthy children (group 3—control) at presentation (initial) and 3–6 months after the wheezing episode (final).

	Group 1—Wheezing N = 20			Group 2—Recurrent Wheezing N = 20			Group 3—Control N = 16		
	Initial	**Final**	***p***	**Initial**	**Final**	***p***	**Initial**	**Final**	***p***
Total leucocyte count (/mm^3)	8150 (6600–14,150)	6700 (5725–7825)	0.041	7800 (4700–9400)	6500 (5800–7700)	0.221	6200 (5175–8650)	6400 (5400–8050)	0.67
Neutrophils (/mm^3)	4650 (2800–6700)	3150 (2650–4125)	0.027	4550 (2850–5725)	3700 (2550–4450)	0.114	3400 (2725–4250)	3700 (2700–4375)	0.08
Lymphocytes (/mm^3)	2600 (1725–3925)	2450 (1550–3187)	0.65	2350 (1725–2900)	2200 (1700–2950)	0.559	2100 (1800–3350)	1900 (1600–2300)	0.59
Monocytes (/mm^3)	650 (500–1325)	650 (500–950)	0.284	600 (600–700)	500 (500–650)	0.105	500 (425–600)	600 (500–900)	0.94
PLT ($\times 10^9$)	275.5 (207.25–347.50)	293 (271–337)	0.905	277.5 (246.50–340.00)	255 (214–290)	0.118	314.0 (248.5–370.5)	290 (224–350)	0.52
MPV (fL)	7.9 (7.32–8.3)	8.2 (7.35–8.4)	0.184	8.85 (8.05–9.67)	8.8 (7.75–9.7)	0.48	8.45 (8.10–9.07)	8.2 (7.8–8.95)	0.45
CRP (mg/L)	1.1 (0.27–3.47)	0.25 (0.1–0.4)	0.005	1.8 (0.72–2.10	0.4 (0.15–0.5)	<0.001	0.10 (0.10–0.20)	0.35 (0.2–0.47)	0.83
ESR (mm/h)	26 (10–37.75)	12 (9–14)	0.006	25 (19.25–28.00)	12 (10.5–19.5)	0.004	9 (6.25–12)	10 (7–11.5)	0.79

PLT: platelets; MPV: mean platelet volume; CRP; C-reactive protein; ESR: erythrocyte sedimentation rate; IL-10: interleukin 10; IL-31: interleukin 31. All values are presented as median and (25–75th) percentiles.

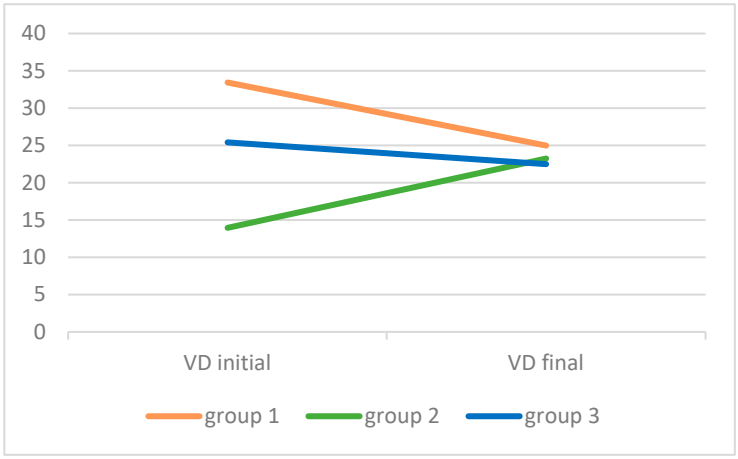

Figure 1. Level of Vitamin D [25(OH)D, VD] in the three study groups of children at the beginning and the end of the study (3–6 months after the wheezing episode). Group 1: single episode of wheezing (N = 20); group 2: recurrent wheezing (N = 20); group 3: control group (N = 16). All values are median.

Table 3. Levels of vitamin D, MPV, ESR, CRP and IL-10 in the three study groups of children on presentation.

	Group 1 (Wheezing, N = 20)	**Group 2 (Recurrent Wheezing, N = 20)**	**Group 3 (Control, N = 16)**	***p***
MPV (fL)	7.9 (7.32–8.3)	8.85 (8.05–9.67)	8.45 (8.1–9.07)	0.033
ESR (mm/h)	26 (10–38)	25 (19–28)	9 (6–12)	0.001
CRP (mg/L)	1.1 (0.27–3.47)	1.8 (0.72–2.1)	0.1 (0.1–0.2)	<0.001
IL10 (pg/mL)	22.03 (16.17–39.54)	17.81 (11.06–37.79)	5.35 (4.32–8.19)	<0.001
IL-31 (pg/mL)	7490.18 (2307.49–11,853.75)	7780.54 (2581.32–11,119.61)	5844.84 (3235.20–10,813.91)	>0.05
vit D level (ng/mL)	33.44 (23.1–46.38)	13.94 (11.92–23.27)	25.42 (21.22–32.95)	<0.001

MPV: mean platelet volume; CRP; C-reactive protein; ESR: erythrocyte sedimentation rate; IL-10: interleukin 10; vit D: 25(OH)D. All values are presented as median and (25th–75th) percentiles.

However, after 3–6 months, no statistically significant difference between the three groups was detected for the aforementioned parameters, as shown in Table 4. Analyzing the evolution of the cytokines, a discordant tendency was noted for IL-31, which increased in children with wheezing and in controls, while in those with recurrent wheezing it decreased, but these differences were not significant between groups or in comparison with basal values.

Table 4. Levels of vitamin D, MPV, ESR, CRP and IL-10 in the three study groups of children at the end of the study (3–6 months after the wheezing episode).

	Group 1 (Wheezing, N = 20)	Group 2 (Recurrent Wheezing, N = 20)	Group 3 (Control, N = 16)	*p*
MPV (fL)	8.2 (7.35–8.4)	8.8 (7.75–9.7)	8.2 (7.8–8.95)	>0.05
ESR (mm/h)	12 (9–14)	12 (10–19)	10 (7–11)	>0.05
CRP (mg/L)	0.25 (0.1–0.4)	0.4 (0.15–0.5)	0.35 (0.2–0.47)	>0.05
IL10 (pg/mL)	22.97 (17.86–41.72)	14.93 (12.67–42.85)	15.75 (12.4–30.12)	>0.05
IL-31 (pg/mL)	8842.82 (2544.73–11,830.15)	7112.48 (2612–9776.42)	9779.97 (3333.17–11,019.28)	>0.05
vit D level (ng/mL)	24.98 (21.95–35.96)	23.25 (17.34–29.34)	24.98 (18.54–31.12)	>0.05

MPV: mean platelet volume; CRP; C-reactive protein; ESR: erythrocyte sedimentation rate; IL-10: interleukin 10; vit D: 25(OH)D. All values are presented as median and (25–75th) percentiles.

5. Discussion

Epidemiological and observational studies have reported a consistent association between vit D deficiency and viral respiratory infections under certain conditions, and interventional studies on vit D supplementation and/or vit D status have produced mixed and sometimes conflicting findings [30]. Wheezing in children is most often caused by acute RTI [31], and recurrent wheezing in children aged under 5 years is a heterogeneous condition, usually associated with recurrent upper RTIs. The wheezing phenotypes proposed by the Task Force of the European Respiratory Society (ERS) in 2008 differ depending on the criteria used for classification, namely episodic viral or multiple-trigger wheezing, depending on symptoms, and transient, persistent and late-onset wheezing, according to time-trend classification [2]. This approach allows individual therapeutic decisions to be made, based on the temporal pattern of symptoms [32]. In daily clinical practice, however, many infants and young children present with wheezing during viral infections, and thus a classification into one of these phenotypes is unrealistic [7]. The identification of those infants with wheezing who are at risk of future recurrence and/or severe progression could help pediatricians to improve their therapeutic decisions.

Deng and colleagues characterized factors that are probably associated with wheezing and asthma in preschool children [29], which include the administration of antibiotics in the first month of life, a personal and/or family history of allergy or atopy, and obesity. In our study, the children in whom the above factors were present were excluded. Regarding vit D, the same authors found significant differences in levels in children who did not present wheezing and those who received vit D supplements [29].

Demirel and colleagues detected lower levels of vit D in children with recurrent wheezing compared with a control group [13]. Other studies have reported that low levels of vit D may be a risk factor for recurrent wheezing [33,34]. In the present study, the median vit D level at baseline was the lowest in the group of children with recurrent wheezing. Also, the median vit D level was higher in the children with a single episode of wheezing than in the control group. This could be explained by the fact that the control group consisted of older children, and in winter the serum level of vit D is reported to be lower in older children [35], as confirmed by the authors in previous studies [36,37]. In our study, significantly lower levels of vit D were found in children with recurrent wheezing than in either of those with a single episode, or in the control group, only at baseline evaluation. At the final time point, the three groups had similar serum vit D levels. The trend of vit D

was different in the studied groups. The children with a single episode of wheezing, and the control group, showed a descending trend of vit D, possibly dependent on the season. A similar descending trend of vit D level was also observed by Forno and colleagues, even in their vit D supplementation group [38]. The study children with recurrent wheezing showed an increasing trend in vit D after the wheezing stopped, even in those without vit D supplementation. We could speculate that after repeated episodes of wheezing, the vit D level returned in a relatively short time to levels similar to those observed in the children with a single episode of wheezing, and then may have followed a similar descending trend, corresponding to the seasonal variation, reaching approximately the same values at the end of the study. This explanation could support the theory that during episodes of wheezing and during infections, the level of vit D decreases due to imbalance between the supply (constant or even low, due to isolation at home) and the increased consumption in the immune processes activated during infection. Unfortunately, we did not predict this possibility, and failed to include in the study an intermediate blood test, 1–2 weeks after the onset of the acute episode.

Relevant studies have suggested that IL-31 may be involved in supporting allergic inflammation and is associated with a specific airway epithelial cell response that may characterize allergic asthma [39–41]. In one study, children with a history of more than three episodes of wheezing before the age of 2 years were later diagnosed with asthma much sooner [42]. The level of IL-31 could therefore be expected to be higher in children with recurrent wheezing. In our study, however, the level of IL-31 was higher in children with recurrent wheezing than in those with a first episode only at the initial point, while at the final time point, it was lower. A plausible explanation would be that the children in our study were older than those in the cited study and were in full health at the final time point, 3–6 months after the episode, with no further infections.

The evolution of IL-31 was discordant, in some groups having an ascending trend and in others a descending one. IL-31 seems to have a dual role in asthma, a pro-inflammatory one in the early stage and anti-inflammatory action in the late one. IL 31 is a cytokine that increases in eosinophilic inflammation, i.e., for RV bronchiolitis [24]. In the present study, children had wheezing or recurrent wheezing but not asthma, so we might speculate that IL-31 may play a role when allergic, eosinophilic inflammation is already settled, without having a role in preventing its development. Another explanation for this non-significance can be based on the fact that cases with clinical signs of allergy were excluded, so children with a possible minimal eosinophilic inflammation were not included in the present study. The present study showed that IL-31 does not influence the evolution of pre-existing asthma conditions in the absence of allergic inflammation.

IL-10 is a cytokine with regulatory properties in the immune response, produced by dendritic cells (DCs) and macrophages. The early production of IL-10 by antigen-presenting cells appears to limit excessive inflammation, and thus possible tissue damage [43]. During acute infections, proinflammatory signals are generated by DCs to recognize pathogen patterns. In this pro-inflammatory context, dendritic cells can promote antiviral T-cell responses responsible for pathogen and infection clearance. The activation of these DCs and of natural killer (NK) cells also results in the production of the IL-10 to balance the inflammatory process [44]. Loebbermann and colleagues demonstrated that respiratory syncytial virus (RSV) infection induced IL-10 production by CD4(+) and CD8(+) T cells in mice [45]. In the present study, the mean IL-10 levels were higher in the initial measurements at the onset of wheezing than at the final phase 3–6 months later, in both groups with wheezing, consistent with the antiviral activity of this cytokine. Bont and colleagues showed that IL-10 levels, measured in the convalescent phase of RSV bronchiolitis in infants 3–4 weeks after the hospitalization, were significantly higher in children who developed recurrent wheezing during the following year than in those without recurrence [46]. Conversely, in our study, IL-10 levels were lower in children with recurrent wheezing, but older age, viral etiology other than RSV and milder severity, not requiring hospitalization, are possible reasons for these differences.

This study highlighted the involvement of vit D in immune modulation and raised the hypothesis of a possible indirect relationship between vit D level and Th2-specific immune response in children with recurrent wheezing who are at possible risk of developing asthma. Vit D positively correlated with IL-31 in the acute phase of wheezing, but not for the long term. It seems that the immune response was normal outside the viral infections, and no minimal variation in IL-10 and IL-31 was noticed in the end of the study. This could be explained by the fact that IL-10 and IL-31 could play a role in augmenting or limiting the inflammation when it is already settled at the beginning of the acute episode, having no role in preventing the development of allergic inflammation long-term.

This study has several limitations. Firstly, the study groups of children were not large. Due to the COVID-19 pandemic, the process of inclusion and monitoring of patients had to be stopped, and many children already recruited were excluded because of the impossibility of final monitoring at the 6-month time-point. In addition, the children in the control group, because of the small numbers of younger children attending the hospital for routine visits, were much older than those with wheezing. Another limitation was the lack of specific virus determination. Only children with viral etiology of wheezing were recruited, excluding those with bacterial infection, but the technical possibility of determination of the type of virus was missing.

6. Conclusions

Recurrent wheezing in children is a common reason for specialist referral. The identification of those infants and young children with wheezing who are at increased risk of recurrence and possible development of asthma could help specialists to improve their therapeutic decisions. Low levels of vit D appear to be detected more frequently in recurrent wheezing, and therefore the determination of serum 25(OH)D may be a useful biomarker in infants and children presenting with wheezing. The routine monitoring of serum 25(OH)D should be considered in children with recurrent wheezing, and only in the case of deficiency should this be corrected. It remains unclear whether the low vit D level in children with wheezing predisposes to relapse, or that the recurrent episodes of wheezing result in lowering the level of vit D. Immune Th2 status reflected by IL-10 and IL-31 levels appears to depend on the wheezing phenotype, on the phase of the disease and on the general health status, but larger studies are needed to further explore their role in children with recurrent wheezing.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Scientific and Ethics Committee of the Hospital Unit of Amaliada, General Hospital of Ileia, Greece (protocol code 71/27th October 2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy and ethical restrictions.

Conflicts of Interest: The authors declare no conflicts of interest.

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Review

Antioxidant and Anti-Inflammatory Actions of Polyphenols from Red and White Grape Pomace in Ischemic Heart Diseases

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Abstract: Grape pomace (GP) represents a very reliable source of polyphenols because it could be found globally as a remnant of the wine industry. During the winemaking process, two types of GP are generated: red GP and white GP, according to the produced wine, red or white. Grape pomace represents a viable source of polyphenols, mainly flavanols, procyanidins anthocyanins, and resveratrol which possess antioxidant and anti-inflammatory activities. Multiple differences were observed between red and white GP in terms of their antioxidant and anti-inflammatory activity in both in vitro and in vivo studies. Although most studies are focused on the antioxidant and anti-inflammatory effect of red grape pomace, there are still many variables that need to be taken into consideration, as well as extensive study of the white GP. It was observed that in both in vitro and in vivo studies, the GP polyphenols have a direct antioxidant activity by acting as a free radical scavenger or donating a hydrogen atom. It also possesses an indirect antioxidant and anti-inflammatory activity by reducing mitochondrial reactive oxygen species (ROS) generation, malondialdehyde (MDA), tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), interleukin-6 (IL-6), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κβ), and inhibitor of nuclear factor kappa-B kinase subunit beta (Iκκβ) levels or nitrate oxide-4 (NOX4) expression and by increasing the levels of antioxidants enzymes like superoxide dismutase (SOD), catalase (CAT) glutathione reductase (GRx) and glutathione peroxidase(GPx). Besides these activities, many beneficial effects in ischemic heart diseases were also observed, such as the maintenance of the ventricular function as close as possible to normal, and the prevention of infarcted area extension. In this context, this review intends to present the actual knowledge of grape pomace's potential antioxidant and anti-inflammatory activity in ischemic heart disease, knowledge gathered from existing in vitro and in vivo studies focused on this.

Keywords: antioxidant; anti-inflammatory; grape pomace; polyphenols; ischemic heart diseases

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1. Introduction

Ischemic heart diseases, also known as coronary heart diseases (CAD), alongside stroke and other cardiovascular diseases, are the causes of approximately 17.9 million deaths annually, which represents 32% of the total deaths in the world [1]. Out of these, more than 75% are registered in low and middle-income countries. Furthermore, in accordance with World Health Organization (WHO), ischemic heart disease is the leading cause of global death, with 16% of worldwide deaths, followed by stroke, which is responsible for 11%, respectively. Ischemic heart disease is characterized by narrowing or blockage of one or

more coronary arteries, most frequently due to atherosclerosis, which is the main factor that reduced cardiac blood flow. It is clinically manifested by pectoral angina and heart attack [2]. The main incriminated risk factors that promote CAD are tobacco, an unhealthy diet with low fruit and vegetable intake, lack of physical activity, metabolic syndrome, and excessive use of alcohol [1,3]. Besides these, other pathologies like obesity, diabetes mellitus, nephrotic syndrome, and hypothyroidism could associate with dyslipidemia, which is characterized by elevated levels of LDL and total cholesterol and a reduced HDL level. Moreover, it was observed that people with different lifestyles, like workers who have permanent night shifts, are more likely to develop dyslipidemia [4].

All of these risks lead to atherosclerosis. In this term, atherosclerosis is defined as a multifactorial inflammatory disease of the innermost layer of an artery called intima, a build-up of cholesterol plaque, and a loss of the arterial wall elasticity [5]. Therefore, a primary target in the treatment of CAD is represented by the prevention of atherosclerosis development. In this regard, the management of CAD includes lifestyle changes like dietary modification, smoking cessation, and weight reduction alongside classical medication (nitrates, beta-blockers, calcium channel blockers, and angiotensin-converting enzyme inhibitors). Additional comorbidities like diabetes, hypertension, and dyslipidemia are controlled via oral antidiabetics or insulin, antihypertensive drugs, and statins, respectively.

Even if there are many efficient ways of reducing the incidence of associated risk factors, among which the pharmacological and surgical ones with proven results, CAD still represents the main cause of death worldwide. That is why this pathology represents a great interest for many researchers and their efforts are needed in identifying new ways to prevent and treat CAD. In this regard, plants have been always an inexhaustible source of discovering new compounds with potent pharmacological activities. Shifting from traditional plant utilization, a great alternative is represented by plant waste valorization. This new direction came along with the introduction of the circular economy, an economic system that proposes a reduced use of raw materials and increased reuse and recycling of different components and products already existing [6] (Figure 1).

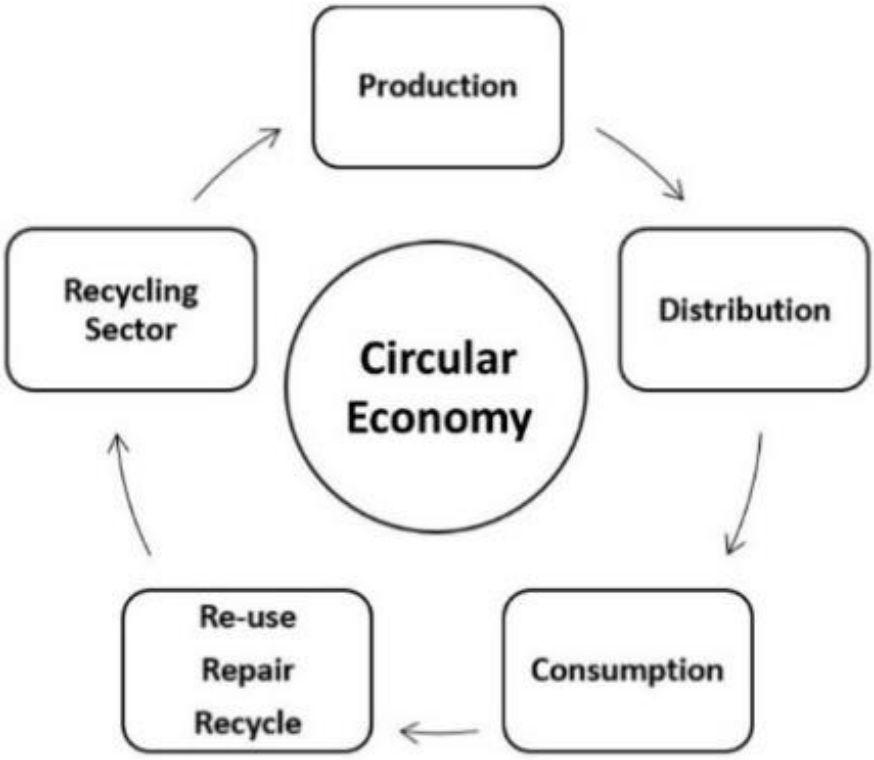


Figure 1. Circular Economy.

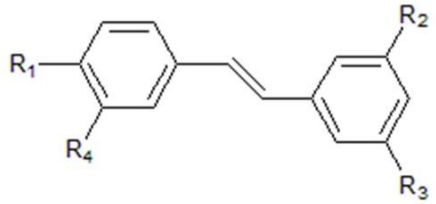
A perfect example of the circular economy's application is represented by the usage of grape pomace (GP). Therefore, it is estimated that annually are used more than 79 million tons of grapes, from which approximately 30% is represented by grape pomace [7,8]. Besides its use as fertilizer or animal feed, another field in which it can be used is the pharmaceutical one, due to the rich amount of bioactive compounds, especially phenolic ones [8]. Thereby, GP is reported to contain high quantities of resveratrol and polyphenols like flavanols: myricetin, quercetin, kaempferol; flavan-3-ols: catechin, epicatechin; cinnamic acids: p-coumaric and benzoic acids: syringic, gallic, and protocatechuic, 4-hydroxybenzoic [9]. It is known that polyphenols, the major compounds in GP waste have well-known antioxidant and anti-inflammatory effects [10]. Previous studies have reported their action on reducing LDL oxidation, inflammation, and platelet activation, all with positive effects in reducing the progression of atherosclerosis [11].

In the present review, we aimed to evaluate the influence of grape pomace through its polyphenols on ischemic heart disease due to its antioxidant and anti-inflammatory activities.

2. Red and White Grape Pomace—Bioactive Compounds

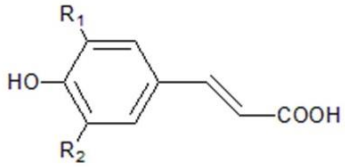
The utilization of grapes has a long history, which dates back to antiquity and spreads to the modern world, especially through their use in the wine industry. That is why there is a variety of literature studies that analyze and characterize grapes, grape derivatives especially wine, and GP composition and content [9,12] (Figure 2). It was observed that red grape pomace (RGP) and white grape pomace (WGP) have different phenolic compound fingerprints and different total phenolic content according to the grape cultivar and terroir. This means that all of the pedological, topographical, and geological aspects of a specific physical environment will alter the physical features of the grapes such as tastes, aromas, textures, and appearances [8]. RGP was found to be rich in stilbenes (resveratrol), phenolic acids (gallic acid, protocatechuic acid), flavanols (epigallocatechin), flavanols (myricetin-3-O-rhamnoside) and anthocyanins (delphinidil-3-O-glucoside, cyanidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside, malvidin-3-O-glucoside) [13]. WGP was reported to have high content of phenolic acids (p-hydroxyphenylacetic acid, vanillic acid, homovanillic acid, homoprotocatechuic acid, gentisic acid, syringic acid, 4-O-methylgallic acid, 3-O-methylgallic acid, dihydro-3-coumaric acid, hydroferulic acid, hydrocaffeic acid, isoferulic acid) and flavanols (catechin, epicatechin, procyanidin B1) [13], flavonoid glycoside (hyperoside, isoquercitrin, rutin, quercitrine), flavonoid aglycons (quercetin, luteolin), and protocatechuic acid [14]. It was also reported that WGP has a high quantity of gallic acid, procyanidin B3-4, epicatechin, and procyanidin gallates [15]. In both RGP and WGP was identified a similar amount of caffeic acid, coumaric acid, catechin, and its isomer epicatechin [14], and similar amounts of total tannins [16]. Overall, numerous studies concluded that RGP contains a higher amount of polyphenols than WGP [17]. However, some studies revealed types of WGP that possessed a greater content of polyphenols than RGP [13].

Stilbenes



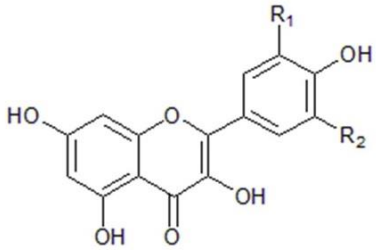
Compound	R1	R2	R3	R4
Resveratrol	OH	OH	OH	H
Piceatannol	OH	OH	OH	OH

Hydroxycinnamic acids



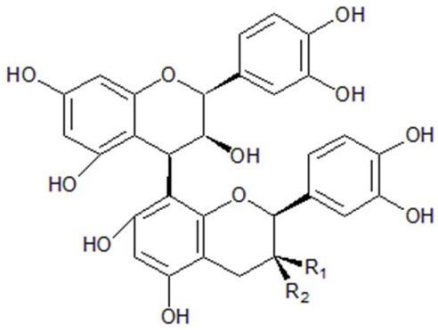
Compound	R1	R2
Cafeic acid	OH	H
Ferulic acid	OCH3	H
Sinapic acid	OCH3	OCH3
p-coumaric acid	H	H

Flavonols



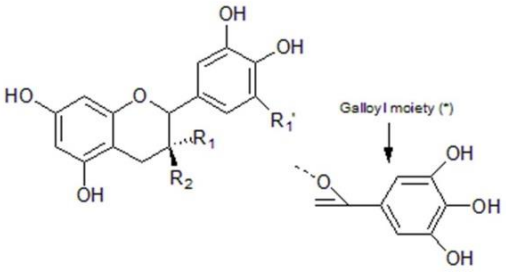
Compound	R1	R2
Myricetin	OH	OH
Quercetin	OH	H
Isorhamnetin	OCH3	H
Kaempferol	H	H

Procyanidins



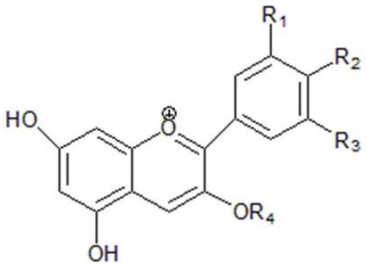
Compound	R1	R2
Procyanidin B1	OH	H
Procyanidin B2	H	OH

Flavanols



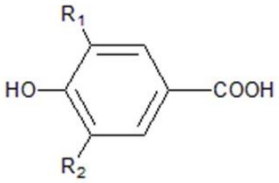
Compound	R1	R2	R1'
(+)-Catechin	H	OH	H
(-)-Epicatechin	OH	H	H
(-)-Epicatechingallat	*	H	H
(-)-Epigallocatechingallat	*	H	OH
(-)-Epigallocatechin	OH	H	OH

Anthocyanins



Compound	R1	R2	R3	R4
Cyanidin	OH	OH	H	H
Malvidin	OCH3	OH	OCH3	H
Petunidin	OCH3	OH	OH	H
Peonidin	OCH3	OH	H	H
Delphinidin	OH	OH	OH	H

Hydroxybenzoic acids



Compound	R1	R2
Gallic acid	OH	OH
Protocatechuic acid	OH	H
Syringic acid	OCH3	OCH3
p-Hydroxybenzoic acid	H	H

Figure 2. The chemical composition of principal polyphenols from grape pomace.

3. Potentially Toxic Effects of Polyphenols from Red and White Grape Pomace

Generally, literature studies focused on GP or GP by-products are emphasizing its health benefits rather than toxic or adverse reactions. Administration of GP may not be an issue in healthy people, but it must be considered in people with certain diseases who are receiving medical treatment [18]. Regarding the toxicity of polyphenols from grape pomace, we could find only one study. Thus, Neag et al., (2019) observed the paradoxical effect of GP polyphenol extract on an animal model of acute kidney injury induced by cisplatin. They reported that when GP, was given alongside cisplatin, for its antioxidant properties, it did not decrease the cisplatin-induced nephrotoxicity, on the contrary, it increased it [19]. The lack of studies reporting the potentially toxic effects of polyphenols from grape pomace could be based on the fact that even if GP presents pro-oxidant activity at a higher dose than the one that presents the antioxidant effect [20,21], that effect is too low to cause changes at the level of an organ or the entire organism, changes that could be highlighted through routine analyses. Thus, this issue should be addressed in terms of precautions rather than acute or chronic toxicity [22].

4. Red and White Grape Pomace—Variability of Total Polyphenols Content and Antioxidant Capacity

It is well-known that GP possesses great antioxidant activity, but it is necessary to find out what are the differences between RGP and WGP to give them an appropriate valorization. Literature studies showed that GP has a strong antioxidant activity, because of the contained phenolic compounds. The antioxidant activity is strongly related to phenolic chemical structures. Thus, the number of existing hydroxyl groups gives them the ability to act as free radical scavengers [23] or to donate an atom of hydrogen [24,25]. Accordingly, several methods for antioxidant activity evaluation have been created over time. The main methods used for GP characterization reported so far were total polyphenol content (TPC), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′–azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS), and ferric reducing antioxidant power assay (FRAP) for antioxidant activity (Table 1).

Total phenolic content represents the reference assay for measuring the polyphenols in plants or other biological samples, by using the Folin–Ciocalteu assay [24,26]. This method involves a reaction between the polyphenols and a redox reagent. Accordingly, the phenolic content is determined using the spectrophotometric measurement of this reaction [27]. Further, the methods used to determine polyphenols' antioxidant activity content are ABTS, DPPH, and FRAP. These assay methods analyze the antioxidant activity via the donation of a hydrogen atom (ABTS and DPPH) or via electron transfer (FRAP) [28,29].

Due to the variety of antioxidant activity methods that are used, it is very difficult to compare data from the literature. This situation leads to the development of a relevant correlation method, which could allow their comparison. In this case, Xu et al., (2016), in their study regarding the phenolic compounds extracted from four GP varieties, identified antioxidants and compounds with antibacterial properties and also developed a correlation method between TPC, DPPH, and ABTS [30]. They observed that between TPC and ABTS there is a significant positive correlation, but none between these and DPPH. A probable cause for this could be the fact that there are differences between the phenolic compounds involved in each method. Thus, it was reported that flavonoids and tannins contributed to the determination of antioxidant activity via ABTS, while in the case of DPPH anthocyanins, they had a major contribution. In comparison to this study, Marchante et al., (2018) observed that in the measurement of antioxidant activity using the DPPH method, a higher contribution was brought by (−)-epigallocatechin, while in the case of the ABTS method by flavan-3-ol monomers. Furthermore, they did not observe any differences between the contribution to the determination of ABTS and DPPH methods for (+)-catechin, (+)-gallocatechin, (+)-epigallocatechin, (+)-catechin gallate, (−)-epicatechin gallate, procyanidin B1, galloylated dimers, flavan-3-ol dimers, flavan-3-ol total oligomers, total flavan-3-ols, and trans-resveratrol-glucoside. Moreover, they also observed that the

compound with the highest antioxidant property was (+)-catechin gallate, followed by (−)-epicatechin gallate, (+)-gallocatechin, (+)-catechin, and (−)-epigallocatechin [31].

Xia et al., (2019), also addressed the necessity of comparing and correlating the values of the different methods used in polyphenols quantification. To eliminate the variations of these values, the authors also chose to determine the antioxidant activity by using all the above-described methods. Thus, Xia et al., (2019), evaluated the TPC and measured the antioxidant assay using ABTS, DPPH, and FRAP of skin and seeds from 31 different cultivars of grapes. Firstly, they observed that the grape seeds have more polyphenols and more antioxidant activity as assayed via DPPH, ABTS, and FRAP than grape skins. Secondly, they observed that the European species have higher antioxidant properties than the American, Asian, or hybrid ones [10].

Even though the majority of studies determined that RGP possesses a higher polyphenolic content and antioxidant activity (Costa et al., 2018, Sagdic et al., 2011, Xu et al., 2016), there is no sufficient evidence yet to affirm that RGP is superior to WGP. Winkler et al., (2015) observed that even though the RGP cultivated in Rhineland-Palatinate, Germany had a higher TPC than WGP, the differences were not significant [32]. Further, Cerda-Carrasco et al., (2015) who investigated GP obtained from *Vitis vinifera* sp. cultivated in Maipo Valley, Chile, observed that two types of white grapes, Sauvignon Blanc and Chardonnay, had higher phenolic content and antioxidant capacity than the two red types, Cabernet Sauvignon and Carménère [15].

Knowing that the differences between RGP and WGP are influenced by the wine-making technologies [15] and also by the terroir [8,32], we can highlight the importance of a correlation method that could help in the appropriate GP waste valorization. To emphasize these differences, the next table presents the variation of TPC and antioxidant capacity identified in different GP varieties.

We can conclude that both GP varieties represent great sources for further valorization, their prior analysis being a key step in directing toward the appropriate use, because of their large variation in terms of phenolic content and antioxidant activity.

Table 1. Comparison of total polyphenols content (TPC) and antioxidant capacity of red and white grape pomace polyphenols extracts.

Grape Pomace (GP)		TPC (mg GAE */g GP)	Antioxidant Capacity			References
			DPPH (µmol TE **/g GP)	ABTS (µmol TE/g GP)	FRAP (µmol F_eSO_4 * $7H_2O$/g GP)	
Vitis vinifera sp. Cultivated in Maipo Valley, Chile						
White	Sauvignon Blanc	19	120	-	-	[15]
	Chardonnay	17	90	-	-	
Red	Cabernet Sauvignon	14	60	-	-	
	Carménère	13	70	-	-	
Vitis vinifera sp. cultivated in Virginia, USA						
White	Vidal Blanc (hybrid variety)	55.5	7.71	334	-	[30]
	Viognier (*Vitis vinifera* sp.)	99.1	3.54	951	-	
Red	Cabernet Franc (*V. vinifera* sp.)	153.8	11.2	1013	-	
	Chambourcin (hybrid variety)	92.0	28.2	378	-	
Vitis vinifera sp. cultivated in Rhineland-Palatinate, Germany						
White	4 varieties of Pinot Blanc and 6 of Riesling	48	-	-	-	[32]
Red	5 varieties of Dornfelder, 5 of Pinot noir and 2 of Portugais bleu	58	-	-	-	
Vitis vinifera sp. cultivated in Blacksburg, Crozet, Floyd VA, USA						
White	Viognier	11.8	-	-	-	[33]
	Vidal Blanc	12.5	-	-	-	
	Niagara	24.8	-	-	-	
	Petit Manseng	32.1	-	-	-	
Red	Petit Verdot	64.8	-	-	-	
	Merlot	35.8	-	-	-	
	Cabernet Franc	36.1	-	-	-	
	Chambourcin	10.4	-	-	-	
White	unknown varieties	90.51	-	-	1619	[34]
Red	unknown varieties	107.40	-	-	1886	

Table 1. *Cont.*

Grape Pomace (GP)		TPC (mg GAE */g GP)	Antioxidant Capacity			References
			DPPH (μmol TE **/g GP)	ABTS (μmol TE/g GP)	FRAP (μmol F_eSO_4 * $7H_2O$/g GP)	
Vitis vinifera sp. cultivated in Cappadocia district of Nevsehir province (Emir), Tokat province (Narince), Sarkoy-Murefte district of Trakya region (Gamay), Ankara province (Kalecik Karasi), Elazig province (Okuzgozu), Turkey						
White	Emir	75.5	-	-	-	[35]
	Narince	138.1	-	-	-	
Red	Gamay	255.4	-	-	-	
	Kalecik Karasi	205.7	-	-	-	
	Okuzgozu	281.4	-	-	-	
Vitis vinifera sp. cultivated in Blackstone, VA, USA						
White	Chardonnay	24.5	-	-	-	[36]
Red	Cabernet Franc	30.4	-	-	-	
Vitis vinifera sp. cultivated in Cantine Cantele, Apulia Region, Southern Italy						
White	Fiano	127.06	-	-	-	[37]
Red	Negramaro	127.87	-	-	-	
Vitis vinifera sp. cultivated in Paros, Greece						
White	Monemvassia	4.49	-	-	0.32	[38]
Red	Mandilaria	5.1	-	-	0.31	
	Aidani mavro	0.25	-	-	0.21	

* GAE—gallic acid equivalent ** TE—Trolox equivalent.

5. Red and White Grape Pomace—In Vitro Antioxidant and Anti-Inflammatory Activities

The fact that many studies investigating GP have shown that it possesses intense antioxidant activity has been a key factor in drawing the attention of researchers to continue the findings and to focus on the beneficial antioxidant and anti-inflammatory activities within the in vitro studies as presented in Table 2. Most literature data reports the antioxidant activity of GP on cells exposed to different oxidative stress factors or/and the anti-inflammatory activity on cells subjected to different proinflammatory factors.

5.1. Red Grape Pomace Antioxidant Activity

Within the in vitro models of oxidative stress induced in different cell lines, hydrogen peroxide (H_2O_2), tert-butyl hydroperoxide, and menadione are the most used chemicals. Physical factors, especially UV radiation, are also often reported. Accordingly, Posadino et al., (2018) studied the antioxidant activity of RGP from *Vitis vinifera* L. Cagnulari cv. from Santa Maria La Palma, Alghero, Italy on H_2O_2-induced oxidative damage in human umbilical vein endothelial cells. They observed that under oxidative stress conditions, the treatment with RGP increased the viability of the cells, mainly due to RGP's capacity to reduce ROS levels [39]. Goutzourelas et al., (2014) investigated the antioxidant capacity of RGP from *Vitis vinifera* L. Batiki Tyrnavou cv. from Greece against oxidative stress. Within this study, the oxidative stress conditions were induced using tert-butyl hydroperoxide in muscle cells (C2C12) and endothelial cells (EA.hy926). The results obtained on muscle cells line indicated that RGP possesses high antioxidant activity demonstrated by TBARS (thiobarbituric reactive substances), ROS (reactive oxygen species), and protein carbonyl level reduction, and by increasing GSH (glutathione) levels. The results obtained from the endothelial cells line had similar results, except for the reduction of ROS levels [40]. The antioxidant effects of RGP from *Vitis vinifera* seeds were investigated by Decean et al., (2016) using UV radiation-induced oxidative stress in human keratinocytes cells (HaCaT cells). They observed that cells pre-treated with RGP had a significantly lower increase in ROS and protein levels that are specific to the apoptosis process. A lower increase in Bax-α pro-apoptotic protein and NF-kB p65 protein levels was also observed [41].

Table 2. In vitro antioxidant and anti-inflammatory activity of grape pomace polyphenols extracts.

Materials	Polyphenols Extracts	Models	Antioxidant and Anti-Inflammatory Activity	References
Grape pomace from different red *Vitis vinifera* species				
GP from *Vitis vinifera* L. Cagnulari cv. from Santa Maria La Palma, Alghero, Italy	Water/ethanol (60:40, *v*/*v*) extract containing: - anthocyanins (malvidin, peonidin-3-O-glucoside, malvidin-3-(6-acetyl)-glucoside, M-3-G)	H_2O_2-induced oxidative damage in human umbilical vein endothelial cells	- increased cells viability - reduced ROS levels	[39]
GP from *Vitis vinifera* L. Batiki Tyrnavou cv. from Greece	Ethanol extract containing: - flavan-3-ols (catechin, epicatechin, epicatechin-3-gallate) - anthocyanidins (malvidin, cyanidin, petunidin, delphinidin) - anthocyanins (peonidin-3-O-glucoside, myrtillin, oenin, kuromanin) - phenolic acids (caftaric acid, gallic acid) - flavanols (quercetin, kaempferol)	Tert-butyl hydroperoxide-induced oxidative damage in muscle cells (C2C12)	- reduced TBARS, ROS and protein carbonyls levels - increased GSH levels	[40]
		Tert-butyl hydroperoxide-induced oxidative damage in endothelial cells (EA.hy926)	- reduced TBARS and protein carbonyls levels - increased GSH levels	
GP from *Vitis vinifera* seeds	-	UV radiation-induced oxidative stress in human keratinocytes cells (HaCaT cells)	- decreased ROS levels - decreased apoptosis proteins levels - decreased Bax-α pro-apoptotic protein levels - decreased NF-kB p65 protein levels	[41]
GP from *Vitis vinifera* from Valea Calugareasca	Acetone extract containing: - flavonoids (catechins, procyanidins, epicatechins) * higher concentration for procyanidin dimer and epicatechin	Intestinal inflammation model: LPS-inflammation induced in Caco-2 intestinal cells Symbiotic combination with *Lactobacillus sp.* as probiotic	- down-regulation of chemokines and cytokines proteins and genes expression - up-regulation of TIMP1 and TIMP2 genes expression - down-regulation of JNK1, ERK1/2, Akt/P70S6K/mTOR, MAPK, NF-κB and Nrf2 expression	[42]

Table 2. *Cont.*

Materials	Polyphenols Extracts	Models	Antioxidant and Anti-Inflammatory Activity	References
GP from *Vitis vinifera* variety Montepulciano from Chieti, Italy	Water extract containing: - gallic acid, caftaric acid, caffeic aicd, syringic acid, coumaric acid, ferulic acid, catechin, epicatechin, chlorogenic acid	H_2O_2-induced oxidative damage in HypoE22 rat hypothalamus cells	- averted the down-regulation of BDNF gene expression - averted up-regulation of COX-2 gene expression and decreased PGE2 levels	[43]
GP from *Vitis vinifera* L. varieties from Emilia Romagna region, Italy	Natural deep eutectic solvents (NaDESs) extract containing: - anthocyanins (malvidin)	Menadione-induced oxidative damage in keratinocyte cells from human skin (HaCaT cells)	- improved cells viability - reduced IL-8 levels	[44]
GP from *Vitis vinifera* L., cv Negramaro from Azienda Agricola Cantele, Guagnano, Lecce, Italy	Methanol/ethanol (80:20, *v*/*v*) extract containing: - caffeic acid, caftraic acid, cutaric acid, gallic acid, catechin, epicatechin, kampferol, oenin, quercetin, rutin, t-resveratrol	LPS and TNF-α-induced inflammation in human colorectal adenocarcinoma-derived intestinal epithelial cells (Caco-2 cells) and human microvascular endothelial cells (HMEC-1 cells)	- decreased IL-6 and MCP-1 levels - down-regulation of MMP-9 and MMP-2 expression - down-regulation of the mRNA levels of the cytokines (IL-1β and TNF-α), the chemokines (CXCL-10 and M-CSF), COX-2 VCAM-1, ICAM-1 - down-regulation of the NF-κB signaling pathways - reduced ROS levels	[45]
GP from *Vitis vinifera* cv Pinot noir from Cautín valley, La Araucanía Region, Chile	Ethanol extract containing: - hydroxybenzoic acids (gallic acid, protocatechuic acid) - flavanol (catechin) - hydroxycinnamic acid (ferulic acid) - flavanols (quercetin, quercetin-3-rutinoside, quercetin-3-galactoside, quercetin-3-glucoside, kaempferol-3-glucoside) - anthocyanins (malvidin-3-glucoside, peonidin-3-glucoside, delphinin-3-glucoside, petunidin-3-glucoside, cyanidin-3-glucoside)	Polycyclic aromatic hydrocarbons-induced cytotoxicity in endothelial cells	- increased cells viability - down-regulation of Nrf2 expression	[46]

Table 2. *Cont.*

Materials	Polyphenols Extracts	Models	Antioxidant and Anti-Inflammatory Activity	References
Grape pomace from different white *Vitis vinifera* species				
GP from *Vitis vinifera* cv Chardonnay from Lowden, WA, USA	-	H_2O_2-induced oxidative damage in human colonic epithelial cells (Caco-2 cells)	- decreased ROS levels	[47]
Red grape pomace versus White grape pomace				
GP from *Vitis vinifera* varieties from Quinta da Cavadinha, Pinhão, Portugal Red: Tinto Cão, Tinta Barroca White: Malvasia Fina, Moscatel Branco	Methanol/distilled water (70:30, *v*/*v*) extract containing: - flavanols (isorhamnetin-3-O-(6-O-feruloyl)-glucoside, quercetin-3-O- glucuronide, quercetin-3-O-rutinoside, kaempferol-3-O-rutinoside, kaempferol-3-O-gluco- side) - cinnamic acid (caftaric acid) - anthocyanins (malvidin-3-O-glucoside, malvidin-3-O-(6-O-caffeoyl)-glucoside, malvidin-3-O-rutinoside) - stilbene (Σ-viniferin)	H_2O_2-induced oxidative damage in human keratinocytes (HaCaT cells)	- increased GSH levels, where WGP from Malvasia Fina had the highest capacity to increase it - decreased ROS levels, where RGP from Tinto Cão had the highest capacity to decrease it - decreased LPO levels, where WGP from Malvasia Fina had the highest capacity to decrease it	[48]

Abbreviations: TIMP1/2—matrix metalloproteinase inhibitors 1/2; MAPK—Mitogen-activated protein kinase; JNK1—c-Jun N-terminal kinase; ERK1/2—Extracellular signal-regulated kinase 1/2; Akt—protein kinase B; P70S6K—ribosomal protein S6 kinase; mTOR—mammalian target of rapamycin; Nrf2—nuclear factor erythroid 2–related factor 2; ROS– Reactive oxygen species; TBARS- Thiobarbituric acid reactive substances; GSH –Glutathione; BDNF—brain-derived neurotrophic factor; PGE2—Prostaglandin E2; LPO—Lipid peroxidation; NF-kB –nuclear factor kappa-light-chain-enhancer of activated B cells; LPS—lipopolysaccharide; TNF-α—tumor necrosis factor; IL-6—Interleukin-6; MCP-1—monocyte chemoattractant protein-1; MMP—matrix metalloproteinases; IL-1β—Interleukin-1-beta; CXCL-10—C-X-C motif chemokine ligand 10; M-CSF—macrophage colony-stimulating factor; COX-2–cyclooxygenase-2; VCAM-1– Vascular Cell Adhesion Molecule 1; ICAM-1– Intercellular Adhesion Molecule 1; H_2O_2—hydrogen peroxide.

5.2. White Grape Pomace Antioxidant Activity

So far, in the literature, we have identified a few studies that investigated the antioxidant activity of WGP. Thereby, Bibi et al., (2017) studied the antioxidant activity of WGP from *Vitis vinifera* cv Chardonnay from Lowden, WA, USA on H_2O_2-induced oxidative damage in human colonic epithelial cells. They observed that WGP played a major role in reducing ROS levels [47]. Furthermore, WGP antioxidant activity was also analyzed in comparison with RGP by Domínguez-Perles et al., (2016). They studied two red types (Tinto Cão, Tinta Barroca) and two white types (Malvasia Fina, Moscatel Branco) from *Vitis vinifera* varieties from Quinta da Cavadinha, Pinhão, Portugal. They highlighted that, on H_2O_2-induced oxidative damage in human keratinocytes (HaCaT cells), all four types possessed antioxidant activity due to a significant decrease in ROS and LPO levels and a significant increase in GSH levels. Of all these four types, the highest decreases in LPO levels and the highest increases in GSH levels were recorded in the case of WGP from Malvasia Fina. On the other hand, the highest decreases in ROS levels were reported for RGP from Tinto Cão [48].

5.3. Red Grape Pomace Anti-Inflammatory Activity

Regarding experimental-induced inflammation in different cell lines in vitro, the most used chemicals are polycyclic aromatic hydrocarbons, TNF-α (tumor necrosis factor- α), and LPS (lipopolysaccharide). Thus, Pistol et al., (2019) investigated the anti-inflammatory activity of RGP from Vitis vinifera grown in Valea Călugărească, România. Within their study, RGP was used as a prebiotic in combination with Lactobacillus sp. used as a probiotic, on LPS-induced inflammation in human colorectal adenocarcinoma-derived intestinal epithelial cells (Caco-2) intestinal cells. This combination led to the down-regulation of the pro-inflammatory chemokines and cytokines genes' expression, and an up-regulation of TIMP1 (matrix metalloproteinase inhibitors 1) and TIMP2 (matrix metalloproteinase inhibitors 1) expression [42]. Moreover, they observed the anti-inflammatory effect by down-regulation of many inflammations signaling pathways such as JNK1 (c-Jun N-terminal kinase), ERK1/2 (extracellular signal-regulated kinase $\frac{1}{2}$), Akt/P70S6K/mTOR (Akt—protein kinase B, P70S6K—ribosomal protein S6 kinase, mTOR—mammalian target of rapamycin), MAPK (Mitogen-activated protein kinase), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and Nrf2 (nuclear factor erythroid 2–related factor 2) [42]. In the study of Calabriso et al., (2022) the anti-inflammatory effects of RGP from *Vitis vinifera* L., cv Negramaro from Guagnano, Lecce, Italy was investigated on LPS and TNF-α-induced inflammation in Caco-2 cells and in human microvascular endothelial cells (HMEC-1 cells). Using the ELISA assay, they observed that the treatment with RGP induced a decrease in IL-6 (interleukin-6) and MCP-1 (monocyte chemoattractant protein-1) levels and down-regulation of MMP-9 (matrix metalloproteinases-9) and MMP-2 (matrix metalloproteinases-2) expression [45]. Moreover, they also highlighted the down-regulation of the NF- κB pro-inflammatory signaling pathways and the down-regulation of the pro-inflammatory proteins such as cytokines (IL-1β and TNF-α) and chemokines. It was also reported that the treatment with RGP decreased ROS levels [45].

5.4. White Grape Pomace Anti-Inflammatory Activity

Like in the case of antioxidant action, the anti-inflammatory activity of WGP is much less studied than RGP. In fact, almost all the studies focused on GP anti-inflammatory activity are done on RGP. We found only one article investigating WGP polyphenol extracts in vitro anti-inflammatory properties. According to this study, Ferri et al., (2017) used a bioluminescent cell-based assay to test WGP water and thanolic polyphenol extracts on human embryonic kidney HEK293 cells. It was observed that the water WGP extract reduced TNFa-induced inflammation by 62% [49].

These results support that GP possesses important antioxidant and anti-inflammatory activities. Although most studies are referring to RGP, there are insufficient data to conclude

that it would be more effective than WGP, and further studies are needed to compare their activities.

6. Red and White Grape Pomace—In Vivo Antioxidant and Anti-Inflammatory Activities

Taking into consideration that several studies have demonstrated a beneficial impact on metabolic syndrome, which is a key factor in many health-related issues [50–55], for reducing cardiovascular disease risk factors such as TMAO (trimethylamine N-oxide) [56], hypertension, and hyperglycemia [57], it is necessary to find out if all of these are supported by in vivo studies. Thus, the literature can provide valuable information about the anti-inflammatory and antioxidant activity that GP possesses in various experimental studies [58–60].

The liver is the main antioxidant site for neutralizing most oxygen-free radicals. For this reason, the liver also plays a key role in maintaining the oxidant/antioxidant balance. This balance can be disturbed by diseases such as atherosclerosis, diabetes, and cancer, which induce oxidative stress. This state occurs when there is an increase in the production of free radicals, which can damage biomolecules such as lipids (lipid peroxidation), proteins (peptide chain fragmentation and electrical charge alteration), and DNA (purine and pyrimidine bases degradation, mutations, translocations or deletion) [61]. An additional measure in combating these pathological changes is brought by antioxidants, including GP, which is known to have a strong antioxidant effect. Thus, many studies have aimed to investigate the effects of GP on liver redox homeostasis.

6.1. Red Grape Pomace Antioxidant Activity

One such study investigated the effect of RGP obtained from *Vitis vinifera* L. var. Moschato from Tyrnavos, Larissa, Greece on a batch of 36 Chios breed sheep [60]. Kerasioti et al., (2017) observed that this extract possesses an intense antioxidant activity demonstrated by increasing GST (glutathione transferase) activity and γ-GCS (γ-synthase glutamyl cysteine) expression in the liver [60]. Both GST and γ-GCS are enzymes involved in the synthesis of GSH (glutathione), which represents an important endogenous antioxidant. The antioxidant and anti-inflammatory activity of RGP obtained from *Vitis vinifera* from Uva'Só, Garibaldi, Rio Grande do Sul state, Brazil was also observed by Souza et al., (2019). Within their study, the effect of RGP was investigated on a model of liver damage induced by Pseudomonas aeruginosa in juvenile grass carps. The study highlighted the fact that the RGP possesses antioxidant activity as demonstrated by the reduction of TBARS levels, NOx, and ROS production [58]. Another study that contributed to the strengthening of the hypothesis that GP has a beneficial role in maintaining the oxidant/antioxidant balance is the study by Chedea et al., (2019). Their research was focused on the effects of RGP from *Vitis vinifera* from Valea Călugărească, România on 20 crossbred TOPIG hybrid pigs. They observed that administration of RGP had antioxidant activity as a result of the increased SOD (superoxide dismutase) activity and reduced TBARS levels [59]. SOD is an antioxidant enzyme that catalyzes the dismutation of the superoxide radical into oxygen and hydrogen peroxide. Even though hydrogen peroxide is still an oxygen-free radical, it is degraded by other antioxidant enzymes such as CAT (catalase) [62], whose activity is also increased by RGP [59,63,64]. Besides the antioxidant activity shown in the liver, studies reported that GP can also act on other organs. Thus, the administration of RGP increased SOD and CAT activity in the spleen and kidney [59], increased SOD; CAT, and GPx (glutathione peroxidase) activity; and reduced TBARS levels in the duodenum [64].

6.2. Red Grapepomace Anti-Inflammatory Activity

It is well-known that oxidative stress, via free radicals, induces damage to different cells and tissues, which leads to an inflammatory response from the organism but, besides this, alternative pathways have also been reported, which could prove the direct anti-inflammatory activity. One such study is that of Boussenna et al., (2016) who investigated

the anti-inflammatory activity of GPs from *Vitis vinifera* from the Rhône valley, France on dextran sodium sulfate-induced inflammatory bowel disease in mice. They underlined that GP reduced polymorphonuclear (PMN) infiltration and attenuated the intensity and breadth of colonic modifications [65]. Another pro-inflammatory pathway where GP seemed to have an antagonistic effect is represented by the NF-κB activation. NF-κB represents an inflammatory factor that possesses a central role in different pro-inflammatory signaling pathways, such as transcriptional induction of cytokines and chemokines; promoting T cells differentiation [66]; activating macrophages which produce pro-inflammatory cytokines (IL-1, IL-6, IL-12, and TNF- α) [67,68]. Thus, Nishiumi et al., (2012) observed that GP from *Vitis vinifera* from Kobe City, Japan inhibited the activation of NF-κB, which led to a suppression of the expression of COX-2 (cyclooxygenases-2) and iNOS (inducible nitric oxide synthase) proteins. iNOS leads to an excess of NO, which could act as a free radical and COX-2 produces prostacyclins and prostaglandins, which are pro-inflammatory mediators. Thus, both suppressive actions demonstrate the anti-inflammatory activity of RGP [69]. The direct anti-inflammatory effect of RGP was observed on obese-induced C57BLK/6J mice [70]. This study suggests that GP anti-inflammatory effects could be enhanced by protective mechanisms other than the antioxidant activity demonstrated in other research works [70].

6.3. White Grape Pomace Antioxidant and Anti-Inflammatory Activities

So far, to our knowledge, the antioxidant and anti-inflammatory activity of WGP was not studied. However, two studies that compared the antioxidant and anti-inflammatory activities of RGP and WGP were identified. Nishiumi et al., (2012) studied RGP and WGP from *Vitis vinifera* grown in Kobe City, Japan. They evaluated the galactosamine and lipopolysaccharide-induced inflammation in 6-week-old Sprague-Dawley males. It was observed that RGP possessed a higher anti-inflammatory activity demonstrated by a stronger NF-κB inhibition as compared to the inhibitory action of WGP [69]. The second one, conducted by Turcu et al., (2020), studied the antioxidant activity of RGP from the Merlot variety and WGP from the Tămâioasă Românească variety. They observed that both 3% and 6% concentrations of both RGP and WGP reduced TBARS levels in tight meat, but only 3% WGP and 6% RGP reduced TBARS levels in breast meat. This could suggest the fact that WGP has higher antioxidant activity, requiring a lower concentration compared to RGP to induce the same effect [71]. The following table (Table 3) presents the antioxidant and anti-inflammatory activity of RGP alone and of RGP in comparison with WGP.

Unfortunately, most studies in the literature focused on the antioxidant and anti-inflammatory activity of GP do not specify whether it comes from red or white grapes. At the same time, in most of the experiments, red grapes were much more often used, so it is difficult to highlight whether there is a difference between the antioxidant and anti-inflammatory activity between RGP and WGP. For these reasons, there is a need for future studies addressing this topic to specify more accurately the origin of the GP. Moreover, it is well recommended that future studies should conduct a comparison between RGP and WGP, in order to highlight whether or not there are differences between their antioxidant and anti-inflammatory activity, and, if there are some, to be able to determine which of the two possesses a more potent activity.

Table 3. In vivo antioxidant and anti-inflammatory activity of grape pomace polyphenols extracts.

Materials	Polyphenols Extracts	Models	Antioxidant and Anti-Inflammatory Activity	References
Grape pomace from different red *Vitis vinifera* cultivars				
Vitis vinifera sp. Cabernet Franc from Blackstone, VA, USA	Ethanol extract	Streptozotocin-induced type 2 diabetes in 6-week-old C57BL/6J male mice	- suppressed the rising of postprandial blood glucose	[36]
Vitis vinifera from Uva'Só, Garibaldi, Rio Grande do Sul state, Brazil	-	Pseudomonas aeruginosa-induced hepatic lesion in juvenile grass carps	- reduced NOx and ROS production - reduced TBARS levels - reduced the increase of SOD and CAT activity through their antioxidant activity - no significant increase in GPx and GST activities	[58]
Vitis vinifera from Valea Calugareasca, Romania	Methanol/Acetone extracts	Organs (liver, spleen, kidney) sampled from 20 crossbred TOPIG hybrid (Landrace & Large White with Duroc & Pietrain) pigs	- increased SOD activity and total antioxidant status in all of these organs - significantly increased CAT activity in kidney and spleen - no significant difference in GPx activity - significantly reduced TBARS levels in liver and kidney	[59]
Vitis vinifera L. var. Moschato from Tyrnavos (Larissa, Greece)	Water extract	36 Chios breed male sheep	- increased GST activity in the spleen and liver - increased γ-GCS expression in liver - no significant difference in SOD activity in both liver and spleen	[60]
Vitis labruscana L. from Korea	Methanol and ethanol extract	Diet-induced hypercholesterolemia in 48 New Zealand white male rabbits	- increased CAT and GPx activity - significantly reduced TBARS levels	[63]
Vitis vinifera from Valea Călugaărească, România	Water extract containing: - procyanidin trimer, procyanidin dimer, gallic acid, gallic acid-glucoside, malvidin-3-O-(6″-coumaroyl-glucoside)	Duodenum and Colon sampled from 20 crossbred TOPIG hybrid (Landrace & Large White with Duroc & Pietrain) pigs	- significantly reduced TBARS levels - increased total antioxidant status - increased SOD activity only in the duodenum - increased GPx and CAT activity	[64]

Table 3. *Cont.*

Materials	Polyphenols Extracts	Models	Antioxidant and Anti-Inflammatory Activity	References
Vitis vinifera from the Rhône valley, France	Ethanol extract containing: - anthocyanins (malvidin-3-glucoside)	Dextran sodium sulfate-induced inflammatory bowel disease in 5-week-old Wistar male mice	- increased SOD activity - decreased PMN infiltration	[65]
Vitis vinifera from Kobe City, Japan	1% acetic acid in methanol extract	Galactosamine and lipopolysaccharide-induced inflammation in 6-week-old Sprague-Dawley male	- suppress the expressions of COX-2 and iNOS proteins by inhibiting NF-κB activation	[69]
Vitis vinifera from Virginia vineyard (Blackstone, VA, USA)	Ethanol 1:10 ratio (*m/v*) extract containing: - catechin, epicatechin, quercetin, trans-resveratrol, caffeic acid, coutaric acid, ferulic acid, gallic acid, p-coumaric acid, p-hydroxybenzoic acid, protocatechuic acid, syringic acid	Obese 6 -week-old C57BLK/6J male mice	- no significant antioxidant activity - anti-inflammatory activity: reduced CRP levels	[70]
Red grape pomace versus White grape pomace				
Vitis vinifera from Kobe City, Japan	1% acetic acid in methanol extract	Galactosamine and lipopolysaccharide-induced inflammation in 6-week-old Sprague-Dawley male	- red grape pomace possessed a higher inhibiting action of NF-κB activation than white grape pomace	[69]
Vitis vinifera from Pietroasa, Buzău county, România PUFA enriched * WGP from Tămâioasă Românească variety * RGP from Merlot variety	3% and 6% dried GP diet	Breast and thigh meat sampled from broilers fed with 3% and 6% grape pomace	- both 3% and 6% diets significantly reduced TBARS levels in tight meat; - just the 3% white GP and the 6% red GP diets significantly reduced TBARS levels in breast meat	[71]

Abbreviations: CRP—C-reactive protein; NOx—Nitrate oxide; ROS—Reactive oxygen species; TBARS—Thiobarbituric reactive substances; SOD—Superoxide dismutase; CAT—Catalase; GPx—Glutathione Peroxidase; GST—Glutathione transferase; PMN—Polymorphonuclear; γ -GCS—γ-synthase glutamyl cysteine; COX-2—cyclooxygenases-2; iNOS—Inducible nitric oxide synthase; NF-kB—nuclear factor - kappa-light-chain-enhancer of activated B cells; PUFA—polyunsaturated fatty acids.

7. Ischemic Heart Diseases—What We Know So Far and What Can Be Improved

When it comes to ischemic heart diseases, the literature refers to them as coronary artery diseases (CAD). Thereby, CAD is characterized as a pathological process caused by the accumulation of atherosclerotic plaque in the intimal wall of the arteries. This accumulation could lead to a complete or incomplete obstruction of the arteries, resulting in an imbalance between myocardial oxygen demand and supply. Other causes that could induce CAD are microvascular dysfunction and a spasm of the coronary arteries. This pathology is considered to be a chronic and progressive disease [2,72].

7.1. Risk Factors

The principal risk factors that contribute to the appearance and progression of atherosclerosis and coronary artery diseases are smoking, diet, weight gain, and physical activity. Among risk factors, tobacco is responsible for more than 8 million death per year. According to WHO, over 80% of tobacco users are from low-middle-income countries, and, taking into consideration that 75% of total deaths of cardiovascular events are registered also in low- and middle-income countries, there is a possible correlation between smoking and cardiovascular death causes. It was observed that smoking cessation leads to a reduction of 36% in CAD-induced mortality. For this matter, besides behavioral counseling, there is also pharmacological support to encourage smoking cessation [72,73]. Another risk factor is represented by an unhealthy diet. For the prevention of several diseases, a dietary plan that includes fruits, vegetables, polyunsaturated fats, fish, and fiber is recommended, along with avoiding a high quantity of refined carbohydrates, saturated, fat and red meat [72]. Nonetheless, physical inactivity represents a major risk factor for CAD and stroke. Both diet and physical activity modulate weight management, which represents one of the most life-long risks of all because a close correlation between body weight and lipid profile has been demonstrated in several studies. Increased body weight may also disturbs the lipid profile, which, could lead to atherosclerosis. According to WHO, over 39 million children under 5 years are obese, and over 1.9 billion adults are overweight, of which over 650 million are obese [74]. Therefore, it was demonstrated that patients who are overweight or obese are more likely to develop cardiovascular diseases than patients with a normal BMI (20–25 kg/m^2) [72]. Considering these, a healthy lifestyle behavior would decrease the risk of cardiovascular events.

7.2. Diagnostics

When it comes to diagnostic methods, there is basic testing, which includes resting ECG, and echocardiography and biochemical tests such as a lipid profile and myocardial injury markers—troponins T and I. In addition to imagistic testing, if the echocardiography is inconclusive cardiac magnetic resonance may be taken into consideration [72]. In the last years, the medical scientific community tried to develop a way for less invasive and less expensive screening for this pathology. In this direction, a risk-estimation system was created and validated, the well-known SCORE system [72].

7.3. Management

The aims of pharmacological management are to reduce the symptoms associated with coronary artery diseases and to prevent major acute cardiovascular events like myocardial infarction. Starting from here, there are two types of therapy: for and for no life-threatening CAD, the second one is usually referred to it as long-term medication. For life-threatening CAD, the gold standard is percutaneous coronary intervention, within 2 h from the appearance of symptoms associated with antiplatelet and anticoagulant therapy. An alternative to percutaneous coronary intervention is fibrinolystic drugs such as alteplase or reteplase. The long-term medication includes numerous classes of drugs such as: anti-ischemic drugs, which include nitrates, beta-blockers, and calcium channel blockers; antiplatelet drugs like aspirin and clopidogrel; anticoagulant drugs, which include heparin and low molecular weight heparins, warfarin, dabigatran. All of these treatments come with side effects

such as hypotension, headache (nitrates), fatigue, bradycardia, bronchospasm, heart block, peripheral vasoconstriction (beta-blockers), headache and ankle edema (calcium channel blockers), and increased risk of bleeding (antiplatelet and anticoagulant drugs) [72,75].

7.4. Potential New Therapy

Taking all of the above into consideration, even with the fact that the major risk factors are well-known and there is a continuous progression in diagnostic methods and pharmacological management, ischemic heart diseases remain one of the major causes of mortality and morbidity so far. Accordingly, there still exists the demand and necessity for alternative therapy to be found. That is why, when taking into consideration the oxidative stress and inflammation associated with all the mechanisms that induce ischemic heart diseases, it is a good premise that the polyphenols from GP could be used as adjuvant therapy. Even though the antioxidant and anti-inflammatory effects of the polyphenols from GP are well-known, there is still a lack of information when it comes to the pharmacological activities, especially their pharmacokinetics and pharmacodynamics. Looking in this direction, it is necessary to conduct studies for this purpose, firstly on animals, and, if the results are promising, to take the next step, and, eventually, to conduct trials on patients with ischemic heart diseases.

8. In Vitro and In Vivo Studies—Grape Pomace Antioxidant and Anti-Inflammatory Actions in Ischemic Heart Diseases

As was already mentioned, GP represents a reliable source of polyphenols that could be used as adjuvant therapies in the treatment of different pathologies characterized by oxidative stress or inflammatory pathophysiological processes. Moreover, GP proved that it could be used in various cardiac diseases due to its antioxidant and anti-inflammatory activities as observed in both in-vitro and in-vivo studies (Table 4). Grape pomace showed beneficial properties both when used as primary prophylaxis, in the control of risk factors, and when used as secondary prophylaxis, in the prevention of complications.

Used as primary prophylaxis, it was observed that the polyphenols from GP presented a protective effect by their anti-atherogenic actions. It is well known that a key factor in the progression of atherosclerosis is the oxidation of LDL cholesterol and the fact that atherosclerosis triggers an inflammatory response in the arterial wall (Figure 3). Despite the existence of lipid-lowering drug classes, statins, and fibrates, which decrease LDL cholesterol, total cholesterol, and triglycerides levels and increase HDL cholesterol levels, there is still a large majority of patients who develop ischemic heart diseases [76,77]. So theoretically, GP due to its antioxidant activity could help in the management of atherosclerosis development and progression by its capacity to prevent LDL cholesterol oxidation and by its anti-inflammatory activity. With this perspective, Rivera et al., (2019) studied the effect of RGP on ischemic heart disease in rats with induced atherosclerosis through an atherogenic diet. They observed that RGP presented anti-inflammatory activity by decreasing TNF-α and IL-10 levels. Moreover, they observed that the RGP added to the diet also increased the concentration of HDL cholesterol, which has an anti-atherogenic effect. Even more, in comparison with the control group, the RGP decreased the size and number of atherosclerotic lesions [78]. Other in vitro and in vivo studies also showed that GP possesses antioxidant activity useful in the management of the prooxidant status, considered a key factor in atherosclerosis development. Thus, GP can increase SOD, CAT, GPx, and/or GRx levels [79–82] and reduce ROS GSH, TBARS, and/or superoxide levels [83–85] being useful in primary prophylaxis.

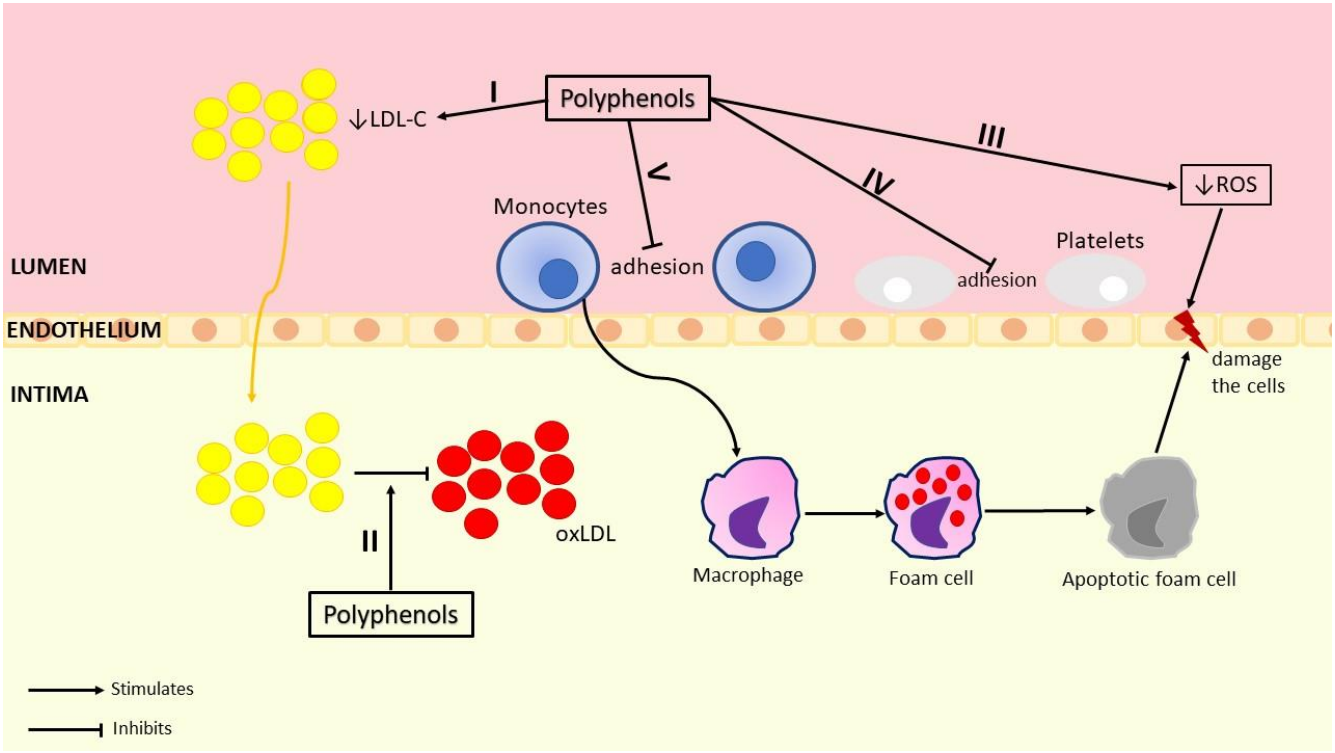


Figure 3. Anti-atherogenic effect of polyphenols from grape pomace (I—Polyphenols reduce LDL levels; II—Polyphenols reduce oxLDL formation; III—Polyphenols reduce ROS production; IV—Polyphenols inhibit platelets activation and adhesion by blocking its agonists; V—Polyphenols reduce monocytes adhesion by reducing the expression of VCAM-1).

Another key factor in the atherosclerosis process is represented by the platelet aggregation induced by endothelium dysfunctions. Muñoz-Bernal et al., (2021) studied the effect of GP from nine types of *Vitis vinifera* on platelet aggregation induced by adenosine diphosphate (ADP), which activates aggregation acting on several receptors like P2Y1, P2Y12, P2 × 1, an ATPC receptor, and by thrombin receptor activating peptide 6 (TRAP-6), which bind to the thrombin receptor. They observed that only the GP from Petit Verdot possesses anti-platelet aggregation, with a 67.1% inhibition of ADP-induced platelets aggregation and a 53.2% inhibition of TRAP-6 [86]. The same inhibitory effect on ADP-induced platelets aggregation (Figure 3) was observed by Bijak et al., (2019), who highlighted not just the anti-platelet effect, but also an anticoagulant effect of GP from *Vitis vinifera* from Hamburg, Germany [87].

Further, if not all the non-pharmacological and pharmacological interventions are successful in atherosclerosis optimum management, in time, the patients are predisposed to develop chronic ischemic heart disease and sometimes acute events. Therefore, patients will need secondary prophylaxis, aiming to prevent the occurrence of complications. Within this context, due to the antioxidant activity presented above, useful in preventing the continuous development of pre-existing atherosclerosis, GP represents a valuable source of polyphenols for CAD secondary prophylaxis.

The prevention of athero-thrombotic episodes that may occur in patients with CAD is also crucial. Therefore, Carrieri et al., (2013) studied the effects of GP from 12 grape varieties on LPS-induced tissue factor (TF) activity [88]. TF is a key factor in the pathogenesis of many thrombotic diseases because of its role in the initiation of coagulation via the extrinsic pathway. TF synthesis is modulated by monocytes and macrophages and activated by different inflammatory factors [89,90]. They observed that GP possessed an inhibitor activity on LPS-induced TF activity and, in general, RGP had the highest activity on both blood and on mononuclear cells. Furthermore, they identified a positive correlation between the concentration of quercetin, cyanidin, and TF inhibition. Moreover, they also identified a negative correlation between the concentration of malvidin, myricetin, petunidin, and TF inhibition [88]. Xuan et al., (2012) observed that in mice with surgical-induced myocardial infarction, resveratrol improves the survival rate from 55% to 80% through several actions. The resveratrol-treated group had a smaller left ventricle infarct size. Moreover, echocardiography highlighted a decrease in cardiac remodeling proven by a smaller left ventricular end-systolic and end-diastolic diameter and a larger left ventricular fractional shortening [91]. Das et al., (2014) also observed the cardioprotective effects of resveratrol in their study performed on mice under ischemic/reperfusion exposure. Besides the antioxidant effects demonstrated by decreased ROS and GSH levels, a reduction in the infarct size and an improvement in left ventricular developed pressure were also observed. Even more, an improvement in the aortic flow, which is an effect of left ventricular improvement, was observed [92]. The same left ventricular improvement was described by Rivera et al., (2019), who observed a restoration in the left ventricle's ejection fraction in the myocardial infarction studied in atherogenic diet-induced mice fed with RGP [78]. The cardioprotective effects as well as antioxidant and anti-inflammatory actions of polyphenols and GP in different experimental settings are summarized in Table 4.

All these antioxidant, anti-inflammatory, and cardioprotective effects are a solid argument for further research concerning GP's potential therapeutic effect in the treatment of patients with ischemic heart disease. In addition to this, it is necessary to identify if it has significant positive effects as primary prophylaxis because it is much more important to prevent the occurrence of the disease by controlling risk factors than preventing the occurrence of complications by controlling the underlying pathology. Thus, it is necessary to compare these effects between RGP and WGP to identify the best substrate that can be used as a future therapy.

Table 4. The cardioprotective effects, antioxidant, and anti-inflammatory actions of polyphenols and grape pomace on different cardiac experimental injuries.

***In Vitro* Studies**			
Materials	**Models**	**Antioxidant and Anti-Inflammatory Effects**	**References**
Resveratrol—100 μmol/L	Neonatal cardiac cells under ischemia/reperfusion exposure	- increased Bcl-2 expression - increased SOD levels - increased cell viability - increased the activity of Ca2+ -ATPase and Na+-K+ -ATPase - reduced the activity of caspase-3 and LDH - reduced apoptotic rate - reduced Bax expression - reduced MDA levels	[79]
Resveratrol—1 mL/2.5 mg/kg food	H_2O_2 exposed cardiomyocytes sampled from Sprague Dawley rats	- averted the activity reduction of CAT and SOD	[80]
Resveratrol—3 μM	Human cardiomyocytes' azidothymidine-induced cardiotoxicity	- decreased the activity of caspase-3, caspase-7 - decreased mitochondrial ROS generation - decreased cardiomyocytes apoptosis	[83]
Resveratrol—3 μM	Neonatal human cardiomyocytes in a medium with endotoxin lipopolysaccharide	- decreased the mitochondrial production of ROS	[84]
Grape pomace from Vitis vinifera L. cv. Barbera, Carignan, Cabernet Sauvignon, Grenache, Merlot, Petit Verdot, Syrah, Tempranillo and Zinfandel from Baja California, Mexico/methanol extract	Human platelets from 6 healthy persons	- GP from Petit Verdot possessed significant anti-platelets aggregation induced by ADP and TRAP-6	[86]
Grape pomace from Vitis vinifera from Hamburg, Germany	Blood samples	- inhibited the ADP-induced platelets aggregation - anticoagulant effect–prolonged activated partial - thromboplastin time (APPT) and prothrombin time (PT)	[87]
12 GP from white (Italia, Baresana, Beogradska, Autumn Seedless), red (Crimson Seedless, Red Globe, Apulia Rose, Supernova) and black (Autumn Royal, Michele Palieri, Summer Royal) from Turi, Italy	Blood samples from healthy donors Mononuclear cells isolated from the blood samples	- on blood: inhibited the LPS-induced TF activity, where RGP had the highest inhibition rate, followed by WGP and BGP - on mononuclear cells: inhibited the PLS-induced TF activity expression, where Supernova (red) had the highest inhibition rate	[88]

Table 4. *Cont.*

In Vitro Studies			
Materials	**Models**	**Antioxidant and Anti-Inflammatory Effects**	**References**
Resveratrol—25 μM	Neonatal rat cardiac cells in a medium with fractalkine	- conserved cardiac cell viability - augmented cardiac cells autophagy - decreased levels of MMP-9, ANP, ICAM-1, TGF-β, FKN, procollagens I and III	[91]
***In vivo* studies**			
Red grape pomace—10% concentration	60 rats with atherogenic diet-induced ischemic heart disease	- decreased levels of IL-10 and TNF-α - decreased the number and dimension of atherosclerotic lesions - increased levels of HDL cholesterol - restored the ejection fraction in the left ventricle	[78]
Grape skin and seed extract from *Vitis vinifera* cultivated in northern Tunisia—4 g/kg	24 Wistar male rats with cardiac injury and oxidative stress induced by arsenic	- increased SOD, GPx and CAT activities - reduced the levels of free iron, ionizable calcium and H_2O_2	[81]
Grape seed proanthocyanidins—100 mg/kg, twice a day	Ischemic-induced left ventricle by a 0.09% NaCl–4oC solution from 32 Rattus Norvegicus rats	- reduced ischemia-related MDA - increased SOD, GPx and CAT levels	[82]
Resveratrol—10 mg/kg IP injection	C57BL/6 mice with endotoxin-induced cardiomyopathy	- reduced the increase of CK and LDH levels	[84]
Resveratrol—10 mg/kg/day	L-NAME-induced malignant hypertension mice	- decreased superoxide anion radical, TBARs, MPO, thiol groups - decreased the damage score of cardiomyocytes - decreased expression of TGF-β - increased NO2 - averted reduction of antioxidant enzymes: SOD, GRx, GPx, CAT	[85]
Resveratrol—20 mg/kg IP injection for 42 days * IP—intraperitoneal injection	C57BL/6 mice with myocardial infarction surgical-induced	- increased the function of the left ventricle - increased survival rate - reduced the infarction size of the left ventricle	[91]

Table 4. *Cont.*

In Vitro Studies			
Materials	**Models**	**Antioxidant and Anti-Inflammatory Effects**	**References**
Resveratrol—2.5 mg/kg for 15 days	Rats under ischemic/reperfusion exposure	- decreased infarction size - reduced ROS and GSH levels - increased aortic flow - increased the development of left ventricular pressure post-ischemia	[92]
Methanolic extract from *Vitis vinifera* seed—125/250 mg/kg	Isoproterenol-induced infarction and streptozotocin-induced diabetes in Wistar mice	- decreased LPO levels - decreased expression of RAGE protein - decreased TNF-α, IL-1β, IL-6, NF- κβ and Iκκβ levels - increased CAT, GPx and SOD levels - increased the activity of Ca2+ -ATPase and Na+-K+ -ATPase	[93]
Resveratrol—30/100 mg/kg for 7 days	Heart sampled from ApoE-KO rats	- decreased levels of ROS and superoxide - decreased expression of NOX2 and NOX 4, with no effect on NOX1 - increased expression of SOD (SOD1, SOD2 and SOD3), catalase and GSH-Px	[94]

Abbreviations: ROS—Reactive oxygen species; ICAM-1—Intercellular adhesion molecule 1; MMP-9—Matrix metallopeptidase 9; FKN—Fractalkine; SOD—Superoxide dismutase; LDH—Lactate dehydrogenase; MDA—Malondialdehyde; CAT—catalase; ANP—Atrial natriuretic peptide; TGF-β—Transforming growth factor beta; GPx/GSH-Px—Glutathione peroxidase; LPO—Lipid peroxidation; IL-10—Interleukin-10; TNF-α—Tumor necrosis factor alpha; HDL—High-density lipoprotein; NF-kb—Nuclear factor kappa-light-chain-enhancer of activated B cells; Ikkb—Inhibitor of nuclear factor kappa-B kinase subunit beta; IL-1b—interleukin-1b; IL-6, interleukin-6; TBARs—Thiobarbituric acid reactive substances; MPO—Myeloperoxidase; GSH—Glutathione; GRx—Glutathione reductase; NOX – nitrate oxide.

9. Conclusions

The potential of polyphenols from GP is well known because of their antioxidant and anti-inflammatory effects. However, the existing data concerning total phenolic content and antioxidant activity according to GP source (red or white grapes) is still limited and there is a clear need to underline the differences between RGP and WGP. Therefore, as future direction, it is necessary to evaluate the geographical, climacteric, and preparation factors of RGP and WGP, using common and standardized bioactive compound quantification methods to establish the differences. These differences are needed to give a proper valorization of them in CAD prophylaxis through antioxidant and anti-inflammatory actions. Furthermore, there are still no sufficient studies to compare RGP and WGP activities in humans, in order to identify the better one to be used in CAD.

In conclusion, knowing that GP presents cardioprotective effects in ischemic heart diseases by modulating and decreasing both atherosclerosis development and atherosclerotic lesions and preserving cardiac function, the development of adjuvant therapy based on GP for patients with cardiovascular diseases would be helpful.

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Predictive Factors for Oral Immune Modulation in Cow Milk Allergy

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Abstract: Aim: The present study analyzed clinical and biological factors that might predict achievement of tolerance in patients with IgE-mediated cow milk allergy (CMA). Method: Seventy patients with IgE-mediated CMA (44.24 ± 24.16 months) were included in the study. The patients were evaluated clinically through skin prick test and sIgE to whole milk, casein, beta-lactoglobulin and alpha-lactalbumin. An eviction diet of 6 months was established, followed by oral food challenge test (OFC) and oral immunotherapy (OIT) with baked milk for 6 months. The tolerance was assessed after 2 years follow up. Results: Thirty percent of patients presented anaphylaxis of different degrees of severity as first manifestation of CMA. Sixty-two patients followed OIT or an accelerated reintroduction of milk. Ten patients (14.28%) did not obtain tolerance to milk within 2 years. A larger wheal in SPT and higher sIgE to milk, casein and betalactoglobulin were noted in patients with positive OFC. A basal level of <2.5 kU/l for sIgE to milk and <11.73 kU/l for sIgE to caseins predicted the occurrence of tolerance in patients with all types of clinical manifestations, including anaphylaxis. Conclusion: Basal levels of sIgE to milk and casein may help to identify patients that could become tolerant to milk.

Keywords: casein; cow milk allergy; oral immunotherapy; oral tolerance

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1. Introduction

Cow milk allergy (CMA) is the most common food allergy in children, with an estimated prevalence of 0.5% to 3% in the pediatric population below 1 year old [1]. Self-reported incidence of CMA is much higher than confirmed allergy in both children and adults [2]. The incidence of self-reported allergy varies between 1.2% to 17%, while the rate of prevalence for milk allergy confirmed by an oral food challenge test is lower, between 0% and 3% [3]. The sensitization to milk is less than 1% in the general population, varying throughout Europe, [4,5]. When the confirmation of the allergy is obtained through skin prick test and specific IgE, the prevalence of CMA is between 2–9% [4–6].

The term cow milk allergy refers to an immune-mediated reaction induced by exposure to cow milk and includes three categories of diseases: IgE-mediated, non-IgE-mediated and

combined (produced by IgE and non-IgE mechanisms). An IgE-mediated cow milk allergy is a type I hypersensitivity reaction, and the clinical manifestations occur within minutes to 2 h after milk ingestions. This form represents almost 60% of CMA cases, but this estimation could vary according to patient age and geographical area [1,7].

The clinical manifestations are variable from acute urticaria or exacerbation of atopic dermatitis to the most severe presentation, which is anaphylaxis [1]. CMA is responsible for 10–19% of all food-induced anaphylactic cases, being the third cause of anaphylactic reactions induced by foods, after peanuts and tree nuts [8]. A positive diagnosis algorithm starts with a careful clinical evaluation, followed by skin prick test and laboratory findings [8]. Skin prick test with fresh milk or standardized extract represents a fast method to detect sensitization but not the allergy [9]. Measurement of specific IgE to cow milk through ELISA, RAST or CAP-FEIA technique is a diagnostic approach with high sensitivity, but sometimes, it may deliver false positive results; thus, they should be analyzed in the clinical history context. A basophil activation test is another useful diagnostic tool for CMA in combination with sIgE, especially in children with atopic dermatitis [10], but it is not frequently used in clinical practice. There are still no equal test assay systems for serum sIgE, which makes for a difficult comparison between studies and techniques. Several studies have tried to describe the predictive values of IgE levels for clinical reactivity [8], but the differences are quite significant mainly due to various selection criteria, age of the patient or different criteria for analyzing the clinical reactivity [9,11,12].

The first measure in the management of CMA is allergen avoidance. A diet without milk and dairy products is recommended until clinical tolerance is induced [2,6,13]. The oral tolerance to cow milk is reached in almost half of the patients by an age of 5 years, increasing the rate up to 75% until teenage years [6,14], but some patients remain with persistent CMA [13,15]. However, the experience of the last 20 years has shown that the natural history of food allergy is changing and that less individuals become tolerant and that a longer time to resolution is needed [13].

Oral immunotherapy (OIT) has shown some promising results in improving patients' quality of life in CMA. It is a therapeutic method that can also be used in young children. Adverse reactions including anaphylaxis may occur during OIT, especially during the escalation phase. The rate of desensitization is variable, with 20–30% of patients remaining with persistent CMA despite OIT [6]. The tolerance to cow milk induced by OIT or achieved naturally may vary from country to country, and it is influenced by the genetic inheritance and the microbiota from the gut [16]. OIT may permit achievement of a rapid tolerance to milk, which allows the children to have normal activities without any restrictions. Standardized protocols of OIT with validated optimal dose and ideal duration, data regarding degree of protection, safety, and efficacy in different ages and populations need to be established [17–19]. There is also an urgent need to establish standardized outcome measures to be applied in food allergy studies, for both prediction of tolerance and for monitoring of OIT [20]. This may allow for a better harmonization of data resulting from different clinical trials.

The aim of the present study was to identify possible clinical and biological predictive factors for achievement of tolerance after OIT in a cohort of patients with IgE-mediated CMA. The second objective was to establish the effectiveness of a modified protocol for oral immunotherapy to milk in obtaining oral tolerance.

2. Materials and Methods

2.1. Patients and Study Design

The study was an analytic, transversal study. The present research analyzed clinical and biological factors that might predict the occurrence of tolerance in patients with cow milk protein allergy.

Seventy-six patients with milk-induced reactions presented for allergological evaluation. The patients were evaluated at the Allergology Department of Regional Institute of Gastroenterology and Hepatology "Prof. Dr. Octavian Fodor" in Cluj Napoca and at the

Almedo Clinic in Cluj Napoca between January 2013–November 2021. Only patients with an unequivocal positive immediate allergic reaction after contact with cow milk as well as documented evidence of sIgE to cow milk protein by blood tests and/or a skin prick test were included. The exclusion criteria were: non-IgE-mediated hypersensitivity reactions induced by cow milk, patients without a definitive positive diagnosis of CMA, patients that refused to sign the informed consent, and patients for which follow up was not performed. Based on these criteria, six patients were excluded from the final analysis.

Seventy patients with IgE-mediated CMA that had presented for allergological evaluation were included in the study. The mean age was 44.24 $\pm$ 24.16 months when the patients were included in the evaluation, and the sex ratio was M:F = 1.41. Diagnosis of IgE-mediated CMA was established according to international guidelines, based on history, clinical evaluation, skin prick test (SPT) and sIgE to milk and components.

The study protocol was approved by the University Ethics Committee of the University of Medicine and Pharmacy (293/28 July 2013), according to the principles from Declaration of Helsinki. Each patient signed the informed consent before the study began.

2.2. Allergological Evaluation

Clinical evaluation was performed at the beginning, when the patients were included in the study (see Figure 1). From anamnesis, the following demographic and clinical data were recorded: age, gender, living area (urban/rural) and clinical picture of the first allergic reaction, onset of disease, duration until first allergological diagnosis, family history of atopy, and other allergic diseases associated.

Skin prick of milk protein mix was performed. Skin prick tests were positive if the wheal diameter was $\geq$3 mm compared to the negative control. Standardized allergen extracts (Hal Allergy, Netherlands) were used. The value in mm was recorded as a medium diameter wheal size.

Serological tests implied determination of total Ig, specific IgE for cow milk (whole extract) and casein, beta-lactoglobulin and alpha-lactalbumin. Laboratory test results were obtained through electrochemiluminescence immunoassay method (ECLIA).

The atopy diagnosis was established through skin prick test at enrollment, according to international guidelines [21]. The skin prick test included the following panel of allergens: house dust mites (Derm. Pteronyssinus and Derm. Farinae), pollens (grasses, cereals, birch and weeds), animal dander (cat and dog) and molds (Alternaria alternata). After the positive diagnosis was established, a diet without milk or dairy products was recommended for 6 months. After 6 months, the remission of symptoms or an accidental exposure to milk were assessed. An oral challenge test with milk 3.5%, baked for 30 min was performed in 62 of patients. The positivity of OFC was established if the patient had a clinical manifestation and if the quantity of milk that induced the reactivity was noted. Simple-blind OFC was not performed in patients if the parents refused to sign the informed consent. The simple blind OFC protocol included 4 steps:

1. 2 mL rice milk (commercially available) as placebo;
2. 0.25 mL baked cow milk plus 1.75 mL of rice milk;
3. 0.5 mL baked cow milk plus 1.5 mL of rice milk;
4. 1 mL baked cow milk plus 1 mL of rice milk.

All the doses were given at 30 min time intervals. The OFC was considered positive if the patient presented clinical manifestations in the aforementioned 4 steps. The OFC was considered negative if the patients tolerated 1 mL milk. If the patients tolerated 1 mL milk during OFC, they continued with an accelerated reintroduction of baked milk (see Figure 1 and Table 1) to reach, in 48 h, the maintenance dose of milk that was used in the protocol of oral immunotherapy. The rapid reintroduction of baked milk was performed in the allergological department under medical supervision until the maintenance dose of 200 mL was reached.

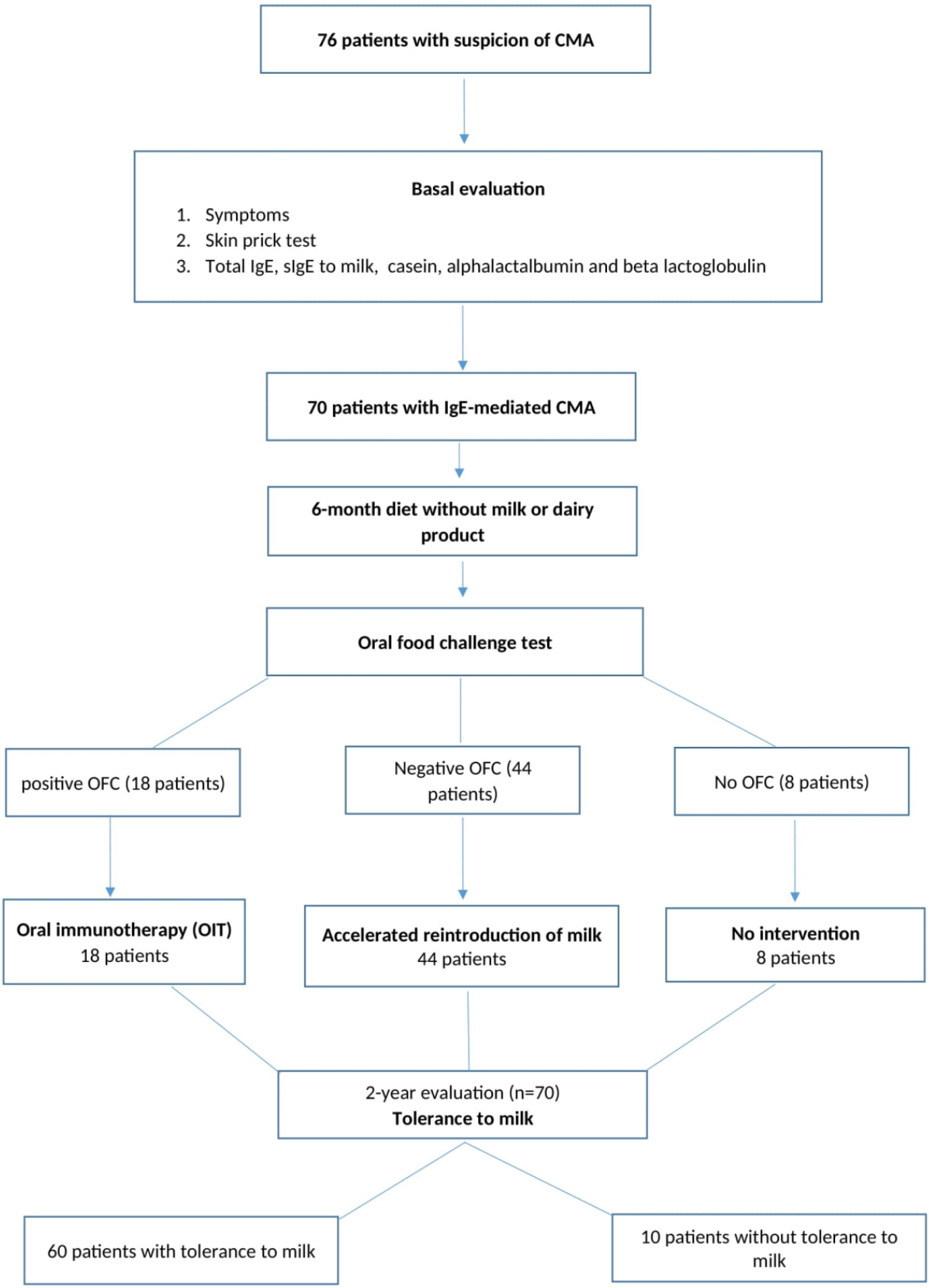

Figure 1. The algorithm of evaluations and therapeutic interventions in patients with cow milk allergy.Abbreviations: CMA, cow's milk allergy; IgE, immunoglobulin E; OFC, oral food challenge.

Table 1. Protocol of rapid reintroduction of milk and oral immunotherapy to milk.

Phases	Rapid Reintroduction of Baked Milk		Oral Immunotherapy	
	Interval of Time between Dose Escalation	**Amount of Baked Milk**	**Interval of Time between Dose Escalation**	**Amount of Baked Milk**
Build up phase	30 min	1 mL	30 min	0.05 mL
	30 min	2 mL	30 min	0.1 mL
	30 min	4 mL	30 min	0.2 mL
	30 min	8 mL	30 min	0.4 mL
	30 min	16 mL	30 min	1 mL
	24 h	25 mL	30 min	2 mL
	48 h	50 mL	24 h	4 mL
			48 h	8 mL
			36 h	16 mL
			1 week	25 mL
			2 weeks	50 mL
Maintenance dose	1 week	100 mL	1 month	100 mL
	1 week	200 mL	3 months	200 mL

2.3. Oral Immunotherapies

A group of patients (18 patients) underwent open oral immunotherapy (see Figure 1). The procedure consisted of the administration of progressively increasing amounts of baked milk 3.5% to induce tolerance and to reduce the allergic symptoms until disappearance. Small amounts of baked milk were administered sublingually initially, with an increasing amount administered orally according to tolerance (build up phase period), to a dose that was given daily (maintenance period) continuously. The initiation of immunotherapy was performed in a specialized allergology unit with existing facilities for emergency assistance if the patients developed adverse reactions. The protocol started with 0.05 mL baked milk, and the maintenance dose of 50 mL was supposed to be reached in 3 weeks. In some patients, the induction phase lasted more than 6 months until the maintenance dose was reached. When the patients were in the maintenance phase, they were allowed to introduce milk substitutes such as yoghurt, cream or ice cream. The protocol of updosing is presented in Table 1. The acquisition of tolerance was established after 2 years follow up.

2.4. Statistical Analysis

Statistical analysis was carried out using the MedCalc Statistical Software version 18.10 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; (accessed on 20 November 2021). Quantitative data were evaluated for normality of distribution using the Kolmogorov–Smirnov test. They were characterized by median and 25–75 percentiles. Qualitative data were expressed as frequency and percentages. Comparisons between groups were performed using the Mann–Whitney or chi-square tests whenever appropriate. The correlation between variables was established using Spearman's correlation. ROC curves were used in order to find cut-off values for quantitative variables that could discriminate between patients with a tolerance to milk and those without. A p value of <0.05 was considered statistically significant.

3. Results

Seventy patients with IgE-mediated CMA were evaluated (Table 2). Most of the patients (62 patients, 88.6%) followed a rapid reintroduction of milk or OIT for milk.

Ten patients (14.28%) did not obtain tolerance to milk within 2 years after the first evaluation and positive diagnosis of cow milk allergy. Only two patients (11.1%) from the group that followed OIT did not gain oral tolerance in this interval of time.

Table 2. Demographic data of patients with cow milk allergy.

Parameter		CMA (*n* = 70)	Patients without Tolerance (*n* = 10)	Patients with Tolerance (*n* = 60)	*p*
Gender	M	41 (58.6%)	8 (80%)	33 (55%)	0.178
	F	29 (49.4%)	2 (20%)	27 (45%)	
Family history of atopy		29 (41.4%)	2 (6.9%)	27 (83.1%)	0.166
Personal history of respiratory allergy		21 (30%)	3 (30%)	18 (30%)	0.918
Personal history of food allergy		20 (28.6%)	3 (30%)	17 (28.6%)	1
Living area	Urban	62 (88.6%)	10 (100%)	52 (83.9%)	0.591
	Non-urban	8 (11.4%)	0	8 (13.3%)	

Demographic data are presented in Table 2.

CMA was noted more frequently in boys than in girls, and more females obtained tolerance after OIT than males (93.1% vs. 80.5%), but the difference was not statistically significant. Twenty-nine patients (41.4%) had a positive family history of atopy, but this did not influence the induction of tolerance compared to patients without a family history of allergy. Personal history of respiratory and/or food allergy were noted in almost one-third of the patients, without any influence in obtaining tolerance. Twelve patients (60%) with other food allergies tested positive to egg, followed by peanuts and other nuts.

The average duration of disease from the onset of the symptoms until the positive diagnosis of CMA was 20 (6.5–40.75) months, and a longer time was noted in patients with persistent allergies compared to those with a tolerance to milk (43.5 (21.5–112.5) vs. 18 (4.5–36), $p = 0.027$). The family or personal history of allergy or the severity of first clinical presentation did not accelerate the presentation to a specialist for evaluation of CMA.

3.1. Clinical Manifestations

The analysis of clinical manifestations revealed that the symptoms occurred, on average, at the age of 9 months (9.72 ± 4.66 years, minimum 1 month, maximum 24 months). The age of onset was higher in patients with persistence of CMA.

Thirty percent of the patients (21 pts) presented anaphylaxis of different degrees of severity. Most of the patients (65.7%) presented cutaneous manifestation such as acute urticaria or aggravation of atopic dermatitis or both (Table 3). The clinical manifestation at the onset of the allergy did not predict the occurrence of tolerance to milk.

Table 3. Primary clinical manifestation of CMA.

Parameter		Patients without Tolerance (*n* = 10)	Patients with Tolerance (*n* = 60)	*p*
Age of symptoms onset (months) *		9 (6–18)	9 (6–11.5)	0.060
Manifestations	Anaphylaxis	3 (14.3%)	18 (85.7%)	0.916
	Acute urticaria	2 (12.5%)	14 (87.5%)	
	Atopic dermatitis	3 (13%)	20 (87%)	
	Digestive symptoms	1 (33.3%)	2 (66.7%)	
	Urticaria + atopic dermatitis	1 (14.3%)	6 (85.7%)	

* Data are expressed as median and percentile.

3.2. Skin Prick Test and sIgE

Skin prick test and specific IgE to milk and major proteins were performed in all cases. The size of the wheal was higher in patients with persistent allergy, but the difference did not reach the level of statistical significance. Basal median values of specific IgE to milk and to casein were significantly higher in patients without oral tolerance (Table 4).

Table 4. Basal results of skin test and laboratory values in patients with cow milk allergy.

Parameter.	Patients without Tolerance (n = 10)	Patients with Tolerance (n = 60)	p
Size of wheal (SPT)	8 (4.75–15.75)	5 (4–8)	0.08
sIgE to milk	12.77 (4.10–86.88)	3.2 (0.6–13.5)	0.039
sIgE to casein	14.3 (1.5–45.72)	0.96 (0.35–5.45)	0.01
sIgE to alpha-lactalbumin	2.3 (0.35–28.02)	2.1 (0.48–7.88)	0.926
sIgE to beta-lactoglobulin	2.42 (0.35–14.9)	1.5 (0.35–5.14)	0.755

Data are expressed as median and 25–27 percentiles.

The basal results of skin prick test and laboratory values were also analyzed in relation to clinical reactivity after OFC. The oral food challenge test was performed after a period of 6 months of eviction diet in order to establish the opportunity of oral immunotherapy. OFC was performed in 62 patients (88.57%), and it was positive for 18 of them (Table 5). The clinical reactivity during OFC was more frequently noted in patients with persistent CMA (p = 0.003).

Table 5. Results of oral food challenge test and correlation with acquired tolerance.

Parameter		Patients without Tolerance (n = 10)	Patients with Tolerance (n = 60)	p
OFC	Negative	1 (10%)	43 (71.67%)	0.003
	Positive	2 (20%)	16 (26.66%)	
	Not done	7 (70%)	1 (1.66%)	

The patients with positive OFC had significantly higher values of specific IgE to milk (p = 0.017), casein (p = 0.006), and beta lactoglobulin (p = 0.011), but not to alpha-lactalbumin (p = 0.083) compared to patients with negative OFC. The size of the wheal at skin prick test was also significantly higher in those patients (p = 0.002).

3.3. Analysis of Patients with Anaphylaxis Induced by Cow Milk Proteins

Twenty-one patients with CMA presented anaphylaxis grade 2 to 4 of severity, from which three patients (14.28%) had a persistent allergy to cow milk. The anaphylaxis as a primary manifestation of CMA was not correlated with a personal history of allergy to other foods or respiratory allergens (p = 1, respectively p = 0.74) and to a familial history of atopy (p = 1). Oral immunotherapy was performed in 18 patients, and all of them obtained tolerance compared to those patients that had a persistent form of CMA and did not follow OIT (p = 0.001). The severity of initial anaphylactic reactions did not predict de-occurrence of oral tolerance (p = 0.792) after OIT.

Specific IgE to milk and casein wase significantly higher in patients with anaphylaxis and persistent allergy to cow milk compared to those who obtained oral tolerance (Table 6).

The ROC curves for basal values of specific IgE for milk and casein were analyzed, and the cut-off values were calculated for these parameters in relation to the presence of tolerance after 2-year follow up after the onset of OIT. The cut off values, AUC, and sensitivity and specificity are presented in Table 7.

Table 6. Basal results of skin test and laboratory values in patients with anaphylaxis induced by cow milk.

Parameter	Patients without Tolerance (n = 3)	Patients with Tolerance (n = 18)	p
Size of wheal (SPT)	15 (5)	7 (5–9)	0.185
sIgE to milk	91.66 (20)	5.3 (1.12–13.1)	0.019
sIgE to casein	74.3 (22.4)	2.3 (0.45–8.87)	0.017
sIgE to alpha-lactalbumin	78.2 (0.9)	2.1 (0.8–8.12)	0.221
sIgE to beta-lactoglobulin	21.8 (0.2)	0.58 (0.35–6.7)	0.534

Data are expressed as median and 25–27 percentiles.

Table 7. ROC curve analysis for oral tolerance at 2-year follow up.

Parameter	AUC	Cut-Off Value	Sensitivity	Specificity	p
sIgE to milk	0.705 (95% CI 0.550−0.860)	2.5 kU/l	45.7% (95% CI 32.7−59.2)	100% (95% CI 69.2−100.0)	0.012
sIgE to casein	0.755 (95% CI 0.581−0.93)	11.73 kU/l	93% (95% CI 83.8−98.2)	60% (95% CI 26.2−87.8)	0.005

During OIT, no severe reactions were noted. Few patients presented mild skin eruptions or perioral contact dermatitis with spontaneous remission or after administration of H1 antihistamines. None of the patients presented bronchospasm, diarrhea or anaphylactic reactions that needed administration of epinephrine.

4. Discussion

The present study assessed the clinical and biological changes in patients with IgE-mediated CMA, showing that both sIgE to milk and casein basal levels could predict the occurrence of oral tolerance after OIT or after rapid reintroduction of milk. The study also demonstrated the efficacy of a modified protocol for oral immunotherapy in inducing oral tolerance to milk.

Cow milk allergy is a common allergy in the pediatric population, being the first food allergy described in the allergic march [2,22]. It may be over- or underdiagnosed, depending on the type of evaluation. Some health care professionals, but especially parents, confuse CMA with lactose intolerance, leading to inappropriate diets. Even if true CMA is diagnosed, the type of elimination diet, substitutive products and the duration of such elimination are not always logical. Complete elimination of cow milk without an appropriate substitution can lead to growth impairment, malnutrition, and deficiencies in nutrients with long term consequences [22]. Food allergies negatively affect quality of life for children and their parents, with a significant disruption in family life and social interactions [23–25]. Both physicians and parents should understand the multifaceted clinical and biological aspects of CMA to know how to manage further diets.

In the present study, the onset of CMA was noted in the first year of life in few patients, with the first symptoms being described afterward, but no later than the age of 2 years. CMA is mostly a disease of infancy and early childhood. Most of the studies reported that affected children presented symptoms within the first 6 months of life and sometimes earlier, usually before 1 month of age and often within 1 week after the introduction of cow milk proteins to their diet [15,22,26]. In the present study, the average onset of CMA was 9 months, later than in the previous studies [27,28], but all of the patients had clinical manifestations within 2 years of life. Boys were more affected by CMA than girls, similar to the EuroPrevall study [29]. The family history of atopy was reported in more than 40% of the patients, as in the EuroPrevall study [29], but the percentage reflects global atopy in mothers and fathers and is not separated by gender.

Eight patients from the countryside are not enough to make proper conclusions about a difference in CMA between patients living in the cities and those living in the countryside. For future studies, an overall online database should be created for doctors from different

departments in order to introduce patients with CMA, especially when small sample sizes are present. Nevertheless, a long period from the first symptoms until the first allergological consultation occurs (median 20 months) is unacceptable. It shows that neither doctors nor the parents are aware of food-induced allergies or comorbidities commonly associated with CMA (e.g., acute urticaria, atopic dermatitis, anaphylaxis, and GERD), and further efforts are needed in order to improve the situation in Romania. Mainly, pediatricians and family doctors must be aware of this topic and should refer probable cases to an allergology department for further evaluation. It is especially important for severe cases presenting clinically with anaphylactic reaction to have proper management and to prevent further acute episodes. Patients with anaphylaxis had earlier presentation to an allergologist (median 10 months), showing that a severe reaction may increase the anxiety of both children and parents and may make them aware of a potential risk. The specialist visit should occur as soon as possible in order to reduce the sequelae, an improper diet, and in order to provide a proper treatment regimen as well as possible oral immunotherapy for patients.

The majority of children with CMA had one or more symptoms that involved one or more organs, mainly the gastrointestinal tract and/or skin. More than half of the patients had skin manifestations (acute urticaria, aggravation of atopic dermatitis, contact dermatitis) as the first manifestation of CMA. Digestive symptoms alone were described only in three patients (4.2%), which is less frequent than in other studies, but digestive symptoms are more common in non-IgE-mediated reactions to milk [29]. Anaphylaxis as the first manifestation was present more frequently in the present analysis (30% of children) compared to previous data [1,8]. An anaphylactic reaction might increase anxiety in the family, allowing parents to be more aware of the risk of a severe reaction. Patients with milder skin reactions probably skip evaluation in the allergology department and are thus treated by a generalist, pediatrician or dermatologist, which may also explain the lower rate of cutaneous manifestations described in this cohort compared to previous data [8,22,29].

Following the ESPGHAN algorithm [30] for the evaluation of children with suspicion of CMA, a simple-blind OFC test was performed in 88.57% of the patients to establish if the patients obtained a tolerance to milk and to assess the opportunity of OIT. OFC should be a part of the routine workup [2,30] along with detailed anamnesis, diagnostic elimination diets, skin prick tests, and sIgE. Lack of OFC in all patients is explained by patients' refusal to partake in it. When cow milk is the only suspected allergen and the only food in the diet, the diagnosis is simpler than in cases where they are already ingesting a variety of foods and OFC could not be a standard procedure. An oral food challenge test was performed in children with more than one food in their diet to confirm a positive diagnosis directly before initiation of OIT. Patients with negative OFC were actually patients with mild CMA that followed a rapid reintroduction of baked milk with an accelerated induction of tolerance.

Oral immunotherapy is a therapeutic method that permits the induction of tolerance and a normal diet after completion of it. OIT to milk is similar to peanut OIT regarding effectiveness in inducing clinical desensitization to the culprit allergen, but with a lower risk of allergic reactions during OIT. Clinical trial data are more limited, and there are no approved formulations for OIT. A significant challenge in determining the efficacy of several therapies for milk and egg allergies is that the natural rate of resolution of these allergies is much higher than for peanuts. In a 2012 meta-analysis of five trials that analyzed milk OIT (including 218 children), milk OIT increased the likelihood of developing full tolerance to milk by 10-fold compared to children without interventions. [17].

The quality of the allergen is critical for both OFC and OIT and may vary in commercial products; thus, it is hard to standardize this method [31]. In the present study, 88.6% of patients followed this procedure with a good response (only 11.1% of them had persistent CMA after 2-year follow up). More patients with an eviction diet who did not follow OIT presented persistent CMA at the end of the follow up period. Garcia-Ara et al. [32] also reported a high successful rate of desensitization after 1-year follow up (88–100%), depending on basal sIgE to milk. In the present study, the patients were not stratified

according to basal evaluation of SPT and sIgE. A lower rate response was also mentioned by Kuitunen et al. (72%) after 6 months of OIT [33]. In another study from Denmark and Australia, patients achieved tolerance in a variable rate (28–77%) at the age of 2 for cow milk, without any interventions [34,35]. This difference could be explained by different inclusion criteria, duration of OIT, or a non-interventional attitude. The present analysis included all the patients with a positive diagnosis of CMA who presented for allergological evaluation. Increasing the duration of OIT and follow up may increase the success rate of it. We did not find associations between tolerance to milk and gender or other food allergies, in accordance with other published results [36,37]. This study sustained the role of OIT in obtaining tolerance, which may permit a normal diet independent from personal or familial history of allergy or from patient gender.

The reported rate of success and the less adverse events of OIT could be explained through a modified protocol of OIT, which used baked milk instead of raw milk until tolerance was induced. The children that obtained tolerance to baked milk after 6–9 months of OIT also tolerated dairy products as a component of the normal diet, or raw milk without any reactions after they switched from heated to unheated milk. Similar results were also reported by Esmaeilzadeh H et al. [38], who demonstrated that introducing baked milk products into the diet of patients with a milk allergy can accelerate the tolerance of unheated milk, but basal sIgE could not predict the success of OIT. Many concerns are raised regarding milk OIT because, unlike most other allergenic foods, milk is typically consumed in diverse forms several times per day, and a total daily dose that could be high may be not tolerated, especially in the presence of anaphylaxis co-factors [39]. Cow milk tolerance can spontaneously occur in the first years of life; thus, the faster tolerance we observed in most of the patients could be a consequence of both immune modulations via OIT with baked milk or may be due to a milder phenotype of CMA. However, this strategy induced a good rate of response to OIT in patients with anaphylaxis as a primary manifestation in the present study; thus, we may suppose that baked milk may accelerate, in a safe manner, the induction of tolerance to milk.

A double-blind placebo0controlled food challenge (DBPCFC) remains the gold standard for a positive diagnosis of CMA, but it is time consuming, expensive, can only be performed under medical guidance, only in specialized clinics, and it has a high risk of inducing severe anaphylactic reactions [9,30]. In addition, the quality of life of patients is affected when they experience a positive challenge test, and for this reason, may refuse to follow an OFC or oral immunotherapy [40]. Development of molecular biology in the last 10 years has permitted an increase in the accuracy of the diagnosis without referring the patient to a DBPCFC. Measurement of sIgE to different allergenic proteins from milk permits an identification of patterns of sensitization in complex polysensitized patients and is useful in identifying different phenotypes of CMA [41].

The present study showed that high levels of sIgE to milk and casein may predict the persistence of CMA despite oral immunotherapy. Previous data showed that patients with persistent CMA have higher values of sIgE to milk than those that can respond to oral immunotherapy [33,42,43], and it may predict the long-term outcome of milk OIT [20,42]. Kuitunen et al. also [33] demonstrated that high basal levels of sIgE to casein, alpha-lactalbumin and betalactoglobulin before the start of OIT were associated with a lower maintenance dose reached at the end of OIT. In addition, Savilahti EM et al. [44] reported that a high level of sIgE to milk and casein could predict a failure to achieve desensitization in milk OIT. It is also important to identify a value of sIge that might predict the resolution of CMA. We calculated a cut off value of 2.5 kU/L for sIgE to milk with 45.76% sensitivity and 100% specificity, but the size of the wheal in the skin prick test was not a predictive marker for OIT outcome. Yavuz ST et al. [45] reported that children with sIgE to milk below 6 kU/L outgrew CMA earlier than those with higher levels. In our cohort, the cut off value for sIgE was lower, but the outcome was to predict the resolution and not the interval of time after which we obtained it.

Component-resolved diagnostics before OIT can help to identify children with a lower probability of a successful OIT outcome. sIgE to casein over 11.73 kU/L predicted a failure of achieving tolerance after OIT, with a sensitivity of 93% and a specificity of 60% in the present study. Kuitunen et al. [33] also reported that sIgE to milk allergens might have a better role in predicting resolution of CMA after OIT compared to other markers. It is essential to establish the role of these markers in order to identify candidates for OIT with a good resolution rate. Patient data derived from modern technology, in combination with a classical approach through the patient's history, can be translated into patient-tailored interventions.

The main strength of the paper is that it presents a clinical and biological analysis of a cohort of patients with IgE-mediated CMA in Romania. The present study offers information from basal evaluation of patients with CMA that might predict the success of a medical intervention in those patients, allowing them to have a better quality of life. The study identifies some aspects that could be improved in the management of CMA. There are also some limitations of the study. First, OFC was not performed in all the patients to measure the exact amount of milk that produces clinical reactivity, and because of this reason, the OIT started in the same way in all included patients. Second, the evaluation of the children was performed at different ages not immediately after the onset of CMA. Most of the parents postponed the evaluation of their children until the moment of entrance in kindergarten or in school to see if they had a risk for severe reactions if an accidental exposure to milk might occur. Third, there was no control group in the present study. It would be interesting to have the possibility to evaluate the patients with mild forms of CMA and to compare the natural resolution of CMA with the active intervention (oral immunotherapy or rapid reintroduction of baked milk). Patients with mild forms of CMA were under pediatrician surveillance, and they followed an eviction diet, which is sometimes a long-term attitude, and they do not benefit from an active intervention.

5. Conclusions

Anaphylaxis with only skin and mucosal involvement represents one of the most frequent manifestations in children with IgE-mediated CMA, although severe anaphylaxis may be present as an initial manifestation of CMA. Basal values of sIgE for milk and casein predict the occurrence of tolerance to milk after 2-year follow up in patients with CMA, including those with anaphylaxis as the first manifestation. OIT, or a rapid reintroduction with baked milk, may be used as an approach for CMA with IgE-mediated mechanisms, and it may result in the induction of tolerance faster and in a higher percentage of patients, allowing for a normal diet without any restrictions.

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Are Markers of Allergic Inflammation in Grass Pollen Allergy Influenced by H1 Antihistamines?

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Abstract: Soluble intercellular adhesion molecule-1 (ICAM-1) and soluble vascular adhesion molecule-1 (VCAM-1) play important roles in allergic rhinitis (AR). Treatment with H1 antihistamines improves AR symptoms and in vitro reduces the levels of adhesion molecules. The aim of the study was to evaluate serum levels of ICAM-1 and VCAM-1 in patients with AR to grass pollen and their response to different H1 antihistamines. Material and methods: A total of 50 patients with grass pollen AR were clinically and biologically evaluated. ICAM-1 and VCAM-1 serum levels were evaluated during pollen season before and after treatment with levocetirizine and desloratadine through the ELISA method. Results: ICAM-1, VCAM-1, eosinophils, and total IgE were elevated in patients with AR, compared with healthy subjects. Both antihistamines improved specific symptoms of AR and increased patients' quality of life during pollen season after one month of treatment. H1 antihistamines reduced VCAM-1, ICAM-1, and total IgE after one-month treatment but not significantly. Patients with increased baseline values tend to remain with increased values after one-month AH1 treatment. Conclusions: ICAM-1 and sVCAM-1 levels are higher in patients with grass pollen-induced AR than healthy controls during pollen exposure. Their serum levels tend to remain at high values during pollen season despite antihistaminic therapy.

Keywords: soluble adhesion molecules; pollen allergy; eosinophils; antihistamines

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1. Introduction

Allergic rhinitis is a common disease affecting 20–30% of the general population in industrialized countries. In central Europe, grass pollen is one of the major allergens during late spring and summer, responsible for symptoms of allergic rhinitis accompanied or not by allergic conjunctivitis and/or asthma [1–3]. Allergic rhinitis is characterized by an IgE-mediated immune response due to exposure to pollen and several cells and mediators could be identified. After allergen exposure, an early phase of allergic inflammation might occur, releasing immediately specific mediators from mast cells, including histamine. These mediators generate a specific inflammatory response, activating cellular adhesion molecules (CAMs) that are involved in eosinophil's migration in the nasal mucosa [4–7]. Vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1), which belong to the immunoglobulin superfamily, are expressed on endothelial cells. Adhesion

molecules are an important part of the inflammatory network in allergic diseases, involved in persistent inflammation in the upper and lower airways [1–8]. IgE and eosinophils increase during pollen season, because of continuous allergen exposure [9–11].

H1 antihistamines (AH1) are the most frequent pharmacological agents used in both intermittent and persistent forms of allergic rhinitis, although more than 50% of the patients did not respond to monotherapy with AH1 [1]. Their anti-allergic effect is related to the blockade of H1 receptors. Research from the last two decades found that the second-generation H1 antihistamines have also an anti-inflammatory effect, decreasing the number of inflammatory cells accumulated in the nasal mucosa and the expression of CAMs [12–19].

The aim of the study was to evaluate the effect of H1 antihistamines during natural exposure to grass pollen and their effects on clinical symptoms, biologic markers, and CAMs. The secondary objective was to identify if there are any differences between 2 commonly used AH1, levocetirizine, and desloratadine.

2. Materials and Methods

2.1. Study Design

In total, 50 patients with grass pollen allergic rhinitis (median age 27.3 (23–37) years and sex ratio M:F = 1:1) that were evaluated in the Allergology Department, were included in the present study. In addition, 30 healthy volunteers were also included in the control group. The first evaluation was carried out in the middle of grass pollen season in Romania, from 15 May to 15 June (2012–2015). The study protocol was approved by the University of Medicine and Pharmacy "Iuliu Haţieganu" Ethics Committee (Approval No. 535/2 September 2011), and all patients signed the informed consent before enrollment. The study protocol and clinical evaluation were performed according to the initial RCT [19], but only allergic patients to grass pollen were included in the present analysis. The intranasal eosinophils were used as a local marker of inflammation. The exclusion criteria were nasal polyps, acute and chronic upper respiratory infections, other systemic inflammations, autoimmune diseases, cardiovascular diseases, administration of intranasal, inhaled, or systemic corticosteroids or H1 antihistamines within the previous 30 days, and administration of immunosuppressive agents.

2.2. Patients' Clinical Evaluation

Diagnosis of AR was conducted according to the ARIA guideline [1], based on history, typically symptoms at pollen exposure, and skin prick test (SPT). From clinical history, the following demographic data were recorded: age, gender, and living area (rural/urban), symptoms (presence and severity). The severity of AR was assessed using the total symptoms score (TSS) and visual analog scale (VAS). Total symptoms score included rhinorrhea, nasal congestion, sneezing, nasal, and ocular itching, the severity of which symptom was assessed on a scale from 0 (absent) to 3 (severe), retrospectively, for 12 h before presentation. TSS was calculated by adding the score for each symptom. It was considered mild rhinitis if TSS was below 6, while a TSS $\geq$ 6 represents a moderate-to-severe form of the disease. VAS scale was also evaluated in order to assess the quality of life (QL), VAS value over 6 points meaning patients with a moderate-to-severe form of AR.

After the baseline evaluation, patients were randomly divided into two groups using adaptive biased-coin randomization. In total, 26 patients formed the first groups, and they were treated with levocetirizine 5 mg/day, while the second group of 24 patients received desloratadine 5 mg/day. The treatment was recommended for 4 weeks, until the end of the pollen season in Romania. The second clinical and biological evaluation was performed at the end of the four weeks of treatment.

The presence of asthma symptoms was assessed after 1.5 years, as previously described [19].

2.3. Skin Prick Tests (SPTs)

Diagnosis of allergy was established through skin prick test, according to international guidelines [20]. The allergen panel included international recommendations and particularities of exposure to allergens in Romania: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, grass pollens mix (*Agrostis stolonifera, Anthoxanthum odoratum, Dactylis glomerata, Lolium perenne, Arrhenatherum elatius, Festuca rubra, Poapratensis, Secale cereale, Holcus lanatus, Phleum pretense*), cereals pollen, birch pollen, hazel pollen, *Artemisia vulgaris,* and *Ambrosia elatior,* cat and dog dander, and *Alternaria Alternata*. Standardized allergen extracts (Hal Allergy, The Netherlands) were used. SPTs were performed at the beginning of the study.

2.4. Biological Evaluation

All the biological parameters were determined before and after 1 month of treatment with H1 antihistamines. The plasmatic level of total IgE was analyzed using the electrochemiluminescence immunoassay method (ECLIA). The obtained values were expressed as UI/mL. A value below <100 UI/mL was considered normal. The intranasal and plasmatic eosinophils (Eos) were manually counted from a slide using May–Grunwald Giemsa staining, and their value was expressed as %. We considered a normal value less than 10% in nasal secretion and less than 4% in the blood.

The serum levels of ICAM-1 and VCAM-1 were determined by the ELISA technique (Quantikinine R&D system, USA). Five ml of blood sample was collected in a tube without anticoagulant and centrifuged within the first hour, followed by serum separation. All the determinations were carried out according to the manufacturers' instructions.

All blood samples were taken on fasting between 8 a.m. and 11 a.m.

2.5. Statistical Analysis

The statistical analysis was performed using SPSS version 21 (Chicago, IL, USA). Data were labeled as nominal, expressed as a percentage, and used continuous variables. The normal distribution for continuous variables was achieved using Kolmogorov–Smirnov test. The influence of different parameters on the evolution of CAM after 1 month was investigated using the ANOVA test for repeating measurements. The Spearman coefficient of correlation was calculated to highlight differences between continuous variables. The level of statistical significance was set at $p < 0.05$.

3. Results

Patients' demographic data are presented in Table 1. There were no statistically significant differences between the two treatment groups.

Table 1. Demographic data.

Parameter		Desloratadine (n = 24)	Levocetirizine (n = 26)	p
Age *		28.05 ± 6.32	29.89 ± 12.17	0.031
Sex ^	male	50% (12)	46.1% (12)	0.263
	female	50% (12)	63.9% (14)	
Living area ^	urban	87.5% (21)	88.5% (23)	0.770
	rural	12.5% (3)	11.5% (3)	
Onset of AR (months) °		24 (6–60)	36 (7.5–68)	0.532
Sensitization ^ to grass pollen	monosensitization	29.2% (7)	23.1% (6)	0.258
	polysensitization	70.8% (17)	76.9% (20)	
Severity ^	mild	25% (6)	23.1% (6)	0.465
	moderate-to-severe	75% (18)	76.9% (20)	
Personal history of allergy		46% (11)	73% (19)	0.5
Familial history of allergy		29.2% (7)	42.3% (11)	0.774

* Data were expressed as mean and standard deviation. ^ Data were expressed as percentage. ° Data were expressed as median and percentiles. Significance $p < 0.05$.

In patients with AR to grass pollen plasmatic levels of ICAM-1 and VCAM-1 were significantly increased compared with the control group during pollen season ($p < 0.001$, respectively $p < 0.001$). Additionally, total IgE, blood, and intranasal eosinophils were increased at baseline towards healthy volunteers (Table 2).

Table 2. Nasal and blood eosinophils, plasmatic values of total IgE and adhesion molecules in healthy volunteers and patients with AR.

Parameter	Healthy Volunteers (n = 30)	Patients with AR (n = 50) Baseline	p
Total IgE (UI/l)	<100	255.8 (56.3–599)	<0.001
Nasal Eo (%)	<10	24 (10–46)	<0.001
Blood Eo (%)	<4	8 (2–15)	0.003
ICAM-1 (ng/mL)	111.21 (100–206.3)	235.11 (209.1–276.6)	<0.001
VCAM-1 (ng/mL)	557 (249–891)	996.19 (832.8–1098.2)	<0.001

Significance $p < 0.05$.

Genetic predisposition for allergic diseases and asthma was evaluated using the accurate family history of the patients. Overall, 19 patients reported a positive familial history of allergy (asthma, allergic rhinitis, or atopic dermatitis). Patients with a family history of asthma had higher values of inflammatory markers, such as blood eosinophils (median value: 5.5% vs. 9%) than patients with no asthma history, but the group of patients was too small to calculate a statistical significance.

Both investigated H1 antihistamines significantly improved all symptoms of AR and increased patients' quality of life during pollen season after one month of treatment. TSS significantly decreased after treatment (median 8.5 (5–12) vs. median 4.2 (0–6), $p = 0.01$), with no differences between levocetirizine and desloratadine ($p = 0.571$) (Table 3). A similar reduction was noticed for VAS. The one-month evaluation revealed a reduction in total IgE level ($p = 0.08$), but this was not statistically significant. The reduction in total IgE was not influenced by the type of treatment, patients' age, sex, living area, or duration of AR ($p > 0.05$).

Table 3. Nasal and blood eosinophils, plasmatic values of total IgE and adhesion molecules initially and after 1 month of AH1 treatment in patients with AR.

Parameter	Patients with AR Baseline (n = 50)	Patients with AR after 1 Month-AH1 Treatment (n = 50)	p
Total IgE (UI/l)	255.8 (56.3–599)	198.7 (49.5–482)	0.08
Nasal Eo (%)	24 (10–46)	18 (10–29)	0.03
Blood Eo (%)	8 (2–15)	5.5 (1–7)	0.03
ICAM-1 (ng/mL)	235.11 (209.1–276.6)	195.42 (124.45–239.89)	0.06
VCAM-1 (ng/mL)	996.19 (832.8–1098.2)	783.19 (689.7–1005.3)	0.09
TSS (score)	8.5 (5–12)	4.2 (0–6)	0.001
VAS (cm)	8.9 (5–10)	3.8 (0–7)	0.001

Significance $p < 0.05$.

The same pattern was also observed after a four-week treatment with H1 antihistamines for plasmatic levels of ICAM-1 ($p = 0.06$) and VCAM-1 ($p = 0.09$), compared with basal values, without reaching the level of statistical significance. The reduction in ICAM-1 and VCAM-1 was observed in 42% and 40%, respectively, while in 14 patients (28%), their levels increased despite the treatment.

There was no difference between levocetirizine and desloratadine in the reduction in CAM plasmatic levels. We observed a significant reduction in VCAM-1 and ICAM-1 levels in patients with moderate-to-severe forms, compared with patients with mild rhinitis ($p = 0.03$, $p = 0.01$, respectively). The reduction in CAM levels was not influenced by patients' age, sex, and type of sensitization. Patients with increased values at baseline tend to remain with increased values after 1-month AH1 treatment ($p = 0.01$).

Intranasal and blood Eo, were significantly reduced after 1-month treatment with AH1 ($p = 0.03$). The reduction in Eo was not influenced by the type of treatment, patients' age, sex, environment, or duration of AR ($p > 0.05$).

After 1.5 years, 10 patients (20%) had asthma symptoms. The evolution of ICAM-1 and VCAM-1 was also retrospectively assessed in these patients. Nine patients had an increased or stationary evolution of ICAM-1 and VCAM-1 during treatment with H1 antihistamines.

4. Discussion

The present study showed a mild anti-inflammatory role of second-generation H1 antihistamines as monotherapy for four weeks of treatment, demonstrated by a reduction in intranasal eosinophils but not on the CAM plasmatic levels in patients with AR to grass pollen during the pollen season. Both desloratadine and levocetirizine improved nasal symptoms and patients' quality of life if they were administered during pollen season in patients with AR. Both TTS and VAS significantly decreased after treatment, with no differences between the investigated drugs.

AR to grass pollen is characterized by the presence of inflammation in the nasal mucosa during the pollen season under allergen exposure. Allergen exposure induces mast cells degranulation and the release of mediators such as histamine, which are responsible for producing the characteristic symptoms of AR (sneezing, nasal and ocular itching, rhinorrhea, and nasal obstruction) [21,22]. In addition to histamine, other mediators are released from mast cells, such as interleukins 4 and 5 (IL-4, IL-5), leukotriene D4, and E4 (LTD4 and LTE4) [23,24]. Those mediators stimulate infiltration of the nasal mucosa with inflammatory cells, mainly eosinophils, which migrate via CAM in the nasal mucosa [25]. The pattern of chronic allergic inflammatory response is represented by eosinophils infiltration in nasal mucosa [17,26]. Commonly, patients with grass pollen allergy have also other types of sensitization (other types of pollens, house dust mites, molds, animal dander), which may induce also an IgE triggered inflammation outside of grass pollen season, maintaining a minimal specific inflammation in the airway. These cells continue to produce other inflammatory mediators, such as cytokines, chemokines leading to persistent symptoms and tissue damages with structural changes [27–29]. Thus, rhinitis persistence and aggravation become more dependent on mediators, which promote infiltration of cells, such as eosinophils and TH2 lymphocytes [21]. AR to grass pollen is a risk factor for asthma development and may appear before or after asthma onset, or during thunderstorm-related asthma [1,2,30–32]. In the present study, 20% of the patients developed asthma symptoms within the next 1.5 year follow-up.

Adhesion molecules such as ICAM-1 and VCAM-1 are surface molecules with immunoglobulin-like structure, involved in intercellular adhesion through interaction with the B2 integrin LFA-1. Their importance resides due to cell-to-cell interaction and eosinophil migration in the nasal mucosa in pollen allergic patients [33,34]. In our study, we evaluated the soluble CAM in patients with AR to grass pollen. In grass pollen allergic patients, the inflammation assessed by CAM is higher than in healthy individuals. After the 4-week treatment, H1 antihistamines decreased the plasmatic levels of ICAM-1 compared to basal values, but the reduction was not significant. Similar results were noticed in patients with perennial allergen exposure to house dust mites [35]. The reduction was not significant probably because traces of pollen remained in the atmosphere even if the 4-week evaluation was performed at the end of the pollen season. Grass pollen is the most common pollen encountered in the temperate area where the center is located. The most abundant allergenic grass pollen in many temperate regions originates from tall grasses, such as *Phleum pratense*, *Dactylis glomerata*, and *Arrhenatherum elatius* [36,37]. It is well known the allergenic cross-reactivity between the members of the Pooideae subfamily grasses of temperate regions (*Lolium perenne, Phleum pratense, Poa pratensis*). A study published by a research group from the same country reported an increased level of grass pollen in the air in May, June, and mid of July in some years [38], while the peak of the season for ragweed and mugwort pollens is noticed in late summer and autumn in Romania [39]. The

patients were evaluated in the middle of grass pollen in Romania, and according to the previous measurement of pollen level in the air, the symptoms corresponding to allergic inflammation were generated by exposure to grass pollen, not to other pollen allergens that are not encountered in the air in the same period of time.

The reduction in CAMs followed the same pattern as in perennial exposure to house dust mites, as Lee et al previously described [35]. Indeed, the treatment that modified pathological mechanism of disease, including the levels of ICAM-1 and VCAM-1 during pollen season is allergen immunotherapy, not H1 antihistamines, which are more symptomatic and pathogenic therapy than an etiologic one [25]. However, long-term treatment with AH1 might contribute to reducing nasal inflammation through inhibition of cytokines and adhesion molecules production and functions. Treatment with H1 antihistamines for one month in this study during the two-month grass pollen season in Romania may not be sufficient in duration to see a statistically significant change in plasma ICAM and VCAM levels. Patients with increased basal values during pollen season tend to remain with increased values despite AH1 treatment, as long as the allergen exposure persists.

There was no difference between investigated compounds, levocetirizine, and desloratadine in the reduction in CAM plasmatic levels. In the present study, CAMs' reduction was more significant in patients with moderate-to-severe AR, compared with patients with mild rhinitis. Other studies that investigated the role of AH1 in other forms of AR induced by mite allergy, showed a decrease in mediators released by systemic and intranasal eosinophils after levocetirizine treatment, but CAM levels were not evaluated [34].

Although CAMs are involved in cells migration, including eosinophils to the site of inflammation, the values of intranasal Eo did not correlate with the serum levels of ICAM-1 and VCAM-1 in the present study. Similar to previous studies [40,41], we found that treatment with H1 antihistamines significantly decreased intranasal eosinophils. Due to difficulty obtaining intranasal CAMs, our study focused on plasmatic CAMs as a proxy [27]. Further studies are needed to investigate levels of CAMs in the nasal mucosa and correlate these levels with local infiltration of Eo.

IgE is the primary molecule in the pathogenesis of allergic diseases. Its synthesis and its level are increased after sensitization and it binds to high-affinity Fcε1 specific receptors expressed on mast cells. Part of it remains free in the serum and can be determined. Total serum IgE is increased in a variety of diseases, and in allergic subjects may remain also normal. In different clinical studies, IgE levels did not correlate with inflammatory markers such as ICAM-1 or TNF-α values, which are higher in asthmatics but not in those with AR [29]. Usually, IgE also increased during pollen season, and decreases after the season, if the patient is not sensitized to perennial allergens. The bound IgE is responsible for recurrent symptoms and inflammation during the pollen season. In the present study, patients with clinical manifestations induced by grass pollen were included, even if some of them were sensitized to several allergens. The inflammatory response in pollen allergy may differ toward house dust mite allergic patients, due to different aspects of the allergens (perennial, dimensions, enzymatic properties) [42–45], but this hypothesis needs further investigation. Another reason for the low reduction in total IgE could be related to polysensitization of the patients, even if they did not report clinical manifestation during the grass pollen season.

The main strength of the present study resides in investigating both clinical and pathophysiological effects of two antihistamines in patients with AR, during natural exposure to an elicited allergen. There are also some limitations of this study. Firstly, a small number of patients were included in the study. Secondly, the count of pollens was not performed in the investigated area, and the level of inflammatory markers could not be correlated with the level of exposure. The third limitation resided in the lack of CAM analysis in the nasal secretion, a determination that could not be accomplished due to technical reasons. It might be interesting to correlate the effect of H1 antihistamines on both nasal and blood CAM and eosinophils.

5. Conclusions

Patients with AR to grass pollen, during the pollen season, have high intranasal eosinophils levels and high serum levels of ICAM-1 and VCAM-1. H1 antihistamines improve symptoms of AR and reduce intranasal eosinophils. Baseline values of CAMs tend to remain higher during pollen exposure and they were not changed significantly despite AH1 treatment.

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nutrients

MDPI

Review

Alternative Fish Species for Nutritional Management of Children with Fish-FPIES—A Clinical Approach

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Abstract: In the Mediterranean region, fish is a common cause of food protein-induced enterocolitis syndrome (FPIES) in children. No laboratory tests specific to FPIES are available, and oral food challenge (OFC) is the gold standard for its diagnosis and testing for achievement of tolerance. Children with FPIES to fish are usually advised to avoid all fish, regardless of the species. Fish are typically classified into bony and cartilaginous, which are phylogenetically distant species and therefore contain less cross-reacting allergens. The protein β-parvalbumin, considered a pan-allergenic, is found in bony fish, while the non-allergenic α-parvalbumin is commonly found in cartilaginous fish. Based on this difference, as a first step in the therapeutic process of children with FPIES caused by a certain fish in the bony fish category (i.e., hake, cod, perch, sardine, gilthead sea bream, red mullet, sole, megrim, sea bass, anchovy, tuna, swordfish, trout, etc.), an OFC to an alternative from the category of cartilaginous fish is suggested (i.e., blue shark, tope shark, dogfish, monkfish, skate, and ray) and vice versa. Regarding the increased mercury content in some sharks and other large species, the maximum limit imposed by the European Food Safety Authority (EFSA) for weekly mercury intake must be considered. An algorithm for the management of fish-FPIES, including alternative fish species, is proposed.

Keywords: food protein-induced enterocolitis syndrome (FPIES); fish; bony fish; cartilaginous fish

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1. Introduction

Food protein-induced enterocolitis syndrome (FPIES), a non-IgE-mediated food allergic disorder, can be induced by a wide range of foods. The rate of fish as the offending food in children with FPIES varies among geographic locations. Many studies have shown that fish is among the frequent causes of FPIES in the Mediterranean basin, where fish is a common dietary constituent [1–11]. In addition, in adults and adolescents, FPIES may be provoked most commonly by seafoods, including fish [12–15]. Observational studies have shown that a certain percentage of people with FPIES caused by one species of fish may tolerate other species [16]. Here, we discuss from the allergist's point of view the different species of fish used for human consumption and review the current evidence on tolerance across fish species, with suggestions on which species would be the most suitable for conducting the first oral food challenge (OFC) in children with FPIES caused by fish.

2. Fish in the Human Diet

Fish is a widely available food, which is highly nutritious due to its rich content of high-quality proteins and polyunsaturated fatty acids (PUFAs). Studies have shown that the regular consumption of fish can enhance health and quality of life (QoL) in various ways, improving vision in childhood and reducing the risk of cardiovascular disease (CVD) [17]. In recent years, there has been a general tendency toward the adoption of a healthier diet, which has led to an increase in fish consumption. According to the Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), sea fish consumption in Europe in 2017 was 24.35 kg/citizen/year [18].

The fish market differs among countries, depending on availability, dietary habits, and economic status. The most commonly consumed fish worldwide are *Gadiformes* (cod, hake), *Salmoniformes* (trout, salmon), *Cypriniformes* (carp), *Clupeiformes* (sardine), *Siluriformes* (catfish), and *Poerciformes* (tuna, mackerel) [19,20]. Carp is among the most popular fish in Asia, while salmon and cod are preferred in northern Europe. Families in Japan and the United States (US) prefer salmon, tuna, and mackerel [21,22]. In terms of preparation, fish can be eaten uncooked, marinated, smoked, fried, grilled, steamed, roasted in the oven, boiled, or baked, according to local customs and individual or familial preferences, and some species are canned. Fish species may differ in their allergenic potency, which is not the same as that of shellfish (e.g., crustaceans, mollusks) [23].

2.1. Classification of Fish

Currently, more than 30,000 species of fish are recognized. According to their biological characteristics, they are divided in two classes: *Osteichthyes* (bony) and *Chondrichthyes* (cartilaginous). Cartilaginous fish account for about 7% of the global biodiversity, and they are further divided in two subcategories, the *Elasmobranchs* (sharks, rays, skates, and sawfish) and the *Holocephali* (chimaeras) [24]. Bony fish or teleost fish consist of 45 orders and more than 430 families; they are divided into *Actinopterygii* and *Sarcopterygii* based on their fin shape [25]. Only a limited number of orders are consumed by humans, specifically, the salmon-like (*Salmoniformes*), cod-like (*Gadiformes*), perch-like (*Perciformes*), herring-like (*Clupeiformes*), carp-like (*Cypriniformes*), catfish-like (*Siluriformes*), and flatfish (*Pleuronectiformes*) [23].

The most obvious difference between bony and cartilaginous fish is in their endoskeleton. The bony fish skeleton is composed exclusively of bones, while that of cartilaginous fish is composed of cartilage. Teleost fish are the largest infraclass in the class *Actinopterygii* (ray finned fish) [26]. They have two types of muscle, which are both used for swimming. The light (white) muscle is used for short bursts and the red (dark) is used for constant swimming [27]. In contrast, the cartilaginous exoskeleton consists of small denticles coated with sharp enamel [24].

The gadiform fish (order *Gadiformes*) belong to the bony fish category, comprising 8 families, 59 genera, and more than 180 species, and they contribute more than one-quarter of the world's marine fish catch [20]. Bony fish that more frequently induce FPIES are cod (*Gadus morhua*), hake (*Merluccius merluccius*), perch (*Perca fluviatilis*), sardine (*Sardina pilchardus*), gilthead sea bream (*Sparus aurata*), red mullet (*Mullus barbatus*), sole (*Solea solea*), megrim (*Lepidorhombus whiffiagonis*), sea bass (*Dicentrarchus labrax*), anchovy (*Anchoa*), tuna (*Thunnus*), swordfish (*Xiphias gladius*), and trout (*Salmo trutta*) [2–6,9,10].

Blue shark (*Prionace glauca*), dogfish (*Scyliorhinus canicular*), monkfish (angelshark—*Squatina squatina*), skates and rays (*Raja*), and tope shark (*Galeorhinus galeus*) are cartilaginous fish that are part of the human diet [28]. In view of the high mercury content of large ocean fish, such as swordfish, sharks, and fresh tuna, several regulatory bodies recommend avoiding the consumption of these fish by pregnant women and young children [29,30]. In this regard, the European Food Safety Authority (EFSA) established a tolerable weekly intake (TWI) for methylmercury of 1.3 μg/kg body weight (bw) and of 4 μg/kg bw. for inorganic mercury [31,32]. However, certain species of shark, including the blue shark, which focus their foraging behavior on prey of the mesopelagic zone, have a lower mercury

content [33]. Taylor et al. showed that for all species, mercury content was directly related to size and age; larger, older specimens had higher concentrations of mercury than smaller, younger sharks and skates [34]. The US Food and Drug Administration (FDA) recommend skate as one of the best choices of fish to be consumed during pregnancy, breastfeeding, and early childhood [29]. As exposure to methylmercury above the TWI is of concern, supervision of children's diet by a specialist and/or dietician is recommended.

2.2. Fish Allergy and Fish Allergens

Food allergy affects around 5% of adults and 8% of children [35]. It is an adverse reaction to foods or food additives, and it can be differentiated into allergic hypersensitivity, which involves an immune mechanism and non-allergic hypersensitivity. It is further differentiated into IgE-mediated and non-IgE mediated [36]. Fish allergy presents in 0.2% to 2.29% of the general population, varying according to regional dietary habits, fish species exposure, and ways of preparation and cooking [19,37]. In children, only a few FPIES caused by both fish and shellfish (crustacean and cephalopods) are reported [3]. In crustacean and cephalopods, the major allergen responsible for ingestion-related allergic reactions is the muscle protein, tropomyosin [37]. Parvalbumins, enolases, and aldolases are present in fish muscles, the first being the major allergen [38,39]. Different fish species have been shown to exhibit various degrees of parvalbumin allergenicity.

2.3. Parvalbumins

There are two subtypes of parvalbumins, according to their phylogenic origin, the α-protein lineage and the β-protein lineage. The β-parvalbumin subtype is encountered in fish and is responsible for almost 95% of IgE-mediated hypersensitivity to fish [38]. The parvalbumins are calcium-binding sarcoplasmic muscle proteins, with a molecular weight of 12 kDa (108–109 amino acids). Fish parvalbumins are highly water-soluble and exhibit resistance to heat, denaturing agents, and extreme pH. They consist of three EF-hand motifs with two high-affinity calcium ion-binding sites [23]. Their ability to buffer calcium (Ca) plays a role in muscle relaxation, and their allergenic potential is significantly reduced when Ca is removed. Ion binding is a key to the parvalbumin stability, and parvalbumins lacking Ca^{2+} bind only weakly to IgE antibodies from fish-allergic patients [38,40]. The parvalbumin found in cartilaginous fish (α-parvalbumin), is characterized as non-allergenic, while the parvalbumin of bony fish (β-parvalbumin) is considered pan-allergenic [27,38]

Fish allergens have been investigated in nearly 40 species, although most European studies have concentrated on common local fish, such as cod, salmon, carp, and tuna. Fish muscle exhibits the greatest allergenicity, and parvalbumin and to a lesser extent enolases and aldolases are the major allergens in fish muscle [39]. Studies have shown that there is variation in the allergenicity of parvalbumin between different fish species [20,27,41–47]. Parvalbumin has been most extensively studied in the following bony fish: Atlantic cod (*Gadus Morhua*), Alaska pollack (*Theragra Chalcogramma*), common carp (*Cyprinus Carpio)*, silver carp (*Hypophthalmichthy Molitrix*), Atlantic salmon (*Salmo salar*), and more recently Asian seabass (*Lates calcarifer*). Regarding cartilaginous fish, the role of parvalbumin has been investigated in blue shark (*Prionance glauca*), salmon shark (*Lamna ditropis*), spotless smooth-hounds (*Mustelus griseus*), and halibut (*Hippoglossus stenolepis*) [47]. There is little evidence on coexisting allergy to α- and β-parvalbumin, which suggests low clinical cross-reactivity between them [38]. The main differences between the lineages are the presence of more acidic amino acid residues in β-parvalbumin and differences in length (≥109 amino acids in α-parvalbumin compared with <109 in β-parvalbumin) [38]. *Chondrichthyes* express low levels of allergenic fast muscle. The variation in the parvalbumin amino acid sequence between different fish species and lineages appears to play an important role in patient sensitization. The difference in amino acid sequence between different parvalbumins could be an indication of the likelihood of clinical cross-reactivity [20].

In a Japanese study, the investigators heated the flesh of seven bony fish (mackerel, red seabream, yellowfin tuna, silver salmon, Japanese sardine, chicken grunt, goldeye rockfish)

and four cartilaginous fish (bigeye thresher, shortfin mako shark, mottled skate, blue shark) to different temperatures and for different times, with the aim of determining the thermostability of fish collagen as an allergen. They found that cartilaginous fish produced less IgE reactivity than bony fish and suggested that the allergenicity of cartilaginous fish collagen is lower than that of bony fish [48]. Kobayashi et al. observed that in bony fish, regardless of fish species, there is less parvalbumin in the dark than in the white muscle. They concluded that the more commonly consumed fish with white muscle is more likely to be allergenic [27]. In tuna, parvalbumin was found in the white muscle but not in the red, and it was also unequally distributed in different parts of the muscle [49]. Another study suggested that the method used to prepare the fish, and the duration of heating, can affect the parvalbumin epitopes, leading to alterations in the allergenicity [46]. Children affected by IgE-mediated fish allergy appear to show a higher tolerance for canned tuna [50]. While heating caused a reduction in antibody reactivity to multimeric forms of parvalbumins in most bony fish, a complete loss of reactivity was observed for cartilaginous fish [46,51]. This is another reason to select cartilaginous fish for the first OFC in a FPIES. Apart from the phylogenetic differences of the allergens, the heating process contributes to the reduced allergenicity of cartilaginous fish. Based on these findings, regardless of the species chosen, we recommend that OFC is conducted with fish baked in the oven at 160 °C for at least 30 min.

3. Food Protein-Induced Enterocolitis Syndrome (FPIES) Caused by Fish

The culprit food differs according to the age of onset of FPIES and depends on the time when the culprit food is introduced in a baby's diet, although cases of adult onset have also been reported [12–15,52–54]. FPIES caused by milk and soy usually develops in the first 3 months of life, while FPIES caused by grains presents between the 5th and 7th month, as grains are more likely to be introduced into the infant's diet at this stage [55]. Fish is usually introduced to children's diet after the 6th month and in certain regions after 12th month, and thus, a delay in FPIES caused by fish is observed [56,57], and children persistently reactive to fish were reported to be diagnosed at a significantly older age than those reactive to milk [9].

From a geographically diverse population of 441 children with FPIES, data provided by caregivers in the International FPIES Association showed that fish was the third lowest in the hierarchy of offending foods [58]. In contrast, reports from Mediterranean countries, specifically Greece [8–10], Italy [2,3], Spain [1,4–7,16,59], and Turkey [60] revealed fish as the first or second most commonly implicated food and a major trigger of solid food protein allergy. It has also been concluded that the resolution of fish-FPIES comes later than that from other foods [61], and many children with fish-FPIES will not overcome the disease during childhood [62]. The current guidelines recommend periodic re-evaluation with supervised OFCs to monitor for resolution [55,57]. Results from studies of children with IgE-mediated fish allergy suggest that they may be able to tolerate fish species other than those associated with the initial onset of symptoms [63]. Observational studies of fish-FPIES show that a certain percentage of children tolerate species other than fish identified as the culprit [16]. As no laboratory analysis or dermatological test is available to predict when tolerance to the offending or alternative fish has been achieved, all children with fish-FPIES should undergo a periodic OFC. Several protocols for OFC in fish-FPIES have been proposed [2,4,5,64,65], and optimal challenge procedures can be unclear to practitioners and underutilized [66]. The suggested OFCs vary regarding the amount of protein/food served per dose, the time between doses, and the duration (one day, or more non-consecutive days). The current consensus and guidelines do not clearly specify whether the OFC should be conducted with the offending fish or with an alternative species, and if the latter, which species [61]. Infante et al. showed that the probability of not presenting a reaction during OFC was four times higher in children with FPIES who received an alternative fish than in children who received the culprit fish; of 32 patients tested to an alternative fish, 27 had a negative OFC [16], and the researchers proposed to challenge first with an alternative fish [67]. In addition to bony fish, blue and tope sharks, skates, and rays

are also commonly used in the Mediterranean cuisine. In our experience, children with both IgE and non-IgE-mediated reactions to hake, cod, or other culprit fish have tolerated cartilaginous fish earlier.

4. The Clinical Approach to Investigation of Tolerance across Fish Species

The observation of differences in allergenicity among fish species has directed researchers toward exploring the tolerance of patients to fish other than the culprit species, with the aim of establishing an alternative option and avoiding a restricted diet [3,10,11,16,68,69].

The various pediatric societies have issued no specific instructions on which fish species should be introduced first to the diet of infants/children. In several countries, hake (*Merluccius merluccius*) is commonly recommended by pediatricians as the fish to be tried first, even before the age of 1 year. It is one of the fish most frequently consumed in Mediterranean countries, but it is also one of the fish most commonly specified as the offending fish in cases of FPIES. It can be found fresh throughout the year, is a small fish, is easy to prepare, and is not very expensive. In addition to its light smell and taste, which makes it more acceptable to children, hake has fewer small bones, so the danger of these being swallowed by babies and children is low.

Most studies do not specify which species of fish is the FPIES culprit under investigation; instead, they refer to it as 'fish', 'cod', or 'codfish' [2,8,70–72]. Data mainly from the Mediterranean countries refer to hake as the most commonly offending fish [1,4–7,11,16]. *Merluccius merluccius* is also one of the most frequently involved fish species in adult-onset FPIES [15]. Hake (*Merluccius merluccius*) belongs to the *Merlucciidae*, a family of cod-like fish of the genus *Merluccius.* Hake and cod are both white-fleshed fish belonging to the *Gadiformes* order. The Atlantic cod belongs to the *Gadidae* family of the genus *Gadus* in the *Actinopterygii* class. Many fish throughout the world that have the word "cod" in their name do not belong to the genus *Gadus* [41]. In Europe, the most commonly consumed bony fish are Gadiforms, such as cod and hake, and the most commonly consumed cartilaginous fish are sharks and rays [19]. Geographical differences are documented in the prevalence of fish allergy and the type of fish causing allergy, possibly due to cultural and dietary differences, and differences in the distribution of fish. In Asia, the most frequently reported causative agents are anchovy and mackerel, while in South Africa, hake (24.8%), yellowtail (32.9%), salmon (15.2%), and mackerel (15.2%) are the most common culprits [19]. In Europe, in children with FPIES, cod and hake are the more frequent offenders. Table 1 shows the current evidence on the distribution of fish found responsible for FPIES in children and the tolerance to other species.

Table 1. Fish species implicated in the presentation of FPIES in children.

Fish Species	Number of Cases (%)	Tolerance to Other Species	Country, City	Publication
hake, whiting, sole, perch, anchovy, monkfish	16	Unspecified	Spain, Alicante	[6]
hake	3 (37.5%)	Unspecified	Spain, Madrid	[7]
hake, sole, megrim, cod, canned tuna, sardine, swordfish	80	canned tuna and swordfish	Spain, Madrid	[16]
cod, perch, sardine, tope, sea bream	56 (56%)	5 subjects tolerated a type of fish other than the culprit species	Greece, Athens	[10]
cod, tope shark, tuna	25 (34.7%)	Unspecified	Greece, Athens	[9]
unspecified	42 (53.8%)	Unspecified	Greece, multicenter	[8]
unspecified fish, white fish, tuna, salmon	12 (5%)	Unspecified	Australia (multicenter)	[52]
hake (14) monkfish (6), sole and megrim (4)	17 (80%)	Unspecified	Spain, La Coruna	[4]
hake, sole, cork float	14	Unspecified	Spain, Madrid	[1]

Table 1. *Cont.*

Fish Species	Number of Cases (%)	Tolerance to Other Species	Country, City	Publication
sole, cod, sea bass, gilthead, anchovy	70	cod, salmon, swordfish, bass, red mullet, anchovy, canned tuna, gilthead, trout	Italy, multicenter	[3]
hake (19), sole (9), monkfish (7), canned tuna (4), salmon (2), swordfish (1), fresh tuna (1), dogfish (1)	44 (54.3%)	Other fish species were not tested.	Spain, Barcelona	[5]
unspecified fish	5 (3.12%)		US, New York	[73]
cod, sole, sea bream, salmon, trout	8 (12%)	three were tolerant to different fish types (salmon + swordfish, cod + tuna, sea bream + cod + perch)	Italy multicenter	[2]
unspecified	102 (57%)	41% reacted to more than one fish species and 78/102 (76%) were avoiding all fish.	Spain, Italy, 12 centers	[11]
unspecified	28 (25%)	Unspecified	Sweden, multicenter	[74]
unspecified	19 (14%)	Unspecified	UK and Ireland	[75]
unspecified	39 (32.5%)	Unspecified	Spain, multicenter	[76]
unspecified	19 (11%)	Unspecified	Australia	[77]

Some studies were able to demonstrate that patients with FPIES caused by certain types of fish could tolerate other fish species [16,67], although Sopo et al. reported that two patients presented with late FPIES symptoms due to the alternative fish, despite having tolerated it well previously [78]. This happened with sole and tuna, respectively, in two patients with initial FPIES to hake and cod, and sole and cod, respectively. It should not be assumed that there will be tolerance to a particular fish when a fish belonging to the same order is well tolerated. The mechanisms behind differential tolerance to fish of the same order are not well understood. It has been suggested that there could be an inappropriate adaptive immune response to the protein component of foods, similar to that encountered in IgE-mediated allergy. In addition, it is not clear which fish allergen triggers the FPIES. In the cases described by Sopo et al., patients suffering from FPIES to one fish had already experienced several uneventful ingestions of the alternative fish prior to the onset of symptoms. It is conceivable that the allergen of the alternate fish is similar to but not exactly the same as that of the offending fish, and thus, it appears initially to be tolerated, but subsequently, the immune system recognizes it as foreign, triggering the allergic reaction. Another possible explanation is that due to the loss of immunological memory, some patients can tolerate the food once (e.g., in the OFC), but they develop symptoms with re-exposure at home, as previously reported [62,78].

The introduction of an alternative species of fish will help to avoid extended dietary restrictions, at least until acquisition of tolerance to the offending fish has been achieved. In addition, it appears that consumption of another type of fish could promote the acquisition of tolerance to the original offender, which otherwise, during the natural course, would develop later. After conducting oral immunotherapy with hake, a child with IgE-mediated fish allergy may be able to tolerate other types of fish [79]. Oral desensitization in egg-induced FPIES has been reported recently, but no data are yet available on the active induction of tolerance in fish-FPIES [80].

Therefore, we consider that following diagnosis of FPIES due to a specific type of fish, an OFC should be conducted with an alternative fish species. As a result of the quantitative and qualitative differences in protein content between bony and cartilaginous fish, and based on our clinical experience, we believe that it is worthwhile to include in the guidelines for OFC the option to conduct a challenge test to cartilaginous fish, in the case of FPIES to bony fish, and vice versa. Based on the clinical experience of each physician and the

familial dietary habits, different other fish species can be used as alternative solutions to a certain offending fish species. We propose an algorithm for the management of FPIES caused by a certain fish, including OFC with alternative fish species (Figure 1).

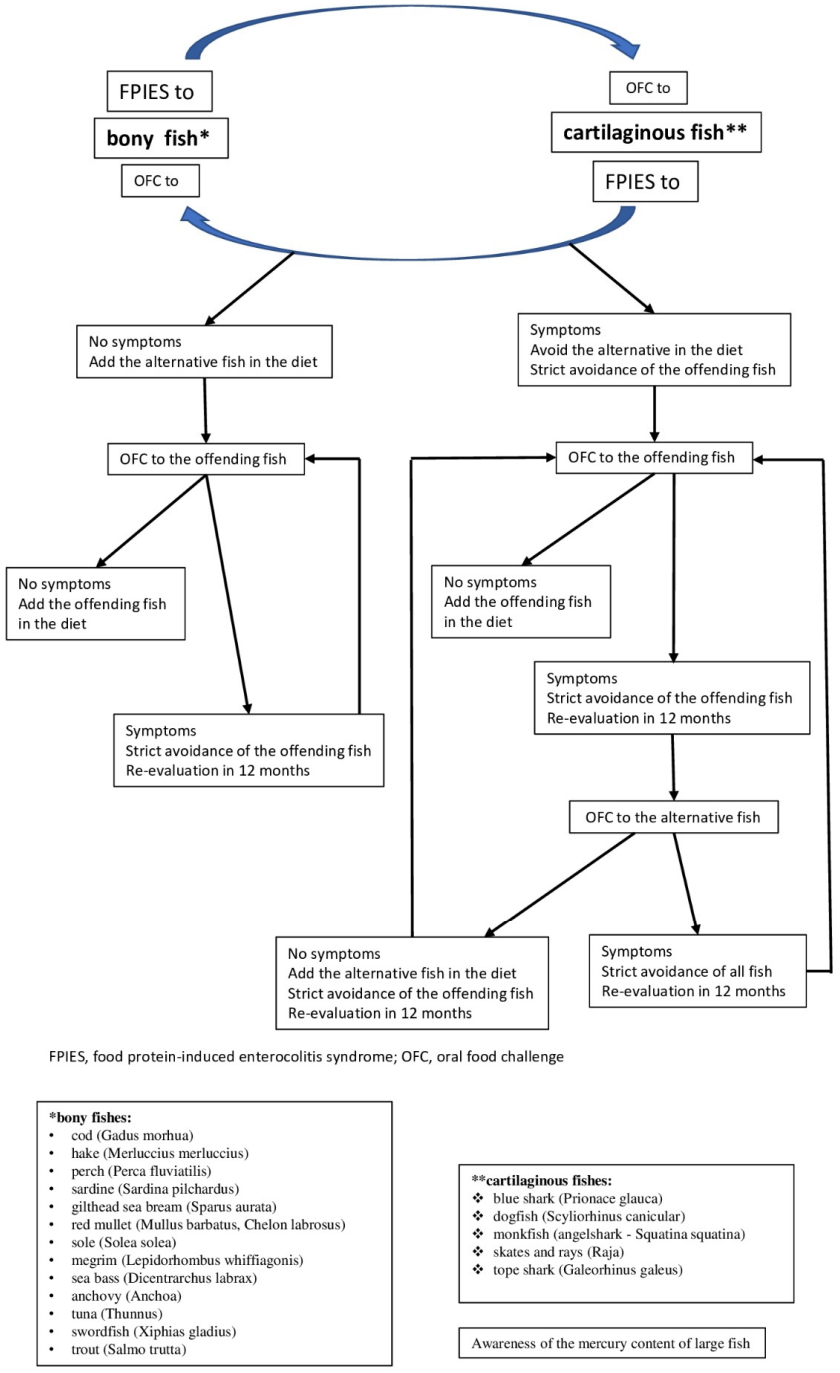

Figure 1. Algorithm for the management of food protein-induced enterocolitis syndrome (FPIES) caused by fish. * bony fishes: cod (*Gadus morhua*), hake (*Merluccius merluccius*), perch (*Perca fluviatilis*), sardine (*Sardina pilchardus*), gilthead sea bream (*Sparus aurata*), red mullet (*Mullus barbatus*, *Chelon labrosus*), sole (*Solea solea*), megrim (*Lepidorhombus whiffiagonis*), sea bass (*Dicentrarchus labrax*), anchovy (*Anchoa*), tuna (*Thunnus*), swordfish (*Xiphias gladius*), trout (*Salmo trutta*); ** cartilaginous fishes: blue shark (*Prionace glauca*), dogfish (*Scyliorhinus canicular*), monkfish (angelshark—*Squatina squatina*), skates and rays (*Raja*), tope shark (*Galeorhinus galeus*) (Information on fish species identification from www.fishbase.org (accessed on 1 November 2021) [28]).

The introduction of other fish species in the diet of children with FPIES caused by a certain species can contribute to the faster acquisition of tolerance to the incriminated species, which is a phenomenon already reported in IgE allergy to fish [79]. Even if it remains to be proven, we believe that this algorithm will be useful to clinicians in managing FPIES and that it will provide the basis for further studies.

5. Conclusions

Children with fish-FPIES are generally recommended fish avoidance regardless of species. No laboratory tests are available to investigate tolerance, and OFC is currently the gold standard to demonstrate tolerance. Testing with fish species other than the type incriminated might identify one or more alternatives that can be tolerated. As a result of their different protein content and/or composition, a logical alternative from the point of view of the allergist would be to conduct an OFC with a cartilaginous fish in the case of allergy to a bony fish and vice versa. Specific consideration should be given in the maximum weekly mercury intake contained in some large species of fish, as imposed by the EFSA. Regular consumption of alternative fish can lead to the acquisition of tolerance for the offending fish.

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Vitamin D Levels in Asymptomatic Children and Adolescents with Atopy during the COVID-19 Era

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Abstract: This study assessed vitamin D status in asymptomatic children and adolescents in Greece, with and without atopy, and possible changes during the coronavirus disease 2019 (COVID-19) pandemic. Serum levels of 25-hydroxy-vitamin D (25(OH)D) and total immunoglobulin E (IgE), and eosinophil count were measured in 340 asymptomatic children and adolescents (155 males, 185 females), mean age 8.6 ± 4.6 years, recruited over a period of 24 months (February 2019–January 2021). Atopy, defined by high level of IgE for age, was associated with vitamin D deficient status ($p = 0.041$). Subjects with and without atopy showed similar rates of insufficient and normal levels of 25(OH)D. The median level of 25(OH)D was significantly higher in subjects recruited during the pandemic, when home confinement rules were observed, than before the pandemic, and significantly more children had normal levels of 25(OH)D ($p < 0.001$), but no differences were noticed for IgE levels or eosinophil count. These results support a link between vitamin D and allergic and infectious inflammations, and specifically the association of vitamin D deficiency with asymptomatic atopy, defined as increased IgE level for age.

Keywords: vitamin D; atopy; COVID-19

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1. Introduction

Atopy is defined as either a personal, familial, or in combination, tendency, to have an immunological response after exposure to allergens, that are usually proteins, leading to differentiation of T helper cells (Th2), synthesis of immunoglobulin E (IgE) antibodies and allergic inflammation [1,2]. Atopic status can be assessed by skin prick tests to aeroallergens, and serum levels of total and specific IgE [3]. A raised serum level of total IgE, despite certain well-established limitations, is included as a diagnostic marker for allergic disease [4]. Epidemiological studies have demonstrated association of various allergic diseases, like asthma and allergic rhinitis, and atopic dermatitis, with an increase in the serum level of total IgE [5]. A high serum level of IgE is a good predictor of atopy and could be considered a marker of allergic airway inflammation in children with allergic disease [6]. Atopy may be asymptomatic [7], and studies have demonstrated airway inflammation in subjects with atopy without clinical manifestations, raising the hypothesis of subclinical inflammation in subjects with asymptomatic atopy [8,9].

The importance of vitamin D in childhood represents a public health pursuit worldwide, especially in developed countries. Low blood levels of vitamin D have been found to be correlated with the development of, apart from skeletal pathology, extra-skeletal problems in childhood, including allergy and autoimmune disorders [10]. The evidence regarding the relationship of the level of 25 hydroxy vitamin D ((25(OH)D) in umbilical cord blood with the development of allergic disease in infancy and early childhood is conflicting [11]. Baïz and colleagues reported an inverse association of cord 25(OH)D level with the risk of transient early wheezing and atopic dermatitis by the age of 5 years, but no relationship respiratory allergies, like asthma and rhinitis [12]. Chiu and colleague demonstrated a relationship between low 25(OH)D level in cord blood and sensitization to milk proteins, but not with asthma, allergic rhinitis or atopic dermatitis in early childhood [13]. Although the optimal level of vitamin D for decreasing the risk of development, and the severity of childhood allergies is still unclear, recent studies reinforce the concept that low levels increase the risk of clinical manifestations of atopy, such as bronchial asthma and allergic rhinitis [14]. Current data on vitamin D effects in inflammation, either infectious or allergic, are still conflicting [14–18]. One experimental study showed that a vitamin D deficient status in early childhood does not affect airway hyperreactivity, but that it aggravates eosinophilic inflammation and the airway remodeling process [19]. Experimental observations, however, need confirmation in human studies, and to date, the findings in clinical trials have been controversial.

The main objective of the present study was to investigate the serum level of 25(OH)D in asymptomatic children and adolescents with atopy. The secondary objective was to evaluate to what degree, if any, the pandemic restrictions imposed for control of COVID-19 have had an impact on the vitamin D status in these groups.

2. Materials and Methods

A 2-year prospective, observational, horizontal study was conducted from February 2019 to January 2021 in the district of Amaliada, in the Peloponnese, in Greece. The study was reviewed and approved by the Hospital Ethics and Scientific Committee of Hospital Unit of Amaliada, and written informed consent was provided by the parents of all the study children. Children who were referred to our laboratory for blood testing were initially screened. Subsequently, healthy children with vitamin D and IgE measurement conducted simultaneously were included in the study. Those children visited the pediatric clinic for regular checkups. The laboratory tests of interest were added to their screening lab test (for anemia and iron deficiency). During the study period, white subjects of both genders were enrolled, aged from 1 to 18 years, with a body mass index (BMI) below the 85th percentile for age and sex and without any symptoms, including those of allergy. The subjects had no underlying illnesses and were taking no vitamin supplementations or any other medications. Exclusion criteria were the administration of vitamin D supplements within the previous 6 months, comorbidities that could affect vitamin D status, chronic health conditions, known atopy, conditions known to increase IgE levels (such as parasitosis), and high BMI (because of the association between obesity and vitamin D level). None of the children had symptoms of infection within 2 weeks before biological evaluation.

Commonly, the term “atopic” is used to describe an “IgE-mediated” disorder [20]. The children with high serum IgE levels for their age were characterized as subjects with atopy. The normal levels of serum IgE for corresponding age were 0–1 y, <15 IU/mL; 2–5 y, <60 IU/mL; 6–9 y, <90 IU/mL; 10–15 y, <200 IU/mL; and over 15 y, <100 IU/mL.

If they had no current or past symptoms of any allergic disease, they were defined as having “asymptomatic atopy”, for comparison with the children without atopy. Criteria for the diagnosis of “asymptomatic atopy” included a free medical history and no clinical findings of any allergic manifestation (e.g., food allergy, atopic dermatitis, allergic rhinitis, and asthma).

Peripheral blood eosinophils were quantified in a venous blood sample, collected on ethylenediaminetetraacetic acid (EDTA), using an automated blood cell counter (XE 2100;

Sysmex xs 1000 hematology analyzer, Norderstedt, Germany) and expressed as a percentage and absolute number. For the determination of 25(OH)D and IgE, the blood samples were centrifuged immediately after collection, and the serum was analyzed. Furthermore, 25(OH)D and total IgE were measured by electrochemiluminescence binding assay, used on a Cobas e-411 immunoassay analyzer. Calibration and quality control were performed according to the manufacturers' recommendations. The laboratory followed the established procedures for corrective measures when the values fell outside the defined limits.

The study sample was divided into the following subgroups. According to age, the subjects were divided into 2 subgroups: infancy and early childhood (1–4 years), and middle childhood and adolescence (5–18 years). Based on the levels of 25(OH)D, 3 subgroups were defined: deficient vitamin D (25(OH)D < 20 ng/mL), insufficient vitamin D (25(OH)D = 20–30 ng/mL), and normal vitamin D (25(OH)D > 30 ng/mL). According to the serum level of total IgE, 2 subgroups were identified: children with atopy (IgE level increased for age) and children without atopy (IgE level normal for age).

Statistical Analysis

Statistical analysis was performed using the SPSS 22 software program. Data were labelled as nominal and continuous variables. The nominal variables were characterized as percentages and frequencies. Normal distribution for continuous variables was tested using the Kolmogorov–Smirnov test. Variables with normal distribution were characterized as mean and standard deviation (mean ± SD), and those with non-normal distribution as median and 25–75 percentiles. Comparisons between groups were performed using Mann–Whitney or chi-square tests, whenever appropriate. Spearman rho coefficient was used for examining correlation between variables. ROC curves were used to find a cut-off value for quantitative variables that could discriminate between subjects with or without atopy. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. The Linkage between Vitamin D and Atopy

During the study period, 340 children and adolescents (155 males and 185 females), with a mean age of 8.6 ± 4.6 years, were recruited. The demographic characteristics, atopic status, and vitamin D status are shown in Table 1.

Table 1. Demographic characteristics and selected laboratory values in the study children with and without atopy *.

Parameter		Children with Atopy * (*n* = 137)	Children without Atopy (*n* = 203)	*p*
Age (years) ^		8 (5–13)	8 (5–11)	0.212
Sex	M	76 (55.5%)	79 (38.9%)	0.03
	F	61 (44.5%)	124 (61.1%)	
Vitamin D status	Deficient	37 (27%)	32 (15,8%)	0.041
	Insufficient	50 (36.5%)	85 (41.9%)	
	Normal	50 (36.5%)	86 (42.3%)	
Eosinophils	Hypereosinophilia **	35 (25.5%)	32 (15.8%)	0.037
	Normal	102 (74.5%)	171 (84.2%)	

* Atopy: based on serum level of IgE for age. ** Hypereosinophilia: absolute number of eosinophils above 500/μL. ^ Data are expressed as median; 25–75th percentile.

The sub-groups of children with and without atopy were of similar age, but atopy was more frequent in boys than in girls ($p = 0.03$). Atopy was associated with vitamin D deficiency ($p = 0.041$), while children with and without atopy showed similar percentages of insufficient and normal serum levels of 25(OH)D. A little less than one quarter of the study

children had hypereosinophilia. Those with atopy had a significantly higher eosinophil count than those without atopy ($p = 0.037$) (Table 1).

The median level of 25(OH)D was slightly higher in the children without atopy than in those with atopy, but the difference did not reach the level of statistical significance ($p = 0.064$) (Figure 1). The ROC curve for patients' vitamin D levels was analyzed and the cut-off values were calculated for these parameters in relation with atopy presence. We calculated a cut-off value of 21.19 ng/mL that could discriminate between atopic and non-atopic patients (sensitivity 34.31 and specificity of 100%).

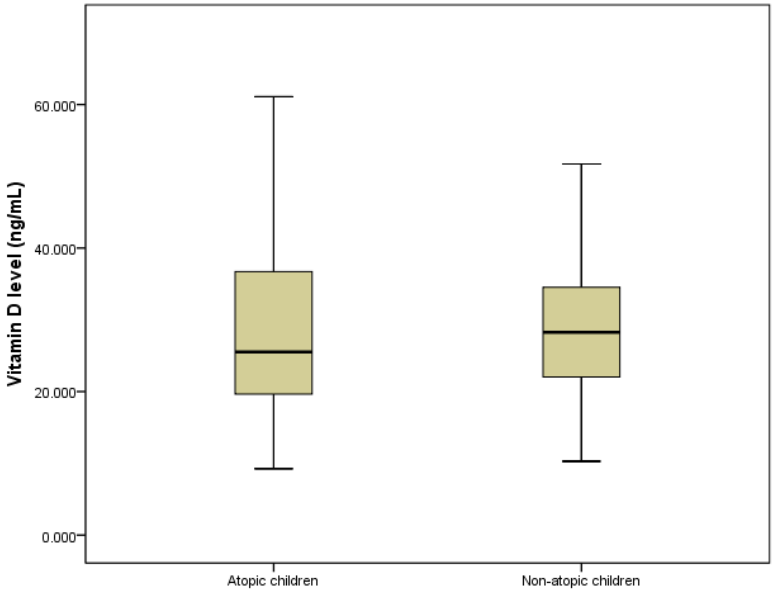

Figure 1. Box plot showing the levels of vitamin D (ng/mL) in children and adolescents with and without atopy ($n = 340$). Data are expressed as median, $p < 0.064$.

The eosinophil counts according to the age, sex, and vitamin D status of the study children are presented in Table 2.

Table 2. Hypereosinophilia (HyperEo) according to the age, sex, and vitamin D status of the study children ($n = 340$).

Parameter		HyperEo * ($n = 67$)	Normal Eosinophil Count ($n = 273$)	p
Age ^ (years)		7 (5–11)	9 (5–12.5)	0.187
Sex	M	33 (49.3%)	122 (44.7%)	0.584
	F	34 (50.7%)	151 (55.3%)	
Vitamin D status	Deficient	14 (20.9%)	55 (20.1%)	0.723
	Insufficient	29 (43.3%)	106 (38.8)	
	Normal	24 (35.8%)	112 (41.1%)	

* Hypereosinophilia: absolute number of eosinophils > 500/μL. ^ Data are expressed as median; 25–75th percentile.

More females than males had a normal eosinophil count, but the difference was not statistically significant. No differences in eosinophil count were found depending on the vitamin D status of the children, and there was no age difference.

The serum level of 25(OH)D was negatively correlated with the age of the children ($R = -0.185$, $p = 0.001$), but was not correlated with the eosinophil count. In children with atopy, total IgE was positively correlated with the eosinophil count ($R = 0.172$, $p = 0.045$), but not with 25(OH)D ($R = 0.096$, $p = 0.172$).

The level of 25(OH)D was higher in the children in whom the measurement was made during summer and autumn compared with winter and spring ($p < 0.001$) (Figure 2).

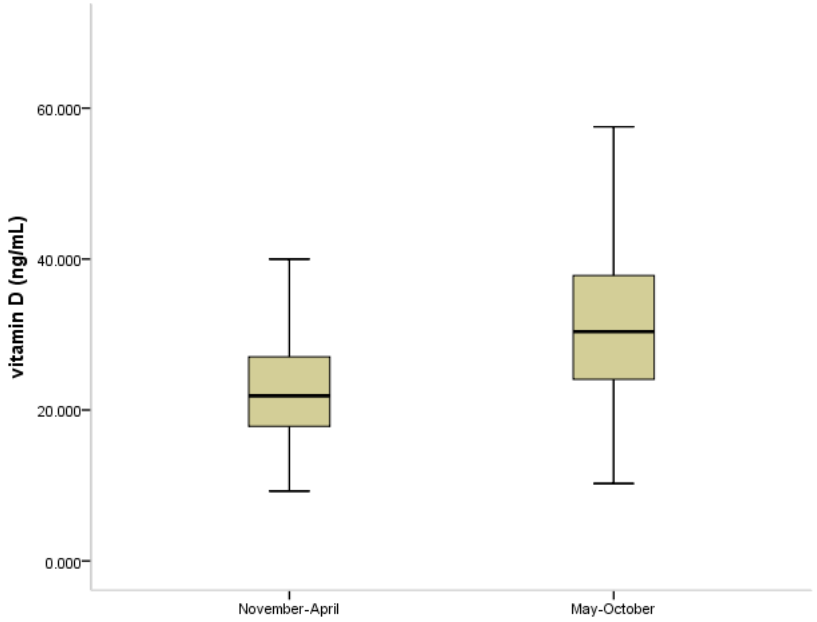

Figure 2. Box plot showing the level of vitamin D (25(OH)D, ng/mL) in children and adolescents with and without atopy ($n = 340$) according to season. Data are expressed as median, $p < 0.001$.

The level of total IgE and the eosinophil count showed no correlation with the season of measurement ($p = 0.827$, $p = 0.658$, respectively).

3.2. Vitamin D Status in COVID-19 Pandemia

The levels of 25(OH)D were also compared for the periods before (2019) and during (2020) the COVID-19 pandemic, in view of the restrictions applied. As shown in Figure 3, the levels of 25(OH)D were significantly higher in 2020 compared to 2019 ($p < 0.001$). Significantly more children and adolescents had normal levels of 25(OH)D during the pandemic ($p < 0.001$). The serum levels of IgE and the eosinophil count showed no significant differences between the two time periods ($p = 0.956$, $p = 0.569$, respectively).

Comparing the level of 25(OH)D in the two age groups prior to and during the COVID-19 pandemic, we found that during the pandemic, less children in both age groups were vitamin D deficient, and significantly more children were vitamin D sufficient (Table 3).

Figure 4 shows the comparison of vitamin D status measured in the different seasons (November–April/May–October) prior to and during the pandemic. Significantly higher levels of 25(OH)D were noted in summer and autumn than in winter and the beginning of spring in both years of study ($p < 0.001$).

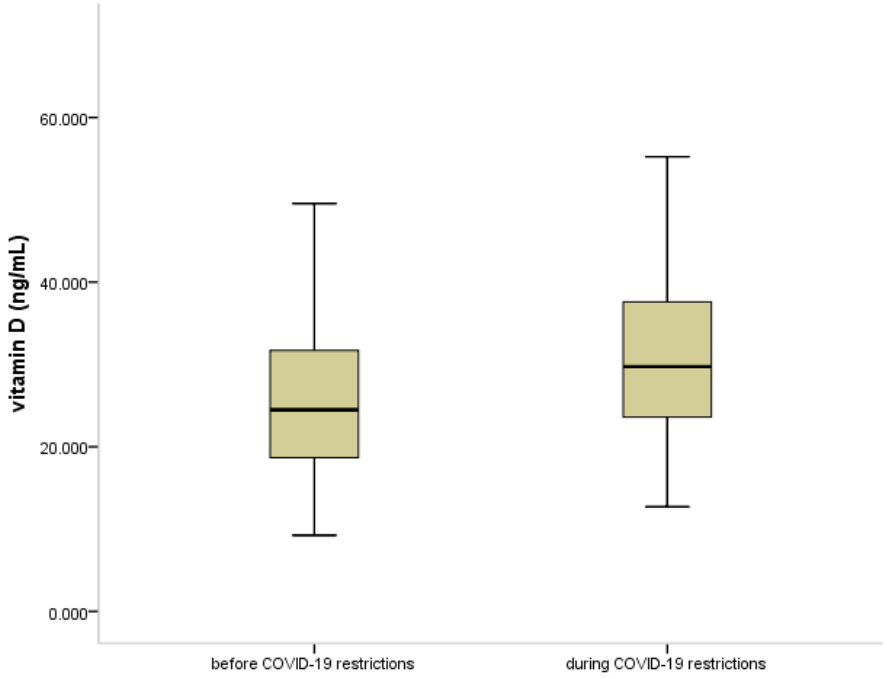

Figure 3. Box plot showing the level of vitamin D (25(OH)D, ng/mL) before and during COVID-19 restrictions. Data are expressed as median, $p < 0.001$.

Table 3. Vitamin D deficiency before and during the COVID-19 pandemic, according to age.

Vitamin D Status	Age Group 1–4 Years (n = 76)			Age Group 5–18 Years (n = 264)		
	before Pandemic	during Pandemic	p Value	before Pandemic	during Pandemic	p Value
Deficient (25(OH)D < 20 ng/mL)	6 (16.2%)	2 (5.1%)	<0.05	44 (33.6%)	17 (12.8%)	<0.001
Insufficient (25(OH)D 20–30 ng/mL)	15 (40.5%)	12 (30.8%)		52 (39.7%)	56 (42.1%)	
Sufficient (25(OH)D > 30 ng/mL)	16 (43.3%)	25 (64.1%)		35 (26.7%)	60 (45.1%)	
Total	37	39		131	133	

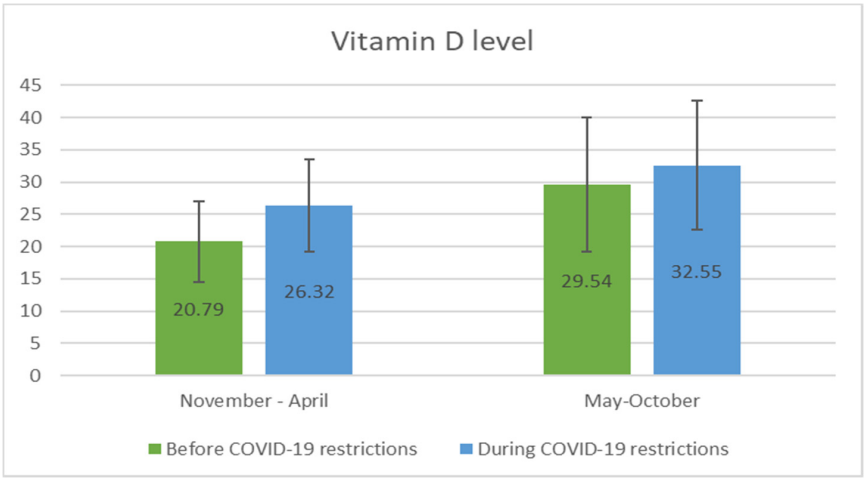

Figure 4. Box plot showing the level of vitamin D (25(OH)D, ng/mL) before and during COVID-19 restrictions, according to season of determination. The differences were statistically significant ($p < 0.001$).

4. Discussion

The present study showed a possible linkage between asymptomatic atopy and vitamin D status in children and adolescents in Greece. The evidence to date concerning the exact role of vitamin D in the immunological mechanism of atopy and allergy is conflicting. Measurement of serum total IgE is a cost-effective test that can be used as a first-line screening tool to identify the status of atopy, and certain health insurance systems require a serum level of total IgE of greater than 100 IU/mL to justify testing for specific IgE [21]. Whether the presence of atopy, defined by a high IgE level for age, turn into clinically manifested allergy depends on a complex interplay of several factors, including the family history of atopy, allergen specific IgE or IgG, IgE against specific epitopes and serum factors that are still unidentified [22]. Vitamin D plays a central role in modulating the immune functions involved in occurrence of asthma and other allergic disorders, but an evident linkage between vitamin D status and allergy or asthma is contradictory. Most of the available experimental and epidemiological data indicate an association between low levels of 25(OH)D and the development of inflammation in atopy, asthma, and other allergic diseases [23–25]. A causal association has been proposed but has not been clearly demonstrated.

In our study, we found that a higher percentage of asymptomatic children and adolescents with atopy had vitamin D deficient status than their peers without atopy (Table 1), clearly indicating that vitamin D deficiency is more common in atopy. In asymptomatic children with atopy, allergic disease is not yet apparent, but allergic inflammation that is already present is likely to progress to allergic disease with various clinical manifestations [9]. Among subjects with asthma, the level of 25(OH)D is inversely associated with the degree of worsening of airflow limitation [26]. Hence, the finding of allergic inflammation in the children with asymptomatic atopy, despite the absence of clinical manifestations might be linked with their low 25(OH)D levels. Furthermore, 25(OH)D could be a biomarker for the presence of allergic inflammation, which has the potential to progress to clinical manifestations of allergic disease. In the case of already established allergic diseases (e.g., asthma, allergic rhinitis, or food allergy), the level of 25(OH)D has been clearly shown to be lower than that in healthy individuals [27,28]. The same relationship has been revealed for other causes of lung inflammation, such as cystic fibrosis [29].

The incidence of atopic manifestations is typically higher among boys [7], and our results are in accordance with this gender difference. As in other studies [30], our study showed an inverse relationship between 25(OH)D level and age in healthy children and adolescents.

In a recently published study from the same geographical region, a small percentage (1.3%) of children with an established food allergy needed administration of a dosage of vitamin D above the recommended age-specific daily requirements to maintain the 25(OH)D level within normal range [31]. Baek and colleagues suggested that low serum vitamin D levels are associated with sensitization to food allergens [32]. They have also shown that vitamin D status represents an independent risk factor for severe atopic dermatitis. Further evidence is needed to define the exact role of vitamin D in allergic inflammation and the progression to overt allergic disease. We found that serum 25(OH)D level was significantly reduced in children and adolescents with asymptomatic atopy (Figure 1), and thus 25(OH)D might be a potential biomarker for the existence of allergic inflammation in the asymptomatic phase of the disease.

In our study no association was observed between 25(OH)D median level and IgE level or eosinophil count. Other studies have also reported lack of association between 25(OH)D level and eosinophil count [26], but Brehm and colleagues reported an inverse relationship between circulating levels of vitamin D and specific allergy markers, like eosinophil count and IgE [33]. Other studies demonstrated no association between vitamin D and levels of IgE in asthmatic children [34]. Raised IgE levels denote the presence of atopy and allergic inflammation. In asymptomatic children with atopy, the absence of clinical manifestations of allergy does not exclude the presence of allergic inflammation; on the contrary, at the molecular level there is already an immune process of balancing

towards the Th2 response. As previous research has shown that vitamin D is involved in Th2 inflammation [35,36], we would expect that in children with atopy, even in the absence of symptoms, the level of 25(OH)D would be low. Asymptomatic children who have low 25(OH)D levels could possibly benefit from correction of the 25(OH) level by vitamin D supplementation, but whether the normalization of the 25(OH)D level will affect the natural evolution of atopy in these children remains to be demonstrated by further studies.

The finding of a lower serum level of 25(OH)D in asymptomatic children with atopy leads us to believe that 25(OH)D is a biomarker of inflammation of the allergic type.

The increased levels of 25(OH)D observed during the pandemic could have several explanations, such as better dietary habits. One important factor could be the reduction in viral infections during that period, due to school closure and social distancing. The absence of infections, and therefore of viral inflammation, could result in higher levels of 25(OH)D in the COVID period compared with the same months of the pre-COVID era. This finding reinforces the idea that 25(OH)D is an index of inflammation in general. The prevalence of atopy, as defined by total IgE level increased for age, and the eosinophil count, did not change after the start of COVID-19 regulations.

The COVID-19 pandemic, apart from being a global public health crisis, created unique infectious epidemiological conditions, difficult to achieve in real life in other circumstances. Following the appearance of the first cases of COVID-19 and the introduction of the first measures to limit transmission, a decrease in respiratory viral infections has been observed in children [37]. During the H5N1 influenza virus epidemic, it was shown that the school closure during the holidays led to a 20–29% reduction in influenza transmission rate [38]. Shortly after the WHO declared the SARS-CoV-2 pandemic, the cases of influenza showed a sudden decrease, and the "flu season" ended earlier than usual. This trend, as the result of wearing facial masks and observing social distancing, has been demonstrated in several studies done in different regions of the world and for different respiratory viruses [39–42].

The connection between vitamin D and infections could be a two-way relationship, both cause and consequence. Viral respiratory infections may lead to a decrease in the vitamin D level, with longer and repeated infections resulting in a lower vitamin D level. Dogru and colleagues showed that in children with recurrent wheezing, the mean level of 25(OH)D was lower than in healthy control children and was negatively correlated with wheezing duration and numbers of wheezing episodes [43]. It is well established that the main cause of recurrent wheezing in childhood is successive viral respiratory infections. Some studies have shown no relationship or even a reverse relation [44]. An explanation for this could be that if the infections are not recurrent, or if the initial vitamin D status is sufficient, infection will not result in 25(OH)D reaching a deficient level. A recent meta-analysis conducted by Martineau et al. reported that vitamin D supplementation protected against acute respiratory infections, especially in patient's vitamin D deficient status [45]. Mitchel, in a recent publication, questioned whether low vitamin D levels are a cause or consequence of respiratory infections [46]. During inflammation, either viral or atopic, there is an increased demand for vitamin D, which is important for local immunomodulation processes. Being practically a hormone [47], the level of vitamin D is regulated by feedback; hence, if the demand is so high that the body's ability to produce it is exceeded, then the serum level of 25(OH)D will decrease. In such cases, it will be necessary to provide vitamin D supplement, and only in these cases, therefore, will supplementation favorably influence the evolution of the disease. This theory is supported by our study, where the absence of viral infections during the pandemic was associated with an increase in 25(OH)D levels (Figure 3), probably due to a decrease in demand for vitamin D. The presence of atopy, on the other hand, led to an increase in demand for vitamin D, resulting in a decrease in serum levels of 25(OH)D.

The UK National Institute for Health and Care Excellence (NICE) COVID-19 guidelines regarding vitamin D recommend that children aged 1–4 years should have a daily supplement containing 10 micrograms (400 units) of vitamin D throughout the year, while for those aged over 4 years this recommendation should be considered if they have little or

no sunshine exposure or have dark skin [48]. In contrast with these guidelines, in Greece no such recommendation was issued by any regulatory body. In our study, as children who had taken vitamin D supplements in the previous months were excluded, the finding of high levels of 25(OH)D is not due to supplementation. In addition, the 25(OH)D levels observed in our study would not lead to such a dramatic decrease in infections observed in the COVID period, which most likely was a result of the COVID measures. We believe that the causal relationship is that less infection results in higher levels of 25(OH)D and not the reverse. In viral respiratory infections, vitamin D is used, locally, by immunomodulatory cells, [47,49] and its consumption results in a decrease in circulating 25(OH)D. Theoretically, the decrease would be greater when the infections are more serious or recurrent. In the COVID period, infections decreased dramatically, and consequently, less vitamin D was consumed, and thus the level of 25(OH)D was higher than in the previous year when common respiratory viral infections were prevalent among children during the winter.

Further investigation will be necessary to determine whether an enhanced inflammation with deficient/insufficient vitamin D status, even in the absence of clinical manifestations of atopy increases the risk of inflammation-related injury. The possible beneficial effect of vitamin D supplementation during asymptomatic allergy related inflammation also remains to be demonstrated.

Strengths and Limitations of the Study

The main strengths of the study are that it presents the first real-life data investigating the link between the levels of 25(OH)D and total IgE in asymptomatic children and adolescents with atopy, and the differences in vitamin D status prior to and during the COVID-19 pandemic and its regulations. One limitation is the small sample size, and the lack of consecutive measurements. In addition, we were not able to investigate the possible influence of dietary habits on 25(OH)D level, time spent outdoors and indoors, and of sports activities, all of which may affect the level of 25(OH)D. In addition, we did not obtain a detailed history of the infections in the subjects, prior to and during the pandemic.

5. Conclusions

The study findings support a link between vitamin D status and different types of inflammation, allergic or infectious, as identified by total IgE status. Vitamin D deficiency was shown to be linked with atopy, defined as increased serum IgE level for age. Overall, the level of 25(OH)D was only slightly decreased in asymptomatic children and adolescents with atopy, compared with their healthy peers. During the pandemic, under the unprecedented circumstances consisting of almost complete absence of other infections, the serum levels of 25(OH)D were higher and fewer children were vitamin D deficient, probably due to a lower demand for vitamin D in immunomodulatory processes. Vitamin D deficient status may not be a risk factor for any types of inflammation, but rather a consequence of atopy or viral infection, through increased metabolic needs and its use by T and B lymphocytes, Th cells and macrophages in immunomodulation processes.

The level of 25(OH)D should be measured in asymptomatic children and adolescents with atopy, and if they are vitamin D deficient, the deficiency should be corrected by vitamin D supplementation, to change the natural history of allergic inflammation. This hypothesis will be of importance for developing preventive strategies for allergy in children.

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Comparative Protective Effect of *Nigella sativa* Oil and *Vitis vinifera* Seed Oil in an Experimental Model of Isoproterenol-Induced Acute Myocardial Ischemia in Rats

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Abstract: The study's aim was to characterize the composition of *Nigella sativa* seed (NSO) and grape seed (GSO) oils, and to evaluate their cardioprotective and anti-inflammatory effect on isoproterenol (ISO)-induced ischemia in rats. *Materials and Methods*: NSO and GSO supplements were physicochemically characterized. Liquid chromatography–mass spectrometry (HPLC-MS), Fourier-transform infrared spectroscopy (FTIR), and gas chromatography–mass spectrometry (GC-MS) analyses were used to determine the phytochemical composition in the oils. Total polyphenol content (TPC) and in vitro antioxidant activity were also determined. Pretreatment with 4 mL/kg/day NSO or GSO was administered to rats for 14 days. The experimental ischemia was induced by a single administration of ISO 45 mg/kg after 14 days. An electrocardiogram (ECG) was performed initially and 24 h after ISO. Biological evaluation was done at the end of experiment. *Results*: The HPLC-MS, GC-MS, and FTIR analyses showed that both NSO and GSO are important sources of bioactive compounds, especially catechin and phenolic acids in GSO, while NSO was enriched in flavonoids and thymol derivatives. Pretreatment with GSO and NSO significantly reduced ventricular conduction, prevented the cardiotoxic effect of ISO in ventricular myocardium, and reduced the level of proinflammatory cytokines and CK-Mb. *Conclusion*: Both NSO and GSO were shown to have an anti-inflammatory and cardioprotective effect in ISO-induced ischemia.

Keywords: black cumin oil; grape seed oil; cardiovascular disease; anti-inflammatory; antioxidant

1. Introduction

Cardiovascular diseases remain one of the leading causes of morbidity and mortality worldwide, regardless of socioeconomic status [1]. Acute myocardial infarction, which is

considered part of the coronary artery diseases spectrum, can be its first manifestation or can occur in its chronic evolution. Myocardial infarction is an acute condition produced by an imbalance between coronary blood supply and myocardial demand, leading to necrosis of the myocardium. Myocardial infarction is associated with an inflammatory response [2], production of oxygen-derived free radicals, and alteration of the extracellular matrix with tissue injury. All these processes lead to fibrosis and myocardial remodeling, responsible for arrhythmias and other cardiac complications [3,4]. Thus, an immediate and long-term treatment is necessary to control pathophysiological mechanisms to preserve a good function of the myocardium, to prevent the extension of myocardial lesions, and to reduce death caused by cardiovascular diseases [2].

Natural products have been used for centuries for the relief and cure of diseases. Natural products are complex and diverse, leading to numerous studies of medicinal plants and great progression in this domain in the past two decades, with more and more bioactive compounds being isolated and pharmacologically characterized. Some of these active compounds are used as drugs, either in their original or in a semisynthetic form, while others are used as natural supplements as classified under the wide umbrella of nutraceuticals [5]. Around 61% of the small-molecule drugs introduced in therapy worldwide between 1981 and 2002 were derived from natural products [6]. Thus, the discovery of new natural compounds with anti-inflammatory and antioxidant properties are still of great interest, especially for the prevention and treatment of cardiovascular diseases, representing the main cause of morbidity and mortality in the world.

Nigella sativa is an annual herbaceous plant that belongs to the botanical Ranunculaceae family. Its seeds contain many active compounds, with anti-inflammatory, anti-ischemic, antihypertensive, hypoglycemic, and cardioprotective effects [7,8]. Compounds from *Nigella sativa* have been shown to have effects on monocyte-derived macrophages, which are prone to take up oxidized low-density lipoprotein (LDL) and augment local inflammation. The effects of *Nigella sativa* in reducing atherosclerotic process were carried out by decreasing the level of proinflammatory mediators, released by primary macrophages. The biologically active compounds thymoquinone and carvacrol are supposedly the most important, which were demonstrated to have anti-inflammatory properties, radical-scavenging ability, and variable antioxidant activity [9,10].

Vitis vinifera (grapevine) is a perennial plant of the Vitaceae family widely used for grape and wine production. Grape seed oil, rich in phenolic compounds, fatty acids, and vitamins, has beneficial properties, mainly detected by in vitro studies. Its beneficial effects include the modulation of antioxidant enzyme expression, protection against oxidative damage in cells, antiatherosclerotic and anti-inflammatory effects, and protection against some cancer types [11,12]. Grape seed oil contains a large amount of phenolic compounds [11], including two stilbenes—resveratrol and piceatannol. The cardiovascular protection given by these derivates is based on the modulation of oxidative processes, inhibition of endothelial dysfunction, and induction of vascular endothelium-dependent vascular relaxation by redox regulation and nitric oxide (NO) production, thus resulting in an antiatherosclerosis effect, as well as on energy metabolism regulation, stress resistance, exercise, and fasting mimetics [13].

The aim of the present study was to characterize the main compounds identified in the oils of *Nigella sativa* seeds and *Vitis vinifera* seeds and their phenolic fraction, and to evaluate their cardioprotective effect on an animal model of ischemia induced by isoproterenol in rats.

2. Results

In the present study, the oils as raw materials, as well as their methanolic extracts, were characterized to have a complete overview of their composition in terms of bioactive compounds with a potential cardioprotective effect.

2.1. NSO and GSO Characterization

2.1.1. NSO and GSO Physicochemical Properties

Some of the relevant physicochemical characteristics of NSO and GSO oil samples such as refractive index, iodine value, free acidity, and peroxide value are presented in Table 1.

Table 1. Physicochemical properties of NSO and GSO.

No	Sample	Refractive Index	Iodine Index g I_2/100 g Oil	Free Acidity (%)	Peroxide Value O_2, mmol·kg^{-1}
1	NSO	1.466	70	1.2	<10
2	GSO	1.478	67	4.4	<10

These parameters, generally used as quality indicators in the case of edible oils, are in the same range as those identified in the literature for the same types of oils [14,15]. The only difference was in the case of the iodine index, having lower values in our study than those reported in the literature. This could be explained by several factors such as the technological process for obtaining the oils, the degree of maturation of the fruits and seeds from which they were extracted, or storage conditions.

2.1.2. NSO and GSO Phytochemicals Characterization

Figure 1 presents the general FTIR spectra (600–3100 cm^{-1}) for NSO and GSO. The tentative peak assignment is shown in Table 2.

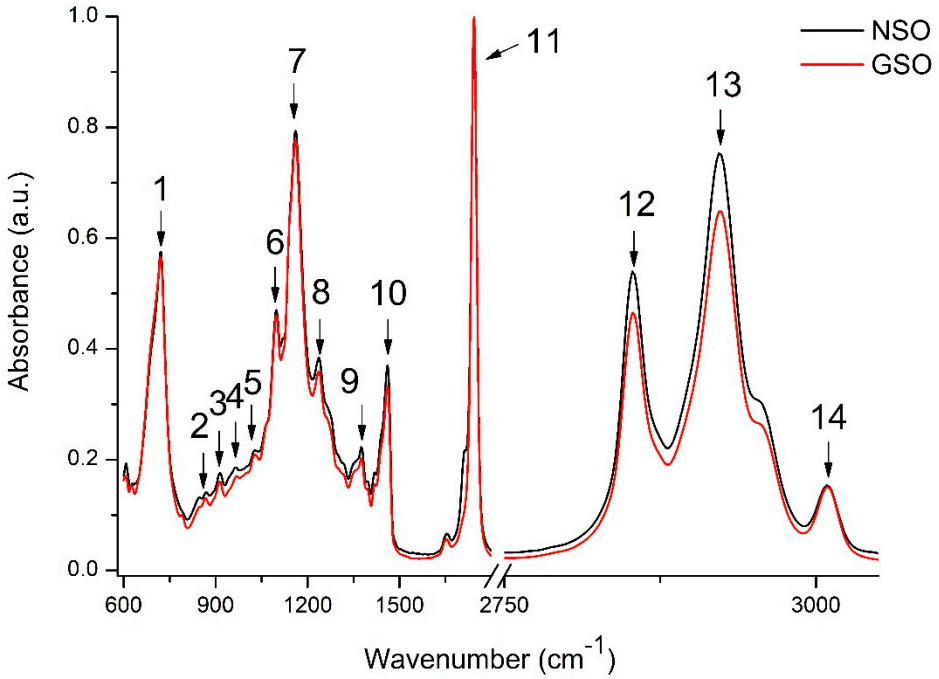

Figure 1. NSO and GSO general FTIR spectra (600–3100 cm^{-1}). For peak assignment, see Table 2.

Table 2. The tentative peak assignment for the general FTIR spectra (600–3100 cm^{-1}) for NSO and GSO.

No	Peak (cm^{-1})	Peak Intensity		Tentative Assignment
		NSO	GSO	
1	719	0.575	0.565	CH=CH– bending out of plane
2	866	0.141	0.131	$=CH_2$ wagging
3	914	0.176	0.161	–C–H bending out of plane
4	968	0.186	0.170	*trans* –CH=CH– bending out of plane
5	1028	0.217	0.209	–C–O stretch
6	1097	0.470	0.460	–C–O stretch
7	1161	0.794	0.776	–C–O stretch; $–CH_2$ bending
8	1236	0.384	0.360	–C–O stretch
9	1375	0.223	0.203	–C–H bending
10	1460	0.369	0.332	$–CH_2$ bending
11	1743	1	1	C=O stretching
12	2852	0.54	0.465	$–CH_2$ asymmetrical stretching
13	2924	0.753	0.648	$–CH_2$ symmetrical stretching
14	3008	0.154	0.151	(*trans* =C–H stretch)

As observed in Figure 1 and Table 2, the principal absorption bands of the FTIR spectra corresponded to the functional groups characteristic for edible fats and oils (peaks 1, 7, 9, 10, 11, 12, 13, and 14) [16–18]. Peaks 5, 6, and 7 could also be attributed to the C–OH stretching vibrations possibly identified in flavonoids [19]. Peak 6 could also be attributed to the absorption bands in the epigallocatechin, epigallocatechin gallate, and epicatechin molecules in the oils [20]. The literature data report that the asymmetric and symmetric stretching vibration of C–O in aromatic and –OH groups in the hydrolyzable tannins can be identified in several spectral regions, e.g., 1050 to 1165 cm^{-1} and 800 to 646 cm^{-1}, respectively [21] or 1326 to 1322 cm^{-1} and 1040 to 1036 cm^{-1}, respectively [21,22]. For proanthocyanidins, the region between 1320 and 1230 cm^{-1} was identified [21].

Furthermore, the chemical composition of the volatile compounds from NSO and GSO was assessed by GC-MS, and the identified molecules are presented in Table 3.

Table 3. Gas chromatography coupled with mass spectroscopy (GS–MS) chemical composition of volatile compounds identified in NSO and GSO.

No	Compounds	Retention Time	Concentration % of Total Peak Area	
			NSO	GSO
1	Hexanal	4.024	0.98	36.68
2	1-Butanol, 3-methyl-, acetate	5.995	-	48.55
3	α-Thujene	7.61	42.97	-
4	α-Pinene	7.853	8.25	3.16
5	Camphene	8.435	0.06	-
6	Sabinene	9.258	2.38	-
7	β-Pinene	9.439	4.96	-
9	Furan, 2-pentyl-	9.907	-	0.43
10	Hexanoic acid, ethyl ester	10.234	-	8.2
12	α-Terpinene	10.912	0.27	-
13	*p*-Cymene	11.227	33.71	-
14	D-Limonene	11.383	2.07	0.75
15	Eucalyptol	11.502	0.06	-
16	γ-Terpinene	12.504	0.64	-
17	Terpinolene	13.56	0.06	-
21	Octanoic acid, ethyl ester	17.874	-	1.34
22	Thymoquinone	19.84	1.9	-
23	Cuminone	20.651	0.4	-

The profile of volatile compounds identified in the oils is in accordance with that reported by the literature data [23,24] but with a different concentration profile. The concentration variability among samples can be explained by several factors, connected with oil extraction and the detection technique or with raw material genetic variability, origin, developmental stage, or environmental conditions. As Table 3 indicates, NSO contained α-thujene as the major compound, followed by *p*-cymene, α-pinene, and β-pinene, accounting for approximately 90% of the total compounds. Fewer compounds were identified in GSO, with hexanal and 1-butanol, 3-methyl-, acetate as the major compounds, representing approximatively 85% of total compounds.

2.2. NSO and GSO Extracts Characterization

The total polyphenol content of the two oils was assessed using the Folin–Ciocâlteu method. Thus, the total polyphenol content determined in the samples was 1.88 ± 0.01 mg gallic acid equivalents (GAE)/100 g NSO and 0.75 ± 0.01 mg GAE/100 g GSO. The total antioxidant activity, measured by the DPPH method, was 12,713.42 ± 156.41 mMT/100 g NSO and 1390.65 ± 1.45 mMT/100 g GSO. The results were in the range reported in the literature for both oils [25–27].

Next, the qualitative and quantitative profiles of the phenolic compounds extracted from NSO and GSO are shown in Figure 2 and Table 4. In total, 13 compounds were identified in the NSO extract and 16 were identified in the GSO extract. The identification was done by comparing compound retention times, UV/Vis absorption spectra, and the $[M + H]^+$ protonated molecules with those reported in the literature [8,24,28–33]. The identified compounds in the oil extracts belonged to various classes such as hydroxybenzoic acids (peak 1, 4), pavine alkaloids (peak 2), flavan-3-ol (peak 3), hydroxycinnamic acids (peak 5, 6, 7), flavonoid glycoside (peak 8), dicaffeoylquinic acids (peak 9, 10), tannins (peak 12,14 17, 22, 23), and monoterpenoid phenol (peak 11, 20, 21) (Table 4).

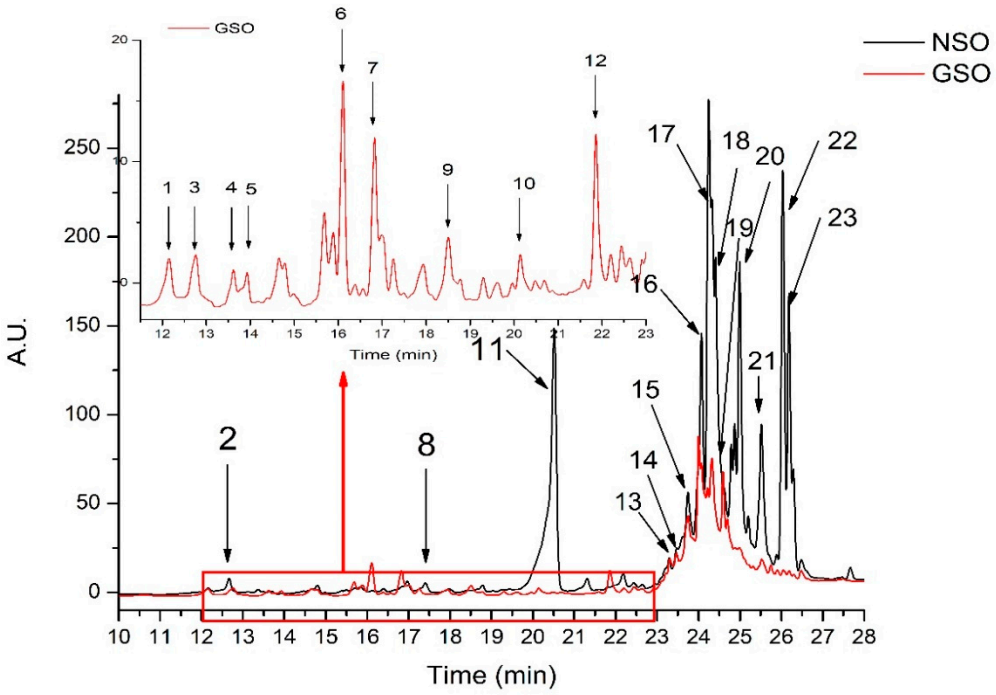

Figure 2. Comparative HPLC chromatograms of NSO and GSO. For peak assignment, see Table 4.

Table 4. Tentative identification, characterization, and concentration of major compounds identified in NSO and GSO extracts.

Peak No.	R_t (min)	UV λ_{max} (nm)	$[M + H]^+$ (*m*/*z*)	Compound	Concentration µg/mL Oil	
					NSO	GSO
1	12.15	275	139	*p*-Hydroxybenzoic acid	0.807	0.971
2	12.66	260	342	Norargemonine	1.167	-
3	12.75	280	291	Catechin	-	0.987
4	13.61	280	169	Vanillic acid	-	0.799
5	13.92	320	181	Caffeic acid	-	0.807
6	16.10	321	165	*p*-Coumaric acid	-	1.749
7	16.82	322	195	Ferulic acid	-	1.519
8	17.40	350, 260	755	Kaempferol-rhamnoside-diglucoside	-	1.118
9	18.77	320	517	Dicaffeoylquinic acid	-	1.110
10	20.13	320	517	Dicaffeoylquinic acid	-	0.872
11	20.51	290	194	Thymol derivative	17.105	-
12	21.86	280	867, 291	Procyanidin trimer possibly C2 (Catechin derivative)	-	1.413
13	23.29	350, 260	755	K	2.191	-
14	23.4	280	867, 291	Procyanidin trimer (Catechin derivative)	-	2.281
15	23.75	280	375	Hydroxymatairesinol	6.687	5.852
16	24.00	280	358	Matairesinol	10.692	4.337
17	24.21	280	1099, 1085	Tanin	14.656	2.748
18	24.32	280	375	Isohydroxymatairesinol	12.076	6.990
19	24.59	280	1120	Tanin (Catechin derivative)	3.927	3.878
20	24.99	290	150	Tymol	10.561	-
21	25.51	280	414	Tymol derivative	8.382	-
22	26.03	280	1142	Tanin	11.364	-
23	26.18	280	1040	Tanin	7.645	-

The next phase of the experiment was the investigation of the cardiac effects of NSO and GSO in the prevention of ISO-induced ischemia (measured by ECG and biochemical parameters), using the 32 rats that were included in the control and experimental groups. During the experimental follow-up, all rats survived.

2.3. The Effect of NSO and GSO on Electrocardiogram Parameters

The analysis of ECG parameters recorded at baseline showed no significant differences in the experiment group ($p > 0.005$) (Table 5). A representative ECG record for all groups is shown in Figure 3.

Table 5. Basal values of ECG parameters recorded at the beginning of the experiment. C (control group), C-ISO (ISO group), NSO + ISO (*Nigella sativa* seed oil and ISO group), GSO + ISO (grape seed oil and ISO group), HR (heart rate).

Group	HR (Beats/min)	RR (ms)	PR	QRS	QT	QTc	R
C	282 ± 19	223 ± 17	42 ± 2	34 ± 2	78 ± 3	65 ± 3	2.1 ± 0.1
C-ISO	287 ± 19	237 ± 16	41 ± 2	34 ± 4	78 ± 4	65 ± 3	2.1 ± 0.1
NSO + ISO	288 ± 15	225 ± 11	42 ± 2	35 ± 4	80 ± 4	65 ± 4	2.1 ± 0.1
GSO + ISO	283 ± 16	230 ± 9	42 ± 2	35 ± 4	78 ± 4	64 ± 3	2.1 ± 0.1

The second ECG evaluation performed on day 14 before ISO administration did not find significant differences between animals that received NSO or GSO for 2 weeks, along with no significant variation compared to basal records ($p > 0.005$).

Figure 3. Normal EGC record from first day of experiment.

Heart rate was significantly increased by ISO in all experimental groups compared to baseline records ($p < 0.001$) (Table 6). ISO administration increased heart rate compared to the control group ($p < 0.001$). Pretreatment with GSO prevented the increase in heart rate induced by ISO ($p \leq 0.036$), but this was not the case for NSO ($p = 0.267$).

Table 6. ECG parameters recorded after MI (day 14). C (control group), C-ISO (ISO group), NSO + ISO (*Nigella sativa* seed oil and ISO group), GSO + ISO (grape seed oil and ISO group), HR (heart rate).

Group	HR (Beats/min)	RR (ms)	PR	QRS	QT	QTc	R
C	271 ± 18	220 ± 16	42 ± 2	34 ± 2	78 ± 3	634 ± 4	2.1 ± 0.1
C-ISO	329 ± 15	186 ± 9	45 ± 2	53 ± 4	104 ± 63	94 ± 6	0.8 ± 0.1
NSO + ISO	315 ± 6	190 ± 4	43 ± 2	53 ± 4	95 ± 4	85 ± 3	1.1 ± 0.1
GSO + ISO	299 ± 15	201 ± 11	43 ± 2	49 ± 7	95 ± 4	82 ± 4	1.2 ± 0.1

PR, QT, and QTc intervals were increased after ISO administration compared to baseline records ($p < 0.001$), while RR interval and R wave amplitude were reduced ($p < 0.001$) (Figure 4). PR interval was increased by ISO administration compared to the control group ($p = 0.004$). Pretreatment did not prevent the increase in PR interval (NSO, $p = 0.152$; GSO, $p = 0.115$). The QRS complex was enlarged in all animals that received ISO compared to the control group ($p < 0.001$). NSO and GSO did not significantly prevent the enlargement of the complex ($p = 0.994$ and $p = 0.204$, respectively).

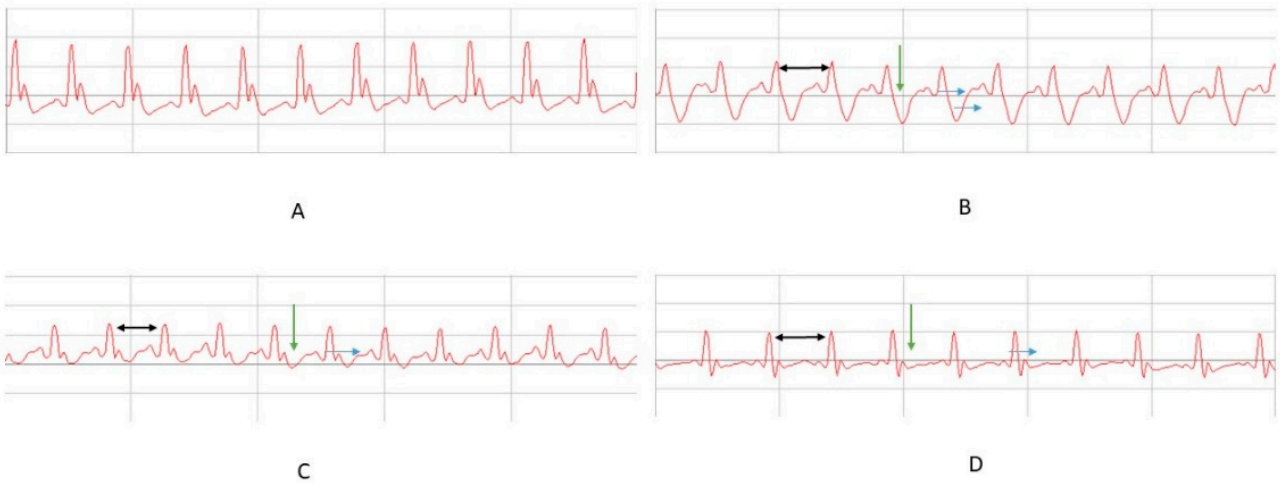

Figure 4. The ECG records in experimental groups with (**A**) C group, (**B**) C-ISO group, (**C**) NSO + ISO group, and (**D**) GSO + ISO on day 15 after MI induction 24 h after ISO administration (groups 2 to 4): increased RR interval (black arrow), ST-segment depression (green arrow), QT interval prolongation (blue arrow). The amplitude of these changes was different in the experimental groups.

The increase in QT and QTc intervals was significantly reduced by NSO and GSO pretreatment compared to the positive control group treated with ISO ($p < 0.001$). There were no differences between experimental substances ($p = 0.998$, respectively $p = 0.504$).

RR interval was significantly reduced in animals that received only ISO ($p < 0.001$). If compared to group 2, after ISO administration, the animals from groups 3 and 4 showed an enlargement of the RR interval, but the enlargement was significant only in the group treated with GSO ($p = 0.036$), not in the case of NSO ($p = 0.818$). Regarding the amplitude of the R wave, it was decreased by ISO administration compared to the control group ($p < 0.001$). Both NSO and GSO significantly prevented the reduction in its amplitude ($p < 0.001$), without differences between them.

2.4. The Effect of NSO and GSO on Biochemical Parameters

Alanine aminotransferase (ALT) level was not influenced by the induction of myocardial infarction or by pretreatment with NSO and GSO ($p > 0.05$) (Figure 5). ALT was significantly increased by ISO administration compared to the control group ($p = 0.001$). Both NSO and GSO significantly reduced the augmentation of aspartate aminotransferase (AST) after induction of acute myocardial infarction ($p = 0.001$, respectively $p < 0.001$), without any difference between NSO and GSO ($p = 0.785$) (Figure 5).

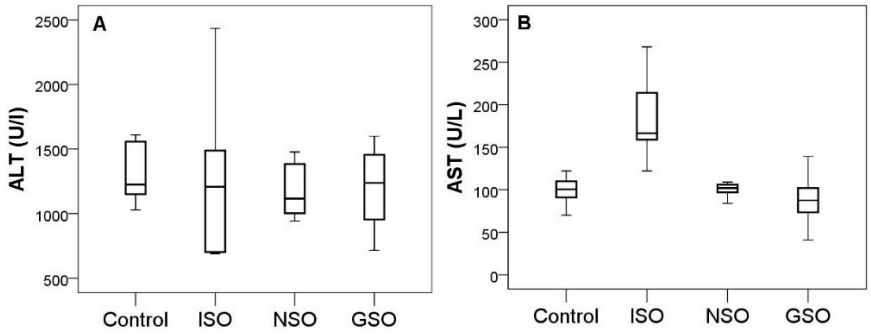

Figure 5. ALT (**A**) and AST (**B**) serum levels in the experimental groups.

2.5. The Effect of NSO and GSO on Cardiac Enzyme Activity

Troponin level was increased by ISO administration in all experimental groups (Figure 6). Pretreatment with NSO or GSO reduced augmentation of troponin, but the differences did not reach the level of statistical significance in experimental groups ($p > 0.05$). The reduction was more pronounced in group 4 that received GSO. The myocardial fraction of creatine kinase (CK-MB) significantly increased at 24 h after ISO administration ($p = 0.001$). Both NSO and GSO prevented the increase in CK-MB ($p < 0.001$), without differences between them ($p = 0.993$).

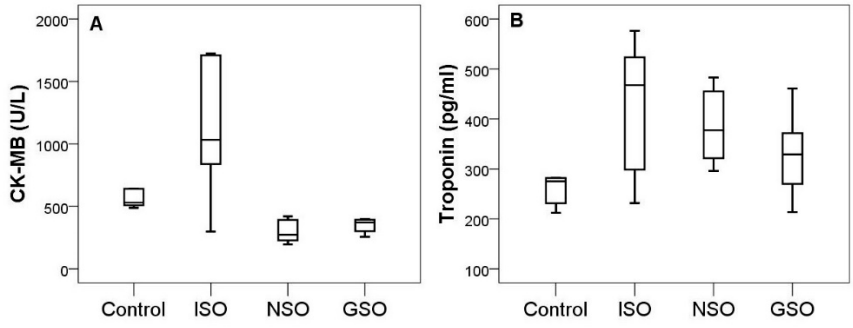

Figure 6. Serum levels of cardiac enzymes CK-MB (**A**) and troponin (**B**) in the experimental groups.

2.6. The Effect of NSO and GSO on Inflammatory Markers

Isoproterenol significantly induced an increase in proinflammatory cytokines compared to the control group (interleukin-6 (IL-6), $p = 0.002$; interleukin-1β (IL-1 β), $p = 0.03$, and tumor necrosis factor-alpha (TNF-α), $p < 0.001$) (Figure 7). NSO significantly decreased the upregulation of IL-1β ($p = 0.024$), IL-6 ($p = 0.016$), and TNF-α ($p < 0.001$) compared to the positive control group that received only ISO. Similar results were also observed in animals treated with GSO, which significantly reduced the inflammatory markers IL-6 ($p = 0.005$), IL-1β ($p = 0.0047$), and TNF-α ($p < 0.001$). There were no significant differences between NSO and GSO in reducing proinflammatory markers ($p > 0.005$ in all comparisons).

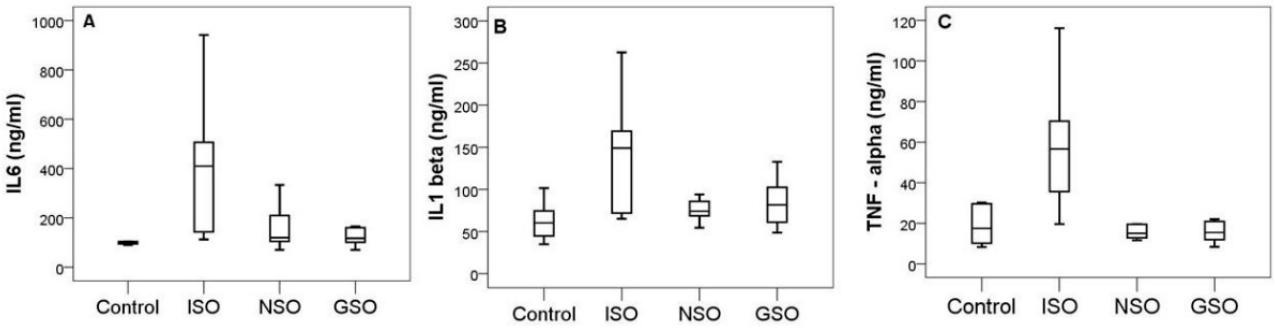

Figure 7. Serum levels of inflammatory markers IL6 (**A**), IL 1beta (**B**) and TNF-alpha (**C**) in the experimental groups.

3. Discussion

Isoproterenol, a nonselective beta-agonist, administered in high doses, causes severe biochemical, functional, and histological alterations in the heart, comparable with those taking place in human myocardial infarction [3,34]. These changes are consequences of oxidative stress and inflammation, which alter tissue antioxidative defense systems in the myocardium [34–36]. Considering the above, antioxidant and anti-inflammatory substances may protect myocardial cells from myocardial infarction damage [34,37].

The isoproterenol ischemic model is characterized by reduced RR interval and increased heart rate [3,38]. It also prolongs the QRS complex, increases QT and QTc intervals, increases duration of the PR segment, and reduces R wave amplitude, thus representing ECG changes that reflect the occurrence of myocardial infarction and conduction disturbances of impulses secondarily to ischemia and due to ISO's cardiotoxic effect [39,40]. Furthermore, the reduction in R wave amplitude reflects a low conduction due to an inflammatory edema associated with ischemia [3]. A prolongation of PR interval reflects a slow conduction in the AV node or a fibrosis in this structure [39]. Isoproterenol also significantly increases cardiac enzymes and proinflammatory markers, sustaining the role of inflammation in cardiac ischemic lesions. Isoproterenol-induced ECG changes specific to acute myocardial infarction could be a result of the loss of action potential in the myocardial cell membrane, because of oxidative stress and inflammation [3].

Pretreatment with GSO and NSO did not influence the duration of the QRS complex or PR interval, but reduced the prolongation of QT and QTc intervals and prevented the reduction in R wave amplitude induced by ISO. This is an argument for the assumption that both investigated oils do not affect the atrioventricular conduction, but partially influence ventricular conduction, thereby preventing the cardiotoxic effect of ISO. GSO, but not NSO, prevented the heart rate increase and RR interval reduction induced by ISO, which may contribute to the cardioprotection conferred by grapes products in acute ischemia.

Few published data investigated the role of NSO and GSO in myocardial ischemia. Most studies evaluated the atherosclerotic process or separately evaluated the pharmacological effects of their main constituents, i.e., thymoquinone from *Nigella sativa* [41] and resveratrol from grapes [42]. For example, long-term treatment with NSO or methanolic extracts (8–12 weeks) reduced atherosclerotic plaque formation in coronary arteries [43,44] and, through this mechanism, showed a possible protective effect in coronary artery disease,

by preventing acute myocardial ischemia. In producing these effects, different mechanisms are supposedly implicated, involving serotonergic, muscarinic, and adrenergic systems [41]. In the present experiment, the heart rate was increased, while RR the interval was reduced by ISO, whereas NSO could not prevent these effects. In Xiao's study [45], thymoquinone, NSO's main constituent, significantly reduced heart rate in a similar experimental model, which might validate the hypothesis of a complex mechanism reported by Ojha et al. [41]. Pretreatment with NSO did not influence QRS complex duration and PR intervals, highlighting the hypothesis that NSO could have a preventative atherosclerosis effect rather than preventing the acute consequences of a beta stimulation of the heart.

Compared to NSO, GSO significantly prevented the heart rate increase and RR interval reduction induced by ISO, which may contribute to the cardioprotection conferred by grapes in acute ischemia.

GSO, like NSO, did not influence the QRS complex and PR interval. In a previous study, Badavi et al. showed that grape seed extract did not influence the heart rate and blood pressure [46], while Tiwari et al. showed that only myricetin, a flavonoid compound extracted from red wine, administered for 21 days significantly inhibited the effects of ISO on heart rate and ECG changes, as reported in the present study [47].

Both NSO and GSO reduced the prolongation of the QT interval and its corresponding value of QTc. Another possible mechanism underlying the cardioprotective effect of *Nigella sativa* could be calcium blockade [48]. Calcium is essential in maintaining the plateau phase of the action potential, and it is a potential contributor to QT interval duration. In the present study, NSO significantly reduced the prolongation of the QT interval in acute myocardial infarction, which might be related to a potential calcium blockade. The cardioprotective effect of GSO was also linked with calcium loading in the myocardium in another two models of cardiotoxicity, induced by doxorubicin [49] and by a high-fat diet [50]. We may raise the hypothesis that the NSO and GSO cardioprotective effects on acute ischemia could be partially related to calcium concentration in the myocardium. Further studies are needed to better understand the exact mechanism of action of NSO and GSO, as well as of their main constituents.

In Al Assom et al.'s study, long-term treatment with *Nigella sativa* induced coronary angiogenesis and had no effect on inducible NO synthase level, demonstrating a possible cardioprotective effect [51]. Olas et al. reported that GSO decreased platelet adhesion in vitro, and the effect was more intense than after resveratrol [52]. Sano et al. showed that GSO reduced oxidized LDL in 61 healthy subjects, suggesting its cardioprotective potential [53]. According to these observations, we suppose that both NSO and GSO may have a cardioprotective effect by modulating endothelial function and platelet aggregation rather than by reducing adrenergic stimulation of the heart.

On the other hand, thymol induced a dose-dependent negative inotropic action on the isolated heart. El-Tahir et al. demonstrated that *Nigella sativa*'s chronotropic and hypotensive effects were mediated centrally either directly or indirectly via mechanisms involving serotoninergic and muscarinic receptors [54].

Increasing evidence supports that cardiovascular diseases have an inflammatory component in their pathophysiology [55]. Several studies have described an increased expression of proinflammatory cytokines IL-1β, IL-6, and TNF-α in ISO-induced myocardial infarction [3,35,55,56]. Beta-blockers have a beneficial effect on myocardial injury, reducing the myocardial expression of proinflammatory cytokines IL-1β, IL-6, and TNF-α [41,57]; thus, proinflammatory cytokines can be used as markers to evaluate the cardioprotective effect of some natural products. Proinflammatory cytokines act as pleiotropic polypeptides that are independently associated with inflammation and oxidative stress and the release of these cytokines leads to myocardial injury through several mechanisms [55].

In the present study, a significant increase in plasmatic levels of IL-1 β, IL-6, and TNF-α was noted after administration of ISO, in agreement with previous studies [3,35,55,56]. Pretreatment with GSO and NSO significantly reduced the level of proinflammatory cytokines, suggesting a clear anti-inflammatory effect in the ischemic heart. The reduction

was more intense in the group with NSO pretreatment than that with GSO pretreatment, for IL-1β and TNF-α.

Similar results were also reported by Ojha et al. for thymoquinone pretreatment in acute ischemia induced by ISO. We may assume that the anti-inflammatory effect of NSO in ISO-induced acute ischemia is a consequence of its main component thymoquinone. Twenty-eight days of pretreatment with grape seed methanolic extract reduced the myocardial expression and levels of IL-1β, Il-6, and TNF-α and decreased the oxidative stress in the infarcted and non-infarcted heart of diabetic rats, suggesting that the cardioprotective effects of grapes are a consequence of anti-inflammatory and antioxidant properties [58]. In the present study, the authors noticed the same reduction in proinflammatory cytokines, but in the serum not in the myocardium, after 14 weeks of therapy with GSO, supporting the anti-inflammatory effect of grapes. A 2 week supplementation with red wine grape pomace reduced premature death, changed TNF-α and IL-10 levels, increased plasma antioxidant activity, and attenuated myocardial infarction and dysfunction, confirming the cardioprotective effect of red grape products in ischemic heart disease [59]. We may conclude that both GSO and NSO exerted their cardioprotective effects partially through their anti-inflammatory actions.

Cardiac tropinins and serum CK-MB are used for the diagnosis of myocardial injury [60]. In the present study, the serum levels of T-troponin and CK-MB were significantly increased in 24 h after the onset of ISO-induced myocardial infarction. Pretreatment with GSO and NSO could not prevent myocardial damage and the corresponding increase in T-troponin, but it significantly reduced CK-MB. These results are different from previously published data. Danaei et al. reported a reduction in troponin level in dianozin-induced cardiotoxicity by thymoquinone treatment for 28 days [61]. The different results could be explained by the use of ISO in inducing the ischemia, which is another type of experimental model, as well as by the use of oil in the present study as compared with its main constituent thymoquinone in Danaei's study. Higher doses are probably needed to obtain an optimal concentration of thymoquinone to prevent myocardial cell damage. Regarding the effect of GSO on troponin level, our results also do not conform to previously published data. A significant reduction in troponin-I and CK-MB levels was reported by Giribabu et al., who used diabetic rats that received ISO after 28 days of pretreatment with methanolic grape extract [58]. By comparison, we determined the level of T-troponin involved in myocardial contraction, not I-troponin, and the duration of therapy was lower. Sun et al. reported that only resveratrol 100 mg/kg and resveratrol nanoparticles conferred a cardioprotective effect by reducing cardiac troponin T (cTnT) levels after ISO-induced ischemia [62].

Previously published data sustained the hypothesis that both NSO and GSO may have cardioprotective effects, but the mechanisms are more complex and depend also on the type of extract, the isolated active compounds, and the route of administration, which might influence their bioavailability. On the basis of these results, we may assume that higher doses of these dietary supplements, over a longer term, are needed to have more consistent results in terms of cardiac damage prevention. We did not evaluate their pharmacokinetics to verify whether obtaining higher levels of active compounds would lead to a different result. In the present study, the complex analysis of NSO revealed the presence of high concentrations of flavonoids and thymol derivatives, while thymoquinone represented only 1.9% of the bioactive compounds identified in NSO. The analysis of GSO identified more catechin and phenolic acids than in the case of NSO. The most notable bioactive property of phenolic compounds is their antioxidative and anti-inflammatory capacities, as demonstrated in the present study. Xia et al. reported that grape seed possesses the greatest antioxidant activity, which is related to its high content of gallic acid, catechin, epicatechin, procyanidins, and proanthocyanidins and their synergistic effect with phenolic compounds [63]. This hypothesis could be sustained by the present study. Even though the total phenolic content was lower for GSO than for NSO, the cardioprotective effect of GSO was more pronounced, and we may assume that this effect is a consequence of

the synergistic effects of catechin, procyanidins, and phenols identified in the used GSO. In contrast, the anti-inflammatory effect was more intense in the case of NSO, and this is probably related to the thymoquinone and thymol derivatives in combination with phenols. Previously published data revealed that both thymoquinone and thymol possess anti-inflammatory activities. We may conclude that in vitro activities do not always correlate directly with in vivo effects, and natural supplements should be analyzed as a function of their total complexity, because interactions between active biocompounds may lead to more significant clinical effects.

The main strength of this study was the comparative analysis of the cardioprotective and anti-inflammatory effects of two natural supplements, as entire compounds, in ISO-induced experimental ischemia. This study also had some limitations. Firstly, a histological examination to confirm the size of ischemic lesions on the myocardium was not performed. Secondly, only the in vitro antioxidant effect of NSO and GSO was determined.

4. Materials and Methods

4.1. Chemicals

Acetonitrile, methanol, and acetic acid were purchased from Merck (Darmstadt, Germany). All other chemicals used for physicochemical oils characterization and sample extractions were of analytical grade and were purchased from Sigma-Aldrich (Steinheim, Germany). Water and isoprenaline hydrochloride (98%) for acute myocardial infarction induction were purchased from Sigma–Aldrich (St. Louis, MO, USA). Commercial dietary supplements, AquaNano Negriol in cold-pressed oil form, extracted from *Nigella Sativa* seeds, and Solaris grape oil, from the cold-pressed seed of *Vitis vinifera*, were purchased from the pharmacy and used as experimental substances.

4.2. Oil Physicochemical Characterization

For the physicochemical characterization, the following parameters were investigated: the refractive index, the iodine index, the free acidity, and the peroxide value.

4.2.1. Refractive Index

The refractive index was directly determined with the Abbe Refractometer using the apparatus according to the Association of Official Analytical Chemists methods. The refractive index is directly proportional to the degree of unsaturation and is also affected by the oxidation, free fatty-acid content, and thermal treatment [64,65].

4.2.2. Iodine Index

The iodine index, free acidity, and peroxide value were determined by titrimetry. The iodine index gives information on the unsaturation degree. The double bonds in fat molecules react with iodine. The amount of iodine in grams consumed by 100 g of oil represents a measure of oil and fat unsaturation. The determination was based on American Oil Chemists' Society methods [65].

4.2.3. Free Acidity

Free acidity represents the number of milligrams of potassium hydroxide needed to neutralize the free acids found in 1 g of sample. The determination was based on American Oil Chemists' Society methods [65].

4.2.4. Peroxide Values

Peroxide values give information regarding an oil's incipient rancidity and conservation state. It is expressed by the number of milliliters of sodium thiosulfate (0.002 N) consumed by 1 g oil according to American Oil Chemists' Society methods [65].

4.3. Oil Phytochemical Characterization

The FTIR analysis and in-tube extraction technique (ITEX) coupled with GC-MS analysis was used to determine the phytochemical composition of the oils as described in Pop et al. [8].

4.3.1. FTIR Analysis

The FTIR spectra of NSO and GSO were measured using a Shimatzu IR Prestige-21 (FTIR) spectrometer. The oils were measured directly on the attenuated total reflectance crystal between 4000 and 650 cm^{-1}. Between measurements, the crystal was cleaned with acetone. The identification of specific IR frequencies of functional groups was done in accordance with literature data.

4.3.2. ITEX–GC-MS Analysis

ITEX–GC-MS was performed using a GC-MS Shimadzu model QP-2010 (Shimadzu Scientific Instruments, Kyoto, Japan) on a ZB-5 ms, 30 m × 0.25 mm i.d. × 0.25 µm capillary column (Phenomenex, Torrance, CA, USA). The volatile oil extraction was done using 0.2 g of oil placed in a headspace vial at 60 °C under continuous stirring (500 rpm) for 20 min. The results were expressed as a percentage of total peak area. The tentative compound identification was performed by comparing the mass spectra and fragmentation patterns of the compounds with those indicated by the software's NIST27 and NIST147 mass spectral libraries. The retention indices were also compared with those indicated by the websites www.pherobase.com (accessed on 12 January 2021) or www.flavornet.org (accessed on 12 January 2021), taking into consideration columns with a similar stationary phase. A minimum of 85% similarity was taken into consideration.

4.4. Oil Extraction

The phenolic compounds in NSO and GSO (2.5 g each) were extracted using 3 mL of *n*-hexane and 4 mL of methanol/water solution (60:40; *v*/*v*). The mixture was vortexed and further sonicated for 15 min using a water bath. Afterward, the samples were centrifuged at 8000 rpm for 5 min. The methanolic phase was further collected and washed three times with 4 mL hexane using the same procedure as described above (vortex and centrifugation). Lastly, the mixture was evaporated to dryness by nitrogen flushing. Right before the quantitative and qualitative determinations, the residue was resuspended in 1 mL of MeOH.

4.5. Oil Extract Characterization

The oil extracts were characterized quantitatively regarding their TPC and antioxidant activity and qualitatively to identify the characteristic phenolic compound composition as previously described [8].

4.5.1. Total Polyphenol Content

TPC was determined using the Folin–Ciocâlteu method [66]. The results were expressed as GAE in mg/100 g oil. Triplicate analysis was performed for each oil extract. The results of triplicate analysis were expressed using their mean values ± standard deviations.

4.5.2. Antioxidant Activity

The antioxidant activity test as determined by the DPPH radical-scavenging activity of the oil extracts was evaluated following the Brand-Williams method [67]. The results were expressed as mM Trolox/100 g oil. The results of triplicate analysis were expressed using their mean values ± standard deviations.

4.5.3. HPLC-MS Analysis

The liquid chromatography–diode array detection–electrospray ionization mass spectrometry (HPLC–DAD–ESI MS) method was used to identify the phenolic compounds as

described in Pop et al., 2020. An Eclipse XDB C18 column (4.6 × 150 mm, 5 µm particle size) (Agilent Technologies, Santa Clara, CA, USA) was used for separation. A gradient elution of two mobile phases was used for compound separation. Mobile phase A was 0.1% acetic acid/acetonitrile (99:1) in distilled water (*v*/*v*), while mobile phase B consisted of 0.1% acetic acid in acetonitrile (*v*/*v*). The mass spectra of the investigated molecules were scanned from 100 to 1000 *m*/*z*. The NSO and GSO compounds were expressed as GAEs (R^2 = 0.99). Triplicate analysis was performed. The compound identification was performed by comparing specific UV/visible spectra, retention time, and mass spectra with authentic standards (when available) and literature data.

4.6. Animals

The protocol of the experiment was approved by the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca (3850/2020) and by the Sanitary-Veterinary and Food Safety Directorate from Cluj-Napoca (206/01.04.2020), following the Helsinki Declaration on Animal Studies. The national and international guidelines referring to animal care and use were followed.

Thirty-two Wistar-Bratislava male rats were randomly divided into four groups of eight animals. The animals with a body weight between 200 and 260 g were kept for the entire experiment at the Biobase of "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca. During the experiment, the animals were kept in standard conditions for accommodation, in polypropylene cages, which were acclimated at standard environmental conditions of 22–24 °C, humidity 55% ± 15%, and a 12 h/12 h light/dark cycle. All animals had ad libitum access to water and standard pellets (Cantacuzino Institute, Bucharest, Romania). Two hours before the experiment, the rats were not fed, but water was available ad libitum.

4.7. Experimental Protocol of acute Myocardial Infarction

The rats were randomly divided into four groups of eight animals. The animals received saline solution, NSO, or GSO for 14 days as follows:

- Group 1 (Control group)—saline solution 0.4 mL/100 g;
- Group 2 (ISO)—saline solution 0.4 mL/100 g;
- Group 3 (NSO)—*Nigella sativa* seed oil 0.4 mL/100 g;
- Group 4 (GSO)—Grape seed oil 0.4 mL/100 g.

Saline solution and experimental substances were administered orally by gavage.

The animals from groups 2–4 subcutaneously received a single dose of ISO (45 mg/kg) on day 14 of the experiment. The dose that was used was previously tested and considered the minimal dose producing acute myocardial infarction with elevation of cardiac enzymes, such as troponin [3,68].

At the end of the experiment, the rats were sacrificed by an overdose of anesthetics.

4.8. Electrocardiography

ECG was recorded initially at the beginning of the experiment, on day 14 before ISO administration and 24 h after ISO-induced myocardial infarction, according to a previously described protocol. Intraperitoneal injections of ketamine (26 mg/kg) and xylazine (2.6 mg/kg) were used to anesthetize the rats. To bind the electrodes on the paw pads of the rat, the animals were fixed in a supine position on a board, 15 min after the induction of anesthesia. From there, at lead II (right forelimb to left hind limb), the ECG was recorded using a Biopac MP36 system.

Calibration at 1 mV/1 cm and a speed of 50 mm/s were set for the ECG apparatus [39]. The Biopac Student Lab 3.7.7 software was used to calculate the RR and QT intervals (ms), PR segment (ms), QRS complex duration (ms), and R wave amplitude (mV) [3] from the ECG recordings.

Furthermore, the heart rate of each rat was calculated from the given RR intervals. For that, the following formula was used: HR = 60,000/RR. In addition, the Bazett formula aided with the calculation of the corrected QT intervals (QTc in ms) [69].

4.9. Biologic Evaluation

The blood samples were taken from retroorbital plexus at the end of the experiment. One milliliter of blood on anticoagulants was preserved, and the serum was obtained through centrifugation within first hour. The obtained serum samples were kept at −80 °C until determinations were performed.

The serum levels of AST, ALT, and CK-Mb were determined through a spectrophotometric method using an automatic analyzer Applied Biosystem (Costa Brava, Barcelona (Spain)).

The serum levels of the inflammatory cytokines TNF-α, IL-6, and IL-1β were measured using the ELISA Stat Fax 303 Plus Microstrip Reader (Minneapolis, MN, USA) with commercially available kits (rat TNF-α, IL-6, and IL-1β ABTS ELISA Development kits, PeproTech EC, Ltd., London, UK). Troponin was also measured using an Elabscience ELISA kit. The determinations were done according to the manufacturer's instructions. For each assay, samples were diluted as needed, and protein levels were calculated using a four-parameter logistic (4-PL) curve fit.

4.10. Statistical Analysis

The statistical analysis was performed using SPSS version 19 (Chicago, IL, USA). Data were labeled as continuous variables. Normal distribution for continuous variables was determined using the Kolmogorov–Smirnov test. The results were expressed as the mean and standard deviation (for variables with a normal distribution) or as the median and 25th–75th percentiles (for variables with non-normal distribution). We used one-way ANOVA with Tukey correction and Spearman's rho correlation coefficient for univariate analysis of continuous variables. The level of statistical significance was set at $p < 0.05$.

5. Conclusions

In the present study, the investigated dietary supplements, NSO and GSO, partially prevented ECG alterations and the modification of biological and inflammatory parameters after ISO-induced myocardial infarction. The effects on ECG changes were more pronounced in animals treated with GSO. Both NSO and GSO were shown to have an anti-inflammatory and cardioprotective effect in ISO-induced ischemia. Both compounds were shown to have good potential for future treatment options in cardiovascular diseases.

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Sample Availability: Samples of the compounds NSO and GSO are available from the authors.

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Could FeNO Predict Asthma in Patients with House Dust Mites Allergic Rhinitis?

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Abstract: *Background and Objectives*: The evolution of allergic rhinitis to asthma is a part of "atopic march". The aim of this study was to analyze possible predictive markers for asthma occurrence in patients with allergic rhinitis to house dust mites (HDM). *Materials and Methods*: Fifty-eight patients with persistent allergic rhinitis (PAR) were included. The clinical, biological evaluation and fractionated exhaled nitric oxide (FeNO) measurement were performed at enrolment. The patients were clinically evaluated after one year to determine asthma occurrence. *Results:* The severity of rhinitis symptoms, levels of total immunoglobulin E (IgE), ICAM-1, VCAM-1, E-selectin and IL-6, but not IL-8 and TNF-α were higher in patients with allergic rhinitis who developed asthma compared to non-asthmatics, but the differences were not significant to considered them as predictive factors for asthma occurrence. The risk of asthma was independently influenced by patients aged over 30 years ((OR-3.74; CI95% 0.86–16.31; $p = 0.07$), a duration of allergic rhinitis over 12 months ((OR-4.20; CI95% 0.88–20; $p = 0.07$) and a basal FeNO over 28 parts per billion (pbb) ((OR-18.68; CI95% 3.79–92.05; $p < 0.001$). *Conclusion*: Clinical and biological parameters may predict asthma occurrence in patients with persistent allergic rhinitis to HDM. Adult patients with a longer duration of rhinitis symptoms and a high level of FeNO have a greater risk to develop asthma.

Keywords: allergic rhinitis; allergic inflammation; asthma; FeNO; house dust mites

1. Introduction

Allergic rhinitis is the most frequent IgE-mediated disease and its prevalence is increasing in the last decades [1,2]. Allergic rhinitis is a risk factor for asthma development and may be clinically relevant before or after asthma diagnosis [3]. Allergic inflammation is the key of understanding these diseases and the evolution of allergic rhinitis to asthma [2,4,5].

Allergen exposure leads to mast cell degranulation in nasal mucosa and the release of mediators, mainly histamine and leukotrienes. Cytokines released from Th2 lymphocytes are responsible for inflammatory cells recruitment in affected tissues via adhesion molecules, like E-selectin, ICAM and VCAM. The recruited cells, eosinophils, neutrophils and Th2 lymphocytes, are responsible for producing more proinflammatory cytokines (IL-1β, TNF-α, IL-3, IL-4, IL-5, IL-6 and IL-8), augmenting the inflammation in airways and injury through formation of toxic reactive nitrogen species [5–8].

The evolution from allergic rhinitis to asthma was reported in several studies, and it is a part of "atopic march" [2,9–11]. Allergic rhinitis is a risk factor for developing asthma [12], especially if the onset is severe and occurs in childhood [13]. Those two diseases often coexist and represent "a single airways allergic disease" [2,9]. This concept of "united airway disease" raises the question "Which patients with allergic rhinitis will develop asthma?"

The relationship between upper and lower airway inflammation is not completely understood yet. Genetic studies show that asthma and allergic rhinitis partly coexist because they share many genetic risk variants that dysregulate the expression of immune-related genes [14]. But not only genetic factors are important, environmental ones (allergens exposure) might also contribute to this evolution. The concept of a "united airway disease" could be explained through the migration of inflammatory cells and mediators from nasal secretions to lower airways, by inhalation and aspiration, acting as triggers of inflammation in the lower part [9,15,16]. Other additional mechanisms along with inflammation might contribute to asthma occurrence, including nasal bronchial reflex and alteration of physical filter function of the nose, which can induce bronchial hyperreactivity even in non-atopic patients [16–18].

The clinical aspect of asthma is variable, from a classical description (chest tightness, dyspnea, wheezing and cough) to only chronic cough or dyspnea to mild physical effort [7,15]. Some patients with allergic rhinitis may present rare, mild asthma symptoms, which are not related to rhinitis severity and could be actually a matter of perception of asthma symptoms, like dyspnea [7].

FeNO is a marker of lower eosinophilic inflammation in allergic diseases, especially in asthma. FeNO measurement is used for asthma diagnosis, to differentiate its phenotype and to monitor treatment response [19,20]. In asthmatic patients, a high nitric oxide is more correlated with the risk of having an asthmatic access rather than a predisposition to have asthma [21]. In patients with allergic rhinitis, measurement of FeNO might also indicate the presence of eosinophilic inflammation and might predict the development of lower airway symptoms [19,22].

The aim of this study was to investigate the risk of asthma development in patients with persistent allergic rhinitis to house dust mites after one year and the role of inflammatory cytokines, adhesion molecules and FeNO in predicting asthma occurrence.

2. Materials and Methods

2.1. Study Design, Site and Ethical Approval

The present study is a post-hoc analysis of an initial randomized control trial (RCT) that included patients with persistent allergic rhinitis [23]. The present research analyzed clinical and biological factors that might predict asthma in patients with allergic rhinitis to HDM. A diagnosis of allergic rhinitis was established according to international guidelines, based on history, clinical evaluation and the skin prick test (SPT) [24].

Fifty-eight patients with persistent allergic rhinitis to HDM (median age 27.5 (23–37) years and sex ratio M:F = 1:1), that were evaluated in Allergology Department, were included in the present analysis. The study protocol was approved by University of Medicine and Pharmacy Ethics Committee (approval no. 535/02.09.2009) and all patients signed the informed consent before enrollment. The study protocol and clinical evaluation was similar to initial RCT [23]. The exclusion criteria were as follows: the presence of asthma or nasal polyps, acute and chronic upper respiratory infections, administration of intranasal or systemic corticosteroids or H1 antihistamines in the previous 30 days. The initial evaluation was performed between February 2009 and November 2011.

2.2. Clinical Evaluation

From anamnesis, we noted the following demographic data: age, sex, living area (urban/rural) and the duration of allergic rhinitis symptoms prior enrollment. Retrospectively, for 12 h, we evaluated the allergic rhinitis' symptoms (rhinorrhea, nasal congestion, sneezing, nasal and ocular itching), and their

severity on a scale from 0 to 3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe). At the end we calculated the total symptoms score (TSS). Based on TSS values we differentiated between mild allergic rhinitis (TSS < 6) and moderate–severe allergic rhinitis (TSS 6).

As we previously mentioned, patients that presented low airways symptoms (dyspnea, cough and wheezing) associated to specific symptoms of allergic rhinitis were excluded from the present analysis. The patients also completed an ENT examination to exclude a possible nasal obstruction of other cause or nasal polyps. Patients with nasal polyps or another ENT disease were also excluded.

After one year, we repeated the clinical evaluation to determine the possible development of asthma. We noticed the occurrence of asthma symptoms (cough, wheezing, dyspnea) or an asthma exacerbation that required specific treatment in this period of time.

Spirometry was performed at enrollment in order to exclude a possible impaired lung function due to asthma presence and after one year. We considered asthma development if one of these clinical or functional criteria were present in the period of one year.

2.3. Skin Prick Tests (SPT)

The atopy diagnosis was established through a skin prick test at enrollment, according to international guideline [25]. The skin prick test included the following panel of allergens: house dust mites (Derm. Pteronyssinus (Der p) and Derm. Farinae (Der f)), pollens (grasses, cereals, birch and weeds), animal dander (cat and dog) and molds (Alternaria alternata). Standardized allergen extracts (Hal Allergy, Netherlands) were used. The value in mm was recorded as a medium diameter wheal size.

2.4. FeNO Measurement

Fractionated exhaled nitric oxide (FeNO) was measured at enrollment, according to international recommendations [26], using NIOXMINO® (Aerocrine, Sweden). The measured values were expressed in parts per billion (ppb). A standardized value of 25 ppb was considered as normal upper limit.

2.5. Biological Evaluation

All the biological parameters were determined at the beginning of the study. Total IgE plasma level was done using the electrochemiluminescence immunoassay method (ECLIA). The obtained values were expressed as UI/mL, considering a normal value <100 UI/mL. The eosinophils (Eo) were manually counted from peripheral blood on a slide and their value was expressed as %. We considered a normal value between 2%–4%.

Plasma levels of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and cytokines (TNF-α, IL-6 and IL-8) were determined at the initial visit. Five milliliters of blood sample was collected and centrifuged within the first hour, followed by serum separation. The obtained serum was stored at −80 °C until the determination was performed. The plasmatic levels of all inflammatory markers were determined by sandwich ELISA technique using an ELISA reader StatFax 303. All the aforementioned determinations were done according to the manufacturers' instructions, using ELISA kits from Quantikinine R&D system, USA. For each assay, samples were prepared according to instructions and protein levels were calculated based on four-parameter logistic (4-PL) curve fit.

2.6. Statistical Analysis

Statistical analysis was carried out using the MedCalc Statistical Software version 18.10 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2018). Quantitative data were evaluated for normality of distribution and variables with abnormal distribution were characterized by median and 25–75 percentiles. Qualitative data were expressed as frequency and percent. Comparisons between groups were performed using the Mann–Whitney (for quantitative data) and chi-square tests (for qualitative data). Spearman rho coefficient was used for examining the correlation between variables. ROC curves were used in order to find out cut-off values for quantitative variables that could

discriminate between patients with asthma and those without. A multivariate binary logistic regression was used for assessment of independent contribution of variables that achieved statistical significance in univariate analysis for asthma onset. A p value < 0.05 was considered statistically significant.

3. Results

From the entire group of patients with allergic rhinitis to HDM, 21 patients (36.2%) developed asthma after one year of surveillance.

Patients' demographic, clinical and biological data are presented in Table 1.

Table 1. Comparison between patients based on asthma diagnosis at one-year follow-up.

Variable		Total (n = 58)	Patients with Asthma (n = 21)	Patients without Asthma (n = 37)	p
Age (Years) *		27.5 (23–37)	33 (24.5–40)	26 (22–31)	0.014
Sex ^	M	50% (29)	57.1% (12)	45.9% (17)	0.5
	F	50% (29)	42.9% (9)	54.1% (20)	
Living area ^	U	82.8% (48)	85.7% (18)	81.1% (30)	0.7
	R	17.2% (10)	14.3% (3)	18.9% (7)	
Onset of AR symptoms (months) *		24 (6–60)	36 (15–66)	12 (3–48)	0.04
Total symptom score *		8.5 (5–11)	9 (5.5–13)	8 (5–11)	0.2
FeNO (ppb) *		24 (16–46)	45 (30.5–68)	19 (16–28)	<0.001
Total IgE (UI/l) *		106.5 (44.55–201.5)	118 (35.4–293)	104 (49.8–233)	0.9
Eosinophils *		0.05 (0.026–0.071)	0.05 (0.02–0.08)	0.04 (0.02–0.06)	0.5
E selectin (ng/mL) *		3.28 (2.35–4.69)	3.45 (2.26–4.81)	2.32 (2.43–4.64)	0.9
ICAM (ng/mL) *		21.53 (18.88–26.55)	22.98 (20.02–27.18)	20.87 (18.39–24.32)	0.1
VCAM (ng/mL) *		48.53 (40.95–59.50)	56.38 (41.34–60.55)	46.81 (40.93–55.95)	0.1
TNF-α (pg/mL) *		1.78 (1.22–2.33)	1.62 (1.01–2.11)	1.93 (1.34–2.41)	0.2
IL-6 (pg/mL) *		1.05 (0.75–1.70)	1.25 (0.71–1.84)	1.05 (0.78–1.65)	0.6
IL-8 (pg/mL) *		5.32 (3.33–9.38)	5.05 (2.35–8.20)	5.32 (3.95–9.51)	0.2
Wheal size of Der p allergen at prick test (mm)		7.23 ± 2.92	7.89 ± 3.24	6.57 ± 2.61	0.052

Data are expressed as * median; 25–75th percentile; ^ Data are expressed as %, n; Tests used: Mann–Whitney (for quantitative data) and chi-square tests (for qualitative data); Significance $p < 0.05$. Abbreviations: AR, allergic rhinitis; F, female; FeNO, fractional exaled nitric oxide; M, male; R, rural; TSS, total symptoms score; U, urban.

Analyzing demographic data, we noticed that asthma occurrence is correlated with patients' age, but not with their gender or living area (see Table 1). More male patients developed asthma compared to females, but the difference was not statistically significant.

Forty patients (68.9%) presented persistent moderate–severe forms of allergic rhinitis, proved by an initial TSS over 6 (median 8.5 (5–11)). The development of asthma was not correlated with a moderate–severe form of allergic rhinitis (p = 0.5), even if more patients with asthma had previously moderate–severe allergic rhinitis (76.2% vs. 64.9%). Initial TSS of allergic rhinitis was higher in patients with asthma, but the difference was not statistically significant. Initial was not correlated with the duration of AR and was not influenced by patients' sex or living area ($p > 0.05$). The duration of allergic rhinitis is significantly higher in patients with asthma after one year of surveillance.

The markers of allergic inflammation, total IgE and eosinophils were higher in patients with asthma after one year, compared with patients with allergic rhinitis without asthma, but the differences were not statistically significant ($p > 0.05$). We noticed similar results for adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and IL-6, but not for TNF-α and IL-8. Only FeNO was significantly higher in patients with allergic rhinitis and asthma compared to those without asthma (see Table 1). The initial values of biological markers were not influenced by patients' age, sex and living area, duration or severity of allergic rhinitis ($p > 0.05$).

Thirty-seven patients (63.8%) were polysensitized to both indoor and outdoor allergens, but the symptoms of rhinitis were present after exposure to HDM, while 21 patients (36.2%) were sensitized only to HDM. All the patients were sensitized to Der p, while 86.20% (50 patients) were sensitized also to Der f. The wheal size of Der p sensitization was higher in patients who developed asthma compared with those without asthma. The asthma development was not correlated with number or type of sensitizations to other allergens, except HDM ($p > 0.05$).

In patients with allergic rhinitis to HDM, we observed a moderate positive correlation between baseline values of TNF-α and IL-8 (r = 0.327, $p = 0.01$), IL-6 and IL-8 (r = 0.437, $p = 0.001$), weak negative correlation between VCAM-1 and IL-8 (r = −0.290, $p = 0.03$) and a weak positive one between TNF-α and IL-6 (r = 0.260, $p = 0.04$).

The ROC curve for patients' age, duration of allergic rhinitis and FeNO were analyzed and the cut-off values were calculated for these parameters in relation with asthma onset after one year of the inclusion visit. The cut off values, AUC, sensitivity and specificity are presented in Table 2.

Table 2. ROC curve analysis for asthma diagnosis at 1-year follow-up.

Parameter	AUC	Cut-Off Value	Sensitivity	Specificity	*p*
Age	0.696 (95%CI 0.56–0.81)	>31 years	61.90% (95%CI 38.4–81.9%)	78.38% (95%CI 61.8–90.2%)	0.007
Duration of AR	0.659 (95%CI 0.52–0.77)	>12 months	76.19% (95%CI 52.8–91.8%)	54.05% (95%CI 36.9–70.5%)	0.02
FeNO	0.79 (95%CI 0.66–0.88)	>28 ppb	85.71% (95%CI 63.7–97.0%)	78.38% (95%CI 61.8–90.2%)	<0.001

Abbreviations: AR, allergic rhinitis; AUC, aria under de curve; CI, interval of confidence; FeNO, fractional exhaled nitric oxide; parts per billion.

In order to find out which parameter was independently associated with asthma' occurrence in patients with allergic rhinitis to HDM, we used a multivariate logistic regression (Table 3). Variables which achieved statistical significance in univariate analysis were introduced in the regression. Our model explained 36.2% of asthma prevalence. Age and allergic rhinitis duration were very close to statistical significance, probably due to the small number of patients. FeNO > 28 ppb was the only independent variable that predicted the onset of asthma at a one-year follow-up (Table 3).

Table 3. Multivariate analysis for asthma occurrence in patients with allergic rhinitis to HDM.

Variables	B	*p*	OR	95% C.I. for OR	
				Min	Max
Age > 31 yo	1.321	0.07	3.746	0.860	16.311
Duration of AR > 12 months	1.437	0.07	4.209	0.885	20.008
FeNO > 28 ppb	2.928	<0.001	18.682	3.791	92.057
Constant	−0.825	0.04	0.438		

Abbreviations: AR, allergic rhinitis; B, CI, interval of confidence; FeNO, fractional exhaled nitric oxide; HDM, house dust mites; Max, maximum; Min, minimum; male; OR, odds ratio; ppb, parts per billion. Test used: binary logistic regression.

4. Discussion

The present study showed a significant association of asthma symptoms in patients with persistent allergic rhinitis to HDM after one year of surveillance, 36.2% of them presenting asthma. Clinical (duration, patient age) and biological data (inflammatory markers) may predict asthma development. FeNO was an independent variable that predicted the onset of asthma at one-year follow-up.

Allergic rhinitis and asthma are considered a single respiratory disease involving two parts of the airways [2,9]. In the present study, the authors found a prevalence of asthma of 36.2% after a one-year follow-up. Similar data were already reported in the literature, with a variable prevalence between 20% and 50% of patients with allergic rhinitis [9,10]. The prevalence found in the present research was higher compared to the study published in 1998, where the prevalence of asthma in patients with allergic rhinitis was lower (21.3%) after 23 years of follow up [27]. But Greisner et al. [27] included all patients with allergic rhinitis at the same age (first year of faculty), not only patients with persistent forms and different ages. The patients from the present research had a median age of 27.5 year, higher than the age of patients from the Greisner study. A similar prevalence of asthma was also established in children (30% in 13–14 years group and 35% in 6–7 years group) [28], but they did not follow prospectively their patient and the prevalence was established retrospectively.

The current diagnose of rhinitis relies on combination of three types of data: historical, clinical examination, and allergy diagnostic testing, which allows differentiation into three subgroups: allergic, infectious, and non-allergic non-infectious rhinitis [24,29]. Bronchial hyperreactivity is commonly present in patients with persistent moderate severe allergic rhinitis and should be suspected if other risk factors are present (allergen and viral exposures, indoor and outdoor pollution, allergic rhinitis duration and severity) [30–32]. The validation of some clinical and biological factors will permit to phenotype and endotype allergic rhinitis in order to find a form of "asthma risk" allergic rhinitis. Maybe a different approach of allergic rhinitis according to its phenotype and endotype could be done in order to prevent asthma development.

In this study, the clinical, biological and inflammatory markers that might influence the appearance of asthma in patients with allergic rhinitis to HDM were evaluated. The authors included only patients with persistent allergic rhinitis in this study, knowing that duration of symptoms and their severity are risk factors for asthma development [30,32]. A duration of allergic rhinitis over 12 months was considered a risk factor for asthma occurrence similar to other previous studies, in both adults and children [3,27]. The severity of disease was not correlated with asthma occurrence in this research. In Bousquet et al.'s and del Curvillo et al.'s studies [30,32], the severity of rhinitis was correlated with asthma development, but they included patients with both intermittent and persistent allergic rhinitis, while in the present research all the patients had persistent forms.

Rhinitis phenotypes were also described in relationship to sensitization pattern [33]. Sensitization to HDM is a risk factor for asthma development because they are perennial allergens [9]. In this study, only patients with sensitization to HDM were included. In addition, the sensitization may influence in different degree the severity of allergic rhinitis and the evolution to asthma, as Vidal's study already mentioned [33]. Vidal et al. reported that severe allergic respiratory disease was associated with higher levels of both total IgE and specific IgE to HDM. The presence of sIgE to both Der p 1 and Der p 2 was associated with asthma among HDM-allergic patients [33]. Our results revealed an almost significant association ($p = 0.052$) in univariate analysis between the size of the skin prick test to Der p and the occurrence of asthma. The mean size of wheal to asthmatic patient was 7.89 mm, which may confirm the presence of clinically manifested symptoms as for both asthma and rhinitis as in Haahtela's study [34]. The authors of this research did not determine the level of sIgE to HDM; they confirmed the sensitization based only on the skin prick test. Probably a large number of patients may give more information regarding the role of wheal size in assessing rhinitis or asthma symptoms.

An important step in implementing the precision medicine in patients with allergic rhinitis is to identify possible biomarkers which characterize the endotypes and may guide us to different therapeutic approaches. In asthma research, the endotypes are already described, based on different biomarkers

(cytokines, cells). Allergic rhinitis might have complex endotypes and the current understanding of cellular and molecular processes may lead to identify certain biomarkers that characterize the endotypes, but studies are still required to confirm them. There are several modulators of endotype expression, such as environment, microbiome, lifestyle and nasal anatomy. In several studies, the following endotypes of rhinitis were proposed: type 2 immune response rhinitis, type 1 immune response rhinitis, neurogenic rhinitis, epithelial dysfunction [26,35,36].

However, in the present study, the authors focused only on type 2 immune response allergic rhinitis with HDM sensitization alone or with other co-sensitizations. Previous studies revealed that some markers are increased in patients with allergic rhinitis to HDM: total serum IgE, blood and intranasal eosinophils, various types of cytokines [18,37] and adhesion molecules [6,38,39]. In this study, the comparative univariate analysis of patients with allergic rhinitis with or without asthma after a one-year surveillance showed that mean values of adhesion molecules and IL-6 were increased in the first subgroup compared to patients without asthma, but the differences did not reach the statistical significance and, because of this, they were not included in multivariate analysis. The plasmatic values of cytokines are extremely variable and more patients should be included in order to confirm them as biomarkers. Additionally, their plasmatic level should be correlated with their level in nasal or bronchial lavage. A previous study in children reported that a cytokine imbalance predicted asthma occurrence after three years of surveillance [40], but they concluded that respiratory viral infections may play a significant role in this imbalance, not only atopy. Therefore, these inflammatory markers should be evaluated in different population subgroups, adults and children, and in conjunction with other risk factors, in order to confirm their potential role as biomarkers.

As a biologic marker, only FeNO was significantly higher in patients with allergic rhinitis and asthma compared to those without asthma. Patients with allergic rhinitis to HDM that developed asthma have an increased systemic inflammation, which was correlated with subclinical inflammation in the lower airways. FeNO is a biomarker of atopy and eosinophilic airway inflammation [41]. Previous studies found a correlation of FeNO level and type of sensitization, in both adults and children. Jouaville et al. found that FeNO level increased with the number of positive skin prick tests (SPTs) in both asthmatics and non-asthmatic subjects, but in cases of an equal level of positive SPTs the FeNO was higher in asthmatic than in non-asthmatic, reflecting its role in asthma diagnoses [42]. The exact role of FeNO in the prediction of asthma in patients with allergic rhinitis is still unclear.

In the present study, patients with allergic rhinitis to HDM with continuous symptoms for more than 12 months and an elevated FeNO at presentation over 28 ppb had 18-fold higher risk for asthma after one year. Malinovschi et al. used FeNO in evaluation of allergic rhinitis persistence and severity in general population of adolescents, but the author did not calculate a cut off value which may predict the persistence of rhinitis [43]. Additionally, they evaluate the persistence of allergic rhinitis symptoms, not the occurrence of asthma ones. Di Cara et al. found that children with allergic rhinitis and elevated values of FeNO over 35 ppb developed asthma after five years of monitoring [44]. Similar results were also reported in children and adults [45,46]. The cut off value in our study was lower than in the Di Cara study [44], but only adults were included, while the Di Cara study included only children.

The factors that lead to asthma in patients with allergic rhinitis are multiple. Further studies are still needed to evaluate the evolution of allergic inflammation. However, from the clinical practice point of view, it is important to evaluate all patients with allergic rhinitis for asthma symptoms [47]. Patients with allergic rhinitis and other risk factors for asthma should be carefully evaluated for the presence of subclinical inflammation in the lower airways, which is the substrate for bronchial hyperreactivity.

This study has a strong value because it emphasized the role of FeNO, a marker of lower eosinophilic inflammation in allergic rhinitis in order to predict the evolution of disease to other manifestations. There are some limitations of this study. Firstly, a small number of patients were included in the study. Secondly, the surveillance period is short and some of the investigated markers may not be able to predict the asthma development so soon. It could be interesting to also evaluate the modulation of these biological markers under different treatments that are recommended in allergic

rhinitis. The authors evaluated the markers in patients with rhinitis induced by HDM, but they could not compare patients with monosensitization with the ones polysensitized because of the small number of subjects included in the present analysis.

5. Conclusions

Clinical and biological parameters may predict asthma occurrence in patients with persistent allergic rhinitis to house dust mites. Adult patients with a longer duration of rhinitis symptoms and a high level of FeNO over 28 have a greater risk to develop asthma. The severity of symptoms and the serum inflammatory cytokines and adhesion molecules are not correlated with asthma occurrence after one year of monitoring. FeNO could become a useful biomarker in predicting a specific endotype of allergic rhinitis with high risk to develop asthma.

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Article

Characterization of Patients with Allergic Rhinitis to Ragweed Pollen in Two Distinct Regions of Romania

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Abstract: *Background and objectives:* Ragweed pollen is a major source of allergen, which has rarely been observed in Romania until now. In this study, we evaluated the symptoms and associated factors in patients with allergic rhinitis to ragweed pollen in two distinct regions of Romania. *Materials and Methods:* We evaluated the records of patients newly diagnosed with allergic rhinitis induced by ragweed pollen in two allergological centers from North-West (NW) and Central parts of Romania between 2013 and 2015. The patients were clinically evaluated regarding disease length, presence, and severity of the allergic rhinitis symptoms and the association with other allergic manifestations (asthma and conjunctivitis). *Results:* The sensitization to ragweed was significantly higher in the NW part compared to the Central part (18.27% vs 4.1%, $p < 0.001$). More patients with monosensitization to ragweed pollen were observed in the NE center (27%) compared to the Central one (20.7%). Patients with monosensitization to ragweed pollen presented more severe forms of rhinitis (70% vs 31.5%, $p = 0.02$) in the NW part compared to polysensitized patients. The total symptoms score was significantly higher in patients from the Central part compared to the NW part (9.21 ± 2.01 vs 5.76 ±1.96, $p < 0.001$). Bronchial asthma was associated at a similar frequency to allergic rhinitis in both centers, but it was more frequently observed in monosensitized patients in the NW center. Allergic conjunctivitis was more frequently reported by patients from the Central part (75.86 vs 41.9, $p = 0.02$), while in the NW region it was noticed more commonly in monosensitized patients (65% vs 33.33, $p = 0.02$). *Conclusions:* Allergic rhinitis to ragweed pollen has been more frequently reported in the NW part of Romania. Patients with severe forms of rhinitis were observed in the central part, while in the NW the severe forms of disease were reported by patients with monosensitization. Ragweed pollen is intensely allergogenic and determines association of ocular and asthma symptoms. Co-sensitization increases the risk of asthma association.

Keywords: allergic rhinitis; ragweed; *Ambrosia artemisiifolia*; sensitization; asthma

1. Introduction

Respiratory allergies, like allergic rhinitis and bronchial asthma, are chronic diseases with high social impact worldwide, and epidemiological data has shown an increase in their incidence and

prevalence [1,2]. Specific clinical manifestations could be extremely unpleasant, affecting the patients' quality of life. In severe forms of allergic rhinitis, other non-nasal symptoms could be associated: ocular symptoms, asthma symptoms, headache, and sleep disturbances [3].

The prevalence of allergic rhinitis to pollen is estimated at 40% in the general population of Europe [1]. Total social costs of allergic rhinitis to pollen, quantified in the number of days of absenteeism from work or school, decreased work productivity, the number of visits to an allergologist and/or to an emergency department, and treatment costs are high enough to consider this disease as a real health problem in Europe and worldwide.

Weeds represent a major source of pollen, *Ambrosia artemisiifolia* being the most allergenic one. *Ambrosia artemisiifolia* (commonly known as ragweed) is an invasive annual herbaceous plant which originated from North America [4]. Ragweed was introduced In Europe in the 19th century, but become widely spread after 1900 due to an import of contaminated grains and seeds, from North America and its distribution is an on-going process [5]. The sensitization rate to ragweed is above 2.5% in general population in Europe [6]. But in some regions like Hungary, the Rhone Valley in France, and Northern Italy, the prevalence of ragweed sensitization varies considerably, reaching up to 60%–70% [6,7].

Ragweed is highly invasive and harmful for human beings because each plant produces a large amount of pollen (<1 billion grains/season) [8], which is highly allergenic; even low exposure may trigger a severe allergic reaction [9]. The peak of the season for ragweed pollen is in late summer and autumn in Romania [4]. Ragweed pollen grains can be transported over hundreds of kilometers by air, so they can induce allergy symptoms in areas where the ragweed plant is not widespread. Allergy to ragweed usually manifests clinically as allergic rhinitis or conjunctivitis, sometimes with associated asthma symptoms [10]. Despite this increasing health problem, there are few studies that have analyzed the impact of ragweed sensitization on the clinical manifestations of allergy, especially in Romania [4,11,12]. Most of the studies focused on determining the pollen level in the air and its correlation with the severity of manifestations. Sometimes it is difficult to measure the pollen amount in the air, so it is necessary to better understand the clinical picture of ragweed allergy.

The aim of the study is to characterize from the clinical point of view the patients with allergic rhinitis to ragweed and to identify possible associated factors that may predict the severity of manifestations, in two distinct centers from Romania.

2. Materials and Methods

2.1. Study Design, Site and Ethical Approval

The study was observational, analytic, and retrospective. The study was conducted in two Allergology Departments, one in Satu Mare, in the North-West (NW) region of Romania, near the borders to Ukraine and Hungary, and the second one in the Central part of the country, in Cluj Napoca. The study protocol was approved as 08.03.2017 by the Ethic Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy (ID number 105/08.03.2017). The study was done in accordance with the Declaration of Helsinki.

2.2. Patient Evaluation

All the patients newly diagnosed with allergic rhinitis between 2013 and 2015 were included in the retrospective analysis. In the first center from Satu Mare, data from 405 patients with allergic rhinitis were analyzed, while in the second group from Cluj Napoca, 706 patients were included. Diagnoses of allergic rhinitis was done using international criteria [3], based on clinical evaluation and the skin prick test.

From patients files, the following data were registered: age of patients at the presentation moment, the length of allergic rhinitis, living area (urban or rural), the presence and severity of allergic rhinitis symptoms, and the associated allergic manifestations: bronchial asthma and allergic conjunctivitis. The diagnoses of asthma and allergic conjunctivitis were clinically established based on patient

symptoms, according to international guidelines [3]. The authors recorded the presence of asthma and/or conjunctivitis symptoms during the ragweed pollen season, when the patients presented rhinitis manifestations.

The patients with allergic rhinitis to ragweed pollen were examined during the pollen season (August-September) when they had clinical symptoms of allergic rhinitis. The specific symptoms of allergic rhinitis-sneezing, rhinorrea, nasal congestion, nasal itching, and ocular itching were evaluated on a scale from 0 (lack of the symptom) to 3 (severe symptom). Based on symptoms scores, the total symptoms score (TSS) was calculated. A TSS < 6 represents a mild form of allergic rhinitis, while TSS ≥ 6 represents a moderate to severe form [3].

The allergological evaluation also included the skin prick test (SPT). The SPT was performed according to international guidelines [13] and the particularities of exposure to allergens in Romania. The tested panel included: house dust mites, mix grass pollen, cereals pollen, betulacee pollen, weed pollen (*Artemisia* and *Ambrosia*), cat and dog fur, *Alternaria alternata*, cockroaches (*Blatella germanica*), and feather mix. Allergen extracts from Stallergens, France were used.

2.3. Statistical Analysis

The statistical analysis was performed using MedCalc Statistical Software version 19.0.3 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2019). Data were labeled as nominal, expressed as percentage, and continuous variables. The normal distribution for continuous variable was done using the Kolmogorov-Smirnov test. Variables with normal distribution were expressed as mean and standard deviation, while variables with abnormal distribution as median and 25–75 percentiles.

The adequate statistic tests according to data distribution were chosen. The differences were assessed within groups by Wilcoxon Signed Rank test and between groups by Mann Whitney test. The χ^2 test was also used for data analysis. Level of statistical significance was set at $p < 0.05$.

3. Results

First of all, the authors calculated the percentage of patients suffering of allergic rhinitis induced by ragweed pollen. The percentage of patients with allergic rhinitis to ragweed was significantly higher in the North-West of Romania compared to the Central region ($p < 0.001$) (see Table 1).

Table 1. Patients with allergic rhinitis to ragweed pollen in investigated allergological centers.

Patients	NW Center	Central Center	*p*
Total no. of patients with AR	405 pts	706 pts	
Patient with AR to ragweed	74 pts (18.27%)	29 pts (4.10%)	< 0.001

Abbreviations: AR, allergic rhinitis; NW, North-West; pts, patients.

Demographic data of patients with allergic rhinitis to ragweed pollen are presented in Table 2.

Allergic rhinitis to ragweed pollen was most frequently observed in females and in patients living in urban areas, without significant differences between centers. The duration of disease was found to be significantly longer in patients from the Central region compared to patients from the NW region (see Table 2).

Table 2. Demographic data.

Parameter		NW Center (n = 74 pts)	Central Center (n = 29 pts)	p
Age (age) *		39.5 (31–47)	42.5 (32.5–54)	0.774
Gender ^	M F	47.3% (n = 35) 52.7% (n = 39)	44.82% (n = 13) 55.17% (n = 16)	0.530
Living area ^	Urban Rural	77.02% (n = 57) 22.97% (n = 17)	72.41% (n = 21) 27.58% (n = 8)	0.808
Severity of AR ^	Mild Moderate/severe	58.10% (n = 43) 41.90% (n = 31)	10.3% (n = 3) 89.7% (n = 26)	<0.001
Disease's duration * (years)		4.25 (2–6)	12 (2–24)	0.009
Family history of allergy		14.90% (11)	17.25% (5)	0.344

* Data are expressed as (median; 25–75th percentile); ^ Data are expressed as (%, n); Abbreviations: AR, allergic rhinitis; F, female; M, male; NW, North-West; pts, patients.

3.1. Sensitization to Ragweed Pollen

Most of the patients presented polysensitization to different allergens. In the NW region, 27% of the patients (20 pts) were sensitized only to ragweed, while in the Central region, 20.7% (6 pts) presented a positive skin prick test only to ragweed pollen, without statistical differences between groups (p = 0.514). Among patients with different sensitization, almost half of them presented co-sensitization to artemisia pollen (48.64% (36 pts) in the NW region and 48.27% (14 pts) in the Central region, p = 0.97), or grass pollen (44.59% (33 pts) in the NW region and 44.82% (13 pts) in the Central region, p = 0.98). Monosensitized patients were considered if they had sensitization only to ragweed pollen.

There is no correlation between environment (rural-urban), age, sex, family, or personal allergic history and the type of sensitization in patients with allergic rhinitis to ragweed pollen.

3.2. Severity of Allergic Rhinitis

In the NW region, more than half of the patients had mild forms of allergic rhinitis, while in the Central investigational centers, most of the patients presented moderate or severe forms of allergic rhinitis (Table 2). The total symptom score was significantly higher in patients evaluated in the Central region compared to patients from the NW region (Figure 1.)

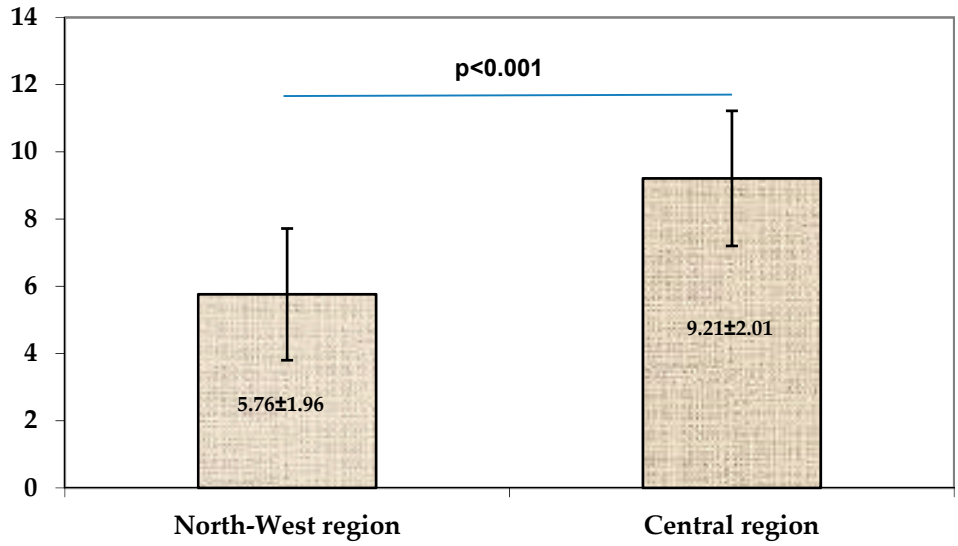

Figure 1. Total symptom scores in both centers (data are expressed as mean ± standard deviation (SD)).

When the severity of allergic rhinitis was analyzed according to type of sensitization, the authors noticed that patients with monosensitization to ragweed pollen presented more severe forms of disease

compared to patients with sensitizations to different pollens in the NW region ($p = 0.02$), but not in the second investigational region ($p = 0.354$) (Table 3). The total symptom score was higher in monosensitized patients in both centers, but the difference reached the statistical significance only in the group from the NW region ($p < 0.001$), not in the Central region group of patients ($p = 0.16$).

Table 3. Severity of allergic rhinitis according to the type of sensitization.

Parameter	NW Center			Central Center		
	Monosens. (n = 20 pts)	Polysens. (n = 54 pts)	p	Monosens. (n = 6 pts)	Polysens. (n = 23 pts)	p
AR severity Mild Moderate-severe	 30% (n = 6) 70% (n = 14)	 68.5% (n = 37) 31.5% (n = 17)	0.02	 0% 100% (n = 6)	 13.8% (n = 4) 86.2% (n = 19)	0.354
TSS	7.05 ± 1.83	5.27 ± 1.09	<0.001	10.33 ± 2.07	8.91 ± 2.19	0.16

Abbreviations: AR, allergic rhinitis; monosens, monosensitization; NW, North-West; polysens, polysensitization; pts, patients; TSS, total symptoms score.

There is no association between environment (rural-urban), age, sex, family, or personal allergic history and severity of allergic rhinitis.

3.3. Association of Other Allergic Manifestations

The authors also analyzed the association of other allergic manifestations: allergic conjunctivitis and bronchial asthma.

Allergic conjunctivitis was noticed in 41.9% of patients in the NW region, but it was more frequently reported by patients from the Central part, where it was noticed in 75.86% of patients ($p = 0.02$). The allergic conjunctivitis was significantly associated to allergic rhinitis in patients with monosensitization compared to patients with polysensitization in the NW region ($p = 0.01$), but not in the Central region ($p = 0.12$) (Table 4).

Table 4. Association of other allergic manifestations.

Parameter	NW Center			Central Center		
	Monosens (n = 20 pts)	Polysens (n = 54 pts)	p	Monosens (n = 6 pts)	Polysens (n = 23 pts)	p
Allergic conjunctivitis	65% (n = 13)	33.33% (n = 18)	0.01	100% (n = 6)	69.56% (n = 16)	0.12
Bronchial asthma	15% (n = 3)	44.44% (n = 24)	0.02	16.7% (n = 1)	30.43% (n = 7)	0.51

Abbreviations: monosens, monosensitization; NW, North-West; polysens, polysensitization; pts, patients.

Twenty-seven (36.5%) patients from the NW region presented bronchial asthma symptoms, while only 27.6% of patients in the second group (8 pts) presented associated bronchial asthma, although the difference did not reach the level of statistical significance ($p = 0.39$). Patients with multiple sensitization presented more frequently asthma symptoms in the NW group compared to monosensitized patients ($p = 0.02$), but not in the Central group ($p = 0.51$) (Table 4).

4. Discussion

The present study characterized the clinical manifestations of allergic rhinitis to ragweed in two distinct regions of Romania, the NW and Central regions. Ragweed has now become an important source of allergens that induces allergic rhinitis in Romania, with an extensive spreading from the West to the Center.

In the present study the sensitization to ragweed pollen was noticed in almost 20% of patients in the NW region, an increased percentage compared to the Central region, knowing that ragweed was not frequently observed in our country until 2007. Ianovici et al. [14] reported a rate of sensitization of 34% in Timisoara in 2009, but this was noticed in the general population, not in patients with clinical manifestations like allergic rhinitis. Recently Florincescu et al. [12] published the first data regarding sensitization to ragweed in patients with allergic rhinitis from Southern part of Romania, reporting a higher percentage of sensitization (48.8%) compared to our study. The highest levels of airborne ragweed pollen in Europe are known to have been recorded in Hungary and Ukraine [6,15], which may explain the higher rate of sensitization from the NW region compared to the Central region. High levels of ragweed pollen have also been recorded in the Black Sea region of Turkey [15,16], which may explain a higher rate of sensitization to ragweed in the Southern region of Romania reported by Florincescu et al. [12]. Looking to all this available data regarding sensitization to ragweed in Romania, we may assume that allergic rhinitis to ragweed could be more frequently observed in Western and Southern parts of the country. Southern and Western parts of Romania are closed to geographical regions rich in ragweed, like Hungary, Ukraine, Bulgaria, and Turkey. The weather conditions (winds, rainfall, average temperatures) in these regions may explain differences in ragweed pollen distribution and secondarily in ragweed sensitization. Ragweed grows intensely on sandy acid soil, which is characteristic for the Southern area of Romania [12,16]. It is an extremely adaptative plant with maximum of germination and multiplication in regions with average temperature around 30 °C in summers [16,17], like in Western and Southern regions of Romania. In summer the wind blows from the West, from Hungary, which may explain a rapid distribution of ragweed pollen from there. In the Central part of the country the average temperature is lower and at the end of summer and autumn the humidity increases, which would explain a reduced growth of the plant and reduced exposure to ragweed pollen [17,18]. Ukraine and Hungary have made extensive attempts to limit ragweed infestation, but Romania only adopted prophylactic measures to limit ragweed spread in 2018.

The distribution of allergy to ragweed could change over the year in all regions of Romania. It would be interesting to analyze the same data over an interval of 5 years to determine the rate of extension of this sensitization, knowing that Florincescu et al. [12] reported an increased number of cases over an interval of 2 years. Actually, an increased rate of sensitization to ragweed was also reported in Germany, the Netherlands, and Croatia [2,19], so a similar increase should be expected in all regions of Romania.

The rate of co-sensitization to other pollens is different in the present research compared to other studies. The co-sensitization to *Artemisia vulgaris* was noticed in almost half of the patients with allergic rhinitis to ragweed, a higher rate than in the Asero study (38%) [20], but significantly lower than the co-sensitization rate observed by Ackermann-Liebrich (82%) [21] in Switzerland. In the present study the co-sensitization to *Artemisia vulgaris* pollen is similar in both centers, an expected result knowing that *Artemisia vulgaris* is a more frequently observed plant in our country compared to the *Ambrosia artemisiifolia*. These results could explain the rapid evolution of sensitization in accordance with the type of exposure to different allergens, old or new, while also considering environmental pollution.

The median age of patients with allergic rhinitis to ragweed was higher in the present study compared to the GAALEN (Global Allergy and Asthma European Network) study [6], but the gender distribution was quite similar that of previously reported data, with this type of allergy being more frequently observed in females compared to males [6,22,23]. An increased median age in the present study is explained by the fact that most of the patients are adults. Children with allergic rhinitis to pollen visit either a pediatric or allergologist specialist, so we may assume that children with allergic rhinitis to ragweed were not included in the present analysis.

In Ackermann-Liebrich study [21], we noticed an increased age of patients with monosensitization compared to those that were polysensitized, but in our study we did not analyze the obtained data on subgroup of patients, due to the small sample size of the groups. Most of the patients lived in urban areas, similar to for previously published data in Europe [3,12,24]. In Florincescu study [12], the rate

of patients living in urban area was even higher than in the present study, but in another study [23] the distribution rate was almost equal. It is well known that in industrialized countries, the allergic rhinitis to pollen is more frequently observed in urban areas, due to extensive pollution [1,3].

In the central part of Romania most of the patients presented moderate-severe forms of allergic rhinitis, similar to in the Florincescu study [12], where almost all the patients presented such kind of forms. But surprisingly in NW Romania, most of the patients presented mild forms of rhinitis. Moderate severe forms of allergic rhinitis were reported especially in monosensitized patients rather than in polysensitized patients in the NW, an observation reflected also by a higher total symptoms score. A similar tendency was also noticed in the central region. Persistent moderate and severe forms of allergic rhinitis were also mentioned in previously published data [25,26]. But in a Gelardi study [27] which included only children and not adults, there was no difference compared to the present study regarding the distribution of mild or moderate severe forms of allergic rhinitis to ragweed pollen and mono or polysensitization. We might assume, based on this observation, that ragweed pollen is an aggressive one, especially in adults and produces severe manifestations, which may explain the significant impairment of patient quality of life. Patients with polysensitization may report severe symptoms during other seasons, which is not always related to ragweed pollination.

The association of allergic conjunctivitis to rhinitis symptoms in patients with allergic rhinitis, irrespective of the type of sensitization, is well known [3,22]. The ocular manifestation was more frequently noticed in patients with monosensitization to ragweed pollen in the NW, and was also more frequently reported in Central region. Florincescu et al. [12] reported a similar association rate of conjunctivitis as in the present study in central Romania, higher than we noticed in the NW center. In the association of ocular symptoms, the type and amount of allergen are not the only important factors. Other environmental factors (airborne particles) may also increase the aggressiveness of pollen grains and the ocular response [26,28]. In a Majkowska-Wojciechowska study [28], allergic conjunctivitis is frequently associated in patients from urban areas, but not in those from rural ones. The study center from the central region is an urban area with an increased population, increased traffic, and is more industrialized compared to NW center, which leads to increased pollution.

Bronchial asthma was frequently associated to allergic rhinitis to ragweed, especially in the NW region and in patients with co-sensitizations. A similar rate of association was also reported in previously published data [12,22,24,29]. Surprisingly, Mark et al. [30] reported a lower rate of associated asthma in Hungary, but they did not distinguish between mono- or poly-sensitized patients. Patients with multiple sensitizations are exposed to allergens for longer, which may explain a persistent inflammation in both upper and lower airways and a higher rate of asthma association.

The main strength of this paper is that it presents the first study that characterized the patients with allergic rhinitis to ragweed pollen in two different regions of Romania, with different levels of exposure to allergens. There are also a few limitations of this study. The number of included patients with allergic rhinitis to ragweed is small, especially in the second group from the Central region. Secondly, the authors could not perform a pollen count, so it was not possible to correlate the clinical manifestations of allergic rhinitis or asthma symptoms with pollen level in the air. Also, while the authors used patient files from two centers of Romania, a larger evaluation is needed to determine the prevalence of sensitization.

5. Conclusions

Allergic rhinitis to ragweed is a common problem in the NW region of Romania, near Hungary and Ukraine. Ragweed pollen is intensely allergogenic and determines severe forms of allergic rhinitis and association of ocular symptoms. Co-sensitization increases the risk of asthma association. This study recommends including the ragweed pollen in the national panel of the skin prick test to identify the sensitization and to evaluate the clinical manifestation of this allergy. Since the disease is so harmful for human health, it is necessary to limit its geographical extension and to inhibit the growth of the plant in order to protect the general population.

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