

# Characterization of the sensitization profile of bee venom allergic patients – Association with the severity of reaction?

*Caracterização do perfil de sensibilização molecular de doentes alérgicos à picada de abelha - Associação com a gravidade da reação?*

Catarina Coutinho<sup>1</sup>, Fernando Pineda<sup>2</sup>, Miriam Castillo<sup>2</sup>, Elisa Pedro<sup>1</sup>, Maria Conceição Pereira-Santos<sup>3,4</sup>

## ABSTRACT

**Introduction:** Bee venom (BV) allergy, a common cause of anaphylaxis in adults, is often associated with severe reactions. The use of component-resolved diagnostics (CRD) increases diagnostic accuracy. **Objectives:** To characterize the sensitization profile of BV allergic patients and a possible correlation with the severity of reaction. **Materials and methods:** We selected patients with a clinical history of BV allergy, positive skin tests, and specific IgE (sIgE) for BV. The allergenic profile was analyzed by both CRD and Western blot using a well-defined and properly characterized BV extract. **Results:** Forty-four patients were included, 30 (68.2%) were men. Mean age was 48.9 (SD 17.9) years. Eleven (25%) had large local reactions (LLRs) and 33 (75%) had systemic sting reactions (SSRs). One patient with negative sIgE for BV had positive sIgE for Api m 1, Api m 5, and Api m 10. The sensitization frequency for BV, Api m 1, Api m 2, Api m 3, Api m 5, and Api m 10 was 97.7%, 75%, 47.7%, 20.5%, 40.9%, and 61.4%, respectively. Five patients (11.4%) were sensitized to all BV components. CRD association showed that 5 patients (11.4%) were sensitized only to Api m 1, 8 (18.2%) to Api m 1/Api m 3/Api m 10, and 16 (36.6%) to Api m 1/Api m 10. Twenty-eight patients (84.8%) with SSRs were sensitized to Api m 1, and concomitant sensitization to Api m 1/Api m 10 was detected in 20 (60.6%). There was a significant difference in Api m 1 between patients with LLRs and SSRs ( $p = 0.0104$ ). Similar profiles were identified by Western blot analysis, with relevance for the detection of Api m 6 in 28 (64%) and Api m 4 in 16 (36%) patients. **Conclusion:** The analysis of the sensitization profile using CRD and the association of several of these components can increase diagnostic accuracy in BV allergy. Our data showed that concomitant sensitization to Api m 1 and Api m 10, detected by both CRD and electrophoretic profile, may be associated with SSRs. We emphasize the identification of sensitization to Api m 6 in >50% of patients, which may be considered a major allergen, and to Api m 4, which may be related to reactions during BV immunotherapy.

**Keywords:** Allergy, bee venom, systemic sting reaction, large local reaction, molecular diagnosis, Western blot.

## RESUMO

**Introdução:** A alergia ao veneno de abelha (VA) é uma causa frequente de anafilaxia em adultos e está muitas vezes associada a reações graves. O diagnóstico por componentes moleculares (CRD) contribui para uma melhor caracterização desta alergia. **Objetivos:** Caracterização do perfil de sensibilização molecular de doentes alérgicos ao veneno de abelha e possível correlação com a gravidade da reação. **Material e métodos:** Selecionaram-se doentes com história de alergia a VA, testes cutâneos e IgE específica (sIgE) positivos para VA. Avaliou-se o perfil alergênico por CRD e por *Western Blot*, utilizando extrato de VA bem caracterizado. **Resultados:** 44 doentes, 30 (68,2%) sexo masculino. Média de idades 48,9 ± 17,9 anos, 11 (25%) com reações locais exuberantes e 33 (75%) com reações sistêmicas à picada (SSR). Um doente tinha sIgE negativa para VA, mas Api m 1, Api m 5 e Api m 10 positivas. A frequência de sensibilização para VA, Api m 1, Api m 2, Api m 3, Api m 5 e Api m 10 foi 97,7%; 75%; 47,7%; 20,5%; 40,9% e 61,4%, respectivamente. Cinco (11,4%) doentes estavam sensibilizados a todos os componentes. Por associação de CRD, detectaram-se 5 (11,4%) doentes sensibilizados apenas a Api m 1, 8 (18,2%) a Api m 1/Api m 3/Api m 10, e 16 (36,6%) a Api m 1/Api m 10. Vinte e oito (84,8%) doentes com SSR tinham Api m 1 positiva e 20 (60,6%) tinham Api m 1/Api m 10 simultaneamente positivas. Observou-se uma diferença estatisticamente significativa para a Api m 1 entre doentes com reações locais exuberantes e sistêmicas ( $p = 0,0104$ ). Os perfis detectados por *Western Blot* foram semelhantes, de referir, à detecção de Api m 6 em 28 (64%) e Api m 4 em 16 (36%) dos doentes. **Conclusão:** A análise do perfil de sensibilização através de CRD e a sua associação aumentam a precisão do diagnóstico de alergia a VA. Sensibilização simultânea a Api m 1 e Api m 10 identificados tanto por CRD como por perfil eletroforético, pode estar associada à ocorrência de SSR. Destaca-se a sensibilização a Api m 6 em > 50% dos doentes, podendo ser considerado um alergénio major, e a Api m 4, possivelmente associado a reações durante a imunoterapia com VA.

**Descritores:** Alergia, veneno de abelha, reação local exuberante, reação sistêmica à picada, diagnóstico molecular, *Western Blot*.

1. Serviço de Imunoalergologia, Hospital Santa Maria, Centro Hospitalar Universitário Lisboa Norte, Lisboa, Portugal.

2. Laboratório Diater, Laboratorio Aplicaciones Madrid, Madrid, Espanha.

3. Clínica Universitária de Imunoalergologia, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal.

4. Lab Imunologia Clínica, Faculdade de Medicina, Instituto de Medicina Molecular, Universidade de Lisboa, Lisboa, Portugal.

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## Introduction

Insect stings by Hymenoptera species are very common. From 56.6 to 94.5% of the general population has been stung at least once in their lifetime.<sup>1</sup> Hymenoptera venom allergy often presents as large local or systemic reactions and is reported as one of the leading causes of anaphylaxis in adults.<sup>2,3</sup> In Europe, it has a prevalence of 20%,<sup>4,5</sup> and more than 95% of allergic reactions are to honeybee (*Apis mellifera*) stings, which are potentially fatal.

Bee venom immunotherapy (bVIT) is a well-established therapy with an efficacy of 77-84%.<sup>1</sup> It has been shown to improve the quality of life of patients with systemic sting reactions (SSRs),<sup>6</sup> and more importantly, to prevent life-threatening reactions following an accidental sting.<sup>7</sup>

In the past, the diagnosis and treatment decisions in bee venom (BV) allergy were based on clinical history (a past SSR), positive skin tests, and specific IgE (sIgE) for the whole venom extract. However, in some patients with a history of anaphylactic reactions, skin tests are negative, sIgE is undetectable, and, frequently, the causative insect cannot be identified. Currently, the use of component-resolved diagnostics (CRD) allows a more accurate diagnosis. CRD uses purified native or recombinant allergens to detect IgE sensitivity to individual allergen molecules, thus allowing the discrimination between primary sensitization and cross-reactivity,<sup>8</sup> particularly in patients with sensitization to both honeybee and wasp venom.

The best characterized venom is that of honeybee (*Apis mellifera*). Twelve allergens have been identified,<sup>9</sup> and the main ones are phospholipase A2 (Api m 1), which is the most potent allergen, and hyaluronidase (Api m 2), both considered major allergens; together with melittin (Api m 4), they make up most of the dry weight of the venom. The basic peptide melittin (Api m 4) is considered to be an allergen of low prevalence. However, its relevance has recently been demonstrated, and it has been proposed as a biomarker of poor tolerance in patients at the initial stages of bVIT.<sup>10-12</sup> The other allergens are present but in much less quantity. They have also been identified as relevant and include acid phosphatase (Api m 3), dipeptidyl peptidase IV (Api m 5), and icarapin (Api m 10). Icarapin is a BV allergen with great relevance in diagnosis, which may be underrepresented in some therapeutic extracts.<sup>10,13</sup>

The present study aimed to characterize the sensitization profile of BV allergic patients by using CRD and to investigate a possible correlation between the sensitization profile and the severity of reaction.

## Materials and methods

We conducted a retrospective study of patients aged >12 years with a clinical history of recurrent anaphylaxis after a bee sting, not subjected to bVIT, with large local reactions (LLRs), defined as edema with an average extension of >10 cm in diameter persisting for at least 24 hours, or grade I to IV SSRs (according to Muller Classification<sup>14</sup>) after a bee sting, and with positive skin tests and/or sIgE (>0.35 kU/L) for the whole BV extract who were followed up in the Immunoallergology Outpatient Clinic of Hospital de Santa Maria, Centro Hospitalar Universitário of North Lisbon.

Exclusion criteria were pregnancy, age ≤12 years, and presence of acute disease.

Data were anonymized to ensure confidentiality. The study protocol was approved by the Ethical Board of Centro Académico de Medicina of Lisbon - Centro Hospitalar Universitário of North Lisbon (approval number 18/19). Written informed consent was obtained from each study participant.

## Skin tests

Skin tests with BV extracts were performed according to the European Academy of Allergy and Clinical Immunology (EAACI) guidelines<sup>1</sup> with Bial-Aristegui/Roxall® extracts at least 4 weeks after the last sting reaction. The skin prick tests were performed using a concentration of 100 µg/mL with both a negative control (0.9% NaCl) and a positive control (histamine 10 mg/mL). Intradermal tests were performed with increasing concentrations from 0.001 to 1 µg/mL, as well as a negative control.

## Evaluation of sIgE

We determined the sIgE antibody levels to the whole BV extract and the recombinant allergens phospholipase A2 (Api m 1), hyaluronidase (Api m 2), acid phosphatase (Api m 3), dipeptidyl peptidase IV (Api m 5), and icarapin (Api m 10) using an immunoenzymatic assay (ImmunoCAP 100™; Thermo Fisher Diagnostics, Uppsala, Sweden), according to the manufacturer's instructions. Values ≥0.35 kU/L were considered positive.

We also analyzed the allergenic profile by IgE-Western blotting with a well-defined and properly characterized BV extract which contained the following allergens and their relative abundances (%): Api m 1 (30%), Api m 2 (1.37%), Api m 3 (0.17%), Api m 4 (35.34%), Api m 5 (1.47%), Api m 6 (3.87%), Api m 7 (2.41%), Api m 8 (0.23%), Api m 9 (0.63%), Api m 10 (1.26%), and Api m 11 (1.50%). We used proteomic tools, including in-gel digestion and liquid chromatography-mass spectrometry (LC-MS)<sup>15</sup>, and the resulting gels were transferred to polyvinylidene fluoride (PVDF) membranes using the Trans-Blot Turbo™ Transfer System (Bio-Rad, Hercules, CA, USA). The binding of IgE antibodies to allergens was analyzed by Western blotting individual patients' sera and anti-human IgE peroxidase conjugate (Southern Biotech, Birmingham, AL, USA). Chemiluminescence detection reagents (Western Lightning® Plus-ECL; Perkin Elmer, Waltham, MA, USA) were added according to the manufacturer's instructions. IgE-binding bands were identified using Bio-Rad Diversity Database software.

### Statistical analysis

Student's *t* test and Wilcoxon test were used to compare differences between variables. Data were analyzed in GraphPad Prism®, version 5.01 (San Diego, CA, USA). A *p*-value <0.05 was considered statistically significant.

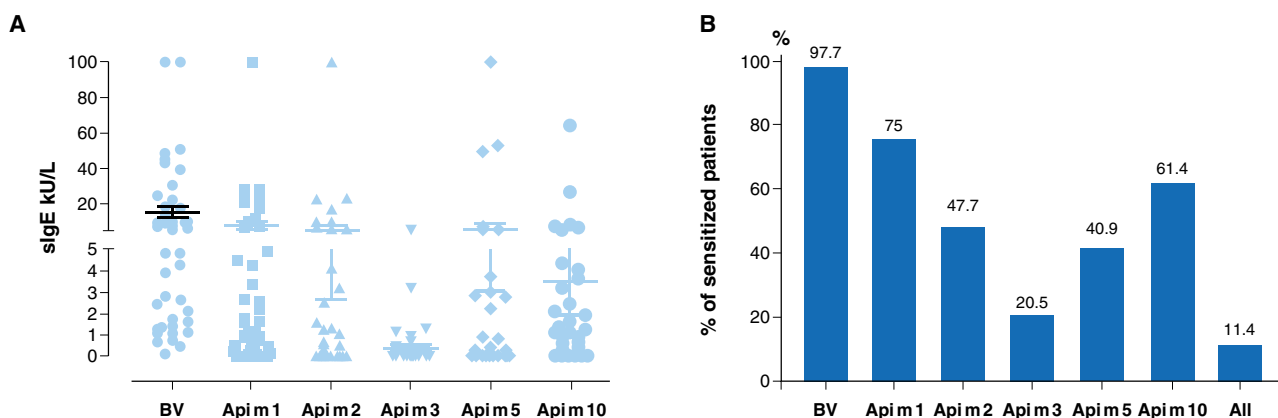
### Results

Forty-four patients were included in the study, 9 were beekeepers. Most patients were men (*n*=30; 68.2%), and mean patient age was 48.9 (SD 17.9) years (ranging from 13 to 82 years). Almost all patients (*n*=43; 97.7%) had positive sIgE for the whole BV extract. The one patient with negative sIgE for BV had positive sIgE for the molecular components Api m 1, Api m 5, and Api m 10. Most patients, including beekeepers, reported SSRs (*n*=33; 75%).

Regarding the concentrations of BV components, the whole BV extract showed the highest concentration (median 7.1 [IQR 1.6-16.3]), followed by Api m 1 (median 1.6 [IQR 0.3-7.5]), Api m 2 (median 0.2 [IQR 0-3.3]), Api m 3 (median 0 [IQR 0-0.3]), Api m 5 (median 0.2 [IQR 0-2.8]), and Api m 10 (median 0.7 [IQR 0.1-2.4]) (Figure 1A).

Regarding the sensitization profile of the study population, patients were also more frequently sensitized to the whole BV extract (*n* = 43; 97.7%), followed by Api m 1 (*n* = 33; 75%), Api m 10 (*n* = 27; 61.4%), Api m 2 (*n* = 21; 47.7%), Api m 5 (*n* = 18; 40.9%), and Api m 3 (*n* = 9; 20.5%) (Figure 1B).

The evaluation of a single species-specific recombinant allergen may be limited in the diagnosis of BV allergy. In our study, all patients were sensitized to at least 1 component, and 5 patients (11.4%) were sensitized to all of them. Also, 5 patients (11.4%) were sensitized only to Api m 1, but the



**Figure 1**

**A** - Mean concentrations of the whole extract and molecular components of bee venom (BV); **B** - Frequency of sensitization to each of the molecular components of BV

combination of Api m 1 and Api m 10 allowed the detection of sensitization in 16 patients (36.6%) and the combination of Api m 1, Api m 3, and Api m 10 allowed detection in 8 patients (18.2%).

We analyzed whether the type of reaction (LLR or SSR) could be associated with the sensitization profile. Eleven patients (25%) reported LLR with a bee sting; of these, 4 were women and mean age was 57.3 (SD 16.5) years. These patients had higher sIgE levels for the whole BV extract, Api m 2, and Api m 5, with mean values of 14.5, 10.5, and 11.1 kU/L, respectively. Thirty-three patients (75%) reported SSR; of these, 9 were beekeepers, 10 were women, and mean age was 46.1 (SD 17.6) years. Most of these patients were sensitized to Api m 1 ( $n = 28$ ; 84.8%). However, it is important to note that concomitant sensitization to Api m 1 and Api m 10 was detected in 20 patients (60.6%). These patients also had higher mean sIgE levels for the whole BV extract (15.2 kU/L) and for Api m 1 (9.6 kU/L), Api m 5 (4.3 kU/L), and Api m 10 (4.2 kU/L). There was a statistically significant difference ( $p = 0.0104$ ) in sIgE levels for Api m 1 between patients with LLRs and SSRs (Figure 2).

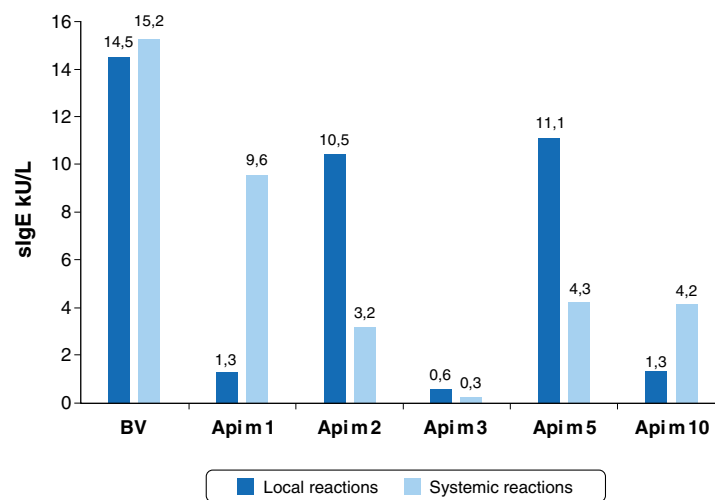
The results of the Western blot analysis of allergenic profile are shown in Figure 3A. The allergenic profiles were similar to those detected by

CRD (ImmunoCAP™): patients were more frequently sensitized to Api m 1 ( $n = 33$ ; 75%) and Api m 10 ( $n = 27$ ; 61.4%), followed by Api m 2 ( $n = 18$ ; 40.9%), Api m 5 ( $n = 13$ ; 29.5%), and Api m 3 ( $n = 10$ ; 27.7%). Nevertheless, the Western blot analysis identified more than half of these patients ( $n=28$ ; 63.6%) sensitized to Api m 6 and 10 (22.7%) and to Api m 4 (Figure 3B). These results are similar to those reported by Vega-Castro et al.<sup>15</sup> in Spain.

## Discussion

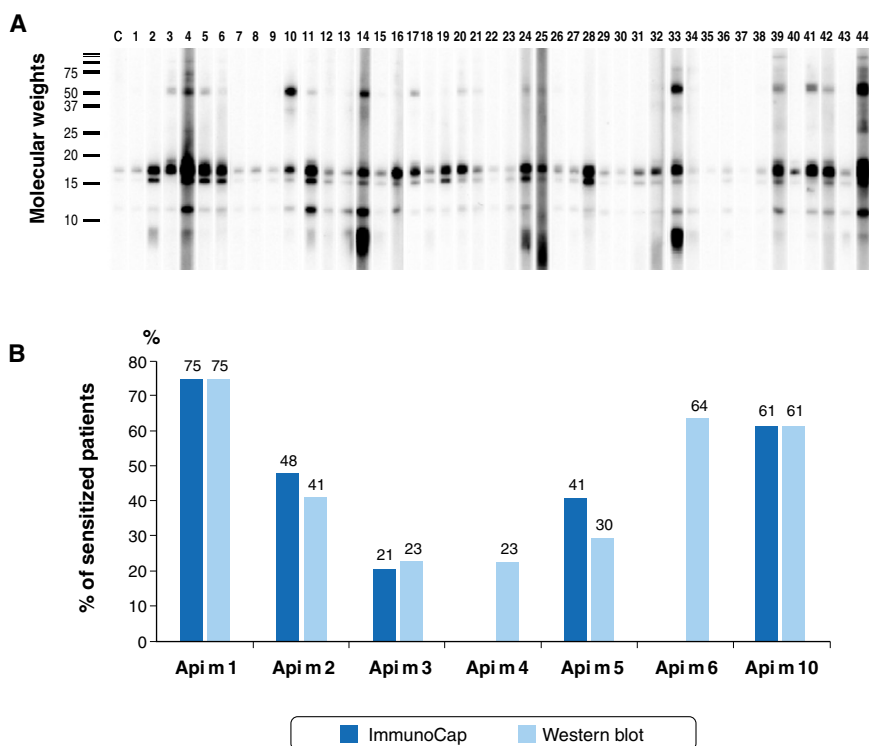
Our results showed that the sIgE levels for the molecular components Api m 1, Api m 2, Api m 3, Api m 5, and Api m 10 add value to the diagnosis of BV allergy. In line with previous reports,<sup>10,16</sup> we showed that the evaluation of more than one molecular component can increase diagnostic accuracy, since the number of identified patients increased with the analysis using combinations: the combination of Api m 1, Api m 3, and Api m 10 detected 8 patients (18.2%) and, even more relevant, the combination of Api m 1 and Api m 10 detected 16 patients (36.6%).

In BV allergic patients, sensitization to Api m 1 ranges from 57 to 97%.<sup>10,17-20</sup> Our study found that 75% were sensitized to Api m 1, which is in agreement



**Figure 2**

Sensitization profile of patients with large local vs systemic sting reactions



**Figure 3**

**A** - Allergenic profile by Western blot analysis in 44 patients; **B** - Comparison between the allergenic profile of the 44 patients as identified by ImmunoCAP™ and by Western blot

with data reported in the literature. Sensitization to Api m 2 ranges from 46 to 52%,<sup>10,17,20</sup> and a rate of 47.7% was found in the present study. Regarding Api m 3 and Api m 5, 20.5% and 40.9% of our patients, respectively, were sensitized to them, rates below those reported in previous studies (38 to 50%<sup>10,21</sup> and 58 to 60%,<sup>10,22</sup> respectively). Sensitization to Api m 10 was present in 61.7% of our patients, which is above the rates reported in previous studies (49 to 52%<sup>10,13</sup>).

Acid phosphatase (Api m 3), icarapin (Api m 10), and, with less expression, melittin (Api m 4) allow the diagnosis of bee sensitization in individuals with negative test to Api m 1. In their absence, it would be considered primary sensitization to wasp venom.<sup>13</sup> In recent years, recombinant BV allergens, such as Api m 4, have been suggested to be associated with a higher frequency of SSRs during bVIT induction,<sup>23,24</sup> or with lower effectiveness of bVIT, such as Api m 10.<sup>25</sup>

Concomitant sensitization to Api m 1 and Api m 10 was associated with the occurrence of SSRs, which is in accordance with the fact that phospholipase

A2 (Api m 1) is considered a major species-specific venom allergen.<sup>10</sup> There was a statistically significant difference ( $p = 0.0104$ ) in sIgE levels for Api m 1 between patients with LLRs and SSRs. In patients with LLRs, Api m 2 and Api m 5, which are non-species-specific components, showed higher sIgE levels, suggesting that the reaction may have occurred due venom toxicity.

More than half of our patients had positive sIgE results for Api m 10, a major BV allergen, which was also associated with the occurrence of SSRs. In our study, 60.6% of patients with SSR had positive results for both Api m 1 and Api m 10. According to the literature, patients with higher sIgE levels for Api m 10 are potentially at increased risk of treatment failure because this allergen is underrepresented or absent in almost all bVIT preparations.<sup>13,25,26</sup> Frick et al.<sup>25</sup> and Pereira Santos et al.<sup>27</sup> showed that patients with increased sIgE levels for Api m 10 were mostly non-responders to bVIT, indicating that sensitization to Api m 10 is the best discriminator of treatment failure.

In the present study, sensitization to Api m 6 was present in more than half of the patients analyzed by Western blot, which may be considered a major allergen. Kettner et al.<sup>28</sup> also identified Api m 6 in 42% of BV allergic patients. Sensitization to Api m 4 was present in 28 (of 44) patients analyzed by Western blot. This allergen has gained importance after its association with the occurrence of SSRs during the build-up phase of bVIT.<sup>23,24</sup>

The present study has limitations that need to be addressed. It is a retrospective study, with a small sample size. Nevertheless, the study is of added interest because it shows the association of double positivity for the 2 more prevalent recombinant allergens in our Portuguese BV allergic patients, Api m 1 and Api m 10, with the severity of reaction. Additionally, by Western blot analysis, we also identified Api m 6 as a potential major allergen in a well-characterized population of BV allergic patients.

We hope that our results can extend the knowledge of recombinant allergens, since these findings could have potential implications for the diagnosis and treatment of severe BV allergic patients.

## Conclusion

This study demonstrated that the analysis of the sensitization profile using molecular components increases diagnostic accuracy in BV allergic patients, allowing a possible correlation with the severity of reaction. Concomitant sensitization to Api m 1 and Api m 10 may be associated with SSRs. We emphasize the identification of sensitization to Api m 6 in more than half of our BV allergic patients, so it may be considered a major allergen. Also relevant is the identification of sensitization to Api m 4, as it may be related to a higher frequency of adverse reactions to bVIT. Further studies are needed to compare the different sensitization profiles in BV allergic patients and their correlation with the severity of reaction.

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Corresponding author:  
Catarina Coutinho  
E-mail: catarinapc@hotmail.com