

# High burden of clonal mast cell disorders and hereditary $\alpha$ -tryptasemia in patients who need *Hymenoptera* venom immunotherapy

Peter Korošec<sup>1,2,3</sup>  | Gunter J. Sturm<sup>4,5</sup>  | Jonathan J. Lyons<sup>6</sup> | Tinkara Pirc Marolt<sup>1</sup> | Manca Svetina<sup>1,7</sup> | Mitja Košnik<sup>1,8</sup>  | Mihaela Zidarn<sup>1,8</sup> | Mark Kačar<sup>1,8</sup> | Nina Freljih<sup>1,8</sup> | Nika Lalek<sup>1,8</sup> | Ajda Demšar Luzar<sup>1,7</sup> | Samo Zver<sup>8,9</sup> | Matevž Škerget<sup>8,9</sup> | Ewa Czarnobilska<sup>10</sup> | Wojciech Dyga<sup>10</sup> | Sanja Popović Grle<sup>11</sup> | Miroslav Samarzija<sup>11</sup> | Lisa Arzt-Gradwohl<sup>4</sup>  | Urban Čerpes<sup>4</sup> | Grzegorz Porebski<sup>10</sup>  | Branko Pevec<sup>11</sup> | Eva Schadelbauer<sup>4</sup> | Peter Kopač<sup>1,8</sup> | Julij Šelb<sup>1,8</sup> | Matija Rijavec<sup>1,7</sup> 

<sup>1</sup>University Clinic of Respiratory and Allergic Diseases, Golnik, Slovenia

<sup>2</sup>Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia

<sup>3</sup>Faculty of Medicine, University of Maribor, Maribor, Slovenia

<sup>4</sup>Department of Dermatology and Venerology, Medical University of Graz, Graz, Austria

<sup>5</sup>Allergy Outpatient Clinic Reumannplatz, Vienna, Austria

<sup>6</sup>Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA

<sup>7</sup>Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

<sup>8</sup>Medical Faculty, University of Ljubljana, Ljubljana, Slovenia

<sup>9</sup>The Department of Hematology, University Medical Center Ljubljana, Ljubljana, Slovenia

<sup>10</sup>Department of Clinical and Environmental Allergology, Jagiellonian University Medical College, Krakow, Poland

<sup>11</sup>Clinic for Respiratory Diseases Jordanovac, University Hospital Centre Zagreb, Zagreb, Croatia

## Abstract

**Background:** In patients who require venom immunotherapy (VIT), there is a need to identify underlying mast cell (MC) disorders since these may affect the risk and severity of future sting reactions and the long-term effectiveness of VIT.

**Methods:** 1319 individuals with Hymenoptera venom allergy (HVA) who needed VIT from referral centers in Slovenia, Austria, Croatia, and Poland underwent examination for *KIT* p.D816V in peripheral blood leukocytes (PBL) using a highly sensitive PCR test and tryptase genotyping by digital droplet PCR. We also included 183 control individuals with large local reactions (LLRs) to Hymenoptera stings and with asymptomatic sensitization to Hymenoptera venoms.

**Results:** 285 of 1319 individuals recommended for VIT (21.6%) were positive for *KIT* p.D816V in PBL, preferably those who present with severe reaction (33.9% [ $n=207$  of 610] with Ring-Messmer grade 3–4 vs. 11% [ $n=78$  of 709] with Grade 1–2;  $p<.0001$ ), whereas only 1.3% ( $n=2$  of 152) of controls with LLR and none with asymptomatic sensitization ( $n=31$ ) had *KIT* p.D816V. *KIT* p.D816V allelic burden was higher in those with severe reaction (median 0.018% [ $n=207$ ] in Grade 3–4 vs. 0.001% [ $n=78$ ] in Grade 1–2;  $p<.0001$ ), and the majority had normal baseline serum tryptase levels (69% [ $n=196$  of 285]). All *KIT* p.D816V-positive individuals ( $n=41$ ) who underwent bone marrow (BM) biopsy were found to have underlying clonal diseases, principally BM mastocytosis. H $\alpha$ T was also associated with severe HVA and symptoms ( $p<.01$ ),

**Abbreviations:** BM, bone marrow; BMM, bone marrow mastocytosis; BST, basal serum tryptase; CMD, clonal mast cell disorders; HVA, Hymenoptera venom allergy; H $\alpha$ T, hereditary alpha tryptasemia; ISM, indolent systemic mastocytosis; MC, mast cell; MMAS, monoclonal mast cell activation syndrome; SM, systemic mastocytosis; TPSAB1, tryptase alpha/beta 1; VIT, venom immunotherapy.

Peter Kopač, Julij Šelb, and Matija Rijavec contributed equally to this study.

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**Correspondence**

Peter Korošec, University Clinic of Respiratory and Allergic Diseases Golnik, Golnik 36, 4204 Golnik, Slovenia. Email: [peter.korosec@klinika-golnik.si](mailto:peter.korosec@klinika-golnik.si)

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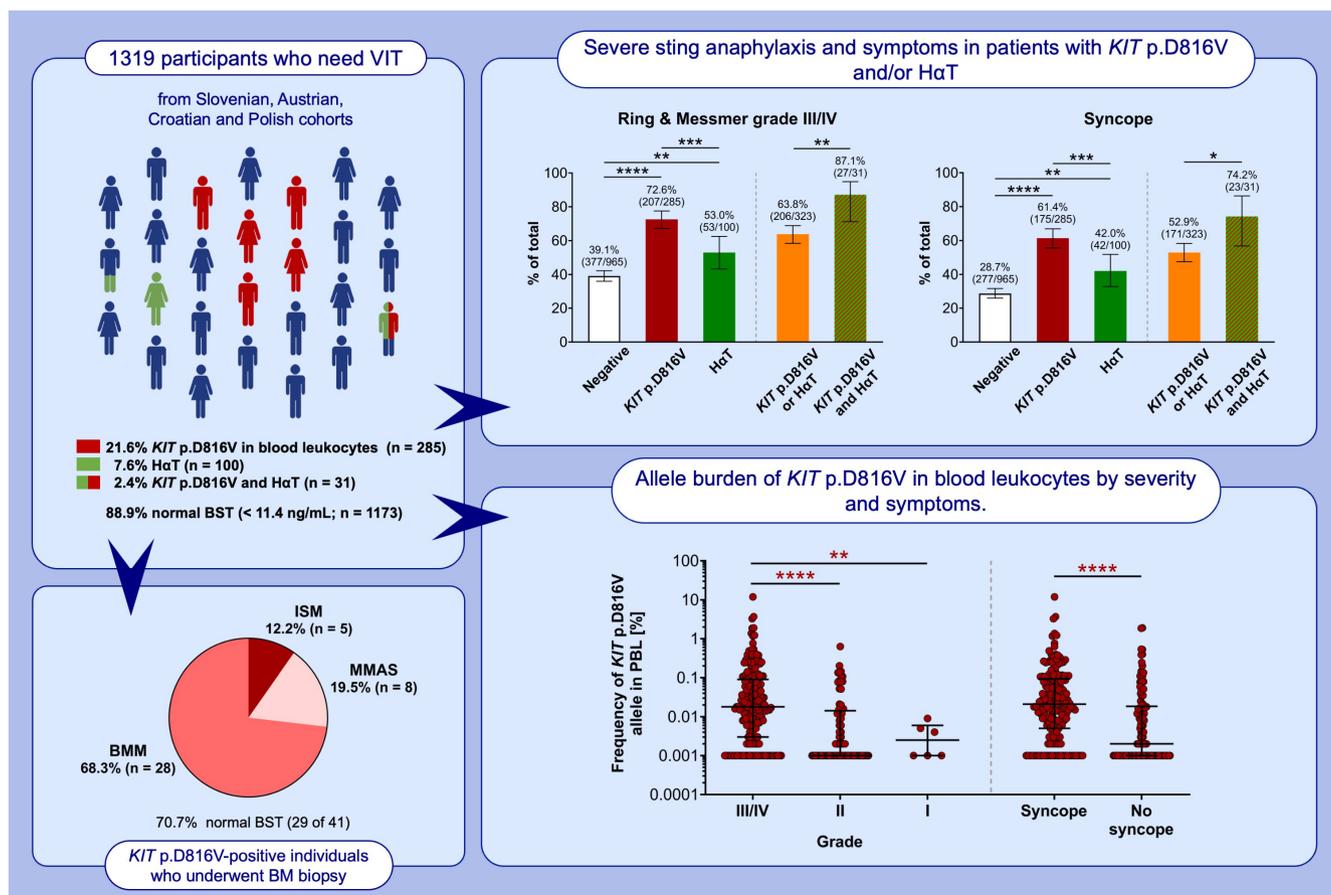
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and remarkably, 31.0% (n = 31 of 100) were found to have concomitant *KIT* p.D816V. Concomitant HαT and *KIT* p.D816V showed an additive effect, and having both was associated with the highest risk for severe HVA, even higher than having either HαT or *KIT* p.D816V alone (OR = 3.8; *p* < .01).

**Conclusions:** By employing prospective universal tryptase genotyping and examination for *KIT* p.D816V in PBL in large HVA populations, we have demonstrated a high burden of clonal MC disorders and HαT in patients who require VIT.

**KEYWORDS**

anaphylaxis, hereditary α-tryptasemia, hypersensitivity, immunotherapy, mast cell, mastocytosis, venom



**GRAPHICAL ABSTRACT**

In this multicenter study, we have demonstrated that clonal mast cell disease and HαT are more prevalent among individuals who need VIT; one or both was found in 27% of individuals overall. Both diagnoses were highly concentrated among individuals with severe anaphylaxis. Higher *KIT* p.D816V allelic burden was associated with more severe reactions. BMM, bone marrow mastocytosis; BST, basal serum tryptase; HαT, hereditary alpha tryptasemia; ISM, indolent systemic mastocytosis; MMAS, monoclonal mast cell activation syndrome; PBL, peripheral blood leukocytes; VIT, venom immunotherapy.

**1 | INTRODUCTION**

In the early 2000s, it was first noted that elevated baseline serum tryptase (BST) levels (>11.4 ng/mL) and systemic mastocytosis (SM) are associated with severe Hymenoptera venom allergy (HVA).<sup>1-3</sup> Further studies demonstrated that even minor increases within normal BST levels (above a concentration of approximately 5 ng/mL<sup>4</sup> or

8 ng/mL<sup>5,6</sup>) were associated with more severe systemic reactions after a field sting, independently of other prognostic variables.<sup>4-7</sup> Patients with insect sting anaphylaxis with elevated or normal BST levels and with the absence of typical skin lesions can be affected by bone marrow mastocytosis (BMM), indolent SM (ISM), or by monoclonal mast cell activation syndrome (MMAS).<sup>8-14</sup> Risk for severe sting anaphylaxis has also been associated with hereditary α-tryptasemia (HαT) resulting

from inherited replications of  $\alpha$ -tryptase-encoding gene copies at *TPSAB1*.<sup>14–18</sup> All of these conditions are associated with elevated BST levels.

The prevalence of HVA in SM is 20%–30%,<sup>19</sup> and HV stings represent the most common trigger of anaphylaxis in adults with SM (22%–60% of cases).<sup>20,21</sup> The reported prevalence of SM in systemic HVA is between 1% and 7.9% and is higher than in the general adult population (10–17 per 100,000 subjects).<sup>22–24</sup> In the US, the reported prevalence of SM among patients with HVA is lower than in European studies,<sup>22–24</sup> with 2.6% of patients with VIT having mastocytosis.<sup>25</sup> However, the prevalence of clonal mast cell disorders (CMD) in HVA may be higher since the diagnosis of clonal disease cannot be excluded on the basis of a normal BST<sup>12</sup> and invasive bone marrow (BM) biopsy requisite for definitive screening for clonal disease is often a major obstacle in fully evaluating larger HVA populations.<sup>6</sup> Indeed, by employing highly sensitive testing for *KIT* p.D816V in peripheral blood leukocytes (PBL)<sup>26–28</sup> we recently found a surprisingly high frequency (18.2%) of *KIT* p.D816V carriers among individuals with severe sting anaphylaxis and normal BST.<sup>14</sup> Recently, EU/US (ECNM-AIM) consensus group guidelines for personalized management strategies in MC disorders<sup>29</sup> emphasized the use of screening for *KIT* p.D816V in PBL, together with BST, as a first step for the evaluation of patients with typical skin lesions and suspected SM or in patients with typical clinical findings (e.g., sting anaphylaxis) without skin lesions.

In clinical practice, allergists frequently treat patients with agents to which they are allergic. Identifying patients at higher risk for severe reactions due to inherited or acquired factors would help drive clinical decision making. In patients who need venom immunotherapy (VIT), there is a need to identify possible underlying CMD or H $\alpha$ T that may affect the risk and severity of future sting reactions, and/or long-term effectiveness of VIT.<sup>6,29</sup> However, routine tryptase genotyping and examination for *KIT* p.D816V in PBL have not been employed prospectively in large HVA populations undergoing VIT.

## 2 | METHODS

### 2.1 | Study cohorts

The study cohorts came from referral centers in Slovenia, Austria, Croatia, and Poland (Table 1). BST levels were determined in all study participants. A clinical history was obtained, and skin and/or blood testing of *Hymenoptera* species was performed as reported.<sup>30,31</sup> Reaction grade was assigned according to the Ring and Messmer grading system<sup>32</sup> and defined as the field sting that had resulted in the most severe systemic reaction according to the individual patient's history.<sup>4</sup> VIT was recommended in all study participants, according to the European guidelines, including those with Grade I reaction with risk factors.<sup>31</sup> All individuals underwent *KIT* p.D816V mutational analysis in PBL and those with BST  $\geq 6$  ng/mL underwent tryptase genotyping by droplet digital PCR (ddPCR). Subjects with large local reactions (LLRs) to *Hymenoptera* stings and subjects with asymptomatic sensitization to *Hymenoptera* venoms<sup>33,34</sup> were

included as controls (Table S1). Further details are provided in the supplementary material—Data S1.

All individuals in the Slovenian cohort and all control subjects provided informed consent on Slovenian National Medical Ethics Committee approved research protocols (KME 150/09/13 and 188/17/4). Informed consent from the Austrian cohort was provided by all individuals in accordance with protocol no. 25-465 ex 12/13 approved by the Institutional Review Board of the Medical University of Graz. For Croatian and Poland study participants, informed consent was provided by all patients on institutional review board-approved research protocols no. 8 1-20/144-2 and no.1072.6120.318.2022, respectively.

### 2.2 | Tryptase quantification

Total BST levels were measured by Phadia 100 or 200 fluorescence enzyme immunoassays (Thermo Fisher Scientific, Waltham, MA). The lower limit of detection for this assay was 1 ng/mL. The normal range for BST in serum is from 1 to 11.4 ng/mL.<sup>35</sup>

### 2.3 | *KIT* p.D816V missense variant and tryptase genotyping in PBL

The detailed methodology is described in the supplementary material—Data S1. Briefly, the activating *KIT* c.2447A>T p.D816V missense variant was determined in PBL using a highly sensitive allele-specific qPCR assay developed by Kristensen et al.<sup>26,27</sup> and recently described in detail.<sup>14,15,28,36,37</sup> Genotyping of *TPSAB1* and *TPSB2* was accomplished as described.<sup>14,15,38,39</sup> ddPCR was performed on a QX200 (Bio-Rad, Hercules, Calif), and all individuals with BST  $\geq 6$  ng/mL underwent tryptase genotyping. No individual has been reported or observed with H $\alpha$ T and BST < 6 ng/mL.<sup>15,18,38,40–43</sup>

### 2.4 | Statistics

Mann-Whitney *U* tests and Fisher's exact tests were used to test the significance of associations as indicated; in all cases, two-tailed tests were used. CIs for prevalence data were determined using exact binomial calculation.

## 3 | RESULTS

### 3.1 | Clonal mast cell disorders are common among patients recommended for VIT

The reported prevalence of SM in systemic HVA is between 1% and 7.9% in Europe,<sup>22–24</sup> and in 2.6% of patients on VIT in the US.<sup>25</sup> However, when all 1319 HVA patients recommended for VIT in the cohorts from referral centers in Slovenia, Austria, Croatia, and

Poland were screened for *KIT* p.D816V in PBL regardless of BST level, 21.6% (285 of 1319; range from 14.3% to 29% for different cohorts) were found to be *KIT* p.D816V positive (Table 1; Figure 1A), an order of magnitude higher than some previous reports.<sup>22–25</sup> This may

result from the efforts of this study to apply a novel algorithm from recent EU/US (ECNM-AIM) consensus group guidelines for personalized management strategies in MC disorders.<sup>29</sup> These data suggest that in addition to BST determination, PBL should be examined for

TABLE 1 Clinical and laboratory characteristics.

Characteristic	All participants (n = 1319)	Slovenian cohort (n = 500)	Austrian cohort (n = 594)	Croatian cohort (n = 125)	Poland cohort (n = 100)
Age [years], median (range)	49 (17–87)	49 (17–79)	48 (17–87)	47 (21–73)	50 (18–77)
Sex, no. (%)					
Male	689 (52.2%)	278 (55.6%)	277 (46.6%)	80 (64.0%)	54 (54.0%)
Female	630 (47.8%)	222 (44.4%)	317 (53.4%)	45 (36.0%)	46 (46.0%)
Reaction severity grade, no. (%) <sup>a</sup>					
I	75 (5.7%)	36 (7.2%)	25 (4.2%)	12 (9.6%)	2 (2.0%)
II	634 (48.1%)	190 (38.0%)	394 (66.3%)	38 (30.4%)	12 (12.0%)
III	592 (44.9%)	272 (54.4%)	169 (28.5%)	70 (56.0%)	81 (81.0%)
IV	18 (1.4%)	2 (0.4%)	6 (1.0%)	5 (4.0%)	5 (5.0%)
Allergy					
Honeybee	349 (26.5%)	157 (31.4%)	97 (16.3%)	56 (44.8%)	39 (39.0%)
<i>Vespinae</i> spp	813 (61.6%)	320 (64.0%)	363 (61.1%)	69 (55.2%)	61 (61.0%)
Honeybee and <i>Vespinae</i> spp	157 (11.9%)	23 (4.6%)	134 (22.6%)	0 (0.0%)	0 (0.0%)
<i>KIT</i> p.D816V, no. (%)					
Positive	285 (21.6%)	137 (27.4%)	85 (14.3%)	34 (27.2%)	29 (29.0%)
Negative	1034 (78.4%)	363 (72.6%)	509 (85.7%)	91 (72.8%)	71 (71.0%)
HαT, no. (%) <sup>b</sup>					
Positive	100 (7.6%)	41 (8.2%)	38 (6.4%)	9 (7.2%)	12 (12.0%)
Negative	1219 (92.4%)	459 (91.8%)	556 (93.6%)	116 (92.8%)	88 (88.0%)
<i>KIT</i> p.D816V and HαT, no. (%)	31 (2.4%)	12 (2.4%)	6 (1.0%)	4 (3.2%)	9 (9.0%)
<i>KIT</i> p.D816V and/or HαT negative, no. (%)	965 (73.2%)	334 (66.8%)	477 (80.3%)	86 (68.8%)	68 (68.0%)
BST level [ng/mL], median (range)	5.0 (<1.0–90.2)	5.2 (<1.0–82.2)	4.7 (1.0–41.7)	4.7 (<1.0–63.5)	5.5 (<1.0–90.2)
<11.4 ng/mL	1173 (88.9%)	444 (88.8%)	542 (91.2%)	108 (86.4)	78 (78.0%)
>11.4 ng/mL	146 (11.1%)	55 (11.0%)	52 (8.8%)	17 (13.6%)	22 (22.0%)
>20 ng/mL	51 (3.9%)	16 (3.2%)	15 (2.5%)	7 (5.6%)	13 (13.0%)
>30 ng/mL	19 (1.4%)	4 (0.8%)	8 (1.3%)	3 (2.4%)	4 (4.0%)
Skin symptoms, no. (%)					
No	490 (37.1%)	192 (38.4%)	245 (41.2%)	24 (19.2%)	29 (29.0%)
Yes	829 (62.9%)	308 (61.6%)	349 (58.8%)	101 (80.8%)	71 (71.0%)
Unconsciousness, no. (%)	471 (35.7%)	237 (47.4%)	118 (19.9%)	44 (35.2%)	72 (72.0%)
REMA score					
≥2	287 (21.8%)	152 (30.4%)	82 (13.8%)	21 (16.8%)	32 (32.0%)
<2	1032 (78.2%)	348 (69.6%)	512 (86.2%)	104 (83.2%)	68 (68.0%)
Cardiovascular comorbidities	204 (26.7%) <sup>c</sup>	134 (26.8%)	9 (23.1%) <sup>c</sup>	25 (20%)	36 (36%)

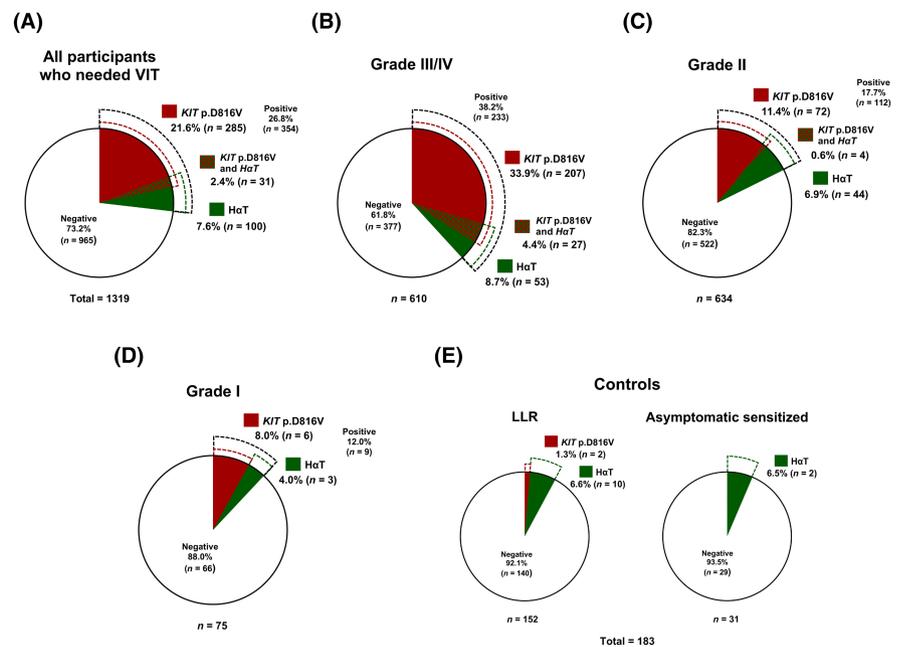
Abbreviations: HαT, hereditary alpha tryptasemia; BST, basal serum tryptase; REMA score, Red Espanola de Mastocitosis scor.

<sup>a</sup>Grades were assigned according to the Ring and Messmer.

<sup>b</sup>Tryptase genotyping was performed in all individuals with BST ≥6 ng/mL; no individual has ever been reported or observed with HαT and a BST <6 ng/mL.

<sup>c</sup>Cardiovascular comorbidities data were not available for 555 individuals of the Austrian cohort. Venom immunotherapy (VIT) was recommended in all individuals according to European guidelines, and at the time of final data analysis, Venom immunotherapy was already started in all individuals of the Slovenian, Croatian and Polish cohorts and in 40% (234 of 594) of individuals from the Austrian cohort.

**FIGURE 1** *KIT* p.D816V and/or HαT prevalence in patients who need VIT and in controls. (A) All participants who need VIT. (B) Grade III–IV HVA. (C) Grade II HVA. (D) Grade I HVA. (E) Controls with LLR to Hymenoptera stings or with asymptomatic sensitization to Hymenoptera venoms. HVA, Hymenoptera venom allergy; HαT, hereditary alpha tryptasemia; LLR, large local reactions; VIT, venom immunotherapy.



*KIT* p.D816V, using a highly sensitive allele-specific PCR test, in all patients with suspected clinical findings (like venom anaphylaxis) as an initial screen regardless of BST results.<sup>6,10,12,29</sup>

Numerous studies have shown that CMD is a high-risk factor for severe sting reactions.<sup>6</sup> We fully confirmed those observations, as *KIT* p.D816V was detected in 33.9% (207 of 610) individuals in Grade III–IV HVA compared with 8% (6 of 75) in Grade I and 11.4% in Grade II HVA (72 of 634) (Figure 1B–D) ( $p < .0001$ ). Moreover, the prevalence of *KIT* p.D816V was comparable between the cohorts (Slovenian vs. Austrian vs. Croatian and Polish) with 39.4% (108 of 274), 26.8% (47 of 175), and 32.3% (52 of 161) in Grade III–IV HVA and 12.8% (29 of 226), 9.1% (38 of 419), and 17% (11 of 64) in Grade I–II HVA being positive, respectively (Figures 2A–D). It is worth noting that overall prevalence of *KIT* p.D816V patients positively correlated with and was dependent upon the frequency of severe reactions that varied substantially between the cohorts (Table 1).

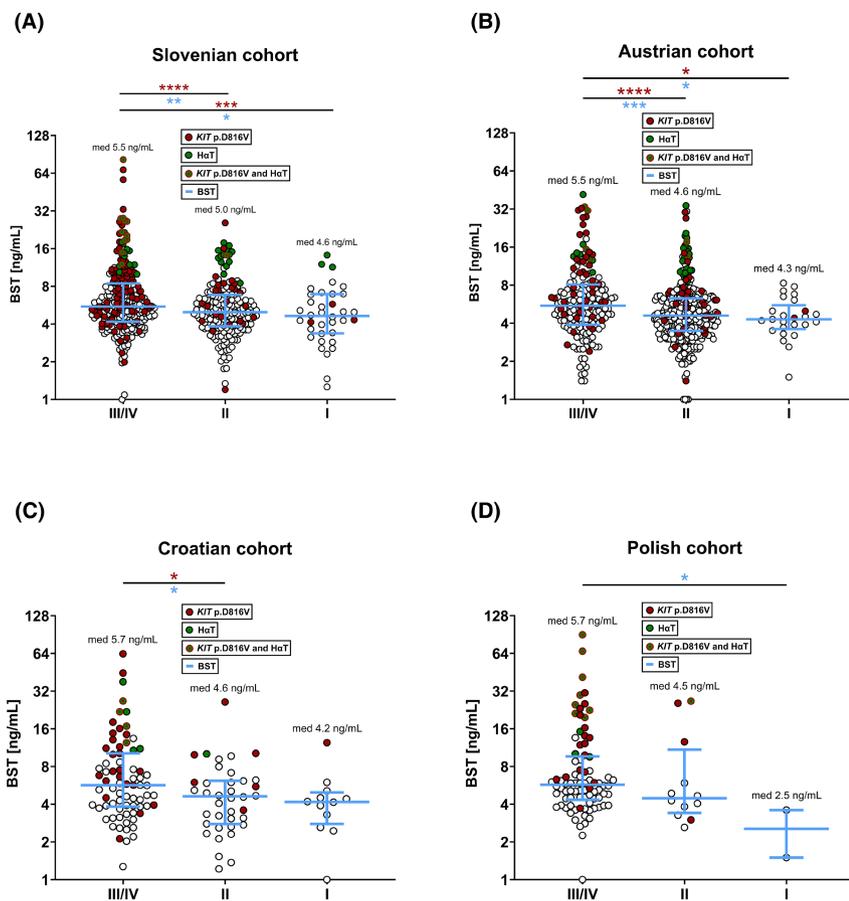
Among individuals with CMD, the prevalence of Grade III–IV HVA was 72.6% (207 of 285) compared with 39.1% (377 of 965) among negative individuals (Figure 3A) ( $p < .0001$ ; odds ratio [OR]=4.1 [95% CI=3.1–5.5]; relative risk [RR]=1.9 [95% CI=1.7–2.1]). Overall, in the case of detectable *KIT* p.D816V, the rate of Grade III–IV versus Grade I–II was roughly 3–1 (207 to 78 of 285), whereas this rate was roughly 1–1 when analyzing all participants (Tables S2A–E) (610 to 709 of 1319) ( $p < .0001$ ).

*KIT* p.D816V was also associated with more severe symptoms. Thus, 61.4% of individuals with *KIT* p.D816V (175 of 285) were characterized by loss of consciousness compared with 28.7% (277 of 965) in negative individuals (Figure 3B) ( $p < .0001$ ; OR=4 [95% CI=3–5.2]; RR=2.1 [95% CI=1.9–2.4]). Further, in 63.5% (181 of 285) of individuals with *KIT* p.D816V, urticaria, and angioedema were absent, while only 30.1% of negative individuals lacked these

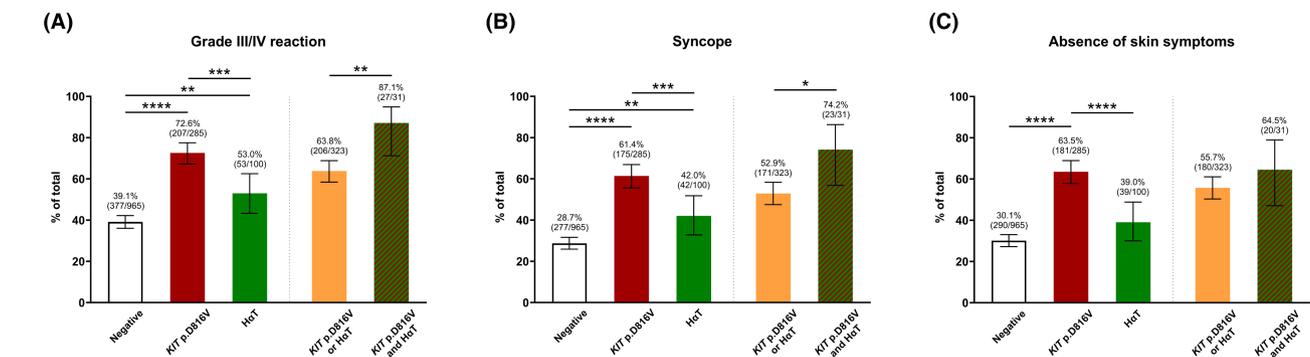
symptoms during reactions (290 of 965) (Figure 3C) ( $p < .0001$ ; OR=4.1 [95% CI=3.1–5.3]; RR=2.1 [95% CI=1.9–2.4]).

Among individuals with severe HVA, those with underlying SM have been reported to frequently be male and older.<sup>6</sup> However, we did not observe major gender differences, but rather similar numbers of males and females within *KIT* p.D816V positive (overall 159 vs. 126; 112 vs. 95 in Grade III–IV; 47 vs. 31 in Grade I–II, respectively) and negative individuals (530 vs. 504, respectively) (Tables S2A–E). However, individuals with *KIT* p.D816V did tend to be older (median 51 years;  $n=285$ ) than those without (median 48 years;  $n=1034$ ;  $p < .01$ ). Nevertheless, the age of Grade III–IV individuals with *KIT* p.D816V (median 51 years;  $n=207$ ) was comparable with those with Grade I–II (median 50 years;  $n=78$ ). No specific Hymenoptera species was associated with HVA in *KIT* p.D816V individuals, and the ratio between honeybee and vespid venom allergy was comparable between those with (30.2% vs. 69.8%; respectively) and without (35.4% vs. 64.6%, respectively) detectable *KIT* p.D816V (Tables S2A–E). Further, we found that the prevalence of cardiovascular comorbidities was highly comparable between *KIT* p.D816V positive and negative individuals (26.2% [54 of 206] vs. 27.5% [142 of 517], respectively; Tables S2A–E). Thus, the association of CMD with severe sting reactions seems to be independent of cardiovascular comorbidities.

As comparators, we obtained 183 control individuals with detectable specific IgE to Hymenoptera venoms; of those controls, 152 have had LLR to Hymenoptera stings, and 31 tolerated previous Hymenoptera stings without a systemic allergic reaction or LLR (Table S1). Among the 152 controls with LLR, 1.2% (2 of 152) had detectable *KIT* p.D816V in their peripheral blood, and among the 31 controls with asymptomatic sensitization, none had *KIT* p.D816V (Table S1; Figures 1E; Figure S1) ( $p < .0001$  in comparison to patients with systemic reactions who needed VIT).



**FIGURE 2** BST levels, *KIT* p.D816V and/or HαT by severity of HVA (Grade I–IV) in patients who need VIT. (A) Slovenian cohort. (B) Austrian cohort. (C) Croatian cohort. (D) Polish cohort. BST, basal serum tryptase; HVA, Hymenoptera venom allergy; HαT, hereditary alpha tryptasemia; VIT, venom immunotherapy.



**FIGURE 3** Severe sting anaphylaxis and symptoms in patients with *KIT* p.D816V and/or HαT. (A) Grade III–IV reaction. (B) Loss of consciousness. (C) Absence of urticaria and angioedema. HαT, hereditary alpha tryptasemia.

### 3.2 | The majority of individuals with clonal mast cell disorders had normal BST

Overall, 69% (196 of 285) of individuals with *KIT* p.D816V in PBL had normal serum tryptase levels (Tables S3A–E). The rate of severe HVA (Grade III–IV vs. Grade I–II) was higher in *KIT* p.D816V individuals with increased BST (>11.4 ng/mL) than those with normal BST (roughly 5–1 [75 vs. 14] compared to 2–1; 132 vs. 64), respectively ( $p < .01$ ). However, in total there were twice as many *KIT* p.D816V individuals with Grade III–IV HVA with normal BST (63.8%; 132 of 207) than those with elevated BST (36.2%; 75 of 207). Furthermore, the majority of individuals with *KIT* p.D816V

and severe symptoms have normal BST (65.7% with syncope; 115 of 175, and 63.5% without skin symptoms; 115 of 181). Moreover, overall 53.7% (153 of 285) individuals with *KIT* p.D816V in PBL had  $BST < 8$  ng/mL,<sup>5,6</sup> and 22.5% (64 of 285) had  $BST < 5$  ng/mL.<sup>4</sup> (Tables S2A–E).

### 3.3 | Higher *KIT* p.D816V allelic burden in PBL is associated with more severe HVA

Improved detection of the *KIT* p.D816V using a quantitative and highly sensitive real-time qPCR assay developed by Kristensen

et al.<sup>26,27,37</sup> has enabled quantification of as few as 0.001% *KIT* p.D816V encoding alleles.<sup>14,15,36</sup> Thus, our examination of large HVA populations with this highly sensitive method enable us to analyze whether allelic burden in PBL correlates with the severity of sting anaphylaxis.

We found that among 285 individuals with *KIT* p.D816V missense variant, those with Grade III–IV HVA (207 of 285) had an 18-fold higher allelic frequency (median 0.018%) compared with the individuals with Grade I–II HVA (78 of 285; median 0.001%;  $p < .0001$ ) (Figure 4A). Moreover, a 10-fold higher *KIT* p.D816V allelic frequency in PBL was found in those with a history of loss-of-consciousness during a sting reaction (175 of 285; median 0.021% vs. 110 of 285; median 0.002%;  $p < .0001$ ) or those without skin symptoms (181 of 285; median 0.022% vs. 104 of 285; median 0.002%;  $p < .0001$ ) (Figures 4B,C). These data suggest the clinical importance of *KIT* p.D816V allelic burden when risk stratifying individuals with HVA.

### 3.4 | *KIT* p.D816V in PBL reliably identifies HVA individuals with BM involvement

The identification of activating point mutations in *KIT* represents one of the minor criteria for the diagnosis of SM, and thus, when identified in a patient, a subsequent BM examination should be performed to classify that individual's CMD.<sup>44,45</sup> However, only a limited number of patients ( $n=41$ ; all Grade III HVA) that had *KIT* p.D816V in their PBL could undergo an extensive work-up that included a BM biopsy (Table S4). Whereas most of these individuals had normal BST (70.7%; 29 of 41), all of these patients had evidence of BM involvement. The majority (68.3%; 28 of 41) were affected by BMM (Figure 5A). BMM is a new SM subtype, defined by fulfilled WHO criteria for SM, involvement of BM, absence of typical skin lesions, no B-Finding, and BST levels  $<125$  ng/mL (Tables S4 and S5).<sup>9,29,45</sup> Five patients (12.2%) were affected by ISM, and 8 (19.5%) were identified as having MMAS (Figure 5A). Individuals with BMM and ISM had a higher frequency of *KIT* p.D816V encoding alleles in PBL (median 0.022% and 0.081%, respectively) compared with MMAS (median 0.003%;  $p < .05$ ); those with ISM also had a higher frequency than BMM (Figure 5B) ( $p < .05$ ). ISM was also associated with higher BST level than BMM (median 13 vs. 8.7 ng/mL, respectively); however

BST in ISM did not reach significance in comparison to MMAS (median 9.1 ng/mL) (Figure 5C).

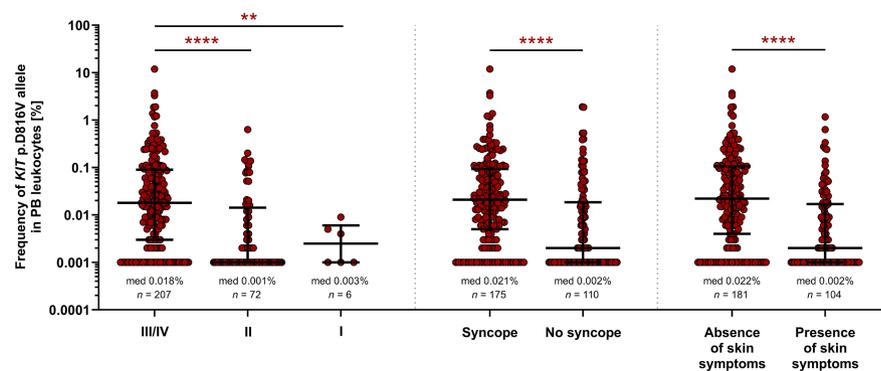
### 3.5 | H $\alpha$ T is associated with increased severity of HVA

Inherited differences in  $\alpha$ -tryptase-encoding copies at *TPSAB1*<sup>16</sup> have recently been linked to severe HVA.<sup>14–16</sup> Among 610 individuals with Ring-Mesmer grade III/IV HVA, we identified H $\alpha$ T in 8.7% (53 of 610) (Figure 1B) compared with a 5.7% prevalence reported for the general populations of the UK, US, and E.U.<sup>46</sup> A similarly high prevalence of H $\alpha$ T in Mueller grade IV HVA was found in previous studies (9.2%,<sup>15</sup> 7.3%,<sup>14</sup> and 8.4% in a combined analysis<sup>16</sup>). On the other hand, the prevalence in Grade I HVA was 4% (3 of 75), and in Grade II HVA was 6.9% (44 of 634) (Figures 1C,D). Among H $\alpha$ T individuals, the rate of Grade III/IV HVA was 53% (53 of 100) compared with 39.1% (377 of 965) among negative individuals (Figure 3A) ( $p < .01$ ; OR=1.8 [95% CI=1.2–2.7]; RR=1.4 [95% CI=1.1–1.7]). H $\alpha$ T was also associated with more severe symptoms; the rate of syncope was 42% of those with the genetic trait (42 of 100) compared to 28.7% of those without (277 of 965) (Figure 3B) ( $p < .01$ ; OR=1.8 [95% CI=1.2–2.7]; RR=1.5 [95% CI=1.1–1.9]).

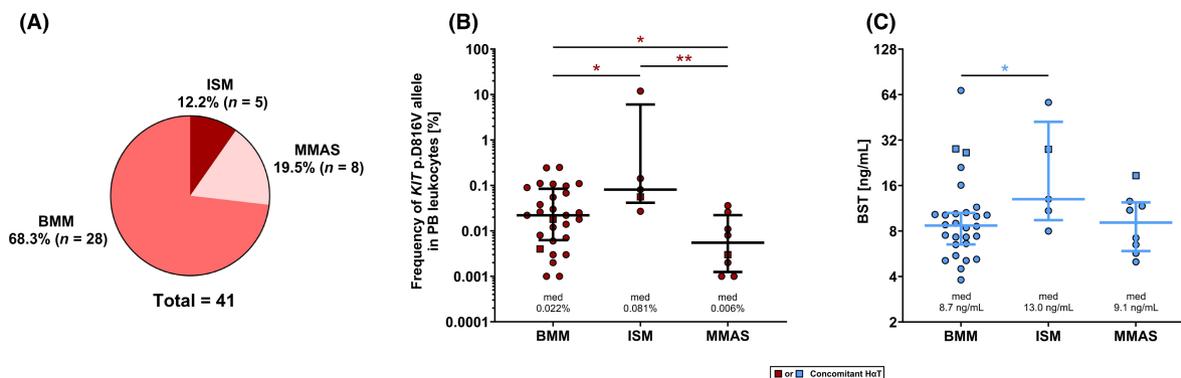
There were no significant differences in gender distribution (58 vs. 42 and 489 males vs. 476 females, respectively), age (median 48 years for both groups), Hymenoptera species (33.6% vs. 66.4% and 35.4% vs. 64.6% ratio between honeybee and vespid venom allergy, respectively), or cardiovascular comorbidities (19.7% vs. 27.5%) between H $\alpha$ T positive and negative individuals who need VIT (Tables S2A–E). Among the controls, a H $\alpha$ T prevalence (6.6% for LLR and 6.5% for asymptomatic sensitized; Table S1; Figures 1E; Figure S1) was comparable to the general population estimates for H $\alpha$ T (of 5.7%).<sup>46</sup>

### 3.6 | Individuals with both H $\alpha$ T and *KIT* p.D816V are at the highest risk for severe anaphylaxis

It is known that H $\alpha$ T and CMD may co-occur,<sup>41</sup> and we previously found a high prevalence of concomitant H $\alpha$ T in HVA patients with SM (14.3%).<sup>15</sup> In this study, 31 individuals were identified as having



**FIGURE 4** Allele burden of *KIT* p.D816V in blood leukocytes by severity of HVA (Grade I–IV) and symptoms. HVA, Hymenoptera venom allergy.



**FIGURE 5** Presence of clonal disease among HVA individuals with detectable *KIT* p.D816V in blood leukocytes and performed BM studies. (A) Classification. (B) Classification by allele burden of *KIT* p.D816V in blood leukocytes. (C) Classification by BST. BMM, bone marrow mastocytosis; ISM, indolent systemic mastocytosis; MMAS, monoclonal MC activation syndrome.

both H $\alpha$ T and *KIT* p.D816V. Thus, the H $\alpha$ T prevalence in individuals with *KIT* p.D816V was 10.9% (31 of 285). Remarkably, nearly one third of individuals who had H $\alpha$ T (31.0%; 31 of 100) were found to have *KIT* p.D816V in PBL (Tables S2A–E). Of those 31 individuals, nearly 9 out of 10 (87.1%; 27 of 31) had Grade III–IV HVA, and that was significantly higher compared to having either H $\alpha$ T or *KIT* p.D816V alone (63.8%, 206 of 323) (Figure 3A) ( $p < .01$ ; OR=3.8 [95% CI=1.3–11.2]; RR=1.4 [95% CI=1.2–1.6]). Conversely, only ~10% (4 of 31) of individuals with both H $\alpha$ T and *KIT* p.D816V had Grade II HVA, and Grade I HVA was never observed when both were present. The prevalence of Grade II HVA in individuals with either H $\alpha$ T or *KIT* p.D816V alone was 33.4% (108 of 323;  $p < .05$ ), and for Grade I HVA was 2.8% (9 of 323) (Tables S2A–E). Finally, having both H $\alpha$ T and *KIT* p.D816V was associated with more severe symptoms, with a higher prevalence of loss-of-consciousness during a sting reaction (74.2%; 23 of 31) when compared to having either H $\alpha$ T or *KIT* p.D816V alone (Figure 3B) (52.9%, 171 of 323;  $p < .05$ ). None of the controls have concomitant H $\alpha$ T and *KIT* p.D816V (Table S1; Figures 1E; Figure S1). These data demonstrate that H $\alpha$ T and CMD have an additive effect on the severity of HVA, and having both is associated with the highest risk for severe outcomes.

### 3.7 | The association between elevated BST and HVA severity results from CMD and H $\alpha$ T

Elevated BST levels (>11.4 ng/mL) were found in 11.1% (146 of 1319) of the HVA patients in this study (Table 1). This is a highly similar prevalence relative to other large studies of systemic HVA (8.4% and 11.6%).<sup>4,10</sup> Overall, BST was elevated in 16.2% (99 of 610) of those with Grade III–IV HVA compared with 6.6% (47 of 709) of those with Grade I–II HVA ( $p < .0001$ ).

Among individuals with elevated BST, 53.4% (78 of 146) had H $\alpha$ T, 61% (89 of 146) had *KIT* p.D816V (31 with H $\alpha$ T), and 6.8% (10 of 146) had normal tryptase copy number and no evidence of clonal disease in PBL (Tables E3A–E). Further, among individuals with markedly elevated BST (>20 ng/mL), 49% had H $\alpha$ T (25 of 51) and 86.3% had *KIT*

p.D816V (44 of 51)—19 of which also had H $\alpha$ T— and only one could not be explained by these two findings. All individuals with both H $\alpha$ T and *KIT* p.D816V have elevated BST. These data demonstrate that, in addition to CMD, H $\alpha$ T accounts for roughly half of the HVA patients with elevated BST, and very often presents with the concomitant clonal disease.

Among the 1173 patients with normal BST levels (<11.4 ng/mL), those with Grade III–IV HVA had modestly but significantly increased BST levels (median 5.1 ng/mL;  $n = 511$ ) compared with those with Grade I–II HVA (median 4.5 ng/mL;  $n = 662$ ) ( $p < .0001$ ). Similar results that minor increases in BST are associated with more severe HVA have been reported previously.<sup>4,7</sup> However, when individuals with *KIT* p.D816V or H $\alpha$ T (median 6.4 ng/mL;  $n = 139$ ) were omitted from the analysis, BST levels among those with Grade III–IV HVA were essentially indistinguishable from those with Grade I–II HVA (median of 4.7 ng/mL [ $n = 372$ ] vs. median 4.4 ng/mL [ $n = 588$ ]).

### 3.8 | The sensitivity of scoring systems in predicting CMD in HVA is limited

The REMA scoring system is based upon a clinical study that identified four elements (male sex, syncopal episodes, absence of urticaria and angioedema, and BST >25 ng/mL) as independent predictive factors for CMD in patients with a history of anaphylaxis without mastocytosis in the skin (Table S2).<sup>47</sup> The initial application of this score in 158 patients with systemic MC activation symptoms reported a sensitivity of 91% and a specificity of 75% in predicting CMD.<sup>47</sup> However, the clinical utility of using the REMA score in large HVA populations has not been evaluated.

We calculated REMA scores for all patients (Table 1) and compared them with the examination of *KIT* p.D816V in PBL. Overall, half of individuals (52.3%, 149 of 285) with detectable *KIT* p.D816V in PBL had REMA score  $\geq 2$ ; among those with Grade III–IV HVA, the frequency of REMA score  $\geq 2$  increased to 69.6% (144 of 207) (Tables S2A–E). Thus, the sensitivity of the REMA scoring system in

predicting CMD among severe HVA patients was 70% compared to PBL examination for *KIT* p.D816V.

## 4 | DISCUSSION

In this multicenter study, we have demonstrated that clonal disease and H $\alpha$ T are more prevalent among individuals who need VIT; one or both was found in 27% (354 of 1319) of individuals overall. Both diagnoses were highly concentrated among individuals with severe anaphylaxis where prevalence increased to approximately 40% (233 of 610) compared to only approximately 15% among those with moderate reactions (121 of 709). Moreover the majority of those with clonal disease had normal BST levels—even those with severe HVA. Finally, an additive effect was demonstrated, where individuals with clonal MC disease and concomitant H $\alpha$ T were at greatest risk and had the most severe outcomes.

To our knowledge, this is the first report of peripheral blood screening for *KIT* p.D816V using a highly sensitive allele-specific PCR test used in conjunction with tryptase genotyping in large HVA populations recommended for VIT. Given the high prevalence of CMD observed within these four large European cohorts (14% to 29%), routine screening for *KIT* p.D816V in PBL should be considered in addition to currently recommended BST determination<sup>30,31</sup> in all patients who are candidates for VIT. Furthermore, our data demonstrated that in patients with CMD the risk of a severe sting reaction is positively correlated with a higher *KIT* p.D816V allelic burden. These data suggest that the level *KIT* p.D816V+ disease burden has an incremental effect on the HVA severity. Whether expansion of a *KIT* D816V+ stem cell clone may modify the severity of anaphylaxis requires additional study since *KIT* p.D816V has been demonstrated to be a relatively weak oncogene regarding its ability to induce MC proliferation.<sup>48</sup> However, it also is worth mentioning that recombinant human stem cell factor (*KIT* ligand) promotes human MC functional activation *in vivo*<sup>49,50</sup> and that targeting *KIT* reduces MC number and reactivity.<sup>51</sup>

It is important to note that patients with SM and HVA, who are protected during VIT, may have very severe, or even fatal, sting reactions after VIT discontinuation.<sup>52</sup> Therefore, when a diagnosis of SM is established in patients with severe HVA, these patients should undergo life-long VIT.<sup>53–55</sup> We identified BM involvement and underlying CMD (BMM in ~2/3, and ISM or MMAS in ~1/3) in all individuals with detectable *KIT* p.D816V in PBL (all had Grade III HVA, and 71% had normal BST (<11.4 ng/mL)), who underwent BM studies. Thus, detection of *KIT* p.D816V in PBL may be considered tantamount to the diagnosis of CMD and warrant consideration of more aggressive work-up<sup>6</sup> and life-long VIT. However, additional studies, including population-based studies, are necessary to evaluate the associations between *KIT* p.D816V in PBL, BM involvement, and CMD.

We have confirmed recent observations<sup>14–16</sup> that increased germline copies of  $\alpha$ -tryptase-encoding sequences at *TPSAB1* are associated with increased severity of venom anaphylaxis, are more prevalent among individuals with CMD, and that having concomitant

CMD and H $\alpha$ T is associated with a greater likelihood of severe sting anaphylaxis, than having either CMD or H $\alpha$ T alone. Currently, there is no clinical data that would suggest that H $\alpha$ T carriers without CMD need life-long VIT. However, follow-up is necessary as some of those patients may develop CMD,<sup>29</sup> and additional studies are needed to determine whether this genetic trait may affect long-term effectiveness of standard VIT.

One third of those with H $\alpha$ T in this study had concomitant CMD. The exact mechanism(s) underlying the association between H $\alpha$ T and *KIT* p.D816V+ CMD remains unknown. Higher relative numbers of  $\alpha$ -tryptase-encoding gene copies have been associated with increases  $\alpha/\beta$ -tryptase heterotetramer generation,<sup>15,17,18</sup> which have a unique activity that may affect acute vascular membrane permeability and potentiate anaphylaxis severity.<sup>15,17</sup> Thus, the presence of H $\alpha$ T, and indeed the presence of any  $\alpha$ -tryptase-encoding gene copy, may increase the severity of allergic reactions,<sup>56</sup> and anaphylaxis is more frequently observed in H $\alpha$ T versus non-H $\alpha$ T mastocytosis patients.<sup>57</sup> Further, the numbers of MCs in the BM and GI mucosae have been reported to be increased in symptomatic individuals with H $\alpha$ T,<sup>40,43,58</sup> suggesting that over-expression of  $\alpha$ -tryptase may impact MC homeostasis through undefined mechanism(s). Additional studies are needed to understand the full nature of these associations and findings.

It is worth mentioning that most patients with detectable *KIT* p.D816V in PBL had a BST level of less than 11.4 ng/mL. Furthermore, one-half of *KIT* p.D816V positive patients had BST of less than 8 ng/mL<sup>5,6</sup> and one-fifth of less than 5 ng/mL.<sup>4</sup> These data suggest that BST, even with cutoffs of 5 ng/mL<sup>4</sup> or 8 ng/mL,<sup>5,6</sup> has a limited ability to predict underlying clonal disease in HVA and that additional studies are needed to clarify reference values for the upper limit of normal BST in HVA.<sup>59</sup>

In conclusion, we provide genetic and clinical evidence of a high burden of clonal mast cell disease and H $\alpha$ T in HVA patients who present with severe reactions, and demonstrate the clinical impact among those considered for VIT. Characterization of these high-risk factors highlights the importance of considering allelic burden when *KIT* p.D816V is detected, identifying the highest risk individuals who have concomitant clonal MC disease and H $\alpha$ T, and validate the usefulness of routine *KIT* p.D816V screening regardless of BST level. These findings expand our understanding of how MC-related disorders affect HVA and will improve our ability to target those at greatest risk with the most effective strategies to prevent anaphylaxis. Furthermore, this multicenter prospective study illustrates the utility and importance of performing these screening assays routinely, and demonstrates how doing so can have a direct impact on clinical management of these patients.

## AUTHOR CONTRIBUTIONS

Peter Korošec made the conception of the project and was responsible for the interpretation of findings and drafting the manuscript. Gunter J. Sturm and Jonathan J. Lyons supervised the interpretation of results with a critical review of the intellectual content of the manuscript. Tinkara Pirc Marolt, Lisa Arzt-Gradwohl, Manca Svetina, and

Ajda Demšar Luzar assisted with the acquisition of data, appraised the data, and performed statistical analysis for this project. Mitja Košnik, Mihaela Zidarn, Nina Frelj, Nika Lalek, Ewa Czarnobilska, Wojciech Dyga, Sanja Popović Grle, Miroslav Samaržija, Urban Čerpes, Grzegorz Porebski, Branko Pevec, and Eva Schadelbauer were responsible for the acquisition of original data and reviewed the interpretation of this results. Samo Zver, Matevž Škerget, Julij Šelb and Mark Kačar were responsible for BM studies and reviewed the interpretation of results. Peter Kopač, Julij Šelb, and Matija Rijavec supervised the acquisition and analysis of data and provided a critical appraisal of the interpretation and manuscript. All listed authors have given final approval of the submitted version of the manuscript and agree to be accountable for all aspects of the work related to its accuracy and integrity.

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### CONFLICT OF INTEREST STATEMENT

G.J.S. reports lecture fees from ALK-Abellö, Stallergens-Greer and Allergopharma. Other authors declare that they have no relevant conflicts of interest.

### DATA AVAILABILITY STATEMENT

Original data are available on request.

### ORCID

Peter Korošec  <https://orcid.org/0000-0002-0835-1599>

Gunter J. Sturm  <https://orcid.org/0000-0002-7245-121X>

Mitja Košnik  <https://orcid.org/0000-0002-4701-7374>

Lisa Arzt-Gradwohl  <https://orcid.org/0000-0002-5489-2070>

Grzegorz Porebski  <https://orcid.org/0000-0002-6146-0188>

Matija Rijavec  <https://orcid.org/0000-0002-2596-4952>

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## SUPPORTING INFORMATION

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