Production of the Invasive Aspergillosis Biomarker Bis(methylthio)gliotoxin Within the Genus Aspergillus: In Vitro and in Vivo Metabolite Quantification and Genomic Analysis

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Gliotoxin (GT) is a fungal secondary metabolite that has attracted great interest due to its high biological activity since it was discovered by the 1930s. An inactive derivative of this molecule, bis(methylthio)gliotoxin (bmGT), has been proposed as an invasive aspergillosis (IA) biomarker. Nevertheless, studies regarding bmGT production among common opportunistic fungi, including the Aspergillus genus, are scarce and sometimes discordant. As previously reported, bmGT is produced from GT by a methyl-transferase, named as GtmA, as a negative feedback regulatory system of GT production. In order to analyze the potential of bmGT detection to enable identification of infections caused by different members of the Aspergillus genus we have assessed bmGT production within the genus Aspergillus, including A. fumigatus, A. niger, A. nidulans, and A. flavus, and its correlation with gtmA presence. In order to validate the relevance of our in vitro findings, we compared bmGT during in vitro culture with the presence of bmGT in sera of patients from whom the Aspergillus spp. were isolated. Our results indicate that most A. fumigatus isolates produce GT and bmGT both in vitro and in vivo. In contrast, A. niger and A. nidulans were not able to produce GT or bmGT, although A. niger produced bmGT from an exogenous GT source. The frequency and amount of bmGT production in A. terreus and A. flavus isolates in vitro was lower than in A. fumigatus. Our results suggest that this defect could be related to the in vitro culture conditions, since isolates that did not produce bmGT in vitro were able to synthetize it in vivo. In summary, our study indicates that bmGT could be very useful to specifically detect the presence of A. fumigatus, the most prevalent agent causing IA. Concerning A. terreus and A. flavus a higher number of analyses from sera from infected patients will be required to reach a useful conclusion.

Keywords: bis(methylthio)gliotoxin, Aspergillus spp., gtmA, invasive aspergillosis, biomarker
INTRODUCTION

More than 20 years ago the first invasive aspergillosis (IA) biomarker, galactomannan (GM), was developed based on an enzyme linked immunosorbent assay (Stynen et al., 1995). It stirred up the diagnosis of this lethal infectious disease, as it allowed to detect the infection when combined with clinical signs and symptoms (Maertens et al., 1999, 2002). During the last few years, the biomarker weaponry has arisen with the development of a system to detect β-D-glucan, Aspergillus PCR and lateral flow device to detect an Aspergillus-derived protein among others (Odabasi et al., 2004; Thornton, 2008; White et al., 2015). New diagnostic approaches were developed based on the increased accuracy of these tests, such as pre-emptive therapy (Wingard, 2007; Riwas and Wingard, 2012). Despite of these advances, IA management continues to be challenging due to the heterogeneous population at risk, the diversity of clinical and radiological presentations and the lack of a gold standard (Lamoth and Calandra, 2017). Thus, at present, it is required to understand the limitations of each biomarker and the corresponding diagnosis test in order to accurately diagnose these challenging infections (Maertens et al., 2016). In this line, the future directions in IA diagnosis research need to focus on the development of new biomarkers, including a clear understanding of their strengths and limitations, along with the assessment of their utility in well-designed clinical trials (Arvanitis and Mylonakis, 2015; Mercier and Maertens, 2017).

In recent years, bis(methylthio)gliotoxin (bmGT) has generated great interest as an IA biomarker (Maertens et al., 2016; Mercier and Maertens, 2017). Its detection in serum by High Performance Thin Layer Chromatography (HPTLC) was shown to be reliable (Domingo et al., 2012). Moreover, it has been clinically validated in a small prospective study in comparison with GM quantification (Vidal-Garcia et al., 2016). Data suggest a good diagnostic performance (61.5% sensitivity and 93% specificity) and importantly, high positive and negative predictive values when used in combination with GM detection (100% and 97.5%, respectively), which suggest a potential utility in pre-emptive approaches. Pending further validation, unlike GM, bmGT detection could be useful in non-immunocompromised populations as it was previously found to be positive in a non-compromised patient suffering from IA that presented negative GM values (Vidal-Garcia et al., 2017). Nevertheless, data regarding the frequency and distribution of bmGT production by different opportunistic molds are scarce and in most cases based on bioinformatics analysis (Bergmann et al., 2007; Andersen et al., 2013; Dolan et al., 2014). These data would be very important for understanding the specificity and the clinical sensitivity of this biomarker to differentiate between species within the Aspergillus genus and, thus, treat this infection more effectively.

Bis(methylthio)gliotoxin is an inactive derivative of gliotoxin (GT). A. fumigatus is, to date, the most important opportunistic fungi producing bmGT (Li et al., 2006; Guimarães et al., 2010; Domingo et al., 2012; Sun et al., 2012; Liang et al., 2014). BmGT serves as a negative regulator of the GT biosynthesis, and it is produced by methylation of GT by an S-adenosylmethionine-dependent bis-thiomethyltransferase (Dolan et al., 2014, 2015, 2017), eliminating the ability of GT to produce toxic reactive oxygen species (ROS) (Dolan et al., 2015). BmGT formation from an exogenous source of GT has been described in A. niger and A. nidulans (Scharf et al., 2014; Manzanares-Miralles et al., 2016). The enzyme responsible for bmGT biosynthesis, which is an S-adenosylmethionine (SAM)-dependent methyltransferase called GtmA, has been characterized in A. fumigatus; and other orthologs have been found on species like A. niger or A. terreus (Dolan et al., 2014; Scharf et al., 2014; Manzanares-Miralles et al., 2016). The best characterized enzyme is GtmA, which is known to be encoded by the gtmA gene, located in the chromosome 2 (Dolan et al., 2014). Bioinformatics analysis of the Ascomycota phylum showed 124 orthologs of GtmA. However, it is known that toxin production is discontinuous among different species and it is not clear which species within Aspergillus genus are able to produce GT and, subsequently, the inactive derivative bmGT (Gardiner and Howlett, 2005; Patron et al., 2007). The aim of the present study was to assess the frequency and species distribution of bmGT within the Aspergillus genus in cultures in vitro as well as in vivo in sera of patients from whom fungi were isolated. We also characterized the ability of different clinical isolates from Aspergillus genus to methylate GT in cultures in vitro, to confirm the presence and activity of methyltransferases in Aspergillus species isolated from probable and proven cases of IA. Our findings indicate that bmGT could be considered as an specific biomarker to detect infections by A. fumigatus, the most common agent causing IA, excluding the presence of A. nidulans and A. niger.

MATERIALS AND METHODS

Gliotoxin and Bis(methylthio)gliotoxin Production

We analyzed GT and bmGT production within 252 Aspergillus spp. isolates. Most A. fumigatus complex (n = 119) were clinical isolates from Canisius-Wilhelmina Hospital, Nijmegen (Netherlands). Eighteen of those isolates were cryptic species from Section Fumigati from Gregorio Marañón University Hospital, Madrid (Spain). Other Aspergillus species were clinical isolates from Miguel Servet University Hospital, Zaragoza (Spain) and corresponded to 36 A. flavus complex, 35 A. terreus complex, 40 A. niger complex, and 22 A. nidulans complex. One milliliter of 12 McFarland conidial suspension (approximately 3–5 x 10^7 conidia/mL) was added to 9 mL of liquid medium (Roswell Park Memorial Institute [RPMI] 1640 + glucose 20 g/L + glutamine 2 mM + HEPES 25 mM) in 50 mL culture flasks and incubated at 37°C for 96 h. A 2 mL sample of supernatant was obtained and frozen at −20°C and subsequently used for GT and bmGT detection and quantification by HPTLC as described below. In those cases where GT and/or bmGT was not detected, fungal isolates were cultured employing Czapek Dox Broth (+ glutamine + HEPES) to confirm that this defect was not specific for RPMI medium. Czapek Dox Broth is a medium of a different composition to RPMI1640, and similarly to
the last one, it is commonly used in Aspergillus cultures in vitro. Thus, we decided to compare both in order to discard effects relative to specific culture media conditions in vitro.

**Bis(methylthio)gliotoxin Production From Exogenous Gliotoxin**

Bis(methylthio)gliotoxin production from an exogenous source of GT was assessed in a total of 35 isolates of the species complexes *A. flavus* (*n* = 12), *A. terreus* (*n* = 9), *A. niger* (*n* = 8), and *A. nidulans* (*n* = 6). Conidial inoculum was prepared as described above and added to 50 mL culture flasks with Czapek Dox Broth (+glutamine + HEPES). These cultures were incubated at 37°C for 45 h. At 45 h, GT was added to a final concentration of 2.5 mg/L and methanol was added as solvent control. At 0, 3, and 6 h, 2 mL aliquots of supernatant were taken and frozen until GT and bmGT analysis.

**Detection of Bis(methylthio)gliotoxin in Sera From IA Patients**

We assessed sera from patients hospitalized in the Miguel Servet University Hospital with probable/proven IA according to the EORTC/MSG definitions (De Pauw et al., 2008). We included in the study those cases with *Aspergillus* spp. strains in clinical samples. Serum were prospectively collected and frozen at −20°C until GT and bmGT detection. All protocols were supervised and approved by the Ethics Committee of Clinical Research from Aragón (CEICA), number PI15/0203.

**Metabolite Identification and Quantification by High Performance Thin Layer Chromatography (HPTLC)**

Gliotoxin and bis(methylthio)gliotoxin detection and quantification were performed both in serum and supernatant samples by HPTLC as described by Domingo et al. (2012). Briefly, GT and bmGT were extracted together using dichloromethane. After agitation and two phase’s separation, non-aqueous phase was added onto silica gel plates. Then, they were developed using a horizontal development chamber (Camag). The mobile phase was a mixture of tetrahydrofuran/n-heptane/acetonitrile (40:58:2 [v/v/v]). After 25 min development, plates were scanned with an ultraviolet scanning densitometer (TL Scanner 3, Camag: λ = 280 and 367 nm; linear scanning). GT and bmGT identification was performed by retention time and spectral analysis and quantification was performed by peak area under curve analysis using Camag’s personal computer software.

**Genetic Detection of gtmA Gene**

Chromosomal DNA of Aspergillus spp. isolates was extracted using cetyltrimethylammonium bromide (Sigma-Aldrich, St. Louis, MO, United States). The species-specific primers used for gtmA detection are summarized in Table 1, along with expected amplicon length. PCR was performed using HotTaq Master Mix (IBIAN Technologies, Zaragoza, Aragón, Spain). An initial denaturation of 2 min at 94°C was followed by 35 cycles at 94°C for 30 s, 56°C for 15 s, and 72°C for 1 min. DNA amplification products were visualized after electrophoresis on 2% agarose gels.

**RESULTS**

**Gliotoxin and Bis(methylthio)gliotoxin Production by Aspergillus spp. Isolates**

The production of GT and bmGT was tested in culture supernatants of 252 Aspergillus isolates from the species complexes *A. fumigatus*, *A. flavus*, *A. terreus*, *A. niger*, and *A. nidulans* after 4 days of incubation. Non-cryptic *A. fumigatus* isolates (*n* = 101) produced GT and bmGT at highest frequencies, 77.23% and 84.16%, respectively. Among the five cryptic species analyzed: *A. calidoustus* (*n* = 1), *A. fumigataffinis* (*n* = 3), *A. lentulus* (*n* = 11), *N. udagawae* (*n* = 2), and *A. novofumigatus* (*n* = 1), all but the last produced GT and/or bmGT. *A. fumigataffinis* seemed to be the most frequent GT and bmGT producing species, as the three tested isolates (100%) produced bmGT. In contrast, just two of the eleven isolates (18%) of *A. lentulus* produced bmGT. BMGT was also more frequently detectable than GT in culture supernatants of *A. flavus*, 11.11% vs. 8.33%, respectively. In contrast, *A. terreus* produced GT more frequently than bmGT (22.86% vs. 2.86% respectively) (Figure 1). Notably, none of the *A. niger* and *A. nidulans* isolates tested produced GT and/or bmGT. All cultures were analyzed by HPTLC, a method to detect GT and bmGT previously optimized and validated versus LC-MS (Domingo et al., 2012), and some of the results in selected culture isolates of *A. fumigatus*, *A. niger*, *A. nidulans*, and *A. flavus*, were confirmed by LC-MS (data not shown). The absence of GT and/or bmGT production was not specific for the culture conditions in vitro since culture...

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**TABLE 1 | Primers.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’−3’)</th>
<th>Amplification length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. terreus Fw</td>
<td>TCG GAG GGC GTA AAC CG</td>
<td>291</td>
</tr>
<tr>
<td>A. terreus Rv</td>
<td>GGA TTC GGA AGT CCA ACA AGG</td>
<td>289</td>
</tr>
<tr>
<td>A. flavus Fw</td>
<td>TCA AGG CTC CAT CAT AC</td>
<td>225</td>
</tr>
<tr>
<td>A. flavus Rv</td>
<td>TCG TCA GGG AAG AGT TTA AAA GC</td>
<td>293</td>
</tr>
<tr>
<td>A. niger Fw</td>
<td>TCT AGT GCC CTT CAT GTG GC</td>
<td>565 to 613</td>
</tr>
<tr>
<td>A. niger Rv</td>
<td>TCG TCA GGG AAG AGT TTG AAC</td>
<td>289</td>
</tr>
<tr>
<td>A. fumigatus Fw</td>
<td>TCC AGG GTA CTC AAC CAC AC</td>
<td>293</td>
</tr>
<tr>
<td>A. fumigatus Rv</td>
<td>CGT CTG GAA AGA TCT GGA AG</td>
<td>289</td>
</tr>
<tr>
<td>ITS 1 Fw</td>
<td>TCC GTA GGT GAG CCT GCG G</td>
<td>293</td>
</tr>
<tr>
<td>ITS 4 Rv</td>
<td>TCC TCC GCT TAT TGA TAT G</td>
<td>289</td>
</tr>
</tbody>
</table>
of GT/bmGT negative fungal isolates employing Czapek Dox Broth yielded similar results (data not shown). In addition, the differences were not due to different fungal growth since cell cultures showed a similar behavior and growth as analyzed by XTT reduction assay. Optimal culture conditions, as well as analytical specificity of the method, were confirmed by employing cell cultures from an A. fumigatus gliP deletion mutant, which is unable to produce GT and bmGT (Sugui et al., 2007).

A. fumigatus isolates also produced GT and bmGT in higher concentration than other Aspergillus spp. did. These differences were statistically significant ($p < 0.05$) (Figure 2). The mean concentration of GT was 2.26 ± 0.40 mg/L and of bmGT was 3.45 ± 0.44 mg/L for A. fumigatus. A. flavus isolates yielded a mean concentration of 0.14 ± 0.13 mg/L of GT and 0.39 ± 0.31 mg/L of bmGT. The mean concentration of GT and bmGT were 0.79 ± 0.34 mg/L and 0.07 ± 0.07 for A. terreus.

**Bis(methylthio)gliotoxin Production From Exogenous Gliotoxin**

In order to confirm the results obtained in cell cultures concerning bmGT production, we analyzed the ability of some fungal isolates to generate bmGT from an external GT source as well as the presence of methyltransferase genes. This is of special utility to find out whether the Aspergillus spp. that did not produce GT and bmGT (A. niger and A. nidulans) are also unable to methylate exogenous GT. This finding would mean that these isolates do not express methyl-transferase activity and, thus, confirm that they are unable to generate bmGT. Moreover, this would indirectly suggest that they are also unable to generate GT, since GT methylation has been proposed as a negative feedback regulatory system, inherent to all GT-producing species.

First, we analyzed if fungal isolates presented GT methyltransferase activity by adding pure GT and monitoring the generation of bmGT. The ability to produce bmGT from an exogenous source of GT was assessed in the isolates of A. flavus, A. terreus, A. niger, and A. nidulans that did not produce GT or bmGT in the previous experiment. All isolates from the A. flavus ($n = 12$) and A. terreus species ($n = 9$) were able to methylate exogenous GT in order to produce bmGT. Among A. niger isolates, this ability was less consistent, nevertheless, 5/8 isolates (62.5%) showed such ability. Finally, none of the A. nidulans isolates ($n = 6$) methylated GT to generate bmGT (Figure 3). This result confirms that A. nidulans does not express methyl-transferase activity and, thus, it is unable to methylate exogenous GT in order to produce bmGT. Among A. niger isolates, this ability was less consistent, nevertheless, 5/8 isolates (62.5%) showed such ability. Finally, none of the A. nidulans isolates ($n = 6$) methylated GT to generate bmGT (Figure 3). This result confirms that A. nidulans does not express methyl-transferase activity and, thus, it is unable to methylate exogenous GT in order to produce bmGT. Indeed, it has been previously shown that the methyltransferase MT-ii is expressed in A. niger and methylates exogenous GT (Dolan et al., 2017). However, since they do not produce GT (Figure 1 and Manzanares-Miralles et al., 2016), this would explain that they are unable to endogenously produce bmGT.
Among bmGT producing isolates, immediately after GT addition \((t = 0 \text{ h})\), this was recovered in a mean concentration of 0.86 ± 0.04 mg/L. None of the isolates produced bmGT at this time (Figure 4). At 3 h after GT addition, GT concentration decreased and bmGT concentration increased. This observation continued at 6 h, when the maximum bmGT and the minimum GT concentrations were detected. There were no differences between mean concentration of GT and bmGT among species \((p > 0.05)\) indicating a similar methylating activity.

**Comparison of Serum bmGT Detection in Vivo With bmGT Production in in Vitro Cultures**

Our results confirm that most *A. fumigatus* isolates and some *A. terreus* and *A. flavus* isolates were able to endogenously and exogenously produce bmGT. However, the frequency of bmGT production within *A. terreus* and *A. flavus* isolates was much less than in *A. fumigatus* isolates. Since the analyses of the genes involved in GT synthesis is difficult due to the complexity of the pathways involved, we decided to analyze if the isolates that did not produce endogenously GT and bmGT in *vitro*, were able to synthesis bmGT in humans *in vivo*. To this aim, we included six cases of probable/proven IA with mycological growth from whom *in vitro* cultures had been established and analyzed. Serum bmGT concentration for these patients, fungal isolation, sample type and fungal ability to produce bmGT *in vitro* (de novo and from exogenous GT) as well as detection of gtmA gene and the MT-ii homolog are summarized in Table 2.

All, but one serum, were positive for bmGT. This serum belonged to a patient with probable IA diagnosed by *A. fumigatus* growth in bronchial aspirate. The other three patients with *A. fumigatus* isolation had positive bmGT (range 0.19–13.68 mg/L). There was a case of IA by *A. flavus* and a case of IA by *A. terreus*. Both had detectable bmGT in serum. Notably, these isolates corresponded to those ones in which we were not able to detect either endogenous GT or bmGT during *in vitro*
TABLE 2 | bmGT production, methylation of exogenous GT and carriage of gtmA or MT-ii genes for Aspergillus spp. isolates from probable and proven invasive aspergillosis cases.

<table>
<thead>
<tr>
<th>Case</th>
<th>IA type</th>
<th>Culture (sample)</th>
<th>Serum bmGT (mg/L)</th>
<th>Specie</th>
<th>Supernatant bmGT (mg/L)</th>
<th>GT methylation ([bmGT]_t – e)</th>
<th>gtmA gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proven</td>
<td>Sinus biopsy</td>
<td>1.66</td>
<td>A. flavus</td>
<td>Not detected</td>
<td>0.93</td>
<td>+ (mt-ii)</td>
</tr>
<tr>
<td>2</td>
<td>Proven</td>
<td>Vitreous, thrombus</td>
<td>0.19</td>
<td>A. fumigatus</td>
<td>0.26 ± 0.05</td>
<td>0.90</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Probable (proven IF)</td>
<td>Bronchial aspirate</td>
<td>6.84</td>
<td>A. fumigatus</td>
<td>0.52 ± 0.05</td>
<td>0.55</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Probable</td>
<td>Sputum</td>
<td>13.68</td>
<td>A. terreus</td>
<td>Not detected</td>
<td>0.16</td>
<td>+ (mt-ii)</td>
</tr>
<tr>
<td>5</td>
<td>Probable</td>
<td>Bronchial aspirate</td>
<td>–</td>
<td>A. fumigatus</td>
<td>0.48 ± 0.14</td>
<td>0.98</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Probable</td>
<td>Sputum</td>
<td>2.6</td>
<td>A. fumigatus</td>
<td>0.18 ± 0.02</td>
<td>0.48</td>
<td>+</td>
</tr>
</tbody>
</table>

**FIGURE 5 |** Detection of gtmA and mt-ii genes in clinical isolates from probable/proven invasive aspergillosis cases.

**DISCUSSION**

Despite recent advances, the lack of a single gold standard technique and the limitations of the available ones, make diagnosis of IA still challenging (Maertens et al., 2016; Mercier and Maertens, 2017). In recent years, new metabolite based diagnostic tools have been under research, such as GT or volatile organic compounds (Lewis et al., 2005a; Chambers et al., 2009).

Regarding GT, its high biological reactivity and its potential ability to interact with cells and tissues (Domingo et al., 2012) make it hard to detect in body fluids (Scharf et al., 2012). This limitation is overcame by bmGT, which is more stable and reliably detected in serum (Domingo et al., 2012). It is known that A. fumigatus produces GT in the highest concentrations and more frequently than other Aspergillus species (Lewis et al., 2005b; Kupfahl et al., 2008). This conclusion has been supported by our results, in which 77% of the A. fumigatus isolates were GT-producers in significantly higher concentrations and frequencies than those obtained for A. flavus and A. terreus species complexes, the other Aspergillus-producing GT species. With reference to bmGT, A. fumigatus was also the most common bmGT producing complex. Notably, and in line with previous findings (De Pauw et al., 2008; Manzanares-Miralles et al., 2016), neither A. niger or A. nidulans strains were able to endogenously produce GT and/or bmGT, although methyltransferase activity was found in A. niger isolates when using exogenous GT. These results are supported by both PCR analyses and bioinformatic studies confirming the absence of the gli cluster in these species. These findings contrast to previous studies in which a high proportion of A. niger isolates were shown to produce GT (Kupfahl et al., 2008). We have no explanation for these contradictory findings although, in line with our findings, Kupfahl et al. (2008) did not detect gliP gene in A. niger, which has been shown to be critical for GT synthesis, at least in A. fumigatus isolates (Sugui et al., 2007).

Some authors have been interested in the secondary metabolite profiles of cryptic species of the Fumigati section. Unlike other authors, we found out that A. lentulus and A. fumigatiaffinis were able to produce GT and bmGT (Larsen et al., 2007; Sugui et al., 2010; Tamiya et al., 2015). Regrettably, we
just analyzed one isolate of *A. calidoustus* and *A. novofumigatus*. The *A. calidoustus* isolate was GT and bmGT producer, but *A. novofumigatus* was not. This result does not rule out the ability to produce GT and bmGT by *A. novofumigatus* since culture conditions (medium, aeration, temperature, sampling time...) affect to secondary metabolite synthesis (Belkacemi et al., 1999; Watanabe et al., 2004). This could explain the low GT and bmGT detection among non-*A. fumigatus* species since all experiments were performed in the same conditions and confirmed employing other culture protocols.

Aiming to avoid such a limitation, we analyzed the ability to produce bmGT from an exogenous source of GT among non-toxigenic isolates. We detected bmGT in culture filtrates of all the *A. flavus* and *A. terreus* analyzed, thus suggesting a consistent ability to produce bmGT. To our knowledge, no specific methyltransferases had been described to date for these species. Nevertheless, it has been described an ortholog and a homolog of GtmA for *A. terreus* and *A. flavus* by bioinformatics analysis but it is the first time in which its expression has been described. In our study, *A. niger* also showed the ability to methylate GT, but less frequently. Curiously, none of the *A. nidulans* isolates analyzed produced bmGT even when it has been described that a specific methyltransferase (and the encoding gene) able to produce bmGT from GT for this species (Manzanares-Miralles et al., 2016). This discrepancy could be due to the known fact that culture conditions do not reflect the genetic potential and that not all the strains of the same species have the same metabolic profile (Bergmann et al., 2007). Indeed, secondary metabolism confers a survival benefit to the producing isolate and the *in vitro* culture conditions are not optimal to activate this survival pathway, depending on the *Aspergillus* spp. and/or isolate.

In order to overcome the limitations of the *in vitro* culture to analyze secondary metabolism, we have employed some clinical isolates from patients with probable/proven IA and compared bmGT production in *in vitro* culture with bmGT in serum from those patients. In this scenario, where fungi has to colonize the host and adapt itself to the new environmental conditions, the fungi will activate secondary metabolism and display all potential virulence factors such a GT (Cramer et al., 2006; Sugui et al., 2007) In these conditions, all but one of the seven probable/proven patients had bmGT detectable in serum, even those that did not produce GT and bmGT *in vitro*. Importantly, all of them were able to produce bmGT from exogenous GT and carried an ortholog of GtmA, MT-ii.

In summary, our findings indicate that bmGT production is useful to diagnose IA caused by *A. fumigatus* and, at some extent, by *A. terreus* and *A. flavus*, although at a much lower frequency, since they present the ability to methylate GT and endogenously produce bmGT *in vitro* and *in vivo*. Moreover, and pending of validation with a higher number of samples, our novel findings indicate that conclusions about the expression of molecules that could be used as potential diagnostic biomarkers based on *in vitro* fungal cultures cannot be reached unless they are confirmed in proper *in vivo* studies employing animal models or patients suffering from IA.

### AUTHOR CONTRIBUTIONS

MV-G and SR carried out the experiments. PM helped with genomic analysis and CC helped with *in vitro* experiments. MD performed the HPTLC analysis. MV-G wrote the manuscript with support from AR, JP, and EG. JM provided and characterized different isolates of *Aspergillus* spp. JP and EG conceived the original idea and supervised the project with the support of AR.

### FUNDING

This work was supported by Fondo Social Europeo (FSE; Gobierno de Aragón) and by grants MAT2011-26851-C02-02, SAF2014-54763-C2-1-R, SAF2014-54763-C2-2-R from Spanish Ministry of Economy and Competitiveness. JP was supported by Aragón I+D (ARAID). MV-G was granted by a Rio Hortega contract of National Institute of Health Carlos III (CM16/00236).

### ACKNOWLEDGMENTS

We kindly acknowledge Dr. J. F. Meis from Canisius-Wilhelmina Hospital, Nijmegen (Netherlands) and Dr. E. Bouza from Gregorio Marañón University Hospital, Madrid (Spain) for providing clinical isolates from several *Aspergillus* species and from cryptic species, respectively. We would like to acknowledge ASPANOÁ (Asociacion de Padres de Niños con Cáncer de Aragón) for its support to develop this work.

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**Conflict of Interest Statement:** MD, JP, and EG are co-inventors of a patent licensed to Blackhills Diagnostic Resources S.L. that protects the use of bmGT to diagnose IA (PCT/EP2012/058,247).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.