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ORIGINAL ARTICLE

Analysis of null deletion polymorphism of glutathione *S*-transferase theta (GSTT-1), associated with anti-GSTT-1 antibodies development in transplantation

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Abstract

Glutathione *S*-transferase theta 1 (GSTT1) is an enzyme involved in phase II biotransformation processes and a member of a multigene family of detoxifying and clearing reactive oxygen species. GSTT1 is polymorphic like other biotransforming enzymes, allowing variability in hepatic conjugation processes. Immunological recognition of the GSTT1 alloantigen, as evidenced by donor-specific antibodies formation, has previously been observed in recipients lacking GSTT1 protein (called GSTT1−, GSTT*0, null phenotype or homozygous for the GSTT1 deletion) who receive liver or kidney transplants from GSTT1+ donors and is a risk factor for the development of de novo hepatitis following liver transplants from a GSTT1 expressing donor. Antibodies against GSTT1 are demonstrated in patients who are GSTT1 null and received a transplant from a GSTT1+ donor. Understanding the local population frequency of the GSTT1 deletion is of value in understanding the potential clinical risk of developing post-transplant complications, which can be attributed to the nonexpression of GSTT1. A population of 173 healthy donors of the Murcia Region in Southeast Spain was evaluated for a null allele of GSTT1 (n = 173). DNA was extracted, and GSTT-1 null allele detection was performed by real-time polymerase chain reaction. The frequency of the null GSTT1 genotype (nonexpression or deletion of the homozygous polymorphism of the GSTT1 protein) was 17.9% (n = 31 null allele GSTT1/173 total individuals). Our data suggest that the frequency of null GSTT1 mutations in our population in Southeast Spain is 17.9%, lower than in other Caucasoid populations. This would convert our recipient population into more susceptible to nonlocal potential organ donors and less susceptible to local donors. All recipients bearing this GSTT1 deletion homozygous would be without the protein and triggering an alloantigen in the case of transplantation with a donor without deletion.

The contributions of Isabel Legaz, Pilar Zafrilla and Manuel Muro are equal, and the order is arbitrary.

KEYWORDS
alelo nulo, frecuencia genica, GSTT1, hepatitis autoinmune, transplante

1 | INTRODUCTION

The enzyme glutathione *S*-transferase theta 1 (GSTT1) is predominantly expressed in the liver, kidney and erythrocytes, although it shows limited expression in other organs and tissues in the human body (Aguilera et al., 2016; Ardesjö et al., 2008; Deakin et al., 1996; Hayes & Strange, 2000; Juronen et al., 1996; Kim et al., 2020; Pemble et al., 1994; Qian et al., 2017; Wang et al., 2010). The GSTT1 is an enzyme involved in phase II biotransformation processes and catalyses the conjugation of reduced glutathione to various electrophilic and hydrophobic compounds (Wang et al., 2010). This enzyme also plays an essential role in detoxifying and clearing reactive oxygen species (ROS). Finally, it is highly polymorphic, which allows high variability in hepatic conjugation processes (Hayes & Strange, 2000). Human glutathione *S*-transferases (GSTs) can be divided into five main classes: alpha, mu, pi, theta and zeta. The theta class includes GSTT1, GSTT2 and GSTT2B. GSTT1 and GSTT2/GSTT2B share 55% amino acid sequence identity and may play a role in human carcinogenesis (Liang et al., 2013; Yang et al., 2014). Glutathione *S*-transferases mu 1 (GSTM1) and GSTT1 null deletion polymorphisms lead to a complete loss of enzymatic activity. Hence, people having GSTs null deletion may have reduced ITCs (isothiocyanates) metabolism (Conaway et al., 2005). The detailed literature review shows that GSTM1 and GSTT1 null deletion vary ethnically and geographically (Senthilkumar & Thirumurugan, 2012).

The GSTT1 gene is genotype-specific and is absent in 38%–50% of Asian populations (Liang et al., 2013; Nakanishi et al., 2022). Alternative splicing of this gene results in multiple transcript variants (Liang et al., 2013). This gene is located on human chromosome 22q11.2, comprises five exons and is 8 kb long (Kerb et al., 2002; Nakanishi et al., 2022). Several diseases have been associated with gene expression and/or polymorphism of the GSTT1 enzyme, and they include pathologies such as sensitivity to mutagens, Type 2 diabetes mellitus, senile cataract, different carcinomas or de novo autoimmune hepatitis, among others (Al-Riyami et al., 2022; Ansari-Lari et al., 2020; Liu et al., 2022; Nassereddine et al., 2021; Nelson et al., 1995; Sobha & Ebenezar, 2021; Stepanova et al., 2021). Moreover, GSTT1 is also involved in activating the p38/MAPKAP kinase 2 (MK2) signal pathway. GSTT1 may prevent cells from tumourigenesis by promoting p38/MK2-mediated apoptosis and senescence (Wang et al., 2016). The homozygous deletion of GSTT1 occurs in approximately 20% of the Caucasian population (Hayes & Strange, 2000), approximately 25% of African Americans and approximately 50% of Asians (Parl, 2005). A recent article has elucidated the function of a null allele concerning the pathologies triggered by its role in inflammation and the null deletion's presence or absence (Kim et al., 2020). Thus, Kim et al. (2020) hypothesized that GSTT1 may promote inflammatory processes in response to high pro-inflammatory cytokines such as interleukin (IL)-22, TNF- and ROS but decrease in response to uncontrolled infections. Thus, after being stimulated by inflammatory cytokines, intestinal epithelial cells, neutrophils and macrophages create ROS (Denning et al., 2002). Cytokines, ROS and IL-22 boost GSTT1 enzyme expression, whereas pathogen-associated molecular patterns such as LPS and flagellin decrease GSTT1 enzyme expression, leading to an increase in ROS and TNF- and a subsequent increase in the IL-22 and IL-22/mucin/antimicrobial peptides (AMPs) axis. However, problems in the GSTT1 synthesis or dimerization caused by GSTT1 protein null mutation result in decreased mucin and AMP production, insufficient to combat pathogens but may result in excessive cytokine production. The deficit that occurs with pathogen clearance and triggers deleterious cycles between altered neutralization of high-level ROS accumulation and cell damage results in an altered epithelial barrier and inflammation, exacerbated by pathogen infiltration, resulting in inflammatory pathology in the intestine.

Furthermore, GSTT1 proteins have been shown to have the potential to act as minor histocompatibility antigens in bone marrow transplantation (BMT) (Parl, 2005). Besides, this GSTT1 enzyme has clear implications for pathologies of different aetiologies, such as inflammatory diseases or cancer (Deakin et al., 1996; Broekman et al., 2014). In this respect, immunological recognition of the GSTT1 alloantigen, with the generation of donor-specific antibodies (Abs), has already been described in recipients lacking GSTT1 protein (GSTT1−) who receive liver or kidney transplants from GSTT1+ donors (Aguilera et al., 2016; Álvarez-Márquez et al., 2009). Anti-GSST1 antibodies are a risk factor for developing de novo immunological hepatitis or chronic antibody-mediated rejection, complicating the transplant process. These anti-GSTT1 autoantibodies may be involved in other autoimmune diseases (Ardesjö et al., 2008). Thus, anti-GSTT1 antibodies are present in GSTT1-null individuals with a history of blood transfusion (GSTT1+ donor) or previous pregnancy (GSTT1+ foetus). The incompatibility of the GSTT1 alleles between the donor of the organ and its recipient in a transplant is generally related to eventual problems in the post-implantation period (Aguilera et al., 2016).

Specifically, in liver transplant recipients, this GSTT1 enzyme has been suggested as a potential alloantigen that could mediate the appearance of autoimmune hepatitis in liver recipients with a genetic load of the null homozygous type for GSST1 of the recipient (does not express the GSST1 protein) and receive a donated liver with a GSTT1+ phenotype (Aguilera et al., 2016). On the other hand, in the case of renal transplantation, with recipients with GSTT1-null genotype who received a GSTT1+ implant, an association has been found between pro-antibody (humoural)-mediated rejection of the kidney and the development and production of anti-antibodies GSTT1 post-transplant (Aguilera et al., 2013). Understanding the local population frequency of the GSTT1 deletion is of value in understanding the potential

clinical risk of developing post-transplant complications, which can be attributed to the nonexpression of GSTT1.

For this reason, our study aimed to address the frequency estimation of the null allele homozygous for the GSTT1 of this important alloantigen in a healthy population of Southeast Spain to know the incidence of this allele in neighbouring European and African populations and its consequences in organ transplantation.

2 | MATERIALS AND METHODS

2.1 | Data on demographics, clinical features and study design

A total of 173 consecutive healthy, unrelated bone marrow donors from different regions of Murcia were evaluated at the University Clinic Hospital 'Virgen de la Arrixaca' (Spain) and analysed retrospectively. Of these, 92 (46.8%) were men, and 81 (53.2%) were women, with a mean age of 34.2 years (SD: 5.42), being the age limit to donate was 40 years.

The inclusion criteria for this study were the acceptance of informed consent by the donor, available DNA sample at an adequate concentration and purity, and knowledge of not having any individual or family history of previous liver disease.

Before taking part in the trial, all patients gave informed consent. The study followed the Declaration of Helsinki 2000, and the Ethics Committee approved the HCUVA protocol (PI15/01370 and PI19/01194).

The samples of bone marrow donors used in this study represent the Murcian population, as they include individuals from most of the municipalities of the Region of Murcia.

2.2 | Genomic extraction

The QIASymphony DNA Mini Kit extracted genomic DNA from human peripheral blood (Izasa). The commercial kit used in this work was the QIASymphony DNA Mini Kit (catalogue number 931236, Izasa). Nanodrop technology (Promega) quantified all extracted samples. The study did not include all samples below 1.5 of A260/280 ratio. Finally, all the samples extracted and quantified were stored at −20°C until later use.

2.3 | Antibodies anti-GSST1 in liver recipients as model

The indirect immunofluorescence (IFI) technique was used for detecting positive antibodies in sera samples from liver allograft recipients (up to a sample dilution of 1/320 with phosphate-buffered saline (PBS) as diluent) and who developed *de novo* hepatitis in the post-transplant period (three months after surgery) and staining with secondary antibody in fluorescein ITC. In the GSTT1-positive donor/GSTT1*0 (this nomenclature means that GSST1 protein is not present in a particular individual and his genotype can be considered null) recipient group (five liver recipients positive in our centre in the last years), we saw IFI in liver tissue sections with staining positive of GSTT1 to establish the presence of these antibodies. We performed IFI on triple rat tissue (liver, kidney and stomach) (Biocientifica, Menarini Diagnostics and Grifols Laboratories) using the automated Zenith HUB2.1 Rev2n software (Menarini). The prepared slides were observed under an LED fluorescence microscope (×100–400 magnification).

Liver and kidney sections and fluorescent conjugate-IMMCO-Diagnostic R plates (Grifols Laboratories) were used as a substrate for the evaluation of autoantibodies. All the samples ($n = 5$) were compared with positive and negative controls from the laboratory supplying the substrate and from our files. The test serum dilution was carried out with PBS (Durviz) and incubated overnight at 4°C in a humidified chamber and the dark. Subsequently, the secondary antibody was added and incubated in the dark, protected from light for at least 1 h at room temperature. Later, we washed three times with PBS for 5 min, observing the fluorescence patterns obtained in a fluorescence microscope (Leica Biosystems).

2.4 | Real-time PCR for GSTT1 polymorphism determination

GSTT1 enzyme null deletion mutation testing was performed in transplant patients who developed anti-GSTT1 antibodies and immune hepatitis post-liver transplant period.

There are two main variants: a normal allele and another null mutant variant. They are caused by point mutations in the GSTT-1 gene and are associated with eventual allogeneic recognition (meaning recognition as a foreign antigen by the recipient) against the transplanted organ.

This genomic study was performed by real-time polymerase chain reaction (rt-PCR) in an Applied Biosystems 7500 (MA) for the detection of the GSTT1 allele by rt-PCR, using a commercial kit with TaqMan probes (Genviset-GVS-GSTT1 −48 kit) (Blackhills Diagnostic Resources) after DNA extraction of peripheral blood samples collected in tubes with ethylenediaminetetraacetic acid anticoagulant, as described before. At the same time as amplifying the GSTT1 gene, the designed method amplifies and detects an internal control gene, in this case, ß-globin, to verify the correct functioning of the rt-PCR assay. The liver allograft donors who generated a humoural response to GSTT1 in GSTT1*0 (GSTT1 null) were all GSTT1 positive.

Briefly, the mixture of the Primer Mix (8 μL) and the Taq polymerase (0.1 μL) (5 U/μL) Promega GoTaq Hot Start (Promega Biotech Iberica) was prepared for n+1 samples. Subsequently, 8 μL of this mixture was pipetted into a 96-well plate, and 2 μL of the test DNA sample or the negative contamination control was added. SE Centrifuged for 1 min at 360 × g and introduced into the ABI7500, with the following protocol: 1 cycle of denaturation at 95°C for 5 min, 50 cycles of 95°C for 15 s, and 60°C for 1 min. We detected cyclic labelling with FAM dye. Samples positive for GSTT-1 were identified with a Crossing Point (Cp) value corresponding to the cycle in which fluorescence was detected.

The beta-globin control results go in the VIC/HEX reading channel. The contamination control (Negative Control) must provide negative results for both GSTT-1 and β-globin, considering *Cp* values >35 as negative results. A positive control sample should provide positive results for GSTT-1 and β-globin.

This kit was also validated in a particular workshop on GSTT1 genetics, with an interest in cases of transplant rejection in collaboration with BDR/Diagnostica Longwood and Spanish Society of Immunology. It can be seen at https://issuu.com/seinmunologia/docs/sei-v37-n1-2018.

2.5 | Statistical analysis

For categorical data, the results have been reported as percentages. Fisher's exact test was employed to compare categorical variables. The Kolmogorov-Smirnov test was used to verify the normality of the data. For all statistical tests, $p = .05$ or p-corrected $= .05$ was considered significant. The graphs and statistical analyses were created using SPSS (version 22) and GraphPad Prism software (version 6). The p-value was corrected in multiple comparisons using the Benjamini–Hochberg or Bonferroni methods. For all statistical tests, $p < .05$ (or p-corrected .05 in the event of multiple comparisons) was considered significant.

3 | RESULTS

3.1 | Example of staining of positive GSTT1 antibodies in liver recipients

Sera samples of liver allograft recipients of our hospital who developed *de novo immune* hepatitis in the early post-transplant period (with the GSTT1-positive donor and GSTT1*0 recipient genetics) as a model of positive GSTT-1 antibodies detection are shown in Figure 1. We have included a clear image that shows the typical pattern of intensely stained IFI in the hepatocytes surrounding the centrilobular vein in liver rat tissue. It is shown the typical staining pattern that, according to previous data from the literature, is termed 'atypical liver kidney autoantibody' (liver staining is cytoplasmic and localized mainly to hepatocytes around the central vein; perimembrane enhancement is seen in hepatocytes within the hepatic lobe; renal tubules stain homogeneously, whereas renal tubules stain vessels and glomeruli are negative) as described before, as previously published [30].

The development of atypical LKM is significantly associated with the GSTT1 donor–recipient mismatch. On the contrary, the typical LKM1 autoantibody positivity appearance has been described in patients developing autoimmune hepatitis after allogeneic stem-cell transplantation, as previously reported (LKM1-positive type 2 autoimmune hepatitis following allogenic hematopoietic stem-cell transplantation [31]).

3.2 | GSTT1 genotypic analysis

An rt-PCR amplification process for detecting the GSTT1 null allele was performed. All the experiments were realized in several successive rounds with all the samples in our study. The frequency of the null GSTT1 genotype (nonexpression or deletion of the homozygous polymorphism of the GSST1 protein) was 17.9% ($n = 31$ null GSTT1/173 total individuals) in our population (Table 1).

Eighty-two per cent of the healthy control population from the Region of Murcia express either two homozygous or one heterozygous GSTT1 allele and thus are expected to express the GSTT1 protein. Conversely, 17.9% of this population show evidence of a null deletion of this allele and, thus, if they received a transplant from a GSTT1+ donor, could develop auto-antibodies to this protein, leading to increased risks of post-transplant complications, including auto-antibody mediated hepatitis.

4 | DISCUSSION

In our present work, we reported that the frequencies of a null allele of GSTT1 in the Murcia Region were smaller than in other Caucasian populations. These data agree with the previous data from neighbouring populations (Garte et al., 2001). However, it is essential to indicate that the frequency of our population is slightly lower than that reported and reported in other populations of Caucasian origin (≈20%).

There is a discreet number of related investigations on the allelic frequencies of the GSTT1 gene referring to different ethnic groups and populations to understand the influence of this polymorphism (Buchard et al., 2007; Garte et al., 2001; Nelson et al., 1995; Zhang et al., 2021).

However, the results of these investigations reveal differences in allele frequencies between our study population and studies (Buchard et al., 2007; Christiansen et al., 2021; Zhang et al., 2021). Thus, in a study by Garter et al. (2001), statistically significant differences were obtained in the allelic frequencies of the GST genotypes among three central populations. Regarding the null allele of GSTT1, a higher frequency was reported in Caucasians (27.6%) compared to our study from the Region of Murcia and significantly higher in Asian populations (64.4%) (Nelson et al., 1995). In this sense, Buchard et al. (2007) found that the composition of frequencies in the Danish population was significantly different compared to the frequencies found in Somali and Greenland populations, whereas there were no significant differences between the last two.

On the other hand, the frequencies of the null allele of GSTT1 in South American populations have a distribution range from 0% to 38.2%, being lower than the frequencies found in neighbouring Asian populations (Yadav et al., 2010), which seems curious since that in other polymorphic genes studied does not occur.

One possible cause for these observed differences is that the variations in allele frequencies found between the different ethnic groups could be due to differences in the distribution of drug

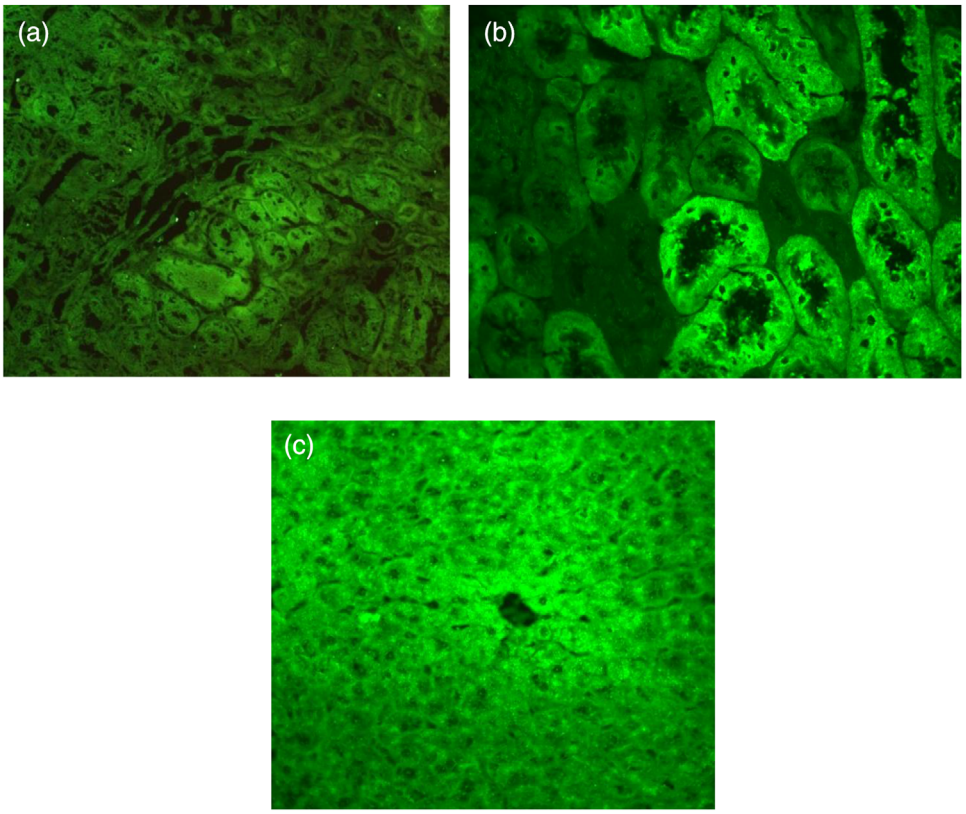

FIGURE 1 In the glutathione *S*-transferase theta 1 (GSTT1)-positive donor/GSTT1*0 recipient group, we used indirect immunofluorescence (IFI) in liver tissue sections with staining positive of GSTT1 to establish the presence of these antibodies. We performed IFI on triple rat tissue (liver, kidney and stomach) (Biocientifica, Menarini Diagnostics) using the automated Zenith HUB2.1 Rev2n software (Menarini Diagnostics). The prepared slides were observed under an LED fluorescence microscope (×100–400). Anti-GSTT1 antibody (1/320 dilution in PBS diluent). Homogeneous green fluorescence cytoplasmic staining of the liver section (a). More intense staining in hepatocytes surrounding the area centrilobular and move decreases towards the area per lobular (a, c). In the case of renal transplant, positivity exists in the tubules of proximal kidneys.

TABLE 1 Results of the genetic analysis by real time-polymerase chain reaction (PCR) of the null allele (deletion polymorphism) of the GSTT1 enzyme in healthy individuals of the population of Caucasoid origin in the Region of Murcia.

	GSTT1 + homo/heterozygote normal	GSTT1 null homozygote mutated	Total
No. of Individuals (*n*)	142	31	173
Frequency (%)	82.1	17.9	100

Abbreviation: GSST-1, glutathione *S*-transferase theta 1.

detoxification genes in the populations (Umamaheswaran et al., 2014). Theoretically, this distribution would not occur randomly but could be influenced by different patterns or profiles, both of geographic location, which would be specific and of ethnic origin (Armengol et al., 2009). In addition, the current mixture that occurs in the different world populations can also contribute to these phenotypic variations (Piacentini et al., 2011).

However, as we have pointed out, not only is the study of this null allele or variant GSTT1 polymorphism functional as a population analysis but its presence and nonexpression of the protein have been implicated in different pathologies (Liang et al., 2013; Masoodi et al., 2012; Qian et al., 2017; Stepanova et al., 2021; Yang et al., 2014).

For example, it has been associated with carcinogenic processes, such as prostate or serous ovarian cancer, associated with resistance to taxol/carboplatin (Masoodi et al., 2012; Yang et al., 2014; Zhang et al., 2021). Also, not only polymorphism but alterations in gene methylation, such as differential methylation over-expressed in the regulatory region, are associated with sudden unexplained death and unexpected death in epilepsy [35] or the additive effect of *Toxoplasma gondii* and genotype GSTT1 in the risk of schizophrenia (Ansari-Lari et al., 2020)

or the increased susceptibility of the GSTT1 polymorphism to develop cardiovascular disease in type 2 diabetes mellitus (Sobha & Ebenezar, 2021) or also with hypertension or asbestosis in populations of Asian origin (Nassereddine et al., 2021).

Thus, the homozygous double null of GSTM1 and GSTT1 was associated with a higher incidence of acute graft-versus-host disease (GVHD) in BMT (hematopoietic stem cell allotransplantation [HSCT]). GSTM1 and double nonnull GSTT1 also increased mortality risk, concluding that GST genotyping prior to HSCT can predict HSCT results (Al-Riyami et al., 2022).

To end this discussion, we report a meta-analysis (Nakanishi et al., 2022) where the PubMed database was searched, including 533 articles and 178,566 people in the analyses. Significant frequency differences existed among different ethnic groups: East Asians have the highest frequencies worldwide for GSTM1 and GSTT1 deletions, which might suggest a higher risk of disorders for this population; in contrast, sub-Saharan Africans had the lowest frequency worldwide, corroborating previous inferences of evolution for other genes encoding metabolic enzymes.

Therefore, a gene first reported in de novo hepatitis with an essential role in the liver or kidney [39, 40] contemplates its role in many different pathologies of different aetiologies and origins. Furthermore, it has previously been reported that the formation and presence of these antibodies can result in hepatic GVHD in patients with the GSTT1+ phenotype who receive HSCT from donors deficient in the GSTT1 enzyme [41].

In patients receiving HSCT for the treatment of congenital haemoglobinopathies, the frequency of rejection in these patients, where the null genotype was significantly higher than in GSTT1+ patients, suggests that a GSTT1 coincidence effect could not be the cause of the facts.

Our study has several limitations, as the sample size used by the donor is relatively small. Therefore, the conclusions of our work should be validated in larger cohorts. In addition, the design of a single-centre study may limit extending our general conclusions.

Finally, we can conclude that, in the Region of Murcia, the frequency of null GSTT1 mutations represents a percentage of 17.9% of the Caucasian population, which, estimating the total population of the Region of Murcia, is 1518,486 inhabitants (according to the National Institute of Statistics-INE, data from the year 2021 [43]), which would mean that with this data on potential organ donors, we would have an approximate number of 271,809 inhabitants of the region who would carry this GSTT1 deletion homozygous with the absence of the protein and triggering an alloantigen in case of transplantation with a recipient without the deletion.

In conclusion, our results on a healthy population show a slightly lower frequency in the region than published studies on other populations of the same Caucasian origin, which would be associated with a lower possibility of post-transplant complications. For this reason, the study and determination of the frequency of the GSTT1-null genotype in our community are critical to discovering the true implication these antibodies could have on kidney, liver, or bone marrow outcomes transplants.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results. The 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.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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