



Detection of wheat allergens using 2D Western blot and mass spectrometry



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ABSTRACT

Background: Wheat allergy is relatively common and the associated clinical manifestations depend on the involved molecular allergens as well as on the way of exposure. Different symptoms have been described: wheat-dependent exercise-induced anaphylaxis (WDEIA), atopic dermatitis (AD) and pollen rhinitis (PR). Traditional diagnostic methods do not allow accurate molecular identification of the allergens that are essential for risk assessment and for the choice of the most adapted treatment.

Methods: Standardized total protein extracts obtained from wheat seeds were separated by 2D electrophoresis. Twenty-one sera with high wheat-specific immunoglobulin E (sIgE) levels were classified into three patients groups based on their clinical profile. These sera were tested by Western blot on 2D separated standardized wheat protein extract and their sIgE sensitization profiles were compared.

Results: Specific sensitization profiles were identified for each phenotype group. For WDEIA, protein spots around 37 kDa (pH 6–9) and 37–50 kDa (pH 5–6) were identified. For AD, spots were observed around 50 kDa (pH 9), 10 kDa (pH 9) and 20 to 75 kDa (pH 3). For PR, specific spots were located around 90 kDa (pH 9). The mass spectrometry (UHPLC-MS/MS) analysis of these identified spots pointed out several potential interesting allergens: Tri a 26, Tri a bA, Tri a 34, Tri a tritin.

Conclusions: The present study allowed the identification of different protein areas specific to these studied groups. The protein spots of interest were identified by UHPLC-MS/MS. It has been possible to establish a link between a specific symptomatology and the newly identified responsible allergens.

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Abbreviations: IgE, sIgE, immunoglobulin E, specific IgE; UHPLC-MS/MS, ultra-high performance liquid chromatography tandem mass spectrometry; MRM, multiple reaction monitoring; WDEIA, wheat-dependent exercise-induced anaphylaxis; BA, baker's asthma; AD, atopic dermatitis; PR, pollen rhinitis; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane with hydrochloric acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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1. Background

Food allergy is a major health problem, affecting 2–3 % of the world population and 6 to 8% of children [1]. Currently, there is no effective treatment except avoidance of the suspected food or long and difficult desensitization. A single food may contain numerous allergens each responsible for different clinical symptoms. Furthermore, depending on the suspected molecular allergen, the symptoms' severity and the strictness of the elimination diet may be highly variable. It is therefore important to identify the suspected molecular allergen(s) in order to accurately assess the potential risks for the patient. It also allows a significant improvement in the quality of life of these patients either by fine-tuning their diet or by a specific desensitization adapted against the responsible allergen extracts.

The standard diagnostic method for identifying food allergies is a careful clinical history and skin prick tests. Concerning available *in vitro* data, total IgE measurement, sIgE measurements and IgG4 measurements can be carried out against total allergenic extracts or against specific molecular allergens available on the market. Currently, concerning wheat, only two molecular allergens are available for routine diagnostic tests: Tri a 19 (omega-5-gliadin) and Tri a 14 (lipid transfer protein, LTP).

Wheat allergy is one of the most frequent food allergies along with milk, egg, peanut and shellfish. Clinical symptoms vary according to the involved allergen and the way of exposure. After consumption of wheat, an allergic patient may present different symptoms: anaphylaxis, exercise-induced anaphylaxis (EIA), baker's asthma (BA), atopic dermatitis (AD), pollinic rhinitis (PR) and urticaria can be cited as examples [1]. Wheat can also be involved in coeliac disease, a gluten-intolerance not to be confused with wheat allergy. Coeliac disease is an autoimmune illness necessitating a strict gluten-free diet, manifesting itself as e.g. intestinal swelling, abdominal pain and diarrhea. Many wheat allergens have been described and their sequences are available on the «Uniprot», «Allergome» and «Allergen» databases [2–4].

Wheat proteins are classified in four main families according to their solubility: albumine (soluble in water), globulins (soluble in diluted saline solutions), gliadins (soluble in alcohol) and glutenins (soluble in alkaline or acid solutions). Gliadins and glutenins are the main components of wheat gluten [5]. The protein composition of wheat matrix is very complex. Moreover, wheat contains a significant number of proteins which are difficult to isolate and identify due to their high starch content. This is why an accurate molecular diagnosis is very challenging. The difficulty in isolating these molecular allergens from each other prevents the measurement of sensitivity of the patient by traditional methods (Immuno-

CAP250, ImmunoCAP Isac). So far, only a few number of allergens have been related to specific clinical symptoms of wheat allergy. Omega-5-gliadin has been linked to EIA and the inhibitor family of amylase/trypsin has been linked to atopic dermatitis in children and adults [1]. During recent years, the use of gluten (gliadins and glutenins) has dramatically increased due to its emulsifying and preserving properties especially in the cosmetic and food industries. For these emerging uses, gluten is chemically-modified, leading to an adaptation or a creation of new allergens called wheat hydrolysates [6] which have been suspected in allergic reactions, especially in Japan, where a new type of WDEIA has appeared. In fact, more than 1300 people developed an allergy when ingesting natural wheat after using a soap containing these hydrolysates [7]. Cases of WDEIA, contact dermatitis, urticaria and anaphylactic shock have all been linked to a preceding sensitization to wheat hydrolysates [5].

Food allergy to wheat is therefore a complex and important subject and the associated elimination diet proposed to sufferers is restrictive and difficult to implement.

Only limited research has been carried out to refine wheat allergy diagnosis. In this study, 2D Western blot (2D WB) is put forward as the methodology of choice in the diagnosis of wheat allergy. Indeed, it can be used to separate complex protein mixtures by two-dimensional electrophoresis according to their isoelectric point and their molecular weight. It can also be used to accurately identify the molecular allergens responsible for allergy in selected patients.

2. Material and methods

2.1. Preparation of standardized wheat extracts

The wheat seeds were supplied by the Agro-Bio Tech faculty (Gembloux, University of Liège, Belgium) and by SCAM (Andenne, Belgium). Several varieties of wheat seeds were extracted and evaluated, including the locally-produced variety of *Triticum aestivum* which showed the widest spectrum of proteins, and which was therefore chosen for this study. Total protein extraction was carried out as follows : 500 mg of wheat seeds were mechanically crushed and proteins were extracted using 8 ml of a buffer containing 2% of sodium dodecyl sulfate (SDS) (Roth, Art.-Nr.CN30.1), 10% of glycerol (Fisher Scientific, G/P450/08), 50 mM dithiothreitol (DTT) (Roth, Art.-Nr.6908.1), 40 mM tris(hydroxymethyl)aminomethane with hydrochloric acid (TRIS-HCl) (Roth, Art.-Nr. 4855.1), pH 6.8. After one hour of incubation at room temperature, the extract was centrifuged at 16 000 g for 10 min, the supernatant was separated and precipitated overnight in cold acetone (–20 °C) and

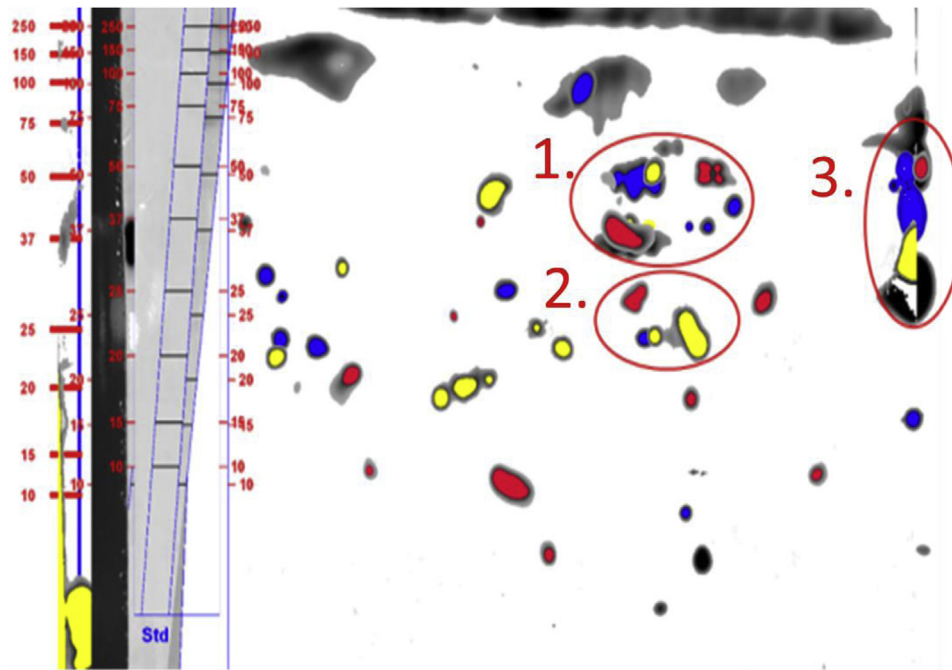


Fig. 1. Overlay of 2D immunoblot of four WDEIA patients : case 1 (black), case 2 (blue), case 3 (yellow) and case 4 (red).

Table 1

Clinical data of the studied patients.

Patients	Sex	Age	Clinical symptoms	F4	F416
1	M	17	WDEIA	/	0.17
2	M	59	WDEIA	0.39	11.3
3	M	40	WDEIA	/	2.84
4	F	3	WDEIA	/	1.52
5	F	3	Severe AD	0.17	/
6	M	26	Eczema	0.19	/
7	F	22	AD	0.54	/
8	M	36	Chronic urticaria	/	0.15
9	F	32	Urticaria	0.23	/
10	M	37	Severe AD	5.67	0.23
11	M	55	Eczema	13.1	/
12	M	28	Eczema	1.6	/
13	M	3	Eczema	0.14	/
14	M	23	Eczema	0.16	/
15	M	31	PR	3.26	/
16	F	32	PR	0.25	/
17	M	55	PR	0.24	/
18	F	22	PR	0.19	/
19	F	27	PR	0.11	/
20	M	51	PR	2.66	/
21	M	11	PR	0.55	/

then centrifuged at 14,000g for 10 min [8]. According to the application, the deposit was re-suspended either in PBS buffer (phosphate buffer saline) for measurement of protein concentration, using the BCA Protein Assay Kit (Pierce BCA Protein Assay Kit, 23227, Thermo Scientific), or in a rehydration buffer for isoelectric focusing (IEF). The rehydration buffer contained 8 M urea (Roth, Art.-Nr. 2317.3), 2% 3-[(3-cholamidopropyl)diméthylammonio]-1-propanesulfonate (CHAPS) (Roth, Art.Nr. 1479.2), 50 mM DTT and 0.2% of biolytes 3-10 (Bio-Rad, Cat.#163-1112).

2.2. Patient selection

Sera from 21 patients with positive sIgE to wheat ($f_4 > 0.1$ KUA/L) were collected from the blood bank of the CHU of Liège, Belgium (Table 1). The analysis was performed by ImmunoCAP250 (Thermo Scientific, Uppsala, Sweden). The patients presenting positive sIgE

to f4 (total wheat extraction) and, in several cases, sIgE to f416 (Tri a 19, omega-5-gliadin), f433 (Tri a 14, LTP), f98 (gliadin), f79 (gluten) and/or g15 (*Triticum sativum* pollen) were selected. Negative controls were also selected based on their negative sIgE to wheat extract and the lack of clinical history of wheat and/or grass pollen allergy. This study was approved by the local institutional ethics committee.

2.3. 2D Western blot

The acetone precipitated wheat extract was dissolved in a buffer for isoelectric focusing (IEF). 175 μ l of the protein sample was mixed with 125 μ l of IEF buffer and was placed on the 7 cm IPG strips, pH 3-10 (Bio-Rad, USA). Proteins were separated at 4000 V for 16 000 Vhr in the PROTEAN[®] i12[™] IEF System (Bio-Rad, USA). Before the subsequent separation of proteins by SDS-PAGE, the IPG strips were first equilibrated in a buffer containing 6 M urea, 2% SDS, 0.375 M TRIS-HCl (pH 8.8), 20% glycerol and 2% TBP, and then in a second buffer containing 6 M urea, 2% SDS, 0.375 M TRIS-HCl (pH 8.8), 20% glycerol and 0.5 g iodoacetamide, both steps for 10 minutes each. The SDS-PAGE separation was carried out using 8–16% Mini-PROTEAN[®] TGX Stain-Free[™] Precast Gels (Bio-Rad, USA) for 20 min at a constant voltage of 200 V using an electrophoresis unit PROTEAN[®] Tetra Vertical Electrophoresis Cell (Bio-Rad, USA). The profile of protein spots was visualized using a ChemiDoc Touch Imaging System (Bio-Rad, USA).

To proceed to the Western blot, 2D separated proteins were transferred onto PVDF membrane (Immobilon, Merck Millipore) for 1 h at room temperature using a Criterion[™] Blotter (Bio-Rad, USA). To reduce non-specific IgE fixations, the membrane was blocked for 1 h at room temperature with a TBST solution (Tris Buffer Saline and Tween 20) containing 10% of milk powder and then incubated overnight at 4 °C with the serum, diluted 10 times in TBST containing 5% of milk powder. After 5 washing cycles in TBST solution, the membrane was incubated for 1 h at room temperature with a solution containing specific secondary antibodies against human IgE conjugated with peroxidase (KPL, USA). The conjugate was diluted 1:1500 in TBST.

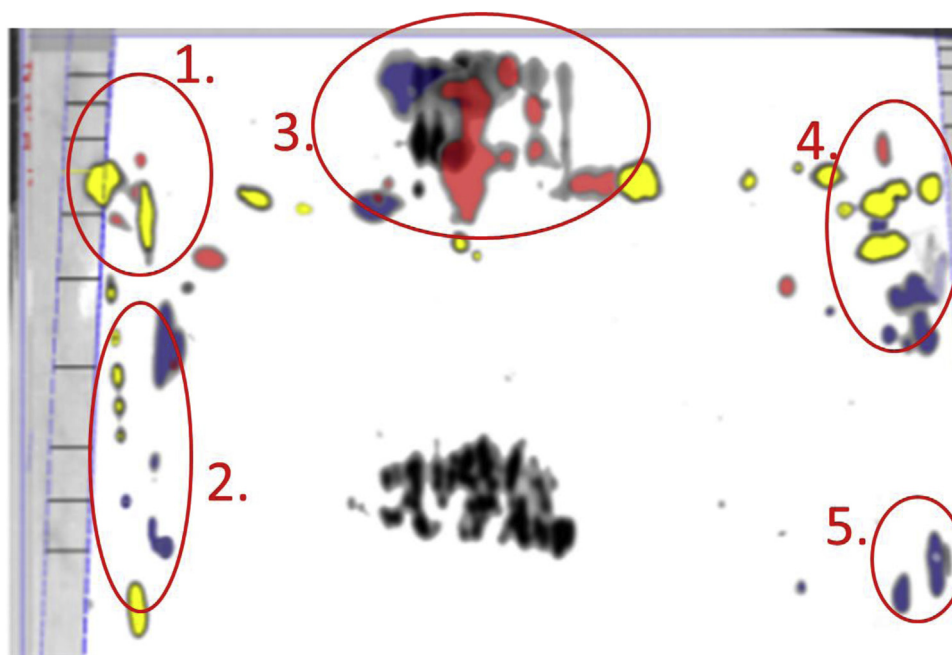


Fig. 2. Overlay of 2D immunoblot of four AD patients : case 6 (red), case 7 (yellow), case 9 (blue) and case 11 (black).

The wheat protein spots recognized by sIgE from the patients' serum were highlighted by ECL Clarity™ Western ECL Blotting Substrate (Bio-Rad, USA) according to the manufacturer's instructions, and visualized using the ChemiDoc Touch Imaging System (Bio-Rad, USA).

2.4. Marker peptide selection

First, wheat protein sequences were selected from the available sequences described in UniProt database. Second, the open source software Skyline was used for *in silico* enzymatic digestion of wheat allergen proteins and multiple reaction monitoring (MRM) design. The selected parameters used were: peptide length of 8–25 amino acids, b or y fragmentation, carbamidomethylation modification, precursor ion charge 2 or 3 and product ion charge 1 or 2.

2.5. Tryptic digestion from SDS-PAGE gel

The protein spots of interest were cut from SDS-PAGE, washed four times during 15 min with 50 mM ammonium bicarbonate (Acros organics, 123360010). After washing, 200 μ l of 50 mM ammonium bicarbonate and 20 μ l of trypsin (Promega, Sequencing Grade Modified Trypsin, Porcine, V5111) (0.1 μ g/ μ l) were added to the gel piece and incubated at 37 °C overnight. Proteolysis was stopped by addition of 30 μ l of 2% formic acid solution (Fisher Chemical, A117-50). Samples were stored at –20 °C until analyzed.

2.6. Protein identification by UHPLC-MS/MS

Digested peptides were then analyzed by UHPLC-MS/MS (Ultra-High Performance Liquid Chromatography tandem mass spectrometry; Waters, Milford, Massachusetts, USA). An acquity system (Milford, Massachusetts, USA) equipped with a c18 Acquity BEH130 Waters column (2.1 \times 150 mm) was used to separate wheat allergen peptides. The column compartment and thermal autosampler were set at 40 °C and 10 \pm 5 °C, respectively. 20 μ l sample was injected. A water (A)- acetonitrile plus 0.1% formic acid (B) gradient was applied for 24 minutes at 0.2 ml/min. Gradient was performed as follows: 0–1 min: 90% A, 1–9 min: 90% A to 60% A, 9–10 min :

60% A to 0% A, 10–12 min: 0% A, 12–13 min: 0% A to 90% A, 13–14 min: 90% A. A Waters Xevo TQS triple quadrupole system with a positive electrospray and MRM mode was used to detect wheat allergen peptides. A 150 l/h cone flow and a 650 l/h desolvation flow of nitrogen were applied. The capillary voltage was set at 3.50 kV and the collision gas flow was set at 0.12 ml/min. The source and desolvation temperatures were set respectively at 150 and 500 °C.

3. Results

3.1. 2D electrophoresis

Several optimizations have been developed for the protein extraction protocol, the buffers composition, the isoelectric separation programs and the percentage of acrylamide in SDS-PAGE gels.

3.2. Analysis of patients' sera using 2D WB

The clinical history of the 21 selected patients was used to classify them into 4 different groups: patients with EIA, patients with isolated atopic dermatitis, patients with pollinic rhinitis and patients with combined clinical symptoms. Most patients of the last group had a combination of atopic dermatitis and pollinic rhinitis.

Each of the 21 sera was evaluated using 2D WB with a standard protein spot profile for wheat obtained by 2D electrophoresis.

3.2.1. EIA group

Four out of 21 patients analyzed using 2D WB showed symptoms corresponding to EIA (Fig. 1) after eating wheat. The sensitization profile obtained for each patient was compared by superimposing Chemidoc Touch images within each patient group.

Fig. 1 shows IgE fixation on different protein spots localized in three areas: the first two are located around 50 kDa and pH 6 (circle 1) and pH 9 (circle 3) and the third is located at 25 kDa and pH 6 (circle 2).

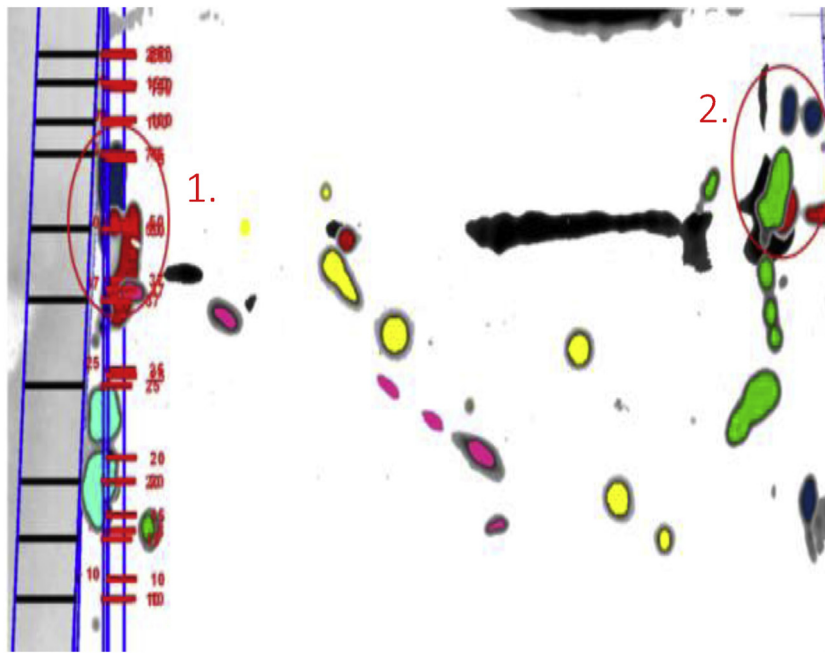


Fig. 3. Overlay of 2D immunoblot of seven PR patients: case 15 (pink), case 16 (black), case 17 (green), case 18 (red), case 19 (dark blue), case 20 (yellow) and case 21 (light blue).

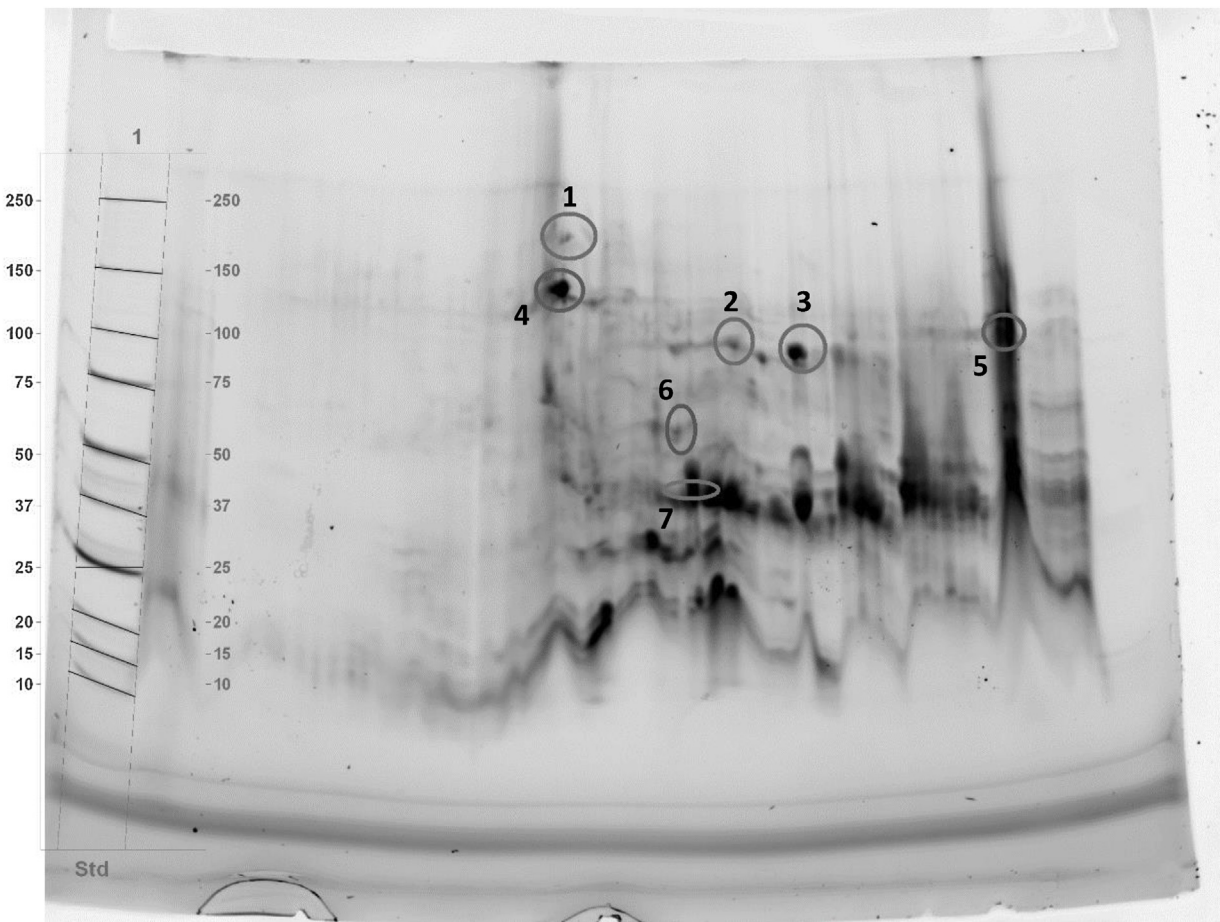


Fig. 4. Identification of proteins of interest (surrounded) by UHPLC-MS/MS. Tri a 26 is identified in the spots of protein numbers 1, 2 and 3. Tri a bA is identified in the spot of protein number 4. Tri a 19 is identified in the spot of protein number 5. Tri a tritin is identified in the spot of protein number 6. Tri a 34 is identified in the spot of protein number 7.

3.2.2. AD group

Ten of the 21 patients presented cutaneous symptoms such as atopic dermatitis, urticaria and/or eczema after wheat consumption. Each serum was analyzed by 2D WB. Sensitization profiles are shown on Fig. 2. However, due to the large number of patients in this group, only four blots have been represented to avoid overloading of the picture (Fig. 2).

The detailed analysis of these 4 WB highlighted five areas of interest with sIgE fixation (Fig. 2). Two areas were located around 50 kDa at pH 3 (circle 1) and pH 9 (circle 4), a third one was at 15 kDa and pH 3 (circle 2), the fourth was located around 75 kDa and pH 5-6 and the last one was around 15 kDa and pH 9 (circle 5).

3.2.3. PR group

Seven of 21 patients presented a seasonal grass pollen allergic rhinitis. Each serum was analyzed by 2D WB. Sensitization profiles are shown on Fig. 3.

The highlighted protein spots were identified as common and specific to this pollinic rhinitis group. These two areas are located around 50 kDa and pH 3 (circle 1) and pH 9 (circle 2) as shown on Fig. 3.

3.3. Identification of proteins of interest by UHPLC-MS/MS

Marker peptides were selected in a few steps. First, the Uniprot database was searched for target proteins of wheat. Second, peptides and transitions were generated by Skyline *in silico* digestion and peptide specificity was controlled with a BLAST analysis to check for sequences homology (Uniprot). Third, digested wheat proteins were analyzed by UHPLC-MS/MS. Several protein spots highlighted by sIgE interaction on 2D WB were identified corresponding to potentially interesting allergens: Tri a 26, Tri a bA, tri a 34, Tri a tritin and Tri a 19. Fig. 4 shows the spots of analyzed and identified proteins (surrounded on the global protein spot profile wheat extract). Table 2 shows the MRM parameters for the identification of aforementioned wheat proteins by UHPLC-MS/MS.

4. Discussion

Wheat proteins allergy is a complex food allergy with various symptoms depending on the allergen responsible. Accurate diagnosis is difficult because wheat contains a multitude of allergenic proteins and, apart from mixtures of either total extract, gliadins or gluten, only two molecular antigens are available on the market for routine sIgE testing.

Around fifty molecular allergens with molecular weight from 9 kDa to 90 kDa have been described for wheat. These allergens have been classified in different families according to their molecular properties and, more precisely, according to their structure homology. A limited number of wheat allergens have been associated with specific clinical symptoms of wheat allergy. Amongst them, the allergic reaction to omega-5-gliadin (Tri a 19) can be mentioned as being responsible for EIA but also the food allergy to wheat in children [9,10]. Moreover, different allergens have been described in baker's asthma: LTP (Tri a 14), Thioredoxin (Tri a 25) and others such as α -amylase inhibitors, prolamins, thaumatine-like proteins (Tri a TLP) [9-14], etc.

Furthermore, some allergens are responsible for cross-reaction between wheat flour and grass pollen: profilin (Tri a 12), LTP (Tri a 14), triosephosphate isomerase (Tri a 31), omega-5-gliadin (Tri a 19), thioredoxin (Tri a 25) and GAPDH [15]. Finally, different modified allergens of wheat hydrolysate allergy have been highlighted: omega-2 and γ gliadins [16,17].

Our results obtained from 2D Western blot analysis clearly show that IgE specificity and hence, sensitization profile of a patient vary with the clinical profile. The limitation of this study is the

small number of patients included. As a consequence, the next step should be to analyze a larger population of patients. Nevertheless, this preliminary study highlights different sensitization profiles associated with the clinical symptoms of each subgroup of patients. The Western blot showed IgE reactive protein spots common to many, if not all, members of each subgroup of patient. Most of these areas of interest did not overlap between the different subgroups and therefore appear to be specific to a given clinical symptomatology. For the EIA group, these specific areas are localized around 50 kDa at pH 6 and around 25 kDa at pH 6. For the AD group, the protein spots are localized around 50 kDa at pH 3 and around 15 kDa at pH 3. For the PR group, only one specific area was identified around 50 kDa at pH 3. This area could be in common with the AD group. It also seems that there is a common area to the three groups of patients located around 50 kDa at pH 9.

Based on wheat proteins already described in the literature, different hypotheses can be advanced concerning the nature of the identified protein areas. The common area for all three subgroups (EIA, AD, PR) could correspond to omega-5-gliadin. For the EIA group, the area around 50 kDa at pH 6 could be serpin (Tri a 33), Tri a 34 or Tri a bA and the area of interest around 25 kDa at pH 6 matches different molecular allergens such as Tri a 20, Tri a 27, Tri a 31 or Tri a 32. For the AD group, the area around 15 kDa at pH 3-4 could correspond to Tri a TLP, Tri a 12, Tri a 36, Tri a 40 or Tri a 42. Concerning the area of interest around 50 kDa at pH 3, no hypothesis can be advanced on the basis of the allergens already described. For the PR group, two areas of interest were identified, the first around 50 kDa at pH 3 and the second around 50 kDa at pH 9. However, these areas may also be found in the EIA and DA groups but given the multitude of molecules located in this range of molecular weight and isoelectric points, it is impossible to know if the same allergens are involved in different symptoms or not.

As the wheat matrix is very complex, it is difficult to confirm accuracy of any hypothesis concerning the identification of the responsible allergens. This link has been established by identification of protein spots detected through sIgE by using mass spectrometry (UHPLC-MS/MS). Effectively, specific sensitization profiles were identified for the three phenotype groups (WDEIA, AD, PR) and several wheat allergens were identified by UHPLC-MS/MS. Thus, the obtained results show that Tri a 26 seems to be specific to the AD patient group. All four AD patients have Tri a 26 specific IgE while patients with different symptomatology do not. Moreover, Tri a 19 seems to be a common allergen to all wheat allergic patients.

5. Conclusions

This study contributed to identify the specific molecular allergens for each wheat allergic subgroup studied: Tri a 26 has been identified as AD specific allergen whereas Tri a 19 sIgE are present in all patients' sera.

To confirm these results, the study should be extended to a larger number of patients and repeated in other wheat-allergic groups of patients, such as baker's asthma patients, or patients with wheat hydrolysates allergy. Finally, it could be interesting to test this method in other food allergies, in order to improve their diagnosis.

Authors' contribution

BQ, ST, RG, EC conceived and designed the experiments. JC and CB performed the experiments and analysis. JC and CB contributed reagents, materials and analysis tools. JC, CB, SE, XVdB, BQ contributed to the writing of the manuscript. RG, EC provided blood sera and patients clinical data, and informed them of the study. JC and CB interpreted the results and drafted the manuscript; JC, CB,

Table 2

Multiple reaction monitoring (MRM) parameters for the identification of wheat proteins by UHPLC-MS/MS. The identified proteins of wheat are Tri a 26, Tri a bA, tri a 34, Tri a tritin and Tri a 19.

Protein of wheat	Peptide	Retention time (min)	Precursor (charge state) (m/z)	Product ion (fragment)
Tri a 26	R.IFWGIPALLK.R	9.85	579,3577++	[b5] - 617,3446+ [b6] - 714,3974+ [b7] - 785,4345+ [b5] - 309,1759++ [y8] - 897,5557+ [y7] - 711,4763+ [y6] - 654,4549+ [y5] - 541,3708+ [y5] - 271,1890++
			386,5742+++	[b3] - 447,2391+ [b4] - 504,2605+ [b5] - 617,3446+ [b5] - 309,1759++ [b7] - 393,2209++ [b8] - 449,7629++ [b9] - 506,3049++ [y6] - 654,4549+ [y5] - 541,3708+ [y4] - 444,3180+ [y9] - 522,8157++ [y8] - 449,2815++ [y5] - 271,1890++
	R.ELQELQER.E	4.41	522,7696++	[b5] - 613,3192+ [b6] - 741,3777+ [b7] - 870,4203+ [y6] - 802,4054+ [y5] - 674,3468+ [y4] - 545,3042+
			348,8489+++	[b3] - 371,1925+ [b4] - 500,2351+ [b5] - 613,3192+ [b6] - 371,1925++ [b7] - 435,7138++ [y5] - 674,3468+ [y4] - 545,3042+ [y3] - 432,2201+ [y7] - 458,2483++ [y6] - 401,7063++
	R.SVAVSQVAR.Q	3.81	458,7642++	[b6] - 572,3039+ [b7] - 671,3723+ [b8] - 742,4094+ [y6] - 659,3835+ [y5] - 560,3151+ [y4] - 473,2831+
			306,1785+++	[b4] - 357,2132+ [b5] - 444,2453+ [b6] - 572,3039+ [b7] - 336,1898++ [b8] - 371,7083++ [y5] - 560,3151+ [y4] - 473,2831+ [y3] - 345,2245+ [y8] - 415,2482++ [y7] - 365,7139++ [y6] - 330,1954++
	R.QVVDQQLAGR.L	4.17	557,3042++	[b5] - 570,2882+ [b6] - 698,3468+ [b7] - 811,4308+ [y8] - 886,4741+ [y7] - 787,4057+ [y6] - 672,3787+

Table 2 (Continued)

Protein of wheat	Peptide	Retention time (min)	Precursor (charge state) (m/z)	Product ion (fragment)
			371,8719+++	[b4] - 442,2296+ [b5] - 570,2882+ [b6] - 698,3468+ [b7] - 406,2191++ [b8] - 441,7376++ [b9] - 470,2483++ [y6] - 672,3787+ [y5] - 544,3202+ [y4] - 416,2616+ [y9] - 493,2749++ [y8] - 443,7407++ [y7] - 394,2065++
Tri a bA	R.DVGATDPDIFYTNR.G	12.24	792,3705++	[b6] - 559,2358+ [b9] - 884,3996+ [b10] - 1031,4680+ [b11] - 1194,5313+ [b6] - 280,1216++ [y8] - 1025,5051+ [y7] - 928,4523+ [y6] - 813,4254+ [y8] - 513,2562++
			528,5827+++	[b6] - 559,2358+ [b7] - 656,2886+ [b8] - 771,3155+ [b6] - 280,1216++ [b11] - 597,7693++ [b12] - 648,2932++ [b13] - 705,3146++ [y8] - 1025,5051+ [y6] - 813,4254+ [y5] - 700,3413+ [y4] - 553,2729+ [y11] - 656,8120++ [y10] - 621,2935++ [y9] - 570,7696++ [y8] - 513,2562++
	R.NIEYLTLGVDDQPLFHGR.T	7.89	1044,0315++	[b10] - 1118,5728+ [b11] - 1233,5998+ [b12] - 1361,6583+ [b12] - 681,3328++ [y11] - 1240,6069+ [y10] - 1183,5854+ [y9] - 1084,5170+ [y6] - 726,4046+ [y6] - 363,7059++
			696,3567+++	[b6] - 734,3719+ [b7] - 847,4560+ [b8] - 904,4775+ [b12] - 1361,6583+ [b12] - 681,3328++ [b13] - 729,8592++ [b14] - 786,4012++ [b15] - 859,9354++ [y8] - 969,4901+ [y7] - 854,4631+ [y6] - 726,4046+ [y15] - 865,9467++ [y14] - 784,4150++ [y13] - 727,8730++ [y6] - 363,7059++
	K.AYDWSAYK.Q	16.61	502,2296++	[b4] - 536,2140+ [b5] - 623,2460+ [b6] - 694,2831+ [y7] - 932,4149+ [y6] - 769,3515+ [y5] - 654,3246+

Table 2 (Continued)

Protein of wheat	Peptide	Retention time (min)	Precursor (charge state) (m/z)	Product ion (fragment)
			335,1555+++	[b3] - 350,1347+ [b4] - 536,2140+ [b5] - 623,2460+ [b6] - 347,6452++ [b7] - 429,1769++ [y5] - 654,3246+ [y4] - 468,2453+ [y3] - 381,2132+ [y7] - 466,7111++ [y6] - 385,1794++
Tri a 34	K.VVISAPSK.D	4.25	400,7475++	[b5] - 470,2973+ [b6] - 567,3501+ [b7] - 654,3821+ [b5] - 235,6523++ [y6] - 602,3508+ [y5] - 489,2667+ [y4] - 402,2347+ [y3] - 331,1976+ [y3] - 166,1024++
			267,5007+++	[b3] - 312,2282+ [b4] - 399,2602+ [b5] - 470,2973+ [b5] - 235,6523++ [b6] - 284,1787++ [b7] - 327,6947++ [y5] - 489,2667+ [y4] - 402,2347+ [y3] - 331,1976+ [y7] - 351,2132++ [y6] - 301,6790++ [y3] - 166,1024++
	R.AASFNIIPSTGAAK.A	6.65	717,8830++	[b7] - 717,3930+ [b8] - 814,4458+ [b9] - 901,4778+ [b10] - 988,5098+ [b7] - 359,2001++ [y10] - 944,5411+ [y9] - 831,4571+ [y8] - 718,3730+ [y8] - 359,6901++
			478,9244+++	[b5] - 491,2249+ [b6] - 604,3089+ [b7] - 717,3930+ [b7] - 359,2001++ [b10] - 494,7585++ [b11] - 545,2824++ [b12] - 573,7931++ [y8] - 718,3730+ [y7] - 621,3202+ [y6] - 534,2882+ [y13] - 646,8459++ [y12] - 603,3299++ [y11] - 529,7957++ [y8] - 359,6901++
	K.VLPELNGK.L	5.34	435,2582++	[b4] - 439,2551+ [b5] - 552,3392+ [b6] - 666,3821+ [y7] - 770,4407+ [y6] - 657,3566+ [y5] - 560,3039+ [y6] - 329,1819++

Table 2 (Continued)

Protein of wheat	Peptide	Retention time (min)	Precursor (charge state) (m/z)	Product ion (fragment)
			290,5079+++	[b3] - 310,2125+ [b4] - 439,2551+ [b5] - 552,3392+ [b6] - 333,6947++ [b7] - 362,2054++ [y6] - 657,3566+ [y5] - 560,3039+ [y4] - 431,2613+ [y3] - 318,1772+ [y7] - 385,7240++ [y6] - 329,1819++
Tri a tritin	K.TDANAPPGK.A	5.59	435,7194++	[b5] - 473,1991+ [b6] - 570,2518+ [b7] - 667,3046+ [b5] - 237,1032++ [b6] - 285,6295++ [y7] - 654,3570+ [y6] - 583,3198+ [y5] - 469,2769+ [y4] - 398,2398+ [y3] - 301,1870+ [y4] - 199,6235++ [y3] - 151,0972++
			290,8154+++	[b4] - 402,1619+ [b5] - 473,1991+ [b6] - 570,2518+ [b5] - 237,1032++ [b6] - 285,6295++ [b7] - 334,1559++ [b8] - 362,6667++ [y5] - 469,2769+ [y4] - 398,2398+ [y3] - 301,1870+ [y8] - 385,1956++ [y7] - 327,6821++ [y6] - 292,1636++ [y4] - 199,6235++ [y3] - 151,0972++
	KLTNVALGR.Q	5.37	422,2560++	[b4] - 428,2504+ [b5] - 499,2875+ [b6] - 612,3715+ [y7] - 730,4206+ [y6] - 629,3729+ [y5] - 515,3300+
			281,8397+++	[b3] - 329,1819+ [b4] - 428,2504+ [b5] - 499,2875+ [b6] - 306,6894++ [b7] - 335,2001++ [y5] - 515,3300+ [y4] - 416,2616+ [y3] - 345,2245+ [y7] - 365,7139++ [y6] - 315,1901++
Tri a 19	K.HQFQESIK.H	3.2	508,7616++	[b4] - 541,2518+ [b5] - 670,2944+ [b6] - 757,3264+ [y7] - 879,4571+ [y6] - 751,3985+ [y5] - 604,3301+
			339,5102+++	[b3] - 413,1932+ [b4] - 541,2518+ [b5] - 670,2944+ [b6] - 379,1668++ [b7] - 435,7089++ [y5] - 604,3301+ [y4] - 476,2715+ [y3] - 347,2289+ [y7] - 440,2322++ [y6] - 376,2029++

Table 2 (Continued)

Protein of wheat	Peptide	Retention time (min)	Precursor (charge state) (m/z)	Product ion (fragment)
	R.HLNPSDQELQSPQQFLEK.T	12.4	1133,5586++	[b3] - 365,1932+ [b10] - 1162,5487+ [b11] - 1249,5808+ [b12] - 1346,6335+ [b3] - 183,1002++ [b11] - 625,2940++ [y16] - 1901,9239+ [y12] - 1474,7536+ [y11] - 1345,7110+ [y10] - 1232,6270+ [y8] - 1017,5364+ [y16] - 951,4656++ [y8] - 509,2718++
			756,0415+++	[b3] - 365,1932+ [b7] - 792,3635+ [b8] - 921,4061+ [b9] - 1034,4901+ [b11] - 1249,5808+ [b3] - 183,1002++ [b11] - 625,2940++ [b14] - 801,8790++ [b15] - 865,9083++ [b16] - 939,4425++ [y16] - 1901,9239+ [y8] - 1017,5364+ [y7] - 920,4836+ [y6] - 792,4250+ [y16] - 951,4656++ [y15] - 902,9392++ [y14] - 859,4232++ [y13] - 801,9097++ [y8] - 509,2718++
	R.TNNSLATPTTIPPATATTIPPATR.TI3.0		1204,1450++	[b7] - 702,3417+ [b11] - 1114,5739+ [b12] - 1211,6266+ [b13] - 1308,6794+ [b14] - 1379,7165+ [b19] - 1866,9807+ [b20] - 1964,0335+ [b7] - 351,6745++ [b11] - 557,7906++ [b12] - 606,3170++ [b19] - 933,9940++ [b20] - 982,5204++ [y17] - 1705,9483+ [y15] - 1507,8479+ [y14] - 1406,8002+ [y13] - 1293,7161+ [y12] - 1196,6634+ [y5] - 541,3093+ [y4] - 444,2565+ [y17] - 853,4778++ [y13] - 647,3617++ [y12] - 598,8353++ [y5] - 271,1583++ [y4] - 222,6319++

Table 2 (Continued)

Protein of wheat	Peptide	Retention time (min)	Precursor (charge state) (m/z)	Product ion (fragment)
			803,0991+++	[b7] - 702,3417+ [b9] - 900,4421+ [b10] - 1001,4898+ [b11] - 1114,5739+ [b12] - 1211,6266+ [b19] - 1866,9807+ [b20] - 1964,0335+ [b7] - 351,6745++ [b11] - 557,7906++ [b12] - 606,3170++ [b17] - 826,9281++ [b18] - 877,4520++ [b19] - 933,9940++ [b20] - 982,5204++ [y17] - 1705,9483+ [y13] - 1293,7161+ [y12] - 1196,6634+ [y10] - 1028,5735+ [y9] - 927,5258+ [y8] - 856,4887+ [y5] - 541,3093+ [y4] - 444,2565+ [y18] - 904,0016++ [y17] - 853,4778++ [y16] - 804,9514++ [y13] - 647,3617++ [y12] - 598,8353++ [y5] - 271,1583++ [y4] - 222,6319++
	RTNNSPATATTIPPAPQQR.F	13.9	932,9792++	[b4] - 417,1728+ [b10] - 959,4429+ [b11] - 1072,5269+ [b12] - 1169,5797+ [b14] - 1337,6696+ [b4] - 209,0901++ [b11] - 536,7671++ [b12] - 585,2935++ [b14] - 669,3384++ [y14] - 1448,7856+ [y11] - 1179,6480+ [y10] - 1108,6109+ [y9] - 1007,5633+ [y7] - 793,4315+ [y6] - 696,3787+ [y4] - 528,2889+ [y14] - 724,8964++ [y7] - 397,2194++ [y6] - 348,6930++ [y4] - 264,6481++
			622,3219+++	[b4] - 417,1728+ [b7] - 686,3104+ [b8] - 757,3475+ [b9] - 858,3952+ [b11] - 1072,5269+ [b12] - 1169,5797+ [b14] - 1337,6696+ [b4] - 209,0901++ [b11] - 536,7671++ [b12] - 585,2935++ [b13] - 633,8199++ [b14] - 669,3384++ [b15] - 717,8648++ [y14] - 1448,7856+ [y8] - 906,5156+ [y7] - 793,4315+ [y6] - 696,3787+ [y4] - 528,2889+ [y14] - 724,8964++ [y13] - 676,3701++ [y12] - 640,8515++ [y7] - 397,2194++ [y6] - 348,6930++ [y4] - 264,6481++

Table 2 (Continued)

Protein of wheat	Peptide	Retention time (min)	Precursor (charge state) (m/z)	Product ion (fragment)
	R.SSQPQQPFSLQPQQPFS,-	12.5	1029,9976++	[b4] - 431,1885+ [b7] - 784,3584+ [b10] - 1115,5116+ [b11] - 1228,5957+ [b12] - 1356,6543+ [b15] - 1709,8242+ [b4] - 216,0979++ [b7] - 392,6828++ [b12] - 678,8308++ [b15] - 855,4157++ [y14] - 1628,8067+ [y11] - 1275,6368+ [y10] - 1178,5840+ [y9] - 1031,5156+ [y6] - 703,3410+ [y3] - 350,1710+ [y14] - 814,9070++ [y11] - 638,3220++ [y6] - 352,1741++ [y3] - 175,5892++
			687,0008+++	[b4] - 431,1885+ [b7] - 784,3584+ [b8] - 881,4112+ [b9] - 1028,4796+ [b12] - 1356,6543+ [b15] - 1709,8242+ [b4] - 216,0979++ [b7] - 392,6828++ [b12] - 678,8308++ [b13] - 727,3571++ [b14] - 791,3864++ [b15] - 855,4157++ [y14] - 1628,8067+ [y11] - 1275,6368+ [y8] - 944,4836+ [y7] - 831,3995+ [y6] - 703,3410+ [y3] - 350,1710+ [y14] - 814,9070++ [y13] - 766,3806++ [y12] - 702,3513++ [y11] - 638,3220++ [y6] - 352,1741++ [y3] - 175,5892++

ST, XVdB, RG, BQ, SE, EC reviewed and approved the final version of the manuscript.

Declaration of competing interests

The authors declare that they have no competing interests

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study was approved by the local institutional ethical board "Comité d'éthique hospitalo-facultaire universitaire de Liège" (707) under the file number B707201836222. All aspects of the study complied with the Declaration of Helsinki. Patients from CHU of Liège were recruited based on an opt-out methodology.

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