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METHODOLOGY

# Quality by Design: Development of Safe and Efficacious Full-Thickness Acellular Dermal Matrix Based on EuroGTPII Methodologies

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**Background:** The activities of tissue establishments are constantly and rapidly evolving. The development of a new type of allograft, full-thickness acellular dermal matrix, with high mechanical properties to be used in tendon repair surgeries and abdominal wall reconstruction, has determined the need for quality by design process in order to assess evidence of quality, safety and efficacy. The EuroGTPII methodologies were specifically tailored to perform the risk assessment, identify and suggest tests in order to mitigate the potential risk consequences of a novel tissue preparation implementation.

**Methods:** The new allograft and associated preparation processes were assessed using the EuroGTP methodologies and characterized to properly evaluate the novelty (Step 1), identify and quantify the potential risks and risk consequences (Step 2), and define the extent of pre-clinical and clinical assessments required to mitigate the risks identified in the assessment (Step 3).

**Results:** Four risk consequences associated with the preparation process were identified: (i) implant failure related with tissue procurement and the reagents used during the decellularization protocol; (ii) unwanted immunogenicity related with the processing; (iii) disease transmission linked with the processing, reagents used, reduction in the reliability of microbiology testing and the storage conditions; and (iv) toxicity related to the reagents used and handling of the tissue during clinical application. The outcome of the risk assessment was a low level of risk. Nevertheless, it determined the need for a series of risk mitigation strategies proposed to reduce each individual risk and to provide additional evidence of the safety and efficacy of full-thickness acellular dermal matrix grafts.

**Conclusion:** EuroGTPII methodologies allow us to identify the risks and ensure the correct definition of pre-clinical assessments required to address and mitigate the potential risk consequences, before proceeding with clinical use of the new allografts in patients.

**Keywords:** EuroGTPII methodology, acellular dermis, risk assessment, quality, safety

## Introduction

The development of novel tissues and tissue-based therapies is driven by the need to improve treatment options for patients or to address unmet clinical needs. The development of new tissue preparations must comply with high quality and safety standards according to the requirements of the European Union Tissues and Cells Directives (EUTCD) to ensure a high level of health protection.[1] The European Good Tissue Practices (EuroGTP) project developed in 2009, for the first time, the guidelines for tissue establishments (TE) on the recovery, processing and preservation of tissues, to ensure that all TE guarantee the highest level of quality and safety of tissues for human application.[2]

However, the tissue preparations are constantly evolving and there is always a risk that any change in the donation, processing or preservation procedures, or in clinical application, can result in harm to the recipient. It is therefore vital to evaluate every potential risk of a process whenever any significant change is considered.[3] Evaluation of the risk resulting

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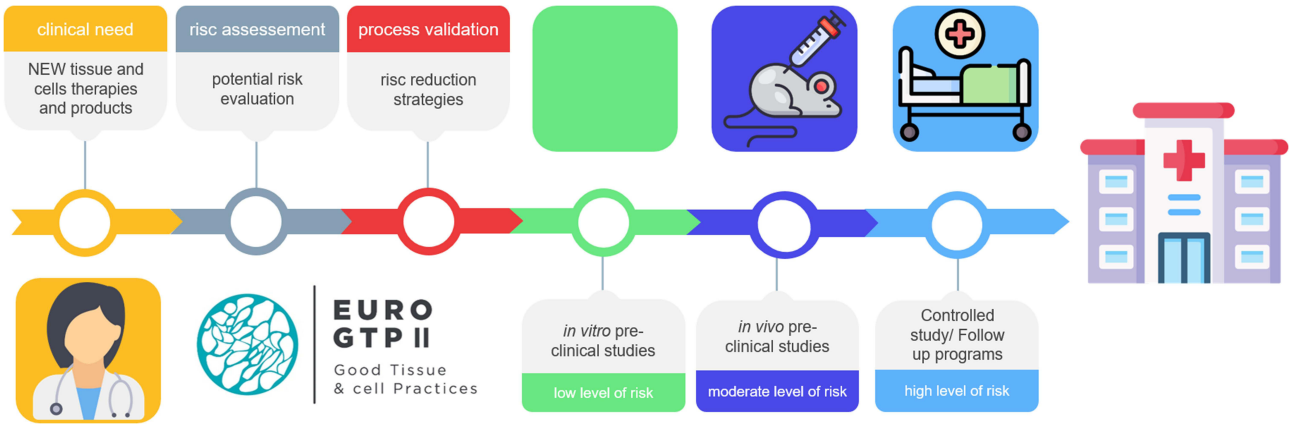
**Graphical Abstract**




from all aspects of the preparation process (from donor to patient) allows the proper design of studies that ensure the safety and quality of the new product. In recognition of the need to perform a risk-benefit analysis, the European Good Tissue and Cells Practices II (EuroGTPII) set up a systematic methodology with regard to pre-clinical and clinical evaluation of Substances of Human Origin (SoHO).[4] This consists in a risk-based mechanism and an Interactive Assessment Tool (IAT: http://tool.goodtissuepractices.site), which allows evaluating if a new or changed tissue preparation has significant novelty; determine the overall risk arising from the novelty; determine an appropriate level of pre-clinical and clinical evaluations to address and assess the risk; implement the result of risk assessment into routine practice and follow up the results.[5]

Since 2019, the EuroGTP II methodology has been applied by the different European TE as a tool to identify, quantify and mitigate the risks associated with the development of novel preparation processes,[6] and changes in any process.[7] This has allowed a harmonized quantification of the risk level associated with the novel SoHO, the adoption of standard strategies to reduce it, and the definition of suitable clinical evaluations, required to demonstrate safety and efficacy.[8]

Tissue banking programmes in Europe started in 1970 and 1980s in response to an expanding clinical need for preserved skin allografts to be used as a coverage in major burns. First activities took place in laboratories stated directly in centres where burned patients were treated.[9] Now, accredited skin banking facilities comply with EU Directives (EU 2004/23/EC) as well as national legislation (in Spain, RD-L 9/2014, 2014), meeting standards in accordance with European Directives 2006/17/EC and 2006/86/EC, as well as the Guide to the Quality and Safety of Tissues and Cells for Human Application (EDQM, 5th Ed.).

Bio-substitutes are widely used, but their high cost and limited accessibility make them unaffordable for some public health systems and inaccessible for patients. Among them, extracellular matrices (ECM) such as acellular dermal matrices (ADM)[10] from different origins are a natural and biocompatible alternative, with successful outcomes in different applications.[11–13] TE have developed dermal matrices of human origin, enabling access to safe and efficacious alternatives for soft tissue regeneration.[14–17] Our TE has previously developed a split-thickness acellular dermal matrix that represents an effective alternative for the treatment of soft tissue loss such as gingival retraction.[18]

Nevertheless, the need for grafts with higher thickness and a wide variety of graft sizes to be applied in tendon reinforcement and closure of large wounds, eg, abdominal wall repair, has prompted the search for full-thickness ADM (ftADM). On one hand, rotator cuff repair is considered successful when there is complete healing of the tear in order to withstand high-tension rates after the repair and avoid re-tears; the use of a scaffold to cover the tear (patch augmentation surgery) enhances the speed and quality of the healing to improve tendon strength.[19,20] In addition to providing a scaffold to support tissue remodeling, augmentation surgery improves mechanical properties in comparison to frail and injured tissue.[21] In this context, it is paramount to ensure appropriate mechanical properties as critical attributes of the graft according to its

final application. The stiffness of a graft, for example, represents the resistance to stretching, and is more representative of the graft performance clinically than tensile modulus. In our experience, greater stiffness is achieved by thicker grafts, while, as reported elsewhere, thinner grafts tend to tear with suture tying.[19] Moreover, for irreparable massive rotator cuff tears, superior capsule reconstruction has recently been described entailing the fixation of a thick graft, achieving pain relief and improving function postoperatively.[22,23] On the other hand, the main objective of ftADM in abdominal wall repair surgeries is to reinforce the tissues. Contrary to ventral hernia repair with synthetic meshes, bioprosthetic meshes like human ADM must be placed on a great deal of tension. If the elasticity in human ADM is inadequately addressed during the repair, laxity of the repair may occur,[24] making ftADM an ideal graft for this type of surgery. Although human ADM has a high number of elastin fibers and is likely to stretch over time, long-term studies conclude that ADM provide durable repair with a low rate of recurrence.[25] Moreover, cells responsible for rejection are removed in the processing of acellular dermal matrices, making ADM a durable scaffold for cellular and vascular ingrowth, promoting tissue regeneration and eventual integration with surrounding tissue, rather than encapsulation. Due to its revascularization capacity and incorporation with surrounding tissue, ADM is associated with lower rates of infection, extrusion, erosion, and adhesion formation compared to synthetic mesh.[26] Hernia reconstruction may also benefit from the use of dermal matrices, not only because of their size and biomechanical properties but also due to their ability to remove bacterial contamination when there is a history of surgical site infection.[27,28]

The development of a decellularization protocol for full-thickness skin is closely related to the maintenance of the structural characteristics of the tissue, requiring the implementation of significant changes in the protocols associated with procurement, processing, decellularization, preservation and clinical application of ADM, previously validated and authorized in our TE.[18] The procedure for preparation of ftADM required adaptation of this protocol to the size, thickness and anatomy of the full-thickness skin starting material.

The present study encompasses the procedures required for the safe implementation of an innovative tissue in the routine practice of our TE, including evaluation of novelty, risk assessment, design and performance of studies to mitigate the potential risks identified, and the definition of a specific Clinical Follow-up Plan (CFUpP), necessary to monitor safety and assess the efficacy of the ftADM allografts in patients.

## Materials and Methods

The evaluation of novelty, risk assessment and definition of studies required to safely implement the ftADM were performed using the EuroGTPII methodologies and interactive assessment tool (http://tool.goodtissuepractices.site/). Briefly, the new product was characterized to properly evaluate the novelty (Step 1); thereafter, the risks associated with the novelty were identified and quantified through a risk assessment (Step 2). The results of this assessment give a Final Risk Score that was used to define the extent of the pre-clinical and clinical evaluation (Step 3). Flowchart is included in the Supplementary Information (Supplementary Figure 1).

### Evaluation of Novelty

The first part of the methodology was intended to identify any change that could significantly affect the quality of the product and/or the safety of the recipients. This evaluation was carried out by answering seven key questions related to all the processes and activities of the supply chain, from donation to clinical application of the novel tissue (Table 1, Step 1, questions A to G).

### Risk Assessment

The risk assessment was focused on identifying the risk factors and quantifying the risk consequences associated with the previously evaluated novelty. Again, all the processes from donor selection to clinical application were evaluated. The risk factors (Table 1, Step 2A) and their respective risk consequences (Table 1, Step 2B) were identified, evaluated in detail and quantified, awarding a score for each one (Table 1, Step 2).[18] For each risk evaluated, a score (risk quantification criteria - Table 1, Step 2C) was defined for the probability of the risk occurring, the severity of the consequences and the ability to detect each individual risk consequence before clinical application. Any relevant data available to support reduction of the calculated risk scores were recorded and documented (Table 1, Step 2D).[6] Supplementary Tables, are provided for the interpretation and quantification of the different parameters of the risk: assessment methodology: probability, severity, detectability and

**Table 1** Summary of EuroGTPII Methodology – Criteria and Scores Used for the Evaluation of Novelty and Risk Assessment[3,4]

| Step 1 | Step 2 | | | | | |
|---|---|---|---|---|---|---|
| **Evaluation of Novelty** | **Step 2A. Risk Factors** | **Step 2B. Risk Consequences** | **Step 2C. Risk Quantification Criteria** | | | **Step 2D. Risk Reduction (RR) Criteria** |
| | | | **Probability (P) Scores** | **Severity (S) Scores** | **Detectability (D) Scores** | |
| A. Has this type of tissue/therapy previously been prepared and issued for clinical use by your establishment?<br>B. Will the starting material used to prepare this tissue/therapy be obtained from the same donor population previously used by your establishment for this type of tissue/therapy?<br>C. Will the starting material for this tissue/therapy be procured using a procedure used previously by your establishment for this type of tissue/therapy?<br>D. Will this tissue/therapy be prepared by a procedure (processing, decontamination and preservation) used previously in your establishment for this type of tissue/therapy?<br>E. Will this tissue/therapy be packaged, stored, and distributed using a protocol and materials used previously in your establishment for this type of tissue/therapy?<br>F. Will this type of tissue/therapy provided by your establishment be applied clinically using an application method used previously?<br>G. Has your establishment provided this type of tissue/therapy for implantation or transplantation into the intended anatomical site and/or same clinical indication before? | • Donor characteristics<br>• Procurement process and environment<br>• Processing and environment<br>• Reagents<br>• Reliability of microbiology testing<br>• Storage conditions<br>• Transport conditions<br>• Presence of unwanted cellular material and/or graft vascularity<br>• Complexity of the pre-implantation preparation and/or application method | • Unwanted immunogenicity<br>• Implant failure<br>• Disease transmission<br>• Toxicity/ carcinogenicity | 1. Rare<br>2. Unlikely<br>3. Possible<br>4. Likely<br>5. Almost certain | 1. Non-serious<br>2. Serious<br>3. Life-threatening<br>4. Fatal | 1. Very high<br>2. Moderately high<br>3. Low<br>4. Very low<br>5. Cannot be detected | 0% - None<br>25% - Limited<br>50% - Moderate<br>75% - Substantial<br>95% - Extensive |

percentage of risk reduction (Supplementary Tables 1–4, respectively) following the definitions and instructions of the EuroGTPII guide – page 37−38.[5]

The outcome of the exercise was a single Final Risk Score, ie, a single overall risk score (ranging from 0 to 100). The possible categories were established: Negligible (0–2), Low (2–6), Moderate (6–22), and High (>22).

Estimation of the Final Risk Score was defined as follows:

$$\text{Final Risk Score} = \frac{\text{Combined Risk Value}}{\text{Highest Possible Score}} \text{x } 100 \quad (1)$$

This Final Risk Score took into account the number of individual risks, defined as the Preliminary Score, and the Combined Risk Value as follows:

$$\text{Preliminary Score} = \Sigma\text{individual risk scores} = \Sigma(\text{SxPxD}) - ((\text{SxPxD})\text{x}(\%\text{risk reduction}) \quad (2)$$

$$\text{Combined Risk Value} = \frac{\text{Preliminary Score x Highest Possible Score}}{\text{Max S} \times \text{Max P} \times \text{Max D} \times \text{number of applicable risk consequences}} \quad (3)$$

Where S is severity, P is probability, and D is detectability. The Applicable Number of risk consequences ranges from 1 to 45, and Highest Possible Risk Score is 4500.

## Risk Reduction Strategies and Definition of the Extent of Clinical Evaluation

The Final Risk Score obtained from the risk assessment (Step 2) determined the corresponding extent of studies required to ensure the safety and efficacy of the novel ftADM. The definition of the extent of studies (Step 3) targeted the mitigation of each individual risk identified during the risk assessment exercise. The studies were designed following the strategies proposed in the EuroGTPII Guide[5] to define the set of pre-clinical assays, including the validation studies, preparation process control strategy and key quality indicators required before clinical application of the newly developed grafts, and the definition of the CFUpP.

# Results

## Evaluation of Novelty

The evaluation of novelty was performed by answering the questions in the first step of the EuroGTPII methodologies (Table 2). The new procedures and ftADM specifications were compared with the previous experience of our TE with ADM and other skin products and procedures.[18] This exercise identified four significant changes in the preparation process and clinical application of ftADM: i) use of a scalpel instead of a dermatome in the procurement procedure; ii) adaptation of the decellularization protocol from ADM, in terms of reagents and incubation times, to ensure the correct processing of skin with different anatomy, size, and thickness; iii) change in the concentration of glycerol as preservation medium; and iv) ftADM application at different anatomical sites, and for different clinical indications.

## Risk Assessment

After evaluation of the novelty, the exercise proposed for Step 2 of the EuroGTPII methodologies was performed to evaluate the risks and their associated consequences for recipients. Table 3 shows the rationale and scoring obtained in this exercise. The risk assessment was performed considering the newly designed decellularization protocol for two different clinical applications: 1) tendon reinforcement and 2) hernia repair/abdominal wall reconstruction. As a result of the algorithm used in the interactive tool, the assessment indicated a low level of risk (Final Risk Score = 5), and that the ftADM grafts were safe and efficacious for clinical use and unlikely to cause harm to recipients.

## Risk Mitigation

The low level of risk calculated determined the need to perform an extensive validation of all new preparation procedures adopted to prepare ftADM grafts. Moreover, to mitigate the potential risk consequences identified, a set of specific pre-clinical studies were established in the following step (Table 4).

**Table 2** Assessment of Novelty of the ftADM Grafts, According to Step 1 of the EuroGTPII Methodologies

| Assessment of Novelty Questions | Yes | No | NA |
|---|---|---|---|
| **A. Has this type of tissue/therapy previously been prepared and issued for clinical use by your establishment?** Our TE has previous experience with the preparation and distribution of ADM and other skin products. | X | | |
| **B. Will the starting material used to prepare this tissue/therapy be obtained from the same donor population previously used by your establishment for this type of tissue/therapy?** The same donor population will be used to obtain ftADM, ADM and skin. | X | | |
| **C. Will the starting material for this tissue/therapy be procured using a procedure used previously by your establishment for this type of tissue/therapy?** Full-thickness skin will be procured using a scalpel instead of the dermatome used to obtain split skin. | | X | |
| **D. Will this tissue/therapy be prepared by a procedure (processing, decontamination and preservation) used previously in your establishment for this type of tissue/therapy?** A new decellularization protocol has been developed to obtain ftADM in our TE. The preparation process is an adaptation of the previous validated ADM protocol.[13] | | X | |
| **E. Will this tissue/therapy be packaged, stored, and distributed using a protocol and materials used previously in your establishment for this type of tissue/therapy?** Packaging and distribution follow the same protocols as for split-thickness ADM. However, the concentration of glycerol as a preservation medium has never been used before in our TE. | | X | |
| **F. Will this type of tissue/therapy provided by your establishment be applied clinically using an application method used previously?** Our TE does not have prior experience with the clinical use of ADM for tendon reinforcement or abdominal wall reconstruction surgeries. | | X | |
| **G. Has your establishment provided this type of tissue/therapy for implantation or transplantation into the intended anatomical site and/or same clinical indication before?** ADM has been previously distributed for soft tissue regeneration in maxillofacial reconstruction. ftADM is intended for use in different clinical applications: tendon reinforcement surgeries (rotator cuff augmentation and superior capsular reconstruction), and abdominal wall reconstruction. | | X | |

## Discussion

EuroGTPII methodologies have identified the processes that represent new or unknown risks associated with clinical application of the newly developed ftADM grafts for tendon reinforcement and abdominal wall reconstruction. The risk factors were associated with each significant change in our original ADM protocol and allowed us to estimate the risk consequences and the overall level of risk. After identifying and quantifying the risks, different mitigation strategies were proposed to reduce each individual risk.

Four significant changes were identified through the use of EuroGTP methodologies, which led to the identification of four risk consequences: implant failure, unwanted immunogenicity, disease transmission, and toxicity/carcinogenicity. The outcome of the risk assessment exercise had a Final Risk Score of 5, corresponding to a low level of risk, and determined the extent of studies required to ensure the safety and efficacy of the ftADM grafts in terms of pre-clinical and clinical evaluation. According to EuroGTPII methodologies, after identifying and quantifying the risks, different mitigation strategies are proposed to reduce each individual risk.

The risk of implant failure was perceived as a risk consequence due to two risk factors: procurement and reagents. During procurement, the retrieval of full thickness skin fragments will be performed using scalpels instead of the dermatomes commonly used in the retrieval of split thickness skin. The newly adopted technique may affect the integrity of the tissue due to the use of a different device. To mitigate this risk, all the staff involved in the procurement of full-thickness skin will be trained and qualified to perform the new retrieval procedure to avoid blade lesions on skin. With regard to the reagents, it has been reported that ECM could be damaged, which may affect the functionality of the allograft. To reduce this risk, the integrity of the ECM will be validated by performing a set of in vitro tests (biochemical

**Table 3** Assessment of Risk Associated with the Implementation and Clinical Use of ftADM

| Risk Factors | | Does it Apply? | Justification | Risk Consequences | P | S | D | PR | RR (%) | Risk Score |
|---|---|---|---|---|---|---|---|---|---|---|
| Donation | Donor characteristics | No | The selection criteria for ftADM donors are the same as for standard skin and ADM donors. These criteria follow the applicable regulation[25–27] and good practices,[1] and do not represent any additional risk associated with donation of the novel tissue. | - | - | - | - | - | - | - |
| Procurement | Procurement process and environment | Yes | Retrieval is performed using a scalpel instead of a dermatome, commonly used in the retrieval of split skin fragments. The newly adopted technique may affect the integrity of the tissue due to the use of a different device. | Implant failure | 3 | 2 | 1 | 6 | 0 | 6 |
| Preparation process | Processing and environment | Yes | The main risk of the decellularization protocol is the probability of not accomplishing complete removal of the cellular content, which may lead to unwanted immunogenicity in the recipient.[28] | Unwanted immunogenicity | 1 | 1 | 5 | 5 | 50 | 2,5 |
| | | | The new preparation process includes several additional manipulation steps and takes longer than previous decellularization procedure. This may increase the risk of contamination of tissues and consequent microbiological infection in the recipient. | Disease transmission | 2 | 2 | 1 | 1 | 0 | 4 |
| | Reagents | Yes | The decellularization protocol requires several reagents (solvents, hypertonic solutions and enzymes) that have been previously used in our TE for other protocols. Despite this prior experience, the new/adapted decellularization procedure includes the use of several reagents, which poses different risks. | | 2 | 2 | 2 | 8 | 0 | 8 |
| | | | ECM could be damaged and this could affect the functionality of the allograft. | Implant failure | | | | | | |
| | | | The use and handling of additional reagents in the preparation process may increase the probability of introducing microbiological contamination in the graft. | Disease transmission | 2 | 2 | 1 | 2 | 0 | 2 |
| | | | If the decellularization reagents are not correctly removed during processing, the remaining residues could induce a toxic/carcinogenic reaction in the recipient. | Toxicity /Carcinogenicity | 2 | 1 | 5 | 10 | 0 | 10 |

(*Continued*)



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**Table 3** (Continued).

| Risk Factors | | Does it Apply? | Justification | Risk Consequences | P | S | D | PR | RR (%) | Risk Score |
|---|---|---|---|---|---|---|---|---|---|---|
| | Reliability of microbiology testing | Yes | The reliability of microbiology tests could be altered due to the presence of reagent remnants in the allograft that mask graft contamination. | Disease transmission | 1 | 2 | 1 | 2 | 0 | 2 |
| | Storage conditions | Yes | The storage conditions, in terms of temperature and preservation media, are similar to those used for ADM. Despite being previously validated in our TE, the concentration of preservation media used to store ftADM has been reduced (compared with ADM) and its effect on microbiological growth during storage has not been tested. | Disease transmission | 1 | 2 | 5 | 10 | 0 | 10 |
| | Transport conditions | No | The transport process for the ftADM has been previously validated for other tissues in our TE. This activity follows the applicable regulation[25–27] and good practices,[1] and does not represent any additional risk associated with preparation of the novel tissue. | - | - | - | - | - | - | - |
| Clinical application | Complexity of the pre-implantation preparation and/or application method | Yes | The additional pre-implantation preparation requires some serial washes with NaCl 0.9% to remove the preservation media before implantation. Potential residual concentrations of preservation media could produce an adverse reaction in the recipient.[29,30] | Toxicity / Carcinogenicity | 1 | 1 | 5 | 5 | 50 | 2,5 |
| Preliminary Score (Σ individual risk scores) | | | | | | | | | | 47 |
| Combined Risk Value ((Preliminary Score x Highest Possible Score)/((Max S × Max P × Max D) × Number of applicable risk consequences) = (47 x 4500)/(100 x 9) | | | | | | | | | | 235 |
| **Final Risk Score** ((Combined Risk Value ×100)/ Highest Possible Score) | | | | | | | | | | **5** |

**Note**: Adapted from the EuroGTP-II guidelines (EuroGTP-II, 2019).
**Abbreviations**: D, detectability; P, probability; PR, potential risk; RR, risk reduction; S, severity.

**Table 4** Risk Mitigation Strategies Associated with the Implementation and Clinical Use of ftADM

| Risk Factors | | Risk Consequence | Justification | Risk Mitigation Strategies |
|---|---|---|---|---|
| Procurement | Procurement process and environment | Implant failure | Retrieval is performed using a scalpel instead of a dermatome, commonly used in the retrieval of standard skin and ADM. The newly adopted technique may affect the integrity of the tissue due to the use of a different device. | Staff training and team qualification |
| Preparation process | Processing and environment | Unwanted immunogenicity | The main risk of the decellularization process is the probability of not accomplishing complete removal of the cellular content, which may lead to unwanted immunogenicity in the recipient.[28] | Validation of the efficacy of the decellularization process |
| | | Disease transmission | The new preparation process includes several additional manipulation steps and takes longer than previous decellularization procedure. This may increase the risk of contamination of tissues and consequent microbiological infection in the recipient. | Set of microbiological quality controls in each step of the procedure. |
| | Reagents | Implant failure | ECM could be damaged, which could affect the functionality of the allograft | Masson's Trichrome Staining<br>Quantification of ECM contents (collagen, elastin, GAGs)<br>Mechanical tensile testing |
| | | Disease transmission | The use and handling of additional reagents in the preparation process may increase the probability of introducing microbiological contamination in the graft. | Set of microbiological quality controls in each step of the procedure. |
| | | Toxicity / Carcinogenicity | If the decellularization reagents are not completely removed during processing, the remaining residues could induce a toxic/ carcinogenic reaction in the recipient. | Cytotoxicity assay |
| | Reliability of microbiology testing | Disease transmission | The reliability of microbiology tests could be altered due to the presence of reagent remnants in the allograft that mask graft contamination. | Validation of the reliability of I analytical microbiology methods |
| | Storage conditions | Disease transmission | The storage conditions, in terms of temperature and preservation media, are similar to those for ADM. Despite being previously validated in our TE, the concentration of preservation media used to store ftADM has been reduced (compared with ADM) and its effect on microbiological growth during storage has not been tested. | Validation of the stability of the ftADM grafts during storage (shelf life) |
| Clinical application | Complexity of the pre-implantation preparation and/or application method | Toxicity / Carcinogenicity | The additional pre-implantation preparation requires serial washes with NaCl 0.9% to remove the preservation media before implantation. Potential residual concentrations of preservation media could produce an adverse reaction in the recipient.[29,30] | Explicit and detailed handling instructions for end users. |

quantifications, Masson's Trichrome histological staining) to evaluate the preservation of major ECM biomolecules (collagen, elastin and GAGs) in the ftADM. Moreover, a uniaxial biomechanical test will be performed to assess the suitability of mechanical properties in the final graft. The results of this set of tests will demonstrate the integrity of the ECM after the decellularization treatment.

The second risk consequence identified was potential unwanted immunogenic reactions in the recipient, caused by incomplete removal of the cellular content during the decellularization process. The efficacy of the decellularization procedure will be assessed through a set of in vitro tests (DNA quantification and Hematoxylin-Eosin histological staining) to determine the presence of cellular remnants in ftADM after decellularization. The results of this mitigation strategy must confirm the absence of cell nuclei in the histology testing, and that the DNA content is below 50 ng/mg in the dry tissue.[29]

The third risk consequence identified was potential disease transmission, related to the new steps in the preparation process, use of reagents, reliability of microbiology testing and the storage conditions. Due to the characteristics of full thickness skin, the ADM preparation process was modified, increasing the length of the procedure by one day, and including several additional manipulation steps. The use of additional reagents during the process may increase the probability of introducing microbiological contamination in the graft. Furthermore, the thickness of the skin may be an obstacle for removal of reagents used during processing, thereby potentially reducing the reliability of microbiology testing due to the presence of remnants that could mask graft contamination in routine quality controls. Moreover, although the storage conditions (temperature and preservation media) are similar to those previously validated for ADM, the concentration of preservation media used to store ftADM will be reduced with respect to ADM and could increase the chance of microbiological growth during storage. These factors may increase the risk of tissue contamination and consequent microbiological infection in the recipient. To avoid these risks, the decontamination efficacy of the entire process will be validated through (i) validation of the efficacy of the antibiotic/antimitotic decontamination cocktail, (ii) validation of the analytical method used as a microbiology test, (iii) implementation of new microbiology controls at different stages of the process, and (iv) implementation of a final filtration step of the reagents involved in the process. In addition, all reagents will be prepared in a closed system and a microbiology test will be performed on each one by the end of the aliquot process. The absence of microbiological growth during storage will be confirmed through accelerated and ongoing in vitro stability assays. To accept the mitigation of disease transmission risk, all validation and implementation procedures must demonstrate that the tests are able to detect each microorganism previously inoculated. Moreover, the results of microbiology tests performed in each reagent used during the preparation, and the results of the stability tests, must be negative for both accelerated and ongoing tests.

The fourth risk consequence is toxicity/carcinogenicity of the final product. This risk consequence is related to the reagents used and the complexity of the handling procedures before clinical application. If the decellularization reagents are not completely removed during processing, the remaining residues could induce a toxic/carcinogenic reaction in the recipient. To mitigate this risk, a series of rinse steps were included in the procedure to eliminate the remaining residues. The cytotoxicity study will be performed following the cell culture model defined in ISO directive 10,993–5. To accept the results of this mitigation strategy, the cell viability must be ≥70%. Furthermore, glycerol preservation requires serial washes with NaCl 0.9% to remove the preservation media before implantation. Potential residual concentrations of preservation media could produce an adverse reaction in the recipient.[30–32] Although our TE cannot directly mitigate this risk, explicit handling instructions will be sent to the clinicians/end users and the document will be added to the ftADM packaging.

The risk reduction process is supported by our prior experience in the preparation and manipulation of ADM, as well as a significant amount of relevant literature.[14,16–18] Nevertheless, despite the low level of risk obtained for ftADM, the use of new procedures and reagents and the graft characteristics determined the need for intensive validation of our internal protocols in order to ensure the correct specifications and the safety of the allograft before its clinical use. The numerous pre-clinical investigations performed aimed either to reduce the probability of risk consequences occurring or to increase the detectability in case of process deviations, namely tissue contamination during the preparation process.

The risk assessment exercise and the results subsequently obtained will support the submission of the preparation process dossier to our competent authority, demonstrating the safety of our newly developed graft. Further CFUpP will address potential risk consequences that were not fully mitigated in the pre-clinical assessments and will focus mainly on

the mandatory reporting of serious adverse reactions and the long-term efficacy of the grafts, as suggested by the EuroGTPII methodologies for grafts/therapies with a low level of risk.

## Conclusion

EuroGTPII methodologies allowed us to identify and quantify the risks associated with the introduction of innovation in our activities. In addition, the use of this standard methodology generates a complete report on the rationale followed during the development and validation of a novel therapy and documents the studies required to address and mitigate the risks, thereby promoting transparency and expediting authorization procedures by competent authorities.

Despite the low level of risk determined for the novel ftADM preparation process, a set of pre-clinical assessments were needed to address and mitigate the potential risk consequences and to guarantee a high level of safety for the clinical application of ftADM. The development of novel grafts and preparation processes through the quality-by-design methodologies proposed by the EuroGTPII tools will lead to optimized and continuous improvement of the products and therapies developed by our TE.

## Data Sharing Statement

No new data were generated or analyzed in support of this research.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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