THE GENETIC PROFILE OF BONE MARROW TRANSPLANTED PATIENTS IN DIFFERENT VESTIGES OF FORENSIC INTEREST

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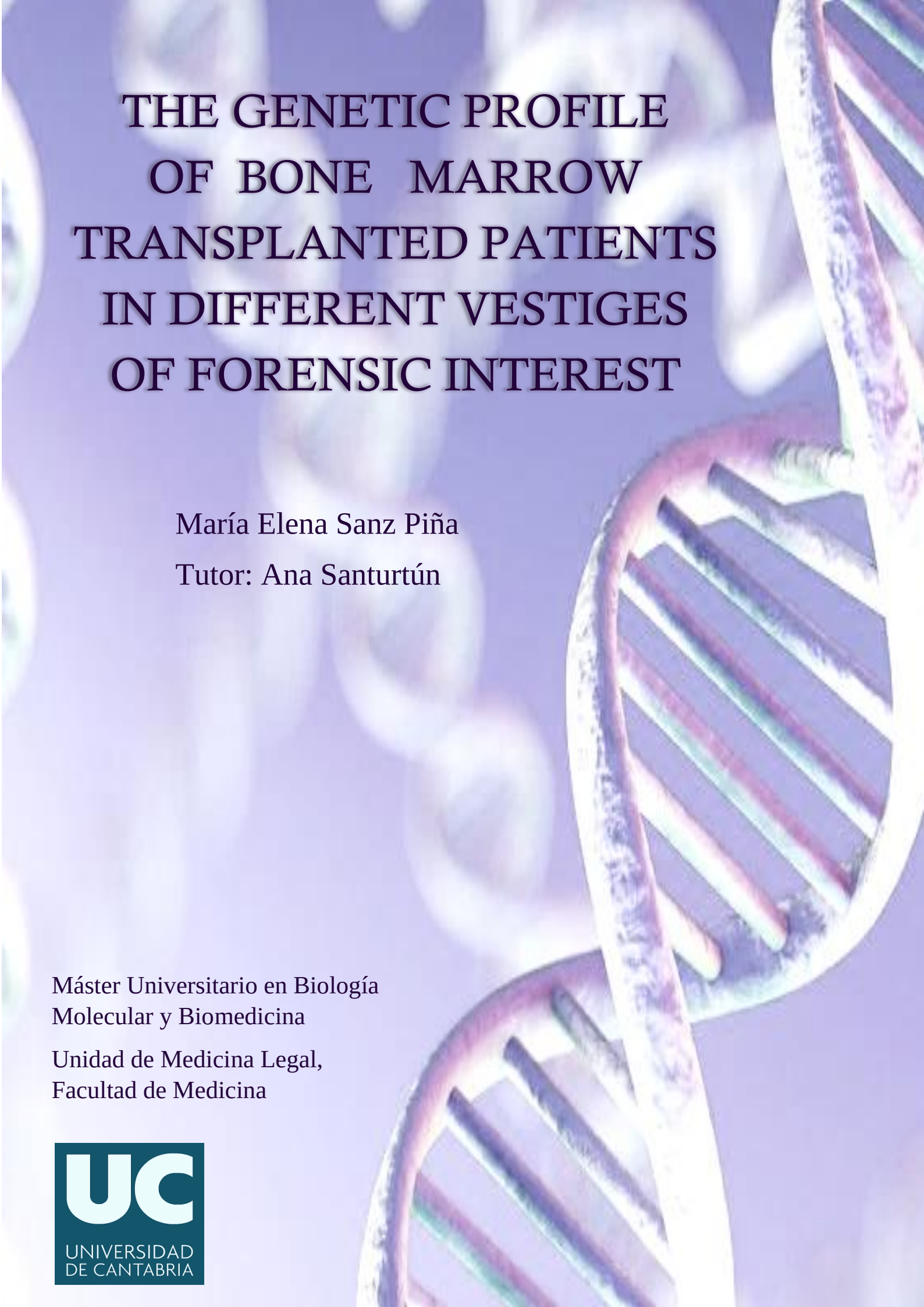

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ABBREVIATIONS

Al: Allele

Bp: Base pair

DNA: Deoxyribonucleic acid

DTT: 1,4-dithiothreitol

EDTA: Ethylenediaminetetraacetic Acid

FISH: Fluorescent *in situ* hybridization

GVHD: Graft-versus-host disease

HLA: Human leukocyte antigen

HSC: Hematopoietic stem cell

HSCT: Hematopoietic stem cell transplantation

InDel: Insertion/Deletion polymorphism

MC: Myeloablative conditioning

PCR: Polymerase chain reaction

RIC: Reduced intensity conditioning

SDS: Sodium dodecyl sulfate

SNP: Single nucleotide polymorphism

STR: Short tandem repeat

Tris-HCl: Tris(hydroxymethyl)aminomethane hydrochloride

VNTR: Variable number tandem repeat

XY-FISH: Fluorescent *in situ* hybridization of sex chromosomes

ABSTRACT

The coexistence of cells with different genetic origins (donor and recipient) in a patient after receiving a hematopoietic stem cell transplantation (HSCT) is called chimerism. The study of chimerism after HSCT allows physicians to know the success or failure of the transplant, to predict the possibility of a relapse and to apply the opportune therapy.

Due to the transdifferentiation capacity of hematopoietic stem cells (HSC) in non-hematological tissues, the vestiges from transplanted patients represent a challenge from a forensic perspective, since the interpretation of the genetic fingerprint can be misleading because of the presence of chimerism.

The objective of this study is to examine the genetic profile in samples of forensic interest (nail and skin epithelial cells) of bone marrow transplanted patients and discuss the forensic and clinical implications.

An observational and descriptive study has been developed in which the genetic profile of nail, epidermal cells and blood samples of patients receiving HSCT has been analyzed by the amplification and sequencing of 38 insertion/deletion polymorphisms (InDels) and 15 short tandem repeat polymorphisms (STRs). In this analysis, the age of patients and donors, the months elapsed from transplantation, the type of conditioning prior to the transplant and whether the patient has suffered graft-versus-host disease (GVHD) have been considered. Finally, the results obtained using the two identification techniques (InDels and STRs) have been compared in blood samples.

The results indicate that chimerism can be detected in the DNA extracted from nail and skin epithelial cells of transplanted patients. The percentage of cells with donor DNA in nail and skin increases with time elapsed from the bone marrow transplantation, but the age of the patient or the donor, the type of conditioning and the presence of GVHD do not influence the proportion of chimerism. Finally, it has been found that, in blood samples of transplanted patients, the use of InDels and STRs for the calculation of chimerism can be used to achieve equivalent results.

Human beings constantly lose epithelial cells, and these biological traces are frequently studied in the context of criminal investigation. In view of these results, it can be concluded that within a judicial context (e.g. when testifying as an expert witness) it is necessary to consider whether we are facing a possible transplanted patient or a person who has been a bone marrow donor.

Key words: Chimerism; Biological vestige; Hematopoietic Stem Cell Transplantation; Forensic Genetics; Human identification

1. INTRODUCTION

In the following project the importance of the hematopoietic stem cell transplantation, as well as its implications at the forensic level will be expounded. This procedure is used to treat many diseases: hematologic malignancies, non-hematologic malignancies and non-malignant disorders (Barriga et al., 2012; Cutler & Antin, 2005).

Cells of the hematological and immune system of the patient are replaced by healthy hematopoietic cells from a donor, which implies the coexistence in the patient of cellular compounds with different genetic origin and therefore, different DNA. This phenomenon is known as chimerism (Santurtún et al., 2017).

The quantitative monitoring of chimerism after transplantation provides information about graft development. For instance, it is possible to perform an early detection of graft rejection or, in the case of hematological malignancies, the detection of receptor cells that can potentially proliferate and lead to cancer recurrence (Khan et al., 2004; Santurtún et al., 2014).

As it will be explained later, the hematopoietic stem cell transplanted patients present a challenge from the forensic perspective since the presence of chimerism in different tissues can complicate identification analyses (Castella et al., 2009; Goodwin et al., 2007).

1.1 HEMATOPOIETIC STEM CELL

Stem cells are undifferentiated cells capable of giving rise to specialized cell types. They divide themselves asymmetrically, creating a differentiated cell type and another stem cell that remains in the tissue, thus maintaining its renewal capacity (Tögel & Westenfelder, 2007).

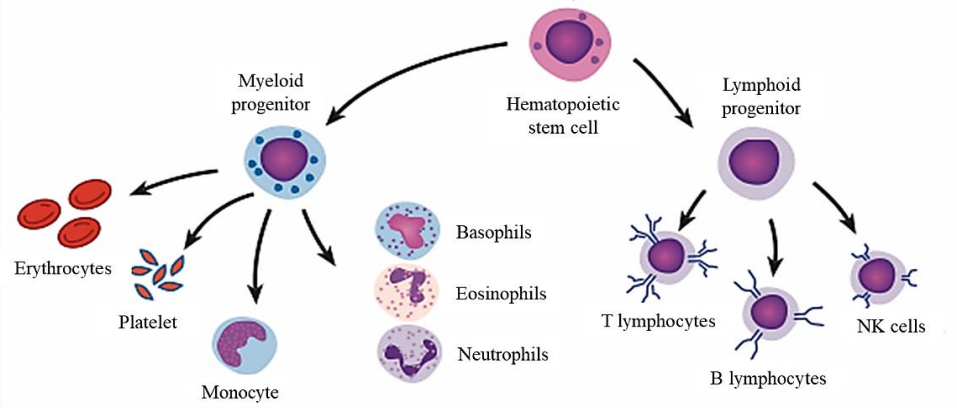


Figure 1. Hematopoiesis: the hematopoietic stem cell is able to produce all blood cells, both myeloid and lymphoid lineages.

Hematopoietic stem cells (HSC), the multipotent stem cells present in the bone marrow, are the precursors for all the blood cells [Figure 1] (Barriga et al., 2012; Tripura & Pande, 2013). The HSC accumulate in the fetal liver during embryogenesis, and then transfer to the bone marrow, where they will be responsible for the definitive hematopoiesis during the whole life of an individual (Barriga et al., 2012).

1.2 HEMATOPOIETIC STEM CELL TRANSPLANTATION

The HSC transplantation (HSCT) is a procedure in which cells of the hematologic and immune systems are replaced by healthy hematopoietic stem cells of a donor. The HSCs can come from three different sources: bone marrow, mobilized peripheral stem cells or placental blood of the umbilical cord (Barriga et al., 2012; Cutler & Antin, 2005; Tripura & Pande, 2013; Walasek et al., 2012). The stem cells of the bone marrow are collected by direct aspiration from the pelvis. To obtain peripheral hematopoietic stem cells, the donor is treated with a colony-stimulating factor or chemotherapy. This induce bone marrow stroma to release hematopoietic stem cells to the peripheral circulation, where they are collected by apheresis. The umbilical cord blood cells are gathered at the time of birth and then cryopreserved (Cutler & Antin, 2005; Tripura & Pande, 2013).

Compared with bone marrow transplantation, the peripheral blood stem cell engraftment is very fast due to the large number of pluripotent stem cells, but it usually produces graft-versus-host disease because of the high number of T cells. On the contrary, transplantation of cord stem cells is much slower (fewer pluripotent cells) but safer (more immune effectors, such as immunomodulatory T-regulatory cells) (Cutler & Antin, 2005; Tripura & Pande, 2013).

1.2.1 Indications

Nowadays, hematopoietic stem cell transplantation is the best treatment for many diseases. The main clinical indication is hematologic malignancies (as leukemia), but HSCT can also treat non-hematologic malignancies (as neuroblastoma) and non-malignant disorders, both congenital and acquired diseases of the hematopoietic system (as some forms of autoimmunity disorders, immunodeficiencies, etc.) (Barriga et al., 2012; Cutler & Antin, 2005; Tripura & Pande, 2013). Therefore, the patient's bone marrow is replaced due to hematopoietic/immune system malfunctions or just because it is necessary to eliminate it to fight some types of cancer.

If the reason of the transplantation was cancer, the HSCT can fail due to relapse of the malignancy. Moreover, in all patients, the HSCT has a significant risk of mortality because of severe graft-versus-host disease (GVHD) and also infections due to immune suppression (Barriga et al., 2012; Clark et al., 2015).

Despite the risk, the use of this clinical technique has increased as a result of: improvements in donor selection (the compatibility between donor and receptor has been studied by the human leukocyte antigen-HLA-system), the use of immunosuppression to prevent GVHD, advances in drugs against infection agents, advances in conditioning regimens and better supportive care (Barriga et al., 2012; Clark et al., 2015; Cutler & Antin, 2005).

Donor types in HSCT

There are two types of HSCT depending on the individual origin of the stem cells:

- **Autologous**

  The self-hematopoietic stem cells are collected from the patient before chemotherapy and then reinfused (Barriga et al., 2012; Cutler & Antin, 2005). This type of transplantation is safer, with a moderate morbidity and low mortality, even in the elderly. This procedure has no autoimmune effects, but it is only used to prolong the survival, as it cannot cure the disorder (Cutler & Antin, 2005).

- **Allogeneic**

  To reconstitute the lymphohematopoietic system of the patient, healthy hematopoietic stem cells are extracted from a different donor and then grafted into the patient, after conditioning regimen. The donor stem cells are free of tumor cell contamination. In addition, they can produce graft-versus-tumor effects and destroy cancer cells of the recipient. However, donor cells can also produce GVHD, in which donor T-lymphocytes attack host tissues. Additionally, the donor must be as compatible as possible with the receptor in order to avoid graft-rejection problems (Barriga et al., 2012; Cutler & Antin, 2005). Donors are selected based on several factors, such as age, sex, serostatus, etc., but mainly by HLA typing. Despite having identical HLA with an unrelated donor, related donors (especially siblings) are much less associated to GVHD than unrelated (Cutler & Antin, 2005).

1.2.2 Stages of Hematopoietic Stem Cell Transplantation

The transplantation process has several stages:

- **Conditioning phase**

  To have a successful transplant, patients undergo a conditioning regimen or immune ablative therapy (chemotherapy and/or radiotherapy) in order to eliminate bone marrow hematopoietic stem cells from them. This supresses the immune system to prevent rejection problems, as well as eliminating as many cancerous cells as possible, thanks to the tumoricidal activity of the therapy agents. The duration varies between 6-10 days (Barriga et al., 2012; Cutler & Antin, 2005; Sastre-Urgellés, 2006). The high-intensity pharmacological immunosuppression of the recipient (myeloablative conditioning) is being replaced by low-intensity immunosuppression (reduced intensity conditioning) because it is more beneficial for patients (Tripura & Pande, 2013).

- **Extraction phase**

  Donor hematopoietic stem cells are removed by cytoapheresis (Sastre-Urgellés, 2006).

- **Infusion of the graft**

  Fresh hematopoietic stem cells extracted from the donor's bone marrow are intravenously transfused to the preconditioned recipient patient (Sastre-Urgellés, 2006; Tripura & Pande, 2013). For autologous transplantation, the previously frozen stem cells, are thawed and transferred immediately (Sastre-Urgellés, 2006).

- **Engraftment and recovery**

  After 7-14 days, the hematopoietic populations of the donor start to appear. The time varies depending on some factors, such as donor-recipient HLA compatibility matching (Sastre-Urgellés, 2006).

- **Patient follow-up by the study of chimerism**

  When the lymphohematopoietic cells of the donor are grafted into the recipient, the result is the presence of non-host origin cells together with host cells in the same organism, a phenomenon known as chimerism (Santurtún *et al.*, 2017). The study of the proportion of donor-host chimerism after the HSCT is an important step as it provides information

about the quality of the graft (rejection or GVHD), as well as the presence of minimal residual disease and the possible relapse or recurrence of malignant cells. In this way, the treatment that would be appropriate for the patient can be determined (Khan et al., 2004; Santurtún *et al.*, 2014).

A "chimera" is an individual who possesses cells from two genetically different origins (Castella et al., 2009; Khan et al., 2004; Thiede, 2004). Natural chimerism occurs in very rare circumstances: fusion of zygotes, blood exchanges between fetuses inside the utero or between fetus and mother, double parental contribution, etc. However, artificial chimerism associated with medical intervention (such as HSCT or blood transfusion) is more common (Castella et al., 2009; Thiede, 2004).

Blood and bone marrow are the most frequent materials to study post-transplantation chimerism. When all the cells in these materials are derived from the donor, it is called "complete chimerism", whereas the presence of both donor and receptor cells is called "mixed chimerism". When a successful conditioning is undergone, all of the patient's hematopoietic stem cells and malignant cells are eliminated, which results in complete chimerism. Therefore, the presence of mixed chimerism is usually associated to disease relapse or inefficient conditioning regimens that maintain a small number of recipient cancerous cells. However, sometimes the detected recipient cells are non-malignant (Khan et al., 2004; Thiede, 2004). With non-malignant disorders, the HSCT conditioning is less aggressive, so the presence of mixed chimerism is quite frequent (Khan *et al.*, 2004).

1.2.3 Chimerism analysis methods

Nowadays, donor cell monitoring is much easier than in the past due to significant improvements in the techniques. One achievement in chimerism analysis was the development of fluorescent *in situ* hybridization of sex chromosomes (XY-FISH), which permits to evaluate the donor cells percentage in a fast and accurate manner; although it is limited to sex-mismatched transplantations (Khan *et al.*, 2004; Santurtún *et al.*, 2014; Thiede, 2004). Another breakthrough was the discovery of the polymerase chain reaction (PCR), an important tool which allowed to study highly polymorphic regions of the genome. It is a fast and highly sensitive technique that requires small quantities of DNA (Thiede, 2004).

Although humans share more than 99.9% of the genome with their peers, there are certain regions that vary between individuals (Goodwin *et al.*, 2007; Pereira *et al.*, 2009). These polymorphic regions can be used as markers, in order to generate a unique profile for each person. These areas must

be not only polymorphic, but also easy to characterize and interpret. Moreover, they cannot be under selective pressure and must have a low mutation ratio (Goodwin *et al.*, 2007).

As a result, differences between polymorphic genetic markers of the donor and receptor can be detected in the post-transplanted patient. Therefore, it is necessary to genotype the specific markers of donor and recipient before the HSCT. Then the post-transplanted patient's DNA profile is analysed, and the amount of donor/recipient cells can be quantified to obtain the chimerism status (Khan *et al.*, 2004).

Some methods are used nowadays to evaluate the chimerism status, as FISH, tandem repeat polymorphisms, single nucleotide polymorphisms (SNP), insertion/deletion polymorphisms (InDel), etc. Due to the high polymorphism of short tandem repeats (STR), they are the most common technique used to analyse chimerism, besides FISH can be only used for analysing sex-mismatched transplantation and SNPs and InDels are less polymorphics (Clark et al., 2015).

Tandem repeat polymorphism

They are also known as length polymorphisms. They include two types: "variable number tandem repeats" or minisatellites and "short tandem repeats" or microsatellites. The length of these polymorphisms depends on the number of repeated units. Each allele has a specific length (Goodwin *et al.*, 2007).

- **Variable number tandem repeat (VNTR)**

  In this type of polymorphism, the length of the repeating units is 6-100 base pairs (bp), and each unit can be repeated a variable number of times, hence its name, creating alleles with lengths from 500 bp to more than 30 kilobases. Large amounts of DNA are required to use VNTRs, so they cannot be used with degraded DNA. In addition, the interpretation of the results can be complicated. VNTRs are a powerful tool, although they are not used too much because of their disadvantages (Goodwin *et al.*, 2007).

- **Short tandem repeat (STR)**

  STR polymorphisms are much more used than VNTRs because they are smaller. In fact, they are the most common polymorphism used in chimerism analysis and forensic genetics because of their advantages, such as being highly polymorphic, being distributed throughout all chromosomes (both autosomal and sexual) and having a low mutation rate. They also have a unit of 2-6 bp (di-, tri-, tetra-, penta- or hexa-nucleotides) that is repeated 10 to 25 times in tandem, resulting in 50-300 bp lengths. STRs are

divided into simple, compound or complex markers. In the simple ones, a same unit is always repeated, although there may be some allele with some variation inserted between the simple repeats. In compound markers, two or more simple units are repeated. Finally, complex markers show several units of different lengths, sometimes with variations between them (Gettings *et al.*, 2015; Goodwin *et al.*, 2007) [Figure 2].

Dinucleotide unit: (AG)(AG)(AG)(AG)(AG)	Simple: (AGT)(AGT)(AGT)(AGT)
Trinucleotide unit: (AGT)(AGT)(AGT)(AGT)	Simple with variation: (AGT)(AGT)TC(AGT)(AGT)
Tetranucleotide unit: (AGTC)(AGTC)(AGTC)	Compound: (AGT)(AGT)(AGT)(AG)(AG)(AG)
...	Complex: (AG)(AG)TC(AGT)GCA(AGTC)(AGTC)

Figure 2. Examples of types of STR markers according to the number of nucleotides of their unit (left) and the organization complexity of the units (right).

To distinguish alleles, it is necessary to separate the DNA according to its size by electrophoresis. These polymorphisms have a high discriminatory power and the results can be easily analysed and compared (Gettings et al., 2015; Goodwin *et al.*, 2007). In conclusion, STRs are an important informative tool in identification studies. However, they may not be ideal with challenged degraded samples of DNA, since they can lead to an incomplete profile typing and their discrimination power decreases (Romanini *et al.*, 2012).

Single nucleotide polymorphism

Single nucleotide polymorphisms (SNP) are differences in just one base of the DNA sequence. During cell meiosis, mutations can appear in the cell genome and one nucleotide can change, which lead to SNPs. They are highly abundant in the genome, but since there are four bases, SNPs usually only have two possible nucleotide variations (A-G or C-T), so they are not very polymorphic. However, the amplification of a large number of SNPs makes it possible to achieve an adequately discriminatory genetic profile, although it also makes the analysis more complicated (Goodwin *et al.*, 2007). Their main advantage is the ability to type degraded DNA as the SNP amplicons usually are smaller than 150 bp. They can be also used in paternity testing due to its low mutation rate (Romanini *et al.*, 2012).

Insertions and deletions polymorphism

Another binary marker used in human identification are insertion/deletion polymorphisms (InDel). InDels are caused by insertions or deletions of one or more nucleotides in the genome. They are originated by a single simple mutation of low frequency and non-recurrent; so its

mutation rate is very low. The insert's length varies a lot (alleles from 2 bp to 10 kb), although the vast majority have a size smaller than 100 bp (Pereira *et al.*, 2009; Pereira & Gusmão, 2012).

InDels are very polymorphic and widely distributed throughout the genome (they comprise approximately 16% of all the DNA). They began to be used in human identification since combine advantages of both STRs and SNPs. Small size InDels (2-5 bp) can be analysed in short amplicons (less than 160 bp), which allows the study of degraded DNA samples. Alleles are detected by separating the fragments by capillary electrophoresis. Therefore, the genotyping is easy, effective and cheap. In addition, this minimizes manipulation, and the risk of external contamination or mixture of samples decreases (Pereira *et al.*, 2009; Pereira & Gusmão, 2012; Romanini *et al.*, 2012; Santurtún *et al.*, 2014).

Therefore, InDels can be especially useful to analyse degraded samples, where the standard STRs sometimes fail (Pereira *et al.*, 2009; Pereira & Gusmão, 2012). In fact, in cases of small chimerism, the complete donor profile has been found using STRs in blood samples, but using InDels, recipient DNA was detected. The InDels system may be more sensitive than STRs for detecting low percentages of chimerism (Santurtún *et al.*, 2014). However, InDels normally are biallelic polymorphisms, as SNPs, whereas STRs can present numerous combinations. Thus, to achieve the same discriminating power of STRs, it is necessary to analyse a large number of SNPs or InDels loci (Goodwin *et al.*, 2007; Pereira *et al.*, 2009; Pereira & Gusmão, 2012; Romanini *et al.*, 2012).

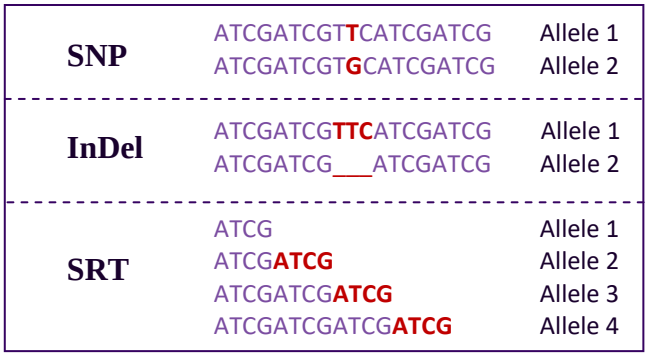


Figure 3. Hypothetical loci of SNPs, InDels and STRs. The SNPs and InDels used as markers normally are biallelic, whereas STR can present many combinations depending on the length of the repeat unit and the number of repeated units.

1.3 DIFFERENTIATION CAPACITY OF THE BONE MARROW HEMATOPOIETIC STEM CELLS

After hematopoietic stem cell transplantation, blood cells are replaced by cells from the donor. In the past, it was thought that donor's cells were limited to hematological tissues (blood and bone

marrow), and that the rest of tissues preserved cells with recipient origin. However, several studies have demonstrated that bone marrow hematopoietic stem cells have an extensive plasticity and a big renewal potential (Li *et al.*, 2014; Tögel & Westenfelder, 2007; Zhou *et al.*, 2011). These characteristics, added to their multilineage differentiation ability, make HSC a suitable tool for cell-based therapy. This marked the beginning of regenerative medicine (Tögel & Westenfelder, 2007; Walasek *et al.*, 2012).

1.3.1 Chimerism analysis in non-hematological tissues

A large number of analyses have been carried out to determine the genetic profile in different tissues of HSC transplanted patients, and donor derived cells have been seen in several non-hematologic tissues. Hair follicles have been found to always contain a complete recipient profile (Li *et al.*, 2014; Zhou *et al.*, 2011; Santurtún *et al.*, 2017), as well as sperm (Li *et al.*, 2014; Santurtún *et al.*, 2017). However, saliva and buccal mucosa analyses show a mixture of DNA from two different sources (recipient and donor) or mixed chimerism (Li *et al.*, 2014; Santurtún *et al.*, 2017; Zhou *et al.*, 2011). The DNA profile in urine was also studied, and the result was mixed chimerism too, although the origin of DNA is unclear. Some patients had shown leukocyturia, implying that in some cases the donor source could be leukocytes present in urine. But chimerism was seen in patients with no leukocyturia too. Therefore, it is also possible that the urinary tract adds donor transplanted HSCs differentiated into epithelial cells (Santurtún *et al.*, 2017).

In conclusion, neither blood, saliva, buccal mucosa nor urine serve as a reliable source to obtain the genetic profile of the recipient patient. This is due to the potential of differentiation of the hematopoietic stem cells transplanted from the donor into hematological and non-hematological tissues. In contrast, donor bone marrow stem cells do not contribute to hair follicle and sperm, so DNA from these two sources can be used as reference to obtain the original genetic profile of the pre-transplant recipient (Li *et al.*, 2014; Santurtún *et al.*, 2017; Zhou *et al.*, 2011).

To know the profile that appears in different samples to which it is easy to access with non-invasive methods has a clinical implication for those patients whose genetic profile was not taken before the transplantation and a subsequent follow-up is required by analysing chimerism. For example, the collection of buccal mucosa samples by swabbing was the traditional method used to obtain the reference profile (of the person before the transplant to follow up) (Goodwin *et al.*, 2007), however, as indicated above, it has been demonstrated that this is not useful since chimerism appears in these samples (Clark et al., 2015). Currently the hair is the main sample collected for this purpose.

1.4 FORENSIC IMPLICATIONS OF THE BONE MARROW TRANSPLANTATION

Forensic genetics is a branch of legal medicine responsible for studying the genetic variation that exists between individuals of the same species, using biological samples recovered from crime scenes. In the case of human samples, they are used to identify people by obtaining their DNA profile, which is the final goal of forensic genetics (Bond & Hammond, 2008; Goodwin *et al.*, 2007; Pereira et al., 2009).

The basis of forensic genetics is that each person possesses a unique DNA sequence, so the forensic analyses of biological samples is a useful tool to identify people. However, individual identification by standard markers can be erroneous in the presence of chimerism. Therefore, some biological samples from patients who have undergone a bone marrow transplant may not be reliable sources for personal identification or paternity testing (Bond & Hammond, 2008; Castella *et al.*, 2009; Goodwin *et al.*, 2007; Li *et al.*, 2014). For instance, after HSCT, blood is completely converted to donor type, rendering it unsuitable. Additionally, as numerous studies indicate, neither urine, saliva nor buccal mucosa are reliable forensic sources due to their mixed chimerism, whereas hair follicles and sperm could be suitable since they show the full profile of the recipient (Li *et al.*, 2014; Santurtún *et al.*, 2017; Zhou *et al.*, 2011).

For all the previously presented information, biological samples from people transplanted from bone marrow can suppose a challenge to legal-medical expertise. Therefore, when evidence is gathering for a biological paternity test or in the context of a criminal investigation, it is important to determine what type of vestiges may show chimerism, since the finding of donor DNA in receptor samples might lead to errors in the interpretation of the results (if an individual potentially involved in the investigation has received an HSCT must be considered, and it is necessary to know which genetic profile will appear in his different vestiges) (Castella *et al.*, 2009; Santurtún *et al.*, 2017)..

Several types of samples have been studied: blood, hair follicles, urine, saliva, etc. However, there is further investigation to be done. For example, it would be interesting to accomplish chimerism analysis in other types of non-hematological samples that have certain forensic potential, such as nails or other epithelial cells that remain attached to surfaces, since these vestiges may be found in a scene of crime.

2. OBJECTIVES

The **main objective** of this work is to analyse the genetic profile in biological vestiges of forensic interest (nail and epithelial cells of the epidermis) of bone marrow transplanted patients in order to establish the percentage of chimerism, which has a forensic and clinical implication.

The **secondary objectives** are:

- To compare the percentage of chimerism found in nails and skin epithelial cells samples with the percentage of chimerism found in blood samples of the same patients.

- To compare the percentage of chimerism in nail and skin epithelial cells samples attending to the age of the patient, the age of the donor, the time elapsed from transplantation and the presence of graft-versus-host disease.

- To analyse the results obtained in the calculation of chimerism after HSCT in blood samples of transplanted patients when it is amplified 38 autosomic InDel polymorphisms or 15 STR polymorphisms, in order to compare their utility in the quantification of chimerism.

3. MATERIALS AND METHODS

An observational and descriptive study has been developed in which it has been analysed the percentage of chimerism in two vestiges of forensic interest (nail and epithelial cells of the epidermis) from patients who are recipients of an allogeneic transplant of hematopoietic stem cells, specifically, bone marrow transplantation.

This study was reviewed and approved by the Clinical Research Ethics Committee of Cantabria. All research participants gave their written consent to participate in the study.

3.1 Study samples

It was analysed a total of 106 samples, corresponding to:

- 61 samples of peripheral blood of donors and receptors of a bone marrow transplantation, before and after the transplant. 18 samples came from donors, 18 from pre-transplanted patients and 25 from post-transplanted patients.
- 20 nail samples and 20 samples of skin epithelial cells given by the transplanted patients (who were being followed-up by the study of chimerism).
- 5 epidermal cells samples isolated from cutaneous biopsies of transplanted patients.

Tips of the patients' nails were cut with sterile scissors and gathered. It was tried to collect skin epithelial cells of the patients, on the one hand, by using an adhesive tape on the internal face of the sock (6 samples) and, on the other hand, rubbing a cotton swab for the armpit and/or friction areas (14 samples).

In order to select the skin biopsies, it was carried out a review to select transplanted patients (that were being followed up by the study of blood chimerism) who would have undergone such intervention for clinical purposes. The samples were collected in the sample bank of the Pathological Anatomy Service of the Marqués de Valdecilla University Hospital (MVUH). Hematoxylin-eosin staining was used to select the non-vascularized area of interest (epidermis) and macrodissect this area on a paraffin block by using a hollow needle of 0.6 mm diameter. The posterior extraction was performed on the generated tissue cylinder.

For subsequent correlation analyses, it was taken into account the age of patients, the age of donors, the type of conditioning prior to transplantation and whether they had suffered graft-versus-host disease.

3.2 DNA extraction and quantification

Total blood DNA extraction was carried out by using the genomic Prep Mini Spin Kit (GE Healthcare, Piscataway, NJ, USA) following the corresponding protocol.

For DNA extraction of nail and epithelial cells it was used a method developed in the laboratory that optimizes the extraction of delicate samples in which it is expected that there is a low concentration of DNA. This protocol consists of: using 500 µL of a home-made cell lysis solution (1.25 mL of Tris-HCl 2M, 2.5 mL of EDTA 0.5M, 10.2 g of sodium acetate and 50 mL of SDS 10% m/v), 20 µL of proteinase K and, in the case of the nail, 40 µL of DTT to dissolve it. After incubation of the sample for 12 hours at 56°C under agitation, it was mixed with 500 µL of phenol: chloroform: isoamyl alcohol 25: 24: 1 (Sigma-Aldrich, Madrid, Spain) to denature the proteins and separate them from the acids nucleic. The proteins remain at the interface while the nucleic acids remain in the upper aqueous phase (UltraPure ™ Phenol…, n.d.). It was centrifuged 45 minutes at 4°C, then the aqueous phase was separated and mixed with 500 µL of cold isopropanol and 50 µL of sodium acetate 3M. After another centrifugation for 15 minutes, the supernatant was removed, and the pellet was mixed with 1 mL of absolute ethanol. Then it is centrifugated again for 5 minutes at room temperature, the ethanol supernatant was completely removed, and the pellet was mixed with 15 µL of distilled water.

The DNA of the cutaneous biopsies was extracted by using the GeneRead DNA FFPE Kit (Qiagen) according to its protocol.

The DNA concentration was measure by Qubit fluorometer (Invitrogen, Foster City, CA, USA) according to the manufacturer instructions.

3.3 Genotyping

To genotype the DNA of epithelial cells presented in the samples of nail, adhesive tape and swab and DNA of blood cells of patients, it was analysed a set of 38 noncoding biallelic InDels previously selected by "Pereira *et al.* (Pereira *et al.*, 2009) [Table 1]. The markers were amplified by multiplex-PCR using the InDelPlex kit (Genomica, Madrid, Spain), according to the manufacturer's instructions. Both normal samples and delicate samples protocols were performed, with no differences in the results.

The DNA of epithelial cells coming from cutaneous biopsy samples was analysed by multiplex-PCR amplification of 15 STR markers [Table 2]. For this, it was used the Identifiler Plus amplification kit (Applied Biosystems) following the established protocol. The work in some

samples with InDels and in others with STRs was motivated by the concentration of DNA obtained during the extraction in each type of vestige.

Table 1. Some information about the 38 biallelic insertion/deletion markers set (Pereira *et al.*, 2009; Pereira & Gusmão, 2012) of the InDelPlex kit.

Int. code	rs Number	Chromosome	Alleles	Amplicon size (S-L)
B01	rs34541393	20	-/AACT	57-61
B02	rs16624	2	-/GT	65-67
B03	rs2307689	19	-/TTC	74-77
B04	rs35769550	8	-/TGAC	89-93
B05	rs2307700	22	-/TCAC	101-105
B06	rs140809	10	-/CAA	115-118
B07	rs3047269	1	-/CTGA	126-130
B08	rs33972805	11	-/CT	135-137
B09	rs33917182	20	-/CA	142-144
B10	rs16402	9	-/TTAT	150-154
G01	rs1610871	5	-/TAGG	61-65
G02	rs2067238	12	-/GCT	71-74
G03	rs2067294	9	-/CTT	80-83
G04	rs2307710	6	-/AGGA	92-96
G05	rs2308242	3	-/CT	106-108
G06	rs2307580	9	-/AATT	120-124
G07	rs1160956	5	-/AGA	128-131
G08	rs34577541	18	-/CTCTT	143-148
G09	rs2307978	7	-/GA	156-158
Y01	rs3051300	17	-/GTAT	63-67
Y02	rs10629077	21	-/AT	74-76
Y03	rs10688868	11	-/CT	81-83
Y04	rs2067208	16	-/GCCAG	93-98
Y05	rs2307579	1	-/ATG	104-107
Y06	rs2308020	15	-/TT	127-129
Y07	rs3080855	18	-/AATT	133-137
Y08	rs1610919	12	-/AT	142-144
Y09	rs2307839	6	-/GA	152-154
R01	Rs2308137	6	-/GA	61-63
R02	Rs36040336	19	-/AT	65-67
R03	Rs1160886	10	-/ACT	75-78
R04	Rs2308026	4	-/CA	83-85
R05	Rs2307526	5	-/ACAC	95-99
R06	Rs34811743	11	-/TG	108-110
R07	Rs2308189	14	-/AACTA	119-124
R08	Rs5895447	8	-/CA	128-130
R09	Rs2308171	13	-/TCTG	135-139
R10	Rs35605984	21	-/TAAAG	151-156

Table 2. Some information about the 15 STR markers set ("AmpFlSTR® Identifiler® Plus" …, 2015) of the Identifiler Plus kit.

Locus designation	Chromosome	Alleles
D8S1179	8	8, 9 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
D21S11	21	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38
D7S820	7	6, 7, 8, 9, 10, 11, 12, 13, 14, 15
CSF1PO	5	6, 7, 8, 9, 10, 11, 12, 13, 14, 15
D3S1358	3	12, 13, 14, 15, 16, 17, 18, 19
TH01	11	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3
D13S317	13	8, 9, 10, 11, 12, 13, 14, 15
D16S539	16	5, 8, 9, 10, 11, 12,13, 14, 15
D2S1338	2	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28
D19S433	19	9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2
vWA	12	11,12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24
TPOX	2	6, 7, 8, 9, 10, 11, 12, 13
D18S51	18	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27
D5S818	5	7, 8, 9, 10, 11, 12, 13, 14, 15, 16
FGA	4	17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2

After PCR, the samples were prepared for sequencing by making aliquots of 8.7 µL of highly deionized formamide (Applied Biosystems, Life Technologies, Madrid, Spain), 0.3 µL of the internal size standard GeneScan 500 LIZ (Applied Biosystems) and 1 µL of amplified DNA from each sample. Then the aliquots were denatured for 4 min at 95ºC and cooled at 4ºC in a thermocycler. The analysis of the DNA profile were performed by capillary electrophoresis in an ABI310 Genetic Analyzer sequencer (Applied Biosystems) using the GENMAPPER 4.0 software (Applied Biosystems). After the analysis, the study of electropherograms was performed.

3.4 Data analysis

Profiles of samples of post-transplant patients were compared with donor and receptor profiles to obtain the level of chimerism by calculating the total percentage of donor DNA with respect to the total DNA. The calculations were made only on the informative markers, that is, the markers that differ in recipient and donor. Although it is possible to work with the peak height of the electropherogram, this only takes into account the amount of PCR product detected in the electrophoresis. Therefore, many authors prefer to work with the area because it is more accurate than height, since it takes into account the peak shape too (Clayton *et al.*, 1998). In this work, it was used the area for calculations.

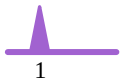
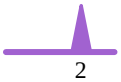
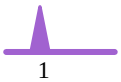
InDels are biallelic markers, so three possible cases can be found. The formulas used in each case ("Quantitative analysis…", 2001) are shown in Table 3:

1. Heterozygous receptor and homozygous donor [Table 3, columns 1 and 2]: The amount of receptor is obtained by dividing the double of the area of the allele that does not share with donor between the sum of the areas of the two alleles. This amount is subtracted to 1 to get the donor amount and multiplied by 100 to get the percentage.

2. Homozygous receptor and heterozygous donor [Table 3, columns 3 and 4]: The donor quantity is obtained by dividing the double of the allele that does not share with the recipient by the sum of the areas of the two alleles. Then the percentage is obtained multiplying by 100.

3. Receptor and donor are homozygous for different alleles [Table 3, columns 5 and 6]: The area of the donor allele is divided by the sum of the areas of the two alleles, and the percentage is obtained multiplying by 100.

Table 3. Formulas used to obtain the level of chimerism by calculating the donor percentage ("Quantitative analysis…", 2001).

Pre-transplantation patient (receptor)						
Donor						
Post-transplantation patient						
Donor percentage	$1-\frac{2\ x\ Allele2}{Al1-Al2}$ x100	$1-\frac{2\ x\ Al1}{Al1-Al2}$ x100	$\frac{2\ x\ Al1}{Al1-Al2}$ x100	$\frac{2\ x\ Al2}{Al1-Al2}$ x100	$\frac{Al2}{Al1-Al2}$ x100	$\frac{Al1}{Al1-Al2}$ x100

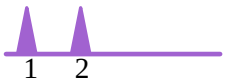
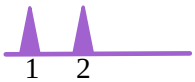
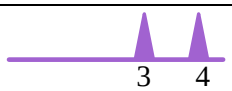
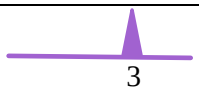
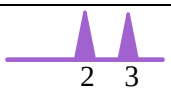
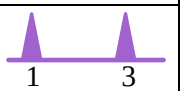
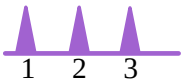
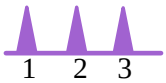
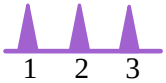
Regarding STRs, since they are not biallelic markers as InDels, we can find more allelic combinations. If only two alleles are present, formulas explained in Table 3 can be applied. If there are more than two alleles, we can find two more cases, whose formulas ("Quantitative analysis…", 2001) will be explained in Table 4:

1 Receptor and donor are homo- or heterozygous and do not share any allele [Table 4, columns 1, 2 and 3]: the area of the allele or alleles of the donor is divided by the sum of the areas of all the alleles, and the percentage is calculated multiplying by 100.

2 Receptor and donor are heterozygous and share one allele [Table 4, column 4]: The area of the donor allele that does not share with the receptor is divided by the sum of the area

of that same allele plus the area of the receptor allele that does not share with donor, then the percentage is obtained multiplying by 100. The area of the common allele is not taken into account for the calculation.

Table 4. Formulas used to calculate the level of chimerism by calculating the donor percentage ("Quantitative analysis…", 2001).

Pre-transplantation patient (receptor)				
Donor				
Post-transplantation patient				
Donor percentage	$\frac{\text{Al } 3 + \text{Al } 4}{\text{Al } 1 + \text{Al } 2 + \text{Al } 3 + \text{Al } 4}$ x 100	$\frac{\text{Al } 3}{\text{Al } 1 + \text{Al } 2 + \text{Al } 3}$ x 100	$\frac{\text{Al } 2 + \text{Al } 3}{\text{Al } 1 + \text{Al } 2 + \text{Al } 3}$ x 100	$\frac{\text{Al } 3}{\text{Al } 2 + \text{Al } 3}$ x 100

After calculating the level of chimerism by the percentage of donor in each marker, the average of them was calculated in each patient to obtain the total percentage of donor in the study sample, as well as the standard deviation.

The Pearson correlation coefficient (*r*) and the Student's t-distribution were used for the statistical evaluation of the results. A $p < 0.05$ was established to interpret the existence of statistical significance.

4. RESULTS

4.1 Comparison between the percentage of chimerism by amplifying InDels and STRs in blood samples

The percentage of donor found in blood samples from 25 bone marrow transplanted patients using the InDels technique was compared with the percentage obtained using the STRs technique. The results are shown in Table 5.

Table 5. Comparison between the donor chimerism percent calculated using InDels and STRs as markers in the 25 post-transplantation patients.

Samples	15 STRs		38 InDels	
	Informative markers	Donor DNA percentage	Informative markers	Donor DNA percentage
1	7	100%	11	100%
2	12	100%	11	100%
3	4	97%	11	93,68%
4	7	100%	14	100%
5	6	100%	16	100%
6	7	100%	11	100%
7	15	100%	15	100%
8	14	99%	17	98,73%
9	4	96%	11	93,80%
10	12	90%	17	89,88%
11	13	100%	29	100%
12	13	100%	14	98,97%
13	13	100%	19	97,80%
14	13	100%	20	98,06%
15	12	100%	16	98,43%
16	15	100%	29	99,28%
17	14	100%	20	99,74%
18	12	89%	17	93,56%
19	13	100%	14	100%
20	9	100%	15	98,43%
21	14	98%	26	99,14%
22	12	91%	17	96,97%
23	4	96%	11	97,24%
24	12	96%	17	97,59%
25	11	98%	13	100%

The results achieved with the use of InDels and STRs are very similar; the relationship between the results found by both techniques is statistically significant (Pearson's r = 0.799, p = 0.000002) [Figure 4].

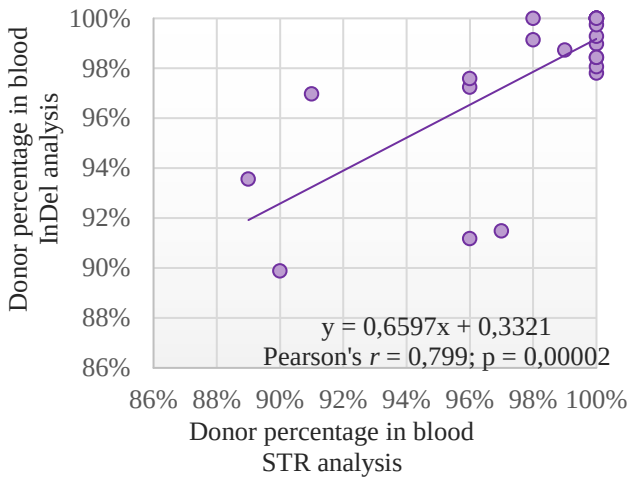


Figure 4. Comparison of the percentage of donor found in 25 blood samples of bone marrow transplanted patients using the InDels and STRs analysis as technique.

Regarding the informativeness of the markers, in the case of STRs technique, the average of informative loci per patient was 10,7 out of 15 (the total number of STRs); whereas the average of informative loci per patient using the InDels technique was 16,4 out of 38 (total number of InDels). Therefore, the average of informative loci per patient using InDels is higher than using STRs, however, the informativeness or the relative percentage of informative loci out of the total loci in each set is much higher in the case of STR (STR = 10,7/15*100 = 70,7%; InDels = 16,4/38*100 = 43,2%) [Figure 5].

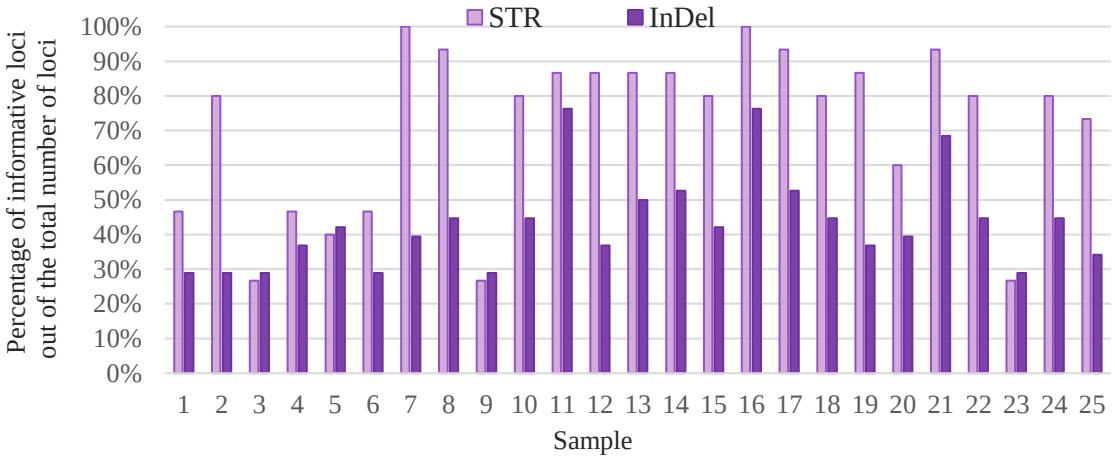


Figure 5. Percentage of informative loci in each blood sample of bone marrow transplanted patient out of the total of available loci in each set (15 in the case of STRs and 38 for InDels).

4.2 Chimerism analysis in blood and nail samples

For blood samples of transplanted patients, the percentage of donor found ranged between 89.9% and 100%; whereas for nail samples, the donor percentage ranged between 0% and 29%. Table 6 shows the percentage in blood and nail, as well as other factors specific to the patient (age, months elapsed from transplantation, type of conditioning type of conditioning that the patient underwent before transplantation and whether he has suffered graft-versus-host disease).

In some cases, more of one sample came from the same patient, since he/she collaborated with the study more than once in different moments after transplantation.

Table 6. Chimerism analysis of 20 blood and nail samples of bone marrow transplanted patients and some information concerning patients.

Samples	Age of patient	Age of donor	Months from transplantation	Type of conditioning	GVHD	Donor DNA percentage	
						Blood sample	Nail sample
1	52	49	3	Non-myeloablative	Yes	100%	0,56%
2	55	31	5	Myeloablative	Yes	100%	1,63%
3	70	62	2	Non-myeloablative	No	93,68%	0,07%
4	42	36	1	Myeloablative	Yes	100%	7,90%
5	53	49	4	Non-myeloablative	Yes	100%	29,46%
6	67	37	4	Non-myeloablative	Yes	100%	1,57%
7	67	-	5	Myeloablative	No	98,73%	19,45%
8	71	62	3	Non-myeloablative	No	93,80%	18,32%
9	59	-	1	Non-myeloablative	No	89,88%	0,68%
10	45	-	1	Myeloablative	No	100%	5,05%
11	57	52	1	Myeloablative	No	98,97%	14,94%
12	65	34	3	Non-myeloablative	No	97,80%	3,20%
13	60	65	1	Non-myeloablative	Yes	98,06%	0,66%
14	68	-	1	Non-myeloablative	No	99,74%	4,06%
15	57	52	1	Myeloablative	No	100%	1,25%
16	55	59	9	Non-myeloablative	No	98,43%	23,31%
17	67	-	4	Non-myeloablative	No	99,14%	21,26%
18	58	-	2	Non-myeloablative	No	96,97%	3,56%
19	70	62	4	Non-myeloablative	No	97,24%	2,28%
20	50	17	3	Non-myeloablative	No	100%	0%

Figure 6 shows some markers extracted from real patient electropherograms that allow visualizing the presence of chimerism in the reading of some post-transplanted patient's samples.

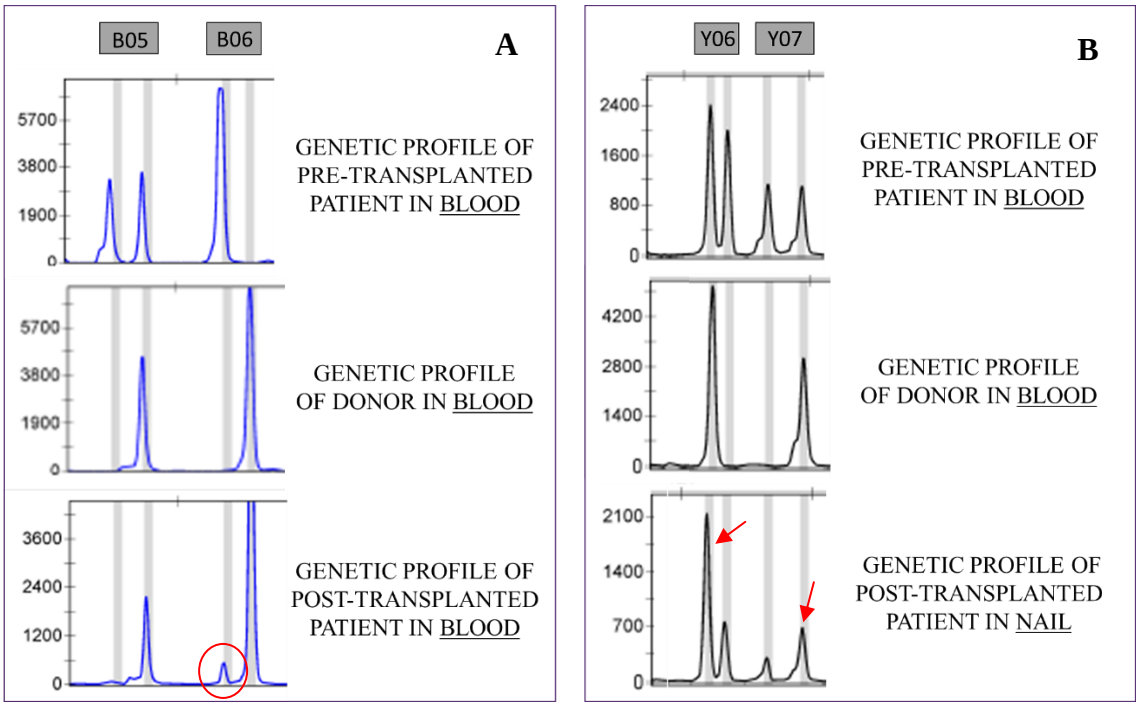


Figure 6. Representative result of InDel analysis. The imagen shows some markers of electropherograms of blood (**A**) and nail (**B**) samples from bone marrow transplanted patients, as well as the donor and receptor profile for these markers. **A.** The blood sample of the post-transplanted patient shows a peak corresponding to the receptor allele (red circle), so this sample contain both receptor and donor DNA. **B.** The nail sample of the transplanted patient shows very disbalanced peaks (alleles that has in common with donor are much higher than the receptor alleles), that indicates presence of donor DNA in this nail sample. Both cases present chimerism.

When it is compared the level of chimerism in blood and nail samples of the same patient, collected at the same time, there is no statistically significant relation between percent donor chimerism in blood and the donor percentage in nail (p>0,05).

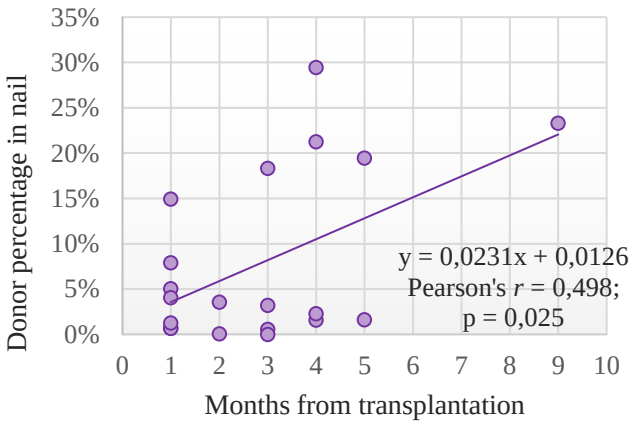


Figure 7. Comparison between the time elapsed from transplantation and the percent donor chimerism found in 20 nail samples of the transplanted patients.

The relationship between the percentage of donor found in the samples and the time elapsed from transplantation was analyzed. No correlation was found for the chimerism in blood (p> 0.05), but it was found statistically significant correlation between the months elapsed from the HSCT and the percentage of donor DNA in nail samples (Pearson's r = 0.498; p = 0.025) [Figure 7].

No statistically significant relationship was found between the age of the patient or the age of the donor and the level of chimerism in the nail ($p > 0.05$).

In addition, the percent donor chimerism found in patients who had been treated with myeloablative conditioning prior to transplantation was compared with percentage in those patients treated with non-myeloablative conditioning by performing the Student's t distribution. No statistically significant correlation was found between the type of conditioning and the percentage of donor DNA in blood or in nail ($p>0,05$). It was also evaluated whether graft-versus-host disease (GVHD) affected the percentage of chimerism, but no statistically significant relationship was found in blood or in nail ($p>0,05$). Table 7 shows how the percentages of donor and receptor in nail samples vary according to the type of conditioning and the presence or absence of GVHD.

Table 7. Donor and receptor DNA percentage in the 20 nail samples from bone marrow transplanted patients according to the type of conditioning and the presence of GVHD.

Type of conditioning	GVHD	Donor percentage in nail samples	Receptor percentage in nail samples
Myeloablative conditioning	Yes	1,63% -7,9%	92,1% -98,37%
	No	1,25% -19,45%	80,55% -98,75%
Non myeloablative conditioning	Yes	0,56% -29,46%	70,54% -99,44%
	No	0% -21,26%	78,74% -100%

4.3 Chimerism analysis in skin epithelial cells samples

Among the 20 samples collected from patients by adhesive tape and cotton swab, it could be only obtained one DNA extraction that allowed the analysis and reading of the genetic profile. This sample was from a rubbish of the armpit of a patient by a cotton swab, and its analysis showed mixed chimerism, with a 19.5% of donor.

In samples of epithelial cells from skin biopsies from post-transplant patients, the analysis was carried out using the STR technique. After calculating the percentage of donor in these samples, a range of ~10-62% was observed [Table 8].

Table 8. Chimerism analysis of cutaneous biopsies and some information concerning patients.

Biopsy sample	Age of patient	Age of donor	Months from transplantation	Donor percentage in epidermal cells samples	Donor percentage in blood samples
1	42	39	29	62,09%	89%
2	60	52	26	43,48%	100%
3	29	22	22	10,32%	100%
4	41	-	9	11,08%	100%
5	60	52	20	30,51%	100%

It was studied the percent donor chimerism found in blood samples of the same patients taken as close as possible to the date of the biopsy, but there is no any correlation between this percentage and the donor percent chimerism found in the samples of epithelial cells of epidermis isolated from biopsies ($p>0,05$).

Figure 8 shows how the reading of an electropherogram is interpreted, by the example of some STR markers of epithelial cells samples (coming from cutaneous biopsies) in which chimerism can be detected.

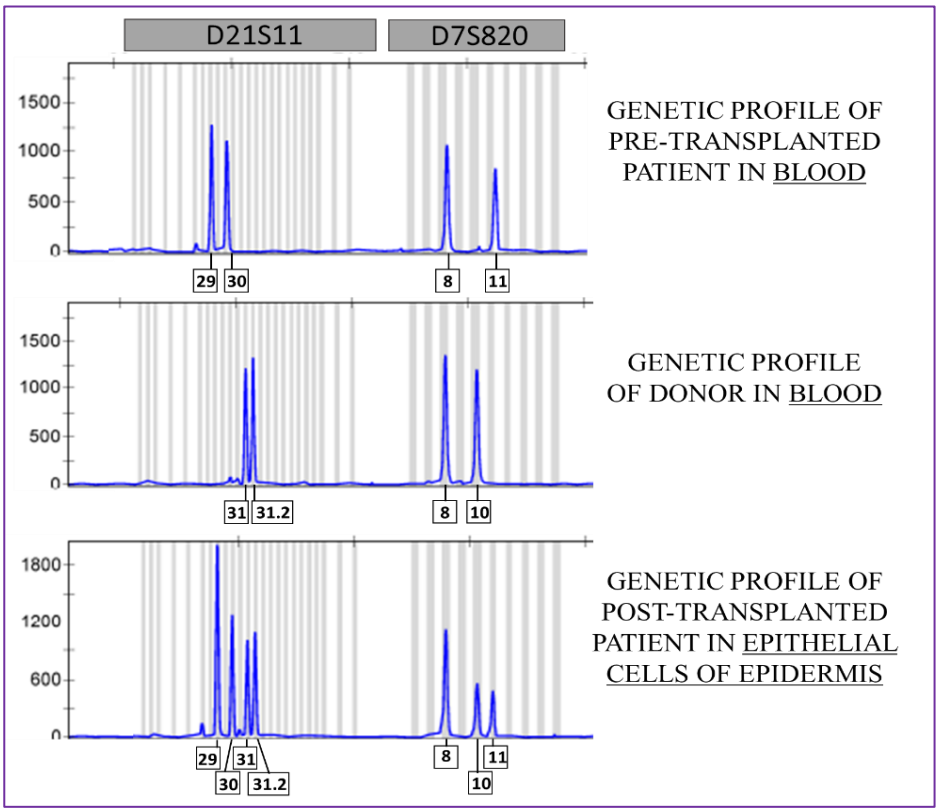


Figure 8. Representative result of STR analysis. The imagen shows two example markers of the electropherogram of epidermal cells sample of one bone marrow transplanted patient, as well as his corresponding donor and receptor profile for these markers. In the sample of epithelial cells of epidermis, the two loci possess peaks of both donor and receptor. Therefore, this sample shows chimerism.

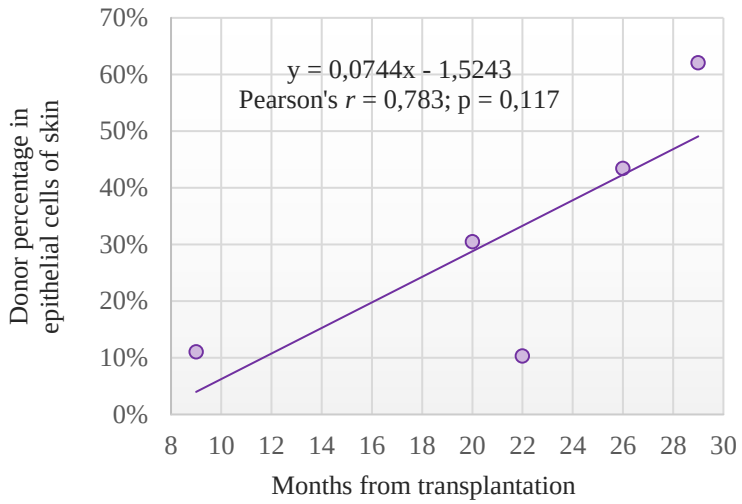


Figure 9. Comparison between the time elapsed from transplantation and the percent donor chimerism in 5 samples of skin epithelial cells isolated in cutaneous biopsy of bone marrow transplanted patients.

When it is analysed the relationship between the time elapsed from the transplant to the biopsy with the level of chimerism in epithelial cells of the epidermis, it is found a correlation, but it is not statistically significant ($p>0,05$, although it is close to the significance (Pearson's r =0,783, p =0,117) [Figure 9]. However, it should be taking into account the low number of samples.

There is not any correlation between the level of chimerism and the age of patients or donors ($p>0,05$).

5. DISCUSSION

The main findings of this study have been: i) InDels and STRs are human identification markers that allow the analysis of chimerism in bone marrow transplanted patients; ii) The nail and skin epithelial cells of bone marrow transplanted patients are biological vestiges that represent a challenge from a forensic perspective, since both of them present chimerism; and iii) The percentage of cells with donor profile in nail and skin increases with time elapsed from the bone marrow transplantation.

One of the objectives proposed in this study was to compare the percentage of chimerism found in blood by the amplification of 15 STRs (markers of choice in forensic genetics) with the percentage found by amplifying the set of 38 biallelic InDels. The results show a strong correlation between both marker kits, which is consistent with the results obtained in previous work in another sample of patients (Santurtún *et al.*, 2014). The higher percentage of informative loci among STRs than InDels out of the total number of available loci in each set was expected since the InDels employed in this set are biallelic markers and the level of polymorphism of STRs is much higher (Pereira *et al.*, 2009; Pereira *et al.*, 2012).

The main purpose of this project was to analyse the genetic profile in nail and skin epithelial cells of bone marrow transplanted patients as they are biological vestiges of forensic interest that might present a challenge in the medico-legal expertise. The results show the presence of donor DNA in both nail and epithelial cells of epidermis.

From an expert witness perspective, the knowledge of what we can find according to the vestige we face is very useful. Due to the natural flaking of the skin and replacement of epithelial cells, they are constantly deposited in the floor or adhered to any surface with which the person has had contact (Goodwin *et al.*, 2007). In criminal contexts it is common to find objects from which it might be relevant to know their origin (for example, to whom a jacket belongs), or who has manipulated them (for example, who has used a knife); in this case, although the number of epithelial cells that is usually found is very low, it can be crucial in the context of the investigation. The commonly used gathering techniques (although they will depend on the surface) are usually the collection of the cells with adhesive tapes, the rubbing with cotton swabs, and the cutting of pieces of cloth (Pérez-Vergara, 2017). In this study, although the epithelial cells samples came mainly from cutaneous biopsies (as presented in the methodology), adhesive tape was used to collect epithelial cells deposited in the internal face of the sock of some transplanted patients; cotton swabs were also used to rub the armpit and friction areas to try collecting epithelial cells that could be removed from skin and remain in these areas; then the genetic analysis was carried out in these samples too. The reason for trying to collect these cells coming from flaking skin is because it resembles

a real situation, and they could be found in a criminal scene. Although enough DNA could only be obtained in one of the armpit swabbing samples to get the profile, it is noteworthy that in this sample a non-negligible percentage of donor was also detected. A possible reason for the lack of usable genetic material could be the insufficient number of cells obtained through these methods or a poor quality of the DNA extracted from the cells, that is, a high degradation in these kinds of vestiges. It is important to highlight the limitations of the results obtained by these procedures since patients collected the samples by themselves. The use of these methods could be potentially optimized, for example, with the help of nurses in the collection of the skin cells.

Body sweat also influences the transmission of skin epithelial cells. Due to the presence of sweat glands throughout the epidermis, fluids are continually secreted and remain at the contact sites (Goodwin *et al.*, 2007; Huynh *et al.*, 2017). Sweat is a source of genetic evidence, since it may contain DNA, as we have seen in this work, although it does not always contain a sufficient number of cells. In addition, sweat remaining on surfaces can be detected by determination of lactate (Huynh *et al.*, 2017).

It is also possible to find traces of skin epithelial cells from a possible aggressor in the body of a victim (Goodwin *et al.*, 2007; Hebda *et al.*, 2014). A fairly common source of epithelial cells are traces that remain under the victim's nails after an assault, due to aggressors being scratched by victims while defending themselves. In this case, if the exogenous material under the nail is sufficient, it can be directly collected by swabbing and analysed to try to identify the aggressor by amplifying standard markers. However, when the exogenous material is insufficient, the best way to collect as much DNA as possible has been shown to be immersing the nail sample collected from the victim in lysis buffer. Therefore, the result would be a mixture of profiles, from which the profile of the victim would be dismissed to try to identify the aggressor (Hebda *et al.*, 2014).

In addition, the use of nails as a vestige for the identification of decomposing corpses, for which the tissues normally analyzed in these cases are bones and teeth, has already begun to be considered. Nails are also preserved, and their analysis presents many advantages; not only can they be collected in a non-invasive way, unlike bone and tooth, but they can also present a concentration of DNA higher than other tissues (Allouche *et al.*, 2008).

Due to all of it is said above, the importance of nails as a forensic vestige is indisputable, and moreover they can be collected non-invasively. However, the existence of chimerism in any of the two vestiges (skin epithelial cells or nail) could lead to errors in the interpretation of the results. For this reason, during expert witness interpretation it is important to take into account whether suspects, victims or persons involved have undergone hematopoietic stem cell transplantation or if they are bone marrow donors.

Besides the impact of the results from a forensic perspective, their clinical implications are also relevant. In some cases, patients start to be monitored by molecular tracking using human identification markers some time after the bone marrow transplantation, without having taken any receptor sample before transplantation to obtain a reference profile in order to carry out the chimerism study. In view of the results of this work, the utility of using nails collected after a transplant as a reference sample of the patient's DNA profile prior to the procedure is discarded.

As discussed in the introduction, only two biological vestiges show a complete profile of the pre-transplanted patient: sperm and hair follicles (Santurtún *et al.*, 2017; Li *et al.*, 2014; Zhou *et al.*, 2011). In the case of sperm, it may be due to the fact that spermatogenesis is a highly conserved process (Li *et al.*, 2014). However, the absence of donor DNA in the hair follicle lead us to expect the profile of the pre-transplanted patient in the nail, since hair and nail tissues share many characteristics: both have an ectodermal origin since they are appendages of the epidermis, both present keratin, in many diseases both tissues are affected at the same time and, furthermore, the niche of the adult nail stem cells is a structure analogous to the hair bulge (niche of the hair stem cells) (Sellhever, 2013). However, the reason for the different plasticity of donor CMH in these tissues is unknown.

The results we obtained are in line with those found by other authors. Imanishi *et al.* (Imanishi *et al.*, 2007) suggested that myeloablative conditioning (MC) regimens before HSCT in these patients could damage the stem cell system of the nail, which somehow would benefit the subsequent infiltration of donor lymphohematopoietic cells after transplantation. This hypothesis is based on the fact that the only patient in this study who underwent a reduced intensity conditioning (RIC) did not show presence of donor DNA in his nail. For this reason, Pearce *et al.* (Pearce *et al.*, 2008) conducted another study in which they compared the presence of chimerism in nails in patients undergoing HSCT after MC with patients transplanted after a RIC. The results showed mixed chimerism in the nail in both groups. However, patients undergoing RIC who were found to have chimerism in the nail had previously presented graft-versus-host disease (GVHD). Therefore, in their research they consider the possibility that the presence of donor cells in nail in this group is due to a local inflammation of the tissue caused by GVHD. These hypotheses prompted us to study both the type of conditioning with which the patient was treated before the transplant and the presence or absence of GVHD, in order to compare the results found in the nail. In our results, the absence of a statistically significant correlation between the percentage of chimerism and the type of conditioning, as well as the absence of correlation with the presence of graft-versus-host disease, suggest the possibility that there are other factors involved. It is important to point out that the previously cited human identification work performed on nail was carried out using the STR standard markers, whereas in the present work the nail of the post-transplanted patients was analyzed using the set of InDels markers.

The age of patients and donors was other factor interesting to investigate since some studies shown that the hematopoietic stem cells are less proliferative with aging, besides it is also produced a decline of hematopoietic growth factors activity (Quaglino et al., 1996). For that reason, the percentage of chimerism in the studied tissues, nail and skin epithelial cells, was compared with the age of patients and donors, in order to determine if aging also influences the capacity of transdifferentiation of the HSCs. However, in our results, the age does not affect the level of chimerism of these two biologic vestiges.

Finally, the presence of donor cells in the nail and epithelial cells of the skin, as well as the significant correlation between the time elapsed from transplantation and the percentage of donor cells in both vestiges, also makes it important to discuss how this finding contribute to the knowledge about the differentiation potential of hematopoietic stem cells. The phenomenon of transdifferentiation (one differentiated cell turns into other type of differentiated cell) is known as "adult stem cell plasticity" (Zhou et al., 2011). The potential differentiation of adult stem cells has a great importance in regenerative medicine. A large number of studies analyse the plasticity of HSCs and their potential to regenerate damaged non-hematological tissues (Tögel & Westenfelder, 2007; Walasek *et al.*, 2012). HSCs have been found to be efficient in the treatment of autoimmune diseases, heart problems, liver failure, and even ischemic conditions, where they have proven to be able to replace dead cells by promoting angiogenesis in the affected tissue (Tripura & Pande, 2013). They have also been effective in repopulating the epithelium of the gastrointestinal tract, so they are still being studied for their use in regeneration treatments of other damaged epithelial tissues (Okamoto *et al.*, 2002).

It is known that HSCs can extensively expand and self-renew themselves in vivo; however, it turns out very difficult when attempting to expand HSC in vitro for medical purposes. The reason is that when hematopoietic homeostasis changes into a hematopoietic stress, as in transplantation, several factors induce HSCs to self-renew and amplify. These factors include hematopoietic growth factors, cell cycle regulators, transcription factors, epigenetic modifiers, etc. Although networks of components and different routes involved in the HSC renewal are being identified, the exact conditions that control the regeneration of the HSC expansion remain unknown (Walasek et al., 2012). Besides studying the way to expand HSCs in vitro, an attempt has been made to find out how to generate HSCs from two different sources: embryonic stem cells and induced pluripotent stem cells (Tripura & Pande, 2013).

Although (to our knowledge) this is the first work that faces the analysis of skin epithelial cells using human identification markers for their forensic interest, in 2003, Tran *et al.* (Tran *et al.*, 2003) demonstrated how stem cells derived from the bone marrow, possibly hematopoietic stem cells, migrated from the marrow to the cheek and was able to differentiate into epithelial cells. Other

studies of chimerism have also shown that cells of the buccal mucosa present both receptor DNA and donor DNA (Santurtún *et al.*, 2017; Li *et al.*, 2014; Zhou *et al.*, 2011). The study by Okamoto *et al.* (Okamoto *et al.*, 2002) proved that bone marrow derived cells could differentiate into epithelial cells of the gastrointestinal tract and repopulate it after tissue damage. None of these studies could attribute the presence of donor cells to the fusion between receptor epithelial cells with donor cells, since this phenomenon rarely occurs (Okamoto *et al.*, 2002; Tran *et al.*, 2003). The results of this work on skin epithelial cell samples agree with all these data, since mixed chimerism is found in the total samples analysed, reaching even a donor percentage of 60%. This allows to infer that donor cells derived from the bone marrow are able to migrate to the epidermis and differentiate into epithelial cells.

At this point, we would like to include some limitations of our study; firstly, as mentioned, despite trying to collect several epithelial cells samples from the skin by using adhesive tape and cotton swab, only one profile could be analysed correctly. As a second limitation, it should be noted that, in the genetic profiles analysis of nail, the low DNA concentration obtained in the extraction made the reading of electropherograms difficult (despite working with InDels instead of STRs) and some loci could not be included, although when working with 38 markers and being several informative in each patient, this did not impede the calculation of the chimerism percentage.

6. CONCLUSIONS AND FUTURE RESEARCH LINES

The main conclusions of the work are:

1. The study of chimerism in blood samples from patients receiving a hematopoietic stem cell transplant is a useful clinical tool in order to know the evolution of the disease and it should be carried out with efficient techniques in terms of the power of discrimination, cost and time. Although STRs analysis is the most used method for this purpose, the InDels analysis is able to achieve very similar results and, in the case of partially degraded samples, where the STRs could not be amplified correctly, they would be very useful.

2. In the analysis of human identification markers of DNA extracted from nail and skin epithelial cells of bone marrow transplant patients, a mixed human profile is obtained (the profile of the donor is amplified), which is a challenge from a forensic perspective.

3. The percentage of cells with the donor profile in nail and skin increases when time elapses from the bone marrow transplantation. However, the age of donor or patient, the type of conditioning prior to the transplant or the presence of graft-versus-host disease do not influence the level of chimerism or the differentiation of haematopoietic stem cells to skin or nail epithelial cells.

As future lines it is proposed to: increase the sample size (for which it would be necessary to count on a bigger period of study to obtain the collaboration of a longer number of transplanted patients); optimize collection and DNA extraction techniques for those delicate supports which contain very few cells, such as adhesive tape and swabs; try to find other factors which influence the percentage of chimerism; analyse the causes of the differences between the hematopoietic stem cells plasticity in hair follicle and nail.

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