VISICORT

Adverse Immune Signatures and their Prevention in Corneal Transplantation

PROJECT FINAL REPORT

Grant Agreement number: 602470

Project acronym: VISICORT

Project title: Adverse Immune Signatures and their Prevention in Corneal Transplantation

Funding Scheme: FP7-HEALTH-2013-INNOVATION-1

Project start date:

Project end date:

Name, title and organisation of the scientific representative of the project's coordinator: Prof. Matthew Griffin, National University of Ireland, Galway

Tel: +353-91-495436

Fax: +353-91-495547

E-mail: matthew.griffin@nuigalway.ie

Project website address: www.visicort.eu

1. Final publishable summary report

1.1 Executive summary

Immune system response is the most complex barrier to long-term success of tissue transplants/implants from allogeneic and bio-artificial sources. While newly developed tissue transplant procedures are not yet performed frequently enough for robust analysis of adverse immune responses in humans, corneal transplantation (CT) is a well-established allogeneic tissue transplant with >100,000 full- and partial-thickness procedures performed annually. Adverse immune responses occur in up to 30% of CT recipients causing rejection and failure. The high levels of CT clinical activity and immune complications create an ideal opportunity to comprehensively profile immune responses associated with adverse tissue transplant outcomes and to develop new approaches for their prevention or early diagnosis.

VISICORT is a multi-disciplinary project with expertise in basic immunology, bio-sampling, systems biology/immune profiling, bioinformatics, clinical tissue transplantation and cell therapy. It brought together twelve partners, with a range of relevant experience. It has provided a heretofore unprecedented level of understanding of the mechanisms involved corneal graft rejection, has completed pre-clinical validation of a novel stromal cell therapy in the treatment of graft rejection and has laid the groundwork for the delivery of a clinical trial of that therapy.

1.2 A summary description of project context and objectives summary

The VISICORT project was designed to create a new level of understanding of adverse immune responses to tissue transplants and implants. It carried out an extensive study of a specific form of tissue transplantation (corneal transplants) in order to better understand and predict adverse immune responses and to improve clinical outcomes. VISICORT has completed the first systematic immune profiling of biological samples from animal and human CT recipients with diverse outcomes. During the project, clinical data and bio-specimens from over 700 CT recipients at five leading transplant centres was centrally collated. Over 50,000 individual tissue samples were collected – including plasma, tears, corneal tissue, aqueous humour, PBMCs, RNA, and DNA. These samples have been catalogued in a custom-designed sample management system (the VISICORT Information System, VIMS), and form the VISICORT Foundation Biobank, which will continue to function as an important scientific resource beyond the end of the VISICORT project. The samples collected have been subjected to multi-platform profiling and bioinformatics analyses at university and SME-based laboratories. A wealth of profiling data and analysis has been produced, which will support better understanding of adverse immune reactions to tissue transplants.

The VISICORT project had planned to use this unique collection of profiling data to develop novel diagnostic and prognostic tests for adverse immune responses in transplant/implant recipients. The project carried out extensive research into the possibility of biomarker assay-based immune surveillance, which would enable early diagnosis and prediction of immunological complications, ultimately underpinning novel approaches to patient-specific tailoring of immunosuppressive therapy. However, from the extensive range of analysis carried out on the samples, candidate biomarkers or biomarker signatures within accessible biological samples including plasma, tears and blood cells with statistically robust associations with transplant rejection did not emerge.

VISICORT was also intended to deliver a clinical trial of immunomodulatory stromal stem cell therapy in high-risk human CT recipients. Extensive research in animal models demonstrated that our preferred therapeutic stromal cell product – prospectively isolated $CD362^{+}$ allogeneic bone marrow mesenchymal stromal cells (allo-hBM-MSCs) was unlikely to achieve clinical efficacy. Instead, highly supportive pre-clinical results were achieved with plastic-adherent allo-hBM-MSCs in high risk corneal transplant model. On the basis of these results, we developed a novel cell manufacturing process and

clinical trial concept and obtained all necessary ethical and regulatory approvals to carry out the planned clinical trial in corneal transplant recipients at high risk of rejection. Delays that resulted from the Covid-19 pandemic and the consequent lockdowns across Europe, meant that, ultimately, we were not able to initiate the clinical trial within the time-frame of the project.

1.3 A description of the main S&T results/foregrounds

1.3.1 WP1 – Adverse immune responses and immunomodulatory stromal cell therapy in PCT

Overall success rates for corneal transplantation (CT) are high but there is a 5-40% risk that a corneal transplant will trigger an acute rejection response by the patient's immune system. Risk of rejection for CT is increased by certain characteristics of the transplant recipient such as presence of inflammation, new blood vessel growth and re-transplantation following a previously failed transplant**.** The risk factors for rejection are not currently well understood, and the treatment options, once rejection takes place, are limited. It was a priority for the VISICORT project to arrive at a clearer understanding of the reasons for the rejection of corneal transplants and the ways in which the risk of rejection might be reduced by an immunomodulatory stromal cell (iSSC) treatment. In order to achieve this understanding and to provide evidence of the effectiveness of the proposed iSSC treatment, WP1 carried out a series of experiments using rat models of corneal transplantation.

In order to define the immune signatures associated with the rejection of CT grafts, experiments were carried out in rats. Blood and tissue samples were collected from different cohorts of rats, including those who had not received a transplant, those who had received a transplant from the same rat species, and those who had received a transplant from a different species. A further group received a transplant and were treated using the corticosteroids most commonly used to handle episodes of rejection post-transplant. Blood samples were obtained from these rats at days 1, 7, 14, and 30 post treatment, and tissue samples, including spleen, lymph nodes, aqueous humour, and corneal tissue were obtained at day 30.

The resulting samples were shipped on dry ice to SYNT, University of Edinburgh, UK. Protocols were developed by SYNT to isolate protein from each sample type for analysis by label-free quantitative mass spectrometry. The resulting data were submitted to FIOS for further bioinformatics analysis. FIOS analysed differences in protein expression across group comparisons and prepared detailed reports.

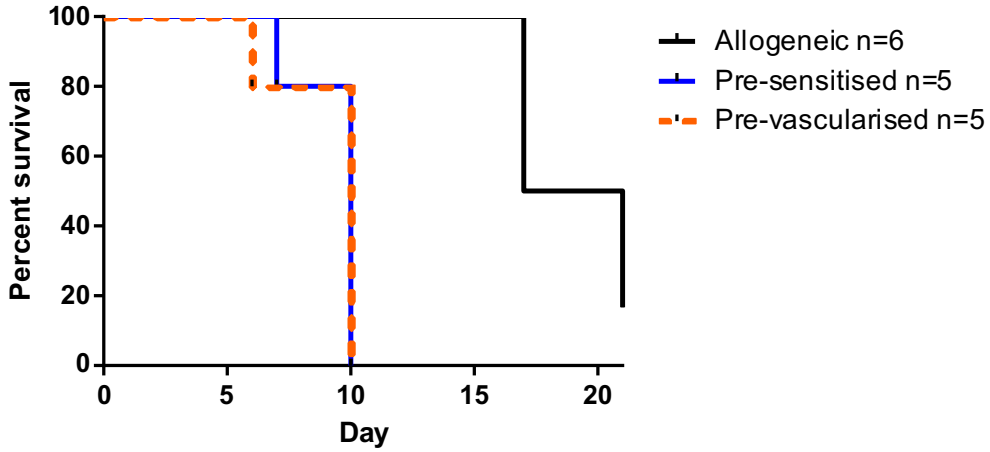
These transcriptomic and proteomic profiling reports were analysed in detail by NUIG after preparation by SYNT and FIOS. One significant result was that the immune response in cornea transplant rejection in the rat model seemed to be more localised than expected, i.e. in the cornea and aqueous humour as opposed to the blood, spleen and lymph node. In the local area, several interesting pathways were observed, including those related to oxidative phosphorylation, complement processes and antigen presentation. A signature of the downstream effects of Interferon-gamma was also noted in the results from cornea and aqueous humour. This included differential regulation of STAT proteins. It was decided to probe this further and a set of experiments was designed to validate that STAT proteins are activated in rejecting corneas and to determine which cells are activating them. Therefore, rejecting and non-rejecting eyes were examined by immunofluorescence using specific antibodies against STAT1, pSTAT1 and immune cell markers CD3 and CD68. These experiments are on-going and will potentially lead to further in vivo studies which could form the basis of a future publication.

A similar range of experiments was carried out on rats who were at high risk of graft rejection. These rats were pre-sensitised to increase their risk of rejecting the transplant. Corneal transplantation was carried out on 12-16-week-old Lewis rats using fully allogeneic Dark Agouti (DA) rat corneas as the donor tissue. In the case of pre-sensitised high-risk corneal transplantation, 1 X 10^7 DA splenocytes

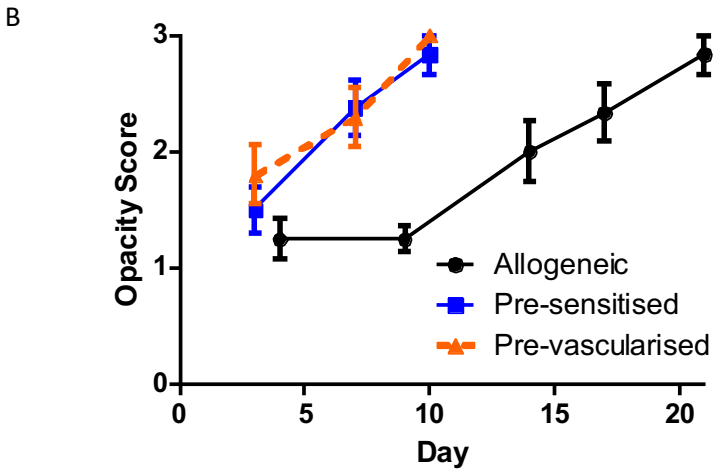
were injected subcutaneously on the back of the recipient 14 days prior to surgery. In the case of pre-vascularised high-risk CT, 2 intra-stromal sutures were placed into the recipient cornea 14 days prior to surgery.

The animals in each high risk group (pre-sensitised and pre-vascularised) rejected their grafts significantly earlier than allogeneic "normal" risk animals (Figure 1A). The allogeneic group rejected on average at day 19, the pre-sensitised group rejected their grafts on average at day 9.4 and the pre-vascularised group rejected on average at day 9.2. This rejection profile was reflected in the opacity curves (Figure 1B), where markedly higher opacity was observed in both the pre-sensitised and pre-vascularised groups at earlier timepoints post-transplant. As the aim of the suture placement in the pre-vascularised group was to induce vascularisation in the recipient it is expected that higher levels of neo-vascularisation would be recorded in this group.

A



B



C

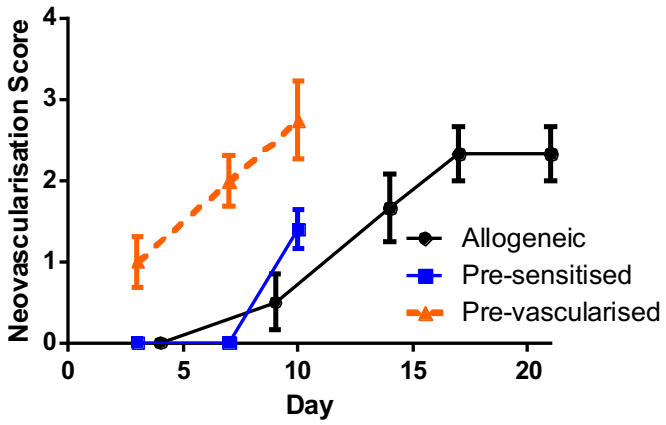


Figure 1: Rejection of high risk corneal transplants

(A) Corneal allograft survival was assessed every 3-4 days under an ophthalmic microscope. (B) Rejection was based on graft opacity, with grafts classified as rejected if they scored two consecutive scores of 2.5 or one score of 3. (C) The infiltration of newly formed blood vessels was also assessed at every observation.

Table 1: *Transplant groups for task 1.2*

Group number	Group Name	Description
1	Allogeneic	No pre-surgical intervention. Unilateral corneal transplant from Dark Agouti to Lewis rat.
2	Pre-sensitised	1 X 10^7 DA splenocytes injected subcutaneously 14 days before unilateral corneal transplant from Dark Agouti to Lewis rat.
3	Pre-vascularised	2 intra-stromal sutures placed in recipient cornea 14 days before unilateral corneal transplant from Dark Agouti to Lewis rat.

Blood sampling was carried out on these animals at days 1, 3, 7, 14 and 21 for isolation of plasma and PBMCs. At the end-point, spleen, draining and non-draining lymph nodes, cornea, AH, PBMCs and plasma were all isolated.

RNA and protein samples from these 3 groups were prepared at NUIG and shipped to SYNT and FIOS. Transcriptomic analysis was performed using Affymetrix rat whole genome microarrays with data analysis reports generated by FIOS. Proteomic analysis was performed by label-free mass spectrometry by SYNT with data analysis reports generated by FIOS.

The proteomic analysis on the spleen samples revealed a significant “Batch Effect” unrelated to the experimental group and unrelated to the Mass Spectrometry run was observed. This resulted in two different groups in the spleen samples termed “left” and “right”.

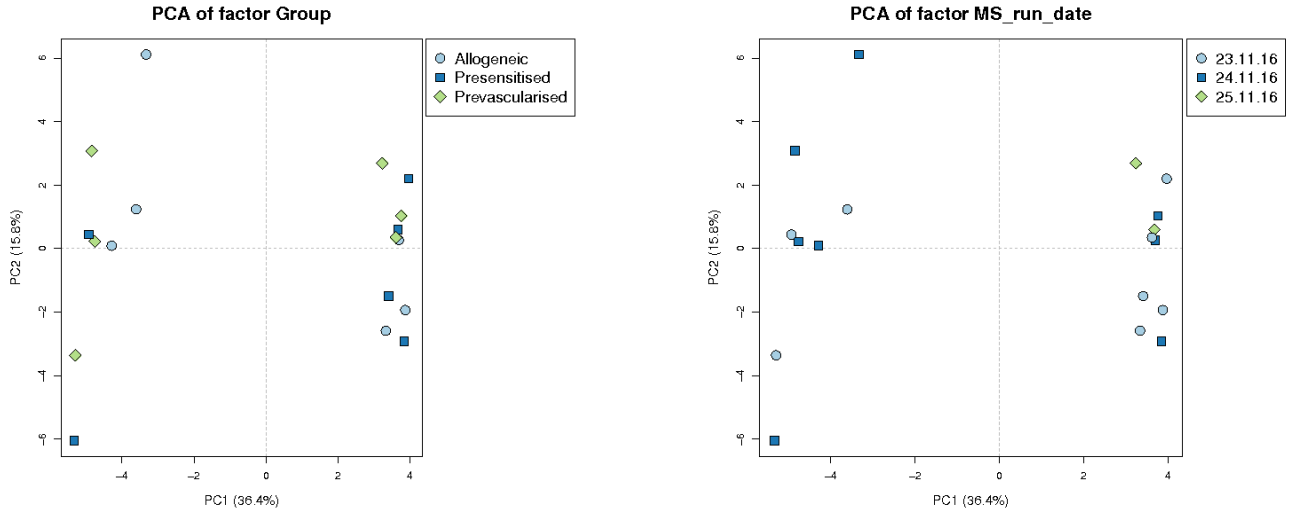


Figure 2: Batch effect observed in spleen samples from Task 1.2
Principle component analyses depicting different groups (left) and different MS run dates (right) showing the unexplained batch effect detected in the analysis.

A left versus right analysis was included in the analysis by FIOS and 43 statistically significantly differentially regulated proteins were detected between left and right. There were fewer differentially regulated proteins detected in the other comparisons as outlined in Table 1.

Table 2: *Significantly differentially regulated proteins detected between different comparisons in spleen in Task 1.2*

Comparison	Significantly differentially regulated proteins
Pre-sensitised v Allogeneic	21
Pre-vascularised v Allogeneic	28
Pre-sensitised v Pre-vascularised	19
Left v Right	43

Spleen transcriptomics analysis revealed several differentially expressed genes. The results focused on the KEGG pathway analysis with some interesting observations. In the pre-vascularised versus allogeneic transplant models the chemokine signalling pathway, graft versus host disease and allograft rejection was downregulated. In the pre-sensitised versus pre-vascularised transplant model Pi3K-Akt and T-cell receptor signalling pathway were upregulated.

Lymph node proteomic analysis was carried out for draining and non-draining cervical lymph nodes in each animal. 1547 total proteins were detected and included in the analysis. Differences were noted between the draining and non-draining lymph nodes in each group.

148 unique proteins were detected and included in analysis from aqueous humour in samples from these rats. The proteomics analysis from aqueous humour samples revealed that samples did not cluster together as well as the rats that had not been pre-sensitised (Figure 3).

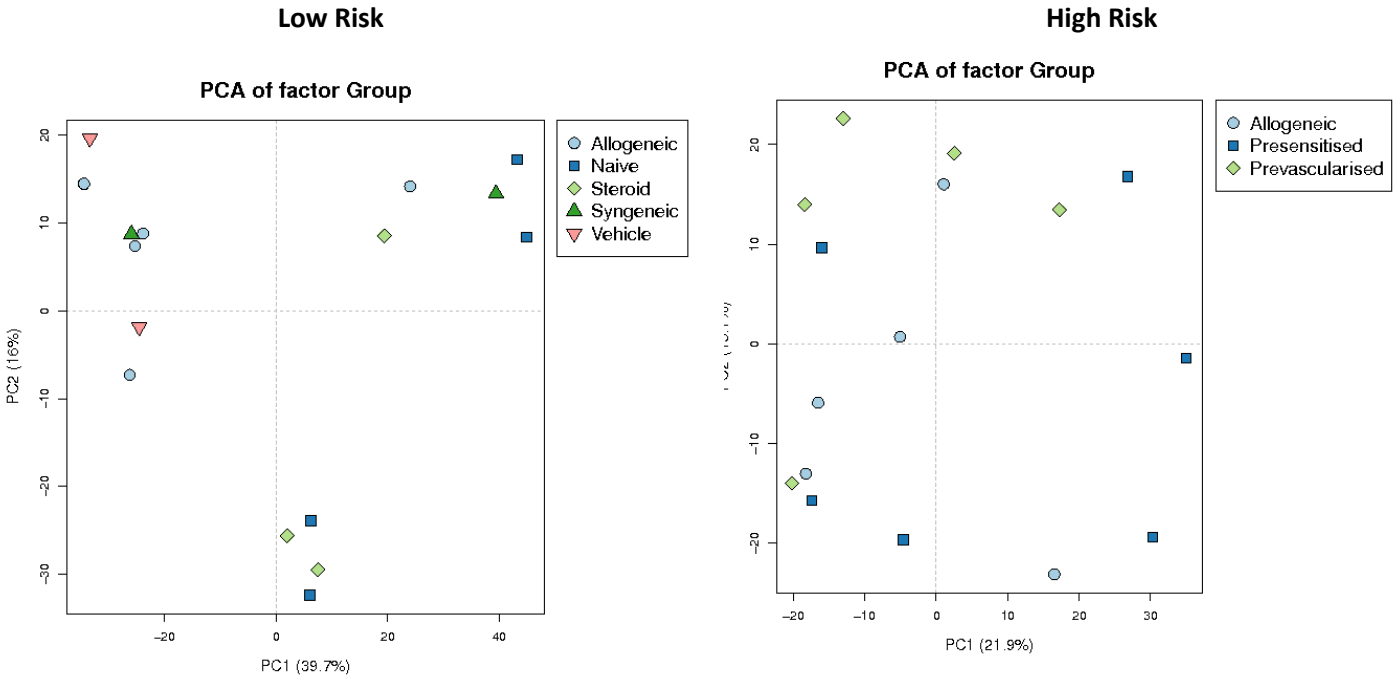


Figure 3: Aqueous Humour Proteomics

Principle component plots from aqueous humour analyses from Task 1.1 (left) and Task 1.2 (right) showing that samples clustered more distinctly based on group in Task 1.1 compared to Task 1.2

Table 3: *Aqueous humour proteomics*

Comparison	Significant genes
Pre-sensitised v Allogeneic	11
Pre-vascularised v Allogeneic	7
Pre-sensitised v Pre-vascularised	27

Table depicts the statistically significantly differentially regulated proteins in aqueous humour between groups in Task 1.2

The proteomic analysis of the cornea samples from the high risk groups identified 1499 proteins which were included in the analysis. Some clustering was observed in principle component plots between pre-sensitised and pre-vascularised groups (Figure 4) and these differences were exemplified by there

being a higher number of differentially regulated proteins between these two groups (Table 3). The allogeneic and pre-sensitised groups appear more similar to each other in this analysis.

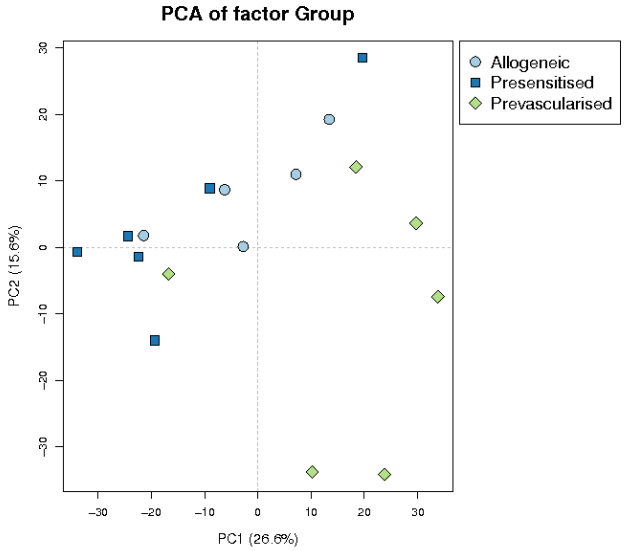


Figure 4: Cornea proteomics

Principle component plots from cornea proteomic analysis from Task 1.2 showing that pre-sensitised and pre-vascularised samples clustered distinctly from each other

Table 4: *Cornea proteomics*

Comparison	Significant genes
Pre-sensitised v Pre-vascularised	45
Allogeneic v Pre-sensitised	4
Allogeneic v Pre-vascularised	17

Table depicts the statistically significantly differentially regulated proteins in cornea between groups in Task 1.2

WP1 was also tasked with developing the protocol for the clinical trial by determining the following variables: the optimum iSSC cell source (donor-derived, recipient-derived or third party), the optimum iSSC subtype (plastic adherent or ORB1*), and the optimal delivery route. The overall aim was to prevent adverse immune responses in high-risk allogeneic CT and eventually translate the rodent therapy into a clinical trial-ready human treatment. Through discussion with the VISICORT clinicians it was decided that the pre-sensitised high risk cornea transplant model would be used to test the utility of iSSC. A protocol was developed which significantly prolongs corneal allograft survival in this model

Corneal grafts were carried out on two groups of pre-sensitised rats. These were treated with plastic adherent donor-derived (Dark Agouti) and third party-derived (Wistar Furth) iSSCs and with $CD362^{+}$ iSSC ($Orb1^{+}$ iSSC), respectively. The grafts were carefully monitored for signs of transplant rejection. These experiments failed to demonstrate any prolonging of corneal allograft survival in the rat model resulting from the administration of $CD362^{+}$ iSSC ($Orb1^{+}$ iSSC). The plastic adherent cells did demonstrate a benefit in prolonging the graft life, and accordingly the decision was taken to progress

the clinical trial using these cells, rather than the ORB1^{+} cells originally planned. WP1 also produced the data required for a regulatory submission for the planned VISICORT clinical trial.

The work carried out in WP1 was fundamentally important for the VISICORT project as a whole, and especially for the planning and preparation for the clinical trial. Beyond this, the pre-clinical work carried out by the work package has provided important insights into the mechanisms of transplant reject ion and the role of immunosuppressive therapies in preventing that rejection.

1.3.2 WP2 - Immune outcomes in human corneal transplantation

Corneal transplantation has been performed for more than 100 years. Until 15 years ago the state-of-the-art type of transplantation was penetrating keratoplasty in which a full-thickness (FT-CT) transplant was performed by cutting out the central 7-8 mm of the recipient cornea and suturing a similar sized donor graft in place. Since the start of this millennium, newly-designed surgical techniques have developed considerably. Today, the vast majority of keratoplasty procedures are performed as delicate lamellar procedures either assisted with fine microkeratomes or femtosecond lasers or using skilled surgical dissection procedures. The most common keratoplasty procedure is today "posterior lamellar keratoplasty" (PL-CT) where diseased cells on the posterior side of the cornea are removed and replaced by healthy cells from a deceased donor.

These advancements have helped patients undergoing keratoplasty to have a much faster visual recovery and a more stable eye with possibly less risk of immunological rejection episodes. Rejection periods and slowly developing failure of the corneal donor endothelium remains a major cause of corneal transplant failure.

The overall goal of WP2 was to develop a cloud-based system to collect relevant, well-defined clinical information and track biological samples, to recruit patients from the five clinical centres, to collect biological samples and clinical data and to follow these patients clinically over several years.

Early in the project, a secure multi-site clinical database of human corneal transplant recipients was established as a combined clinical database with a biological sample management system in a collective VISICORT Information Management System (VIMS). The system allowed all partners global access to the system, provided they have an Internet connection. The VISICORT Project Coordinator was therefore able to manage and coordinate the project more effectively and all key project partners could instantly see the status of: sample procurement in all centres, samples in central storage and samples distributed to test sites in real-time and take corrective action when required if targets were behind schedule.

Standard Operating Procedures (SOPs) describing clear definitions of variables and clinical definitions and diagnoses were also established in order to ensure consistency in reporting data across all clinical centres. In addition to the main variables describing group allocation, variables related to patient age, sex, original diagnosis, time since keratoplasty, whether the other eye had been transplanted, general health, specific eye and corneal related descriptors (thickness, endothelial cell count), and immunosuppressive treatment were defined. Changes in the status of general health, eye and corneal variables over time could also be recorded. The actual selection of variables, including definition of variables were agreed upon after consultation with the other clinical sites (UBR, CUB, NANT, RCSI). Before selection of variables, registration practices in four major Corneal Transplant Registries (Irish Corneal Transplant Registry, Swedish Corneal Transplant Registry, The Australian Corneal Graft Registry, UK Ocular Tissue Transplant Registry) were thoroughly evaluated.

The first part of the clinical studies also involved a multi-centre, cross-sectional clinical analysis and bio-sampling study of 420 prevalent human FT- and PL-CT recipients and control subjects with varying current immunological status. The main aim of the study was to collect bio-samples from a variety of patients who already had been transplanted for corneal disease. Non-transplanted patients participated and normal subjects without any corneal disease were enrolled as well. The final type and number of patients recruited to this cross-sectional study are shown in Table 5.

Table 5

Group March 2020	AUH	CUB	UBR	RCSI	NANT	Total	AIM
1. FT, Acute rejection ≤ 3 yr post-transplant	10	3	8	17	7	45	50
2. FT, Stable ≤ 3 yr post-transplant	17	16	4	17	1	55	50
3. FT, Long-term (> 3 yr) rejection-free survival	11	22	3	10	6	52	50
4. FT, Chronic graft injury (> 3 yr post-transplant)	4	15	3	1	15	38	50
5. PL, Acute rejection ≤ 3 yr post-transplant	15	2	3	3	1	24	30
6. PL, Stable ≤ 3 yr post-transplant	16	18	3	7	0	44	30
7. PL, Long-term (> 3 yr) rejection-free survival	17	7	3	4	0	31	30
8. PL, Chronic graft injury (> 3 yr post-transplant)	3	2	2	2	1	10	30
9. Non-transplanted corneal disease	16	10	6	10	2	44	50
10. Healthy adult volunteer	14	11	19	19	7	70	50
Total	**123**	**106**	**51**	**89**	**40**	**404**	**420**

In most groups it was possible to recruit the planned number of patients. It was, however, difficult to enrol patients for Group 8 (posterior lamellar grafts with chronic graft failure). This may be explained by a quite good survival of posterior lamellar grafts and that posterior lamellar grafting has only been performed on a large scale for less than 10 years. Group 1 and Group 5 consisted of patients with an acute graft rejection episode. A total of 69 patients with an acute rejection episode were enrolled, bio-sampled at the time of rejection and most of these patients attended a follow-up visit 3 months after rejection at which time bio-sampling was repeated. The analyses result of the bio-samples from this cross-sectional study are presented in WP5 & WP6. Recruitment went on until October 2018 as it then was considered that the number of patients recruited would provide a sufficient number of bio-samples for analyses in WP5 and WP6.

Patient recruitment for VISICORT's multi-centre, prospective study of immunological outcomes started in February 2015. The total number of recruited patients is shown in Table 6.

Table 6

Group March 2020	AUH	CUB	UBR	RCSI	NANT	Total	AIM
1. FT, low-risk	7	9	3	29	15	63	75
2. FT, high-risk	17	27	8	19	9	80	75
1. PL, low-risk	27	79	10	13	0	129	100
2. PL, high-risk	16	23	6	11	4	60	50
Total	**67**	**138**	**27**	**72**	**28**	**332**	**300**

All centres involved a study nurse, study physician or study optometrist to help in patient recruitment, data collection, bio-sampling, pre-processing of samples and organising transport to the storage facility, Biostór in Ireland.

The number of patients finally recruited to group 1 (FT Low Risk group) was slightly less than planned (63 instead of 75 and only 60 were operated), but this number is considered acceptable. Difficulties recruiting patients to this group were caused by a general decrease in the number of keratoconus patients needing a CT and by the fact that there has been a trend towards performing anterior lamellar CT for this disease. Recruitment exceeded the target in Groups 2, 3 and 4. This arose because recruitment was simultaneous across 5 sites and we accepted that exceeding the target would facilitate later analysis in the event of some patients withdrawing from the study for any reason. We have been very satisfied with recruitment to the two high risk groups, given their importance to the overall study objectives and the planned VISICORT clinical trial. Recruitment to the study was stopped in January 2017 in order to have a full 2-year follow-up for all patients in January 2019. It was considered sufficient to focus on a full 2-year follow-up as most immunological events will take place within this post-operative period. When the project was allowed to continue for an additional 18 months (March 2019), the clinical sites were, however, encouraged to also complete a 3-year follow-

up for all patients. The number of patients with complete follow-up decreased through the study as some grafts had failed and some patients were not able to attend the planned follow-up visits. The overall follow-up was 86% at 6- and 12-month, 75% at 2-years, but only 37% at 3-years. Therefore, clinical data up to 2-years after surgery were used for D2.3.

The main focus of the VISICORT prospective study was on immunological related graft rejection episodes. The main findings of WP2.2 are summarized in the following.

Graft rejection episodes

A graft rejection episode during the first two years was noted in 37 of the 325 eyes undergoing CT (11%). It was surprising to note that rejection episodes occurred at similar rates in PL and FT grafts, as well as in high- and low-risk groups. Higher and longer use of immunosuppressive treatment in the high-risk group and lower and shorter use in the low-risk and PL group was a possible explanation.

In the PL groups, patients who had undergone DMEK CT (very thin corneal graft), experienced rejection episodes at a similar rate compared with DSAEK (thin corneal graft) transplanted patients.

It was analysed whether a number of factors was associated with development of rejection episodes. Presence of systemic disease in the recipient, recipient gender, donor age and gender, donor endothelial cell density, graft diameter, re-bubbling in PL grafts and suture method in FT grafts had no significant association with graft rejection. Recipient age was, however, significantly lower in patients experiencing a rejection episode, and this was solely caused by a lower patient age in the FT CT groups. This finding suggests that more intensive and longer use of immunosuppressive therapy in younger patients undergoing FT CT is recommended.

Graft failure

Graft failure occurred in 28 of the 325 grafts (9%) during the first two years after surgery, but only 7 failures (2.2%) were attributed to immunological causes and 5 (1.5%) to slowly progressive endothelial failure, which may be caused by un-detected rejection episodes. Due to the low frequency of failures and wide array of possible predictors for immunological failure, detailed statistical analysis was not possible, but was a focus in D2.4 comprising more than 900 CT patients.

Immunosuppression

Immunosuppression therapy was also monitored in the study. Overall, therapy was fairly limited as topical therapy was predominant, and after one year, it was almost entirely FT High-Risk treated patients that were receiving immunosuppressive therapy. In PL grafts, rejection episodes were almost exclusively observed in patients who had stopped the use of immunosuppression. Similarly, it was interesting to note that graft rejection episodes occurred at a similar rate in high- as well as low-risk FT and PL groups. These findings support long-term use of topical immunosuppression even in PL Low-risk patients.

The report on the 2-year outcomes from multi-site prospective biological sampling and clinical follow-up of FT- and PL-CT recipients has led to considerable knowledge on possible confounders and bias related to immune-profiling of CT recipients in the various well-characterised CT recipient groups. The report otherwise confirms what is known in relation to preoperative diagnoses, surgical methods, and immediate postoperative adverse events. The final deliverable on adverse immunological outcomes from FT- and PL-CT (D2.4) includes more than 900 CT's followed for up to 3 years after surgery and allows a more statistically powerful complete assessment of the importance of risk factors and post-operative events.

Although WP2 and its associated deliverables focused on clinical outcomes, the concurrent collection of pre-operative, peri-operative and post-operative samples from the recipients (tears, aqueous humour, blood) was essential to the accomplishment of the wider VISICORT project. The samples

constitute a unique resource, and will continue to play an important role in other immunological profiling/biomarker identification projects.

A combined clinical database with the sample management system was developed early in the project. The cloud-based system, VISICORT Information Management System (VIMS) allowed partners to collect and register clinical data and to track bio-samples from the clinical sites to collaborators doing the actual analyses.

As described for Tasks 2.1 and 2.2 in D2.1, a number of standard operating procedures (SOPs) were agreed upon among the clinical sites to fully describe the clinical characteristics of grafted patients, including definitions of diagnoses and risk factors.

VISICORT also carried out a study to establish the clinical variables associated with favourable and adverse immunological outcome during the first 3 years after FT- and PL-CT. This coordinated broad survey of clinical variables, immunosuppression practice, clinical monitoring, complications and graft and patient outcomes among all incident CT recipients within the VISICORT network of centres has prospectively analysed clinical outcomes in 928 enrolled patients. This number was only slightly lower than the 1,000 planned.

Beyond confirming known factors associated with an adverse immunological outcome after FT- and PL-CT such as the influence of primary diagnosis and type of surgery as risk factors, the clinical factors study revealed factors which merit further investigation. Identification of a re-bubbling procedure (repeated injection of air to keep a PL graft in place) as an important risk factor for development of a later acute rejection episode is important as re-bubbling is necessary in a relatively large number of CT-PL procedures (15-25%). The finding that presence of loose sutures after CT-FT is a significant risk factor for acute graft rejection is not surprising, but further confirms that patients undergoing CT-FT must be followed closely.

Concerning graft failure, it is worth noting that the presence of type 2 diabetes was identified as a significant risk factor for graft failure after CT-FT. This may be due to a partially neurotrophic disease state in many patients with diabetes and this has to be taken into account when deciding to perform CT-FT and in the clinical follow-up of recipients with diabetes, which must be meticulous.

The outcome of work carried out in WP2 will impact other EU-funded projects, especially the European Cornea and Cell Transplantation Registry (ECCTR). The objectives of the ECCTR were to build a common assessment methodology and establish an EU web-based registry and network for academics, health professionals and authorities to assess and verify the safety quality and efficacy of (new) human tissue transplantations and cell-based therapies in ophthalmic surgery. The clinical definitions from the VISICORT project have, to a large extent, been implemented in the ECCTR. The clinical outcomes findings of the VISICORT project have the potential to generate hypotheses, which can be tested, over time, on the large number of patients entering the ECCTR. Similarly, the ECCTR will, without doubt, create findings which can be explored further using the biological samples collected in the VISCORT project.

1.3.3 WP3 Bio-sampling in human corneal transplantation

Introduction

Over the period, 2014-2021, the VISICORT project has acquired >60,000 bio-specimens (blood, tears, peripheral blood mononuclear cells (PBMC), donor tissue, recipient tissue, aqueous humour) with matched clinical data from 1,314 patients at five different European Research Centres: Aarhus University Hospital, Denmark (AUH), Bristol Eye Hospital, UK (UBR), Charité-Universitätsmedizin Berlin, Germany (CUB), Nantes University Hospital - France (NANT), Royal Victoria Eye and Ear, Dublin (RCSI).

The VISICORT Information Management System (VIMS).

At the outset of the project, the VISICORT Information Management System (VIMS) was designed, protocolised and implemented by BIOS, in consultation with the other partners. The system provides tracking and management of VISICORT's extensive and assorted collection of samples as well as supporting the collection and storage of associated clinical data (Figure 5). VIMS has been essential for almost every other aspect of the VISICORT project, and it has met and exceeded user requirements and grown with the project to support a wide range of use cases.

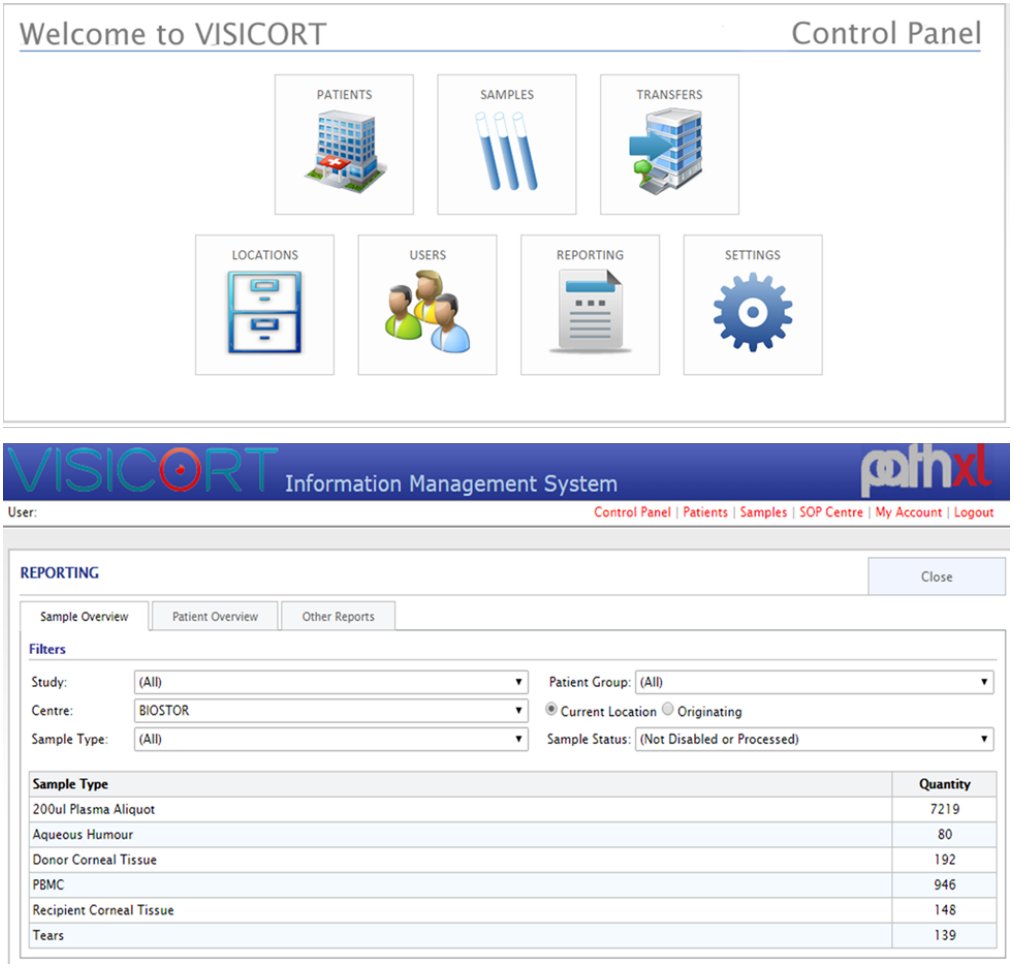


Figure 5: *Representative images of VISICORT Information Management System (VIMS) screen functions in support of bio-sample and clinical data management and tracking for all VISICORT studies.*

VIMS has successfully tracked all the required clinical observations for the: Cross-Sectional, Prospective, and Clinical Variables studies described in the report for WP2. No system anomalies with respect to the logging of clinical observations from the 1,341 enrolled patients or tracking the ~60,000

samples from these patients have been reported. As of March 2021, there are 51,994 samples stored in the VISICORT Foundation Biobank (VFB), as outlined below.

Table 7: *Samples in the VFB as of 31st March 2021*

Sample Type	**Number**
PBMC	4,646
PAXgene DNA	726
PAXgene RNA	3,087
Aqueous Humour	284
Donor Corneal Tissue	759
80µl RNA Cryovial	1299
Recipient Corneal Tissue	711
Tears	1,884
Plasma	38,598
Total	**51,994**

Figure 6: *Montage of representative images from the VISICORT Foundation Biobank established and located at Biostór Ireland Ltd., Rosslare, Co. Wexford, Ireland.*

To date, **2,435** PAXgene RNA samples & **1,708** PBMC samples have been transferred to NANT for RNA/Flow Cytometry analysis, **1,031** plasma and **198** tear samples transferred to SYNT for Proteomic analysis. **70** PAXgene DNA samples transferred to a collaborating company, Epimune in Germany for epigenetic analysis and **9** Corneal Tissue samples sent to a collaborating academic scientist in The MRC Human Genetics Unit at the University of Edinburgh, for genetic analysis.

Based on the sampling carried out by the VISICORT partners, the VISICORT Foundation Biobank (VFB) has been established with a biospecimen count of >50,000 samples made up of nine different biospecimen types.

The VFB has been promoted to relevant user communities and the biospecimens it contains have been registered on The Scientist Web Portal (www.scientist.com) a global marketplace for the promotion of products and services to the international scientific community. In addition, BIOS has connected with the following biospecimen providers to promote the VFB samples: Tissue4Research, Trans Hit Biomarkers, Tissue Solutions, Biosample Hub, Ispecimen, BioIVT & Boca Biolistics.

1.3.4 WP4 – Multi-platform immune response profiles of human corneal transplantation

WP4 focused on profiling the immune-response of human corneal transplantation using a multi-platform approach, investigating the pathways associated with current and subsequent adverse immune response to CT and to define the baseline immune activities associated with adverse outcomes from CT.

Biological samples collected from all human subjects (groups 1-10) within the VISICORT Cross-sectional Study (WP2) were analyzed using multiple technology platforms. Peripheral blood mononuclear cells (PBMC) were subjected to Immune Profiling Assays (NANT), whole genome microarrays (FIOS) and label-free proteomics (SYNT). Transcriptomic and proteomic approaches were applied to plasma and tear samples.

Throughout the project, **FIOS** coordinated the transcriptomic data profiling for the cross-sectional study and analyzed the transcriptomic and proteomic data sets. Few markers appeared to be significantly differentially expressed between the patient groups using recommended statistical thresholds. To facilitate pathway analyses, a relaxed statistical cut-off was used throughout. Analysis of the PBMC samples identified a biological signal primarily in the Posterior Lamellar Corneal Transplant (PL-CT) chronic injury group linked to TGF-beta signalling, which is up-regulated in the PL-CT chronic injury group. However, this is also the smallest group in the study with four subjects; hence the results should be treated with some caution.

SYNT carried out proteomic analysis of the complete set of tears samples from the cross-sectional study. A sample preparation proteomics workflow for the analysis of non-pooled tears fluid was developed. In addition, the plasma samples from the cross-sectional study were depleted and digested in solution followed by a chromatographic cleanup step prior to LC-MS analysis.

Proteomic profiling of tears samples revealed significant differences when comparing Posterior Lamellar Corneal Transplant (PL−CT) chronic vs Non−transplanted, Full Thickness Corneal Transplant (FT−CT) acute vs Healthy volunteers and FT−CT acute vs stable. The up-regulated proteins were primarily shown to be involved in pathways previously linked to cardiomyocyte activity, e.g. Adrenergic signalling in cardiomyocytes, dilated cardiomyopathy and viral myocarditis.

From the proteomic profiling of plasma, significant differences were identified for most comparisons. The comparisons that produced significantly differentially abundant proteins at the most stringent statistical cut-offs have commonalities between the identified proteins. Insulin growth factor proteins are generally down-regulated in the transplant groups whereas fibrinogen chain proteins are up-regulated in the transplant groups. The coagulation and complement cascades pathway is significantly up-regulated in FT−CT stable vs Healthy volunteers, FT−CT chronic vs Healthy volunteers, PL−CT acute vs Healthy volunteers and PL−CT long−term vs Healthy volunteers.

NANT performed an extensive flow cytometry of the cryopreserved PBMC samples obtained from the cross-sectional study (described below) and from the sequential clinical visits from patients enrolled in the prospective study. From these flow cytometry data, a unique database of immune-profiling of CT recipients was established and used to finalize the statistical analysis plan. NANT published several reports in which the panel of markers used for the immune-phenotype and the bio-informatic pipeline of analysis were used, including reports focusing on solid-organ transplantation (kidney, lung). The manuscript presenting the key findings of the cross-sectional study is in preparation (see figures in the next section). Finally, **two collaborative projects** have been initiated: 1. Characterization of transcriptome and miRNA profiles in patients with different clinical outcome of CT (PI C.Murphy, RCSI Dublin); 2. Collaboration with Epimune (defining adverse immune signatures of CT outcome using epigenetic approaches and cross-validation using spectral flow cytometry). The project has benefit from the recent development of high-dimensional panels using spectral flow cytometry (AURORA,

Cytek). The development of spectral flow cytometry panels has been made possible thanks to a ANR Flash grant COVARDS and a BPI grant POLYCOR, in which NANT CRTI Team 4 was WP leader and coordinator respectively and obtained in the framework of the French government support to COVID-19 pandemic related programs.

Initial steps for the flow cytometry analysis involved the design, optimization and validation of panels of monoclonal antibodies as well as the setup of new compensation matrices to ensure optimal, accurate and reliable recording of the data. Using a 4 laser-flow cytometer, the following 4 panels were applied to 552 PBMC samples from the cross-sectional study:

1. **B cell matrix**. Viability, CD19, CD24, CD27, CD38, IgD and IgM
2. **Regulatory CD4 T cells.** Viability, CD3, CD4, CD45RA, CCR7, CD25, CD127, CD45RA, Foxp3 and Helios
3. **Memory CD8 T cells.** Viability, CD3, CD8, CD45RA, CCR7, CD27, CD28, GZMb, PERF, CD57, T-bet and Eomes
4. **Memory CD4 T cells.** Viability, CD3, CD4, CD45RA, CCR7, CD27, CD28, CD38 and HLA-DR

Data collected with the different panels were gathered to build a unique database of immune-profiling of CT recipients which was then linked with the VISICORT clinical database, VIMS, to enable the statistical analysis as shown in Figure .

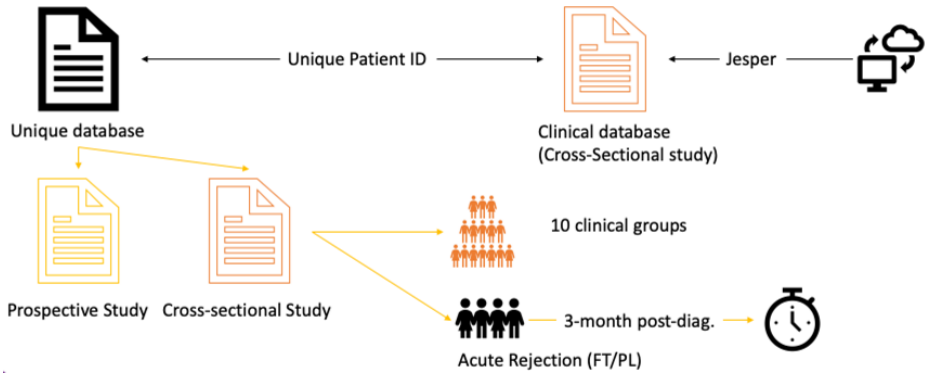


Figure 7: *Development of an integrated immune and clinical database*

A state-of-the-art, automated supervised analysis of up to 165 B and T lymphocytes in order to avoid the inherent biases related to human subjectivity and to include quality control steps to validate each file. The FlowClean and OpenCyto packages (under R studio) were used to compute the frequency of the different T/B lymphocyte subsets as shown below:

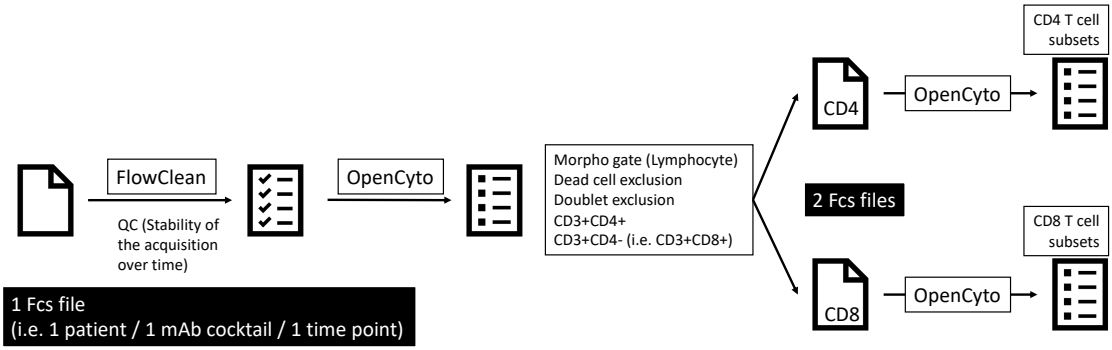


Figure 8: *Cross-Sectional Study – Flow cytometry data analysis platform*

Based on initial comparative analysis, it was decided to merge results for the 2 types of surgical procedures (FT + PL) and for patient groups with stable transplant function for <3 years and >3 years. Thus, the major comparisons were made between CT recipients with acute rejection, CT recipients with stable graft function and non-transplanted control subjects.

These analyses provided a number of insights that are illustrated in Figures 3 and 4 below and can be summarized as follows:

The main immune cell populations (B cell, CD4 T cell, CD8 T cell and ratio CD4/CD8 T cell) were distributed similarly between CT with acute rejection, Stable CT and non-transplanted patients (Figure 9A).

CT recipients with acute rejection exhibit increases frequency of naïve B cells and transitional B cells and decreased frequency of switched B cells compared to Stable CT . Similar findings were obtained when CT patients with an acute rejection were compared to control patients whereas no difference could be evidenced between Stable patients and control patients showing that the modulation in B cell compartment was restricted to patients with an acute rejection (Figure 9B).

Whereas the frequencies of CD4 and CD8 T cell subsets (naïve, EM, CM, TEMRA and Early intermediate memory) were equally distributed across the different clinical groups (Figure 9C and E), CT patients with acute rejection had increased frequency of CD4 regulatory T cells (Treg) as compared to Stable CT (Figure D). The expression of the Ikaros family member Helios by Foxp3+ CD4 Treg was not different between the group suggesting that the stability and regulatory properties of CD4 Treg were similar in CT patients undergoing or not acute rejection (Figure 9D).

The frequencies of activation, differentiation, and cytotoxic functional markers (TBET, EOMES, CD57, PERF1, GZMB) by T cells were not different across the CT patients with acute rejection or stable graft function (Figure 9F) suggesting that the overall impact of local acute rejection of CT results in minor modifications to the T cell compartment.

Finally, analysis of PBMC samples collected 3 months after acute rejection for some patients was used to investigate the impact of the modification of clinical therapy on the B cell and Treg abnormalities associated with acute rejection. This analysis indicated that the immune signature of acute rejection was not significantly different following treatment and clinical follow-up of acute rejection with the exception of an expected decrease in transitional B cells.

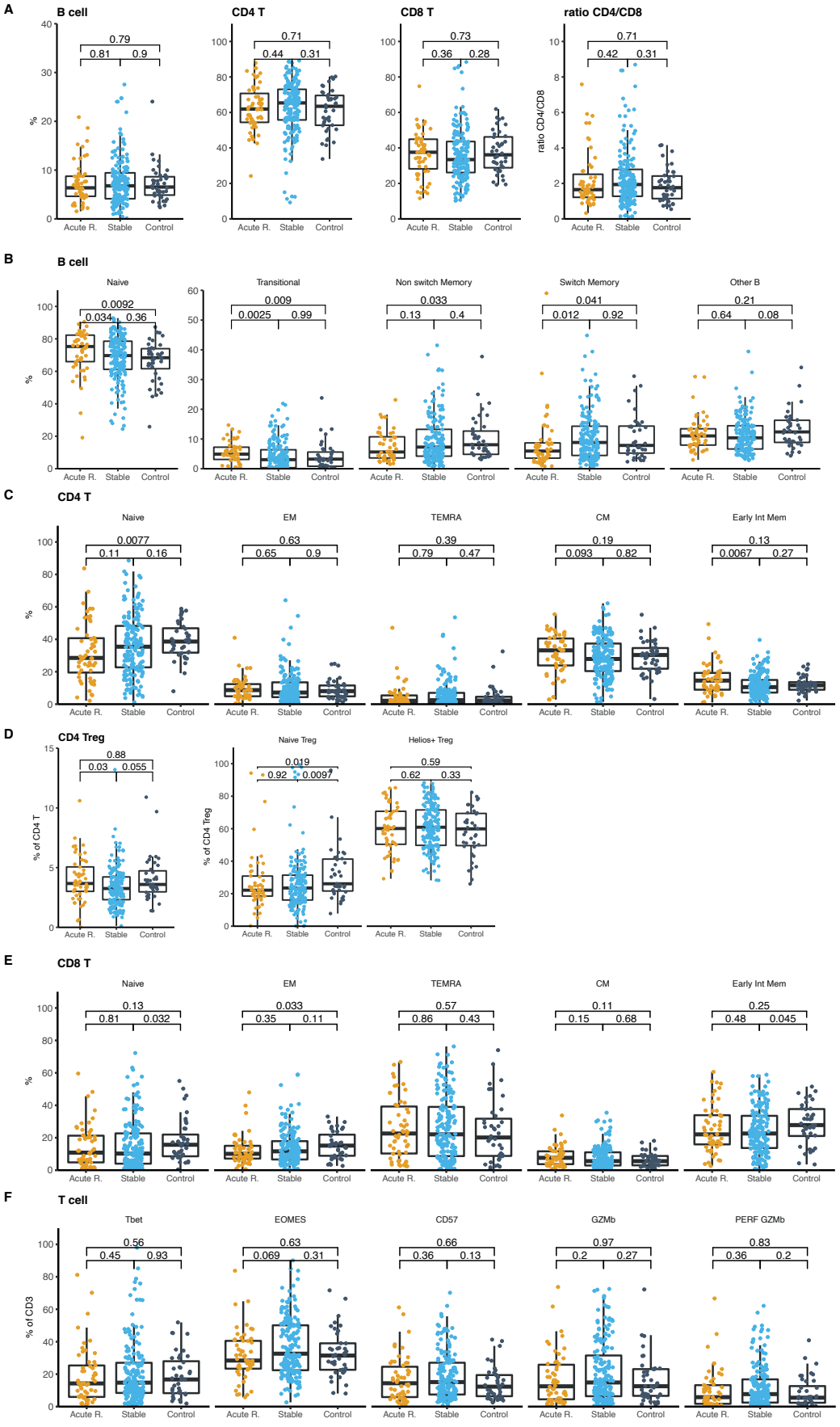


Figure 9: *Immune signature of acute rejection.*

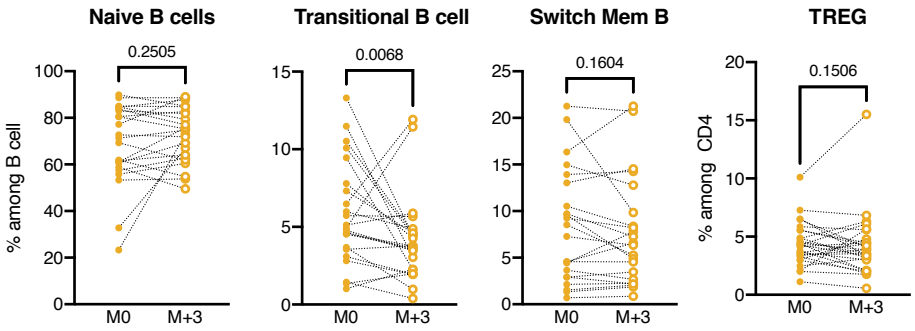


Figure 10: *Immune signature of acute rejection is not modified by therapy adjustment.*

Conclusion

Extensive transcriptomic and proteomic analyses of biological samples of various types from the different groups of a large cross-sectional study of CT recipients provided some insights into biological pathways that are differential active locally (tears) or systemically (plasma, PBMC) in the context of rejection. Overall, however, no gene/protein signatures or pathways were identified that robustly distinguished rejection from stable corneal transplant status within the samples types analysed.

The flow cytometry results from the cross-sectional study are, to our knowledge, the first to investigate using high-dimensional techniques and a large European cohort of CT patients the peripheral blood adaptive (T and B cell) immune response in the blood from CT patients. These analyses demonstrate that the immune cell repertoire is stable over time in CT patients with stable graft function. The analyses revealed subtle changes including unexpected increased frequencies of regulatory associated immune cell population (CD4 Treg, transitional B cells) and in naïve B cells in patients with acute rejection - which were not consistently reversed following treatment of rejection. Our results favour the conclusion that the magnitude of the systemic immune response leading to acute rejection is much lower in CT patients compared to other solid organ transplantation (e.g. kidney, lung, heart, liver). Nevertheless, these changes may provide insight into distinctive immunological events occurring at the time of clinical tissue transplant rejection.

1.3.5 WP5 – Human corneal transplant immune biomarkers

The diagnosis and treatment of corneal transplant (CT) immunological complications is dependent on patient reporting of symptoms and on expert clinical examination of the cornea. Without clinically-applicable biomarkers for adverse immune response, intervention at a preventative stage is not currently possible.

This work performed for WP5 focussed on the data generation and analysis of molecular markers (transcriptomic, proteomic and flow cytometric data) of samples from a longitudinal study of corneal graft rejection. Samples were collected before surgery, six months after surgery and twelve months after surgery. Peripheral blood mononuclear cells (PBMC) and plasma samples collected from the patients at these time-points were profiled using gene expression (PBMC), proteomic profiling (plasma) and multicolour flow cytometry (PBMC). Demographic and extensive clinical outcome data (e.g. rejection) were also collected as described in the report for WP2.

The primary goal of WP5 was to identify biomarker panels and pathways linked to corneal transplant rejection; both for the purpose of early diagnosis or prediction of rejection events as well as to understand the molecular mechanisms involved.

Wp5 used the data and source material collected at the clinical centres and was responsible for the following tasks:

- Proteomic profiling of the plasma samples and microarray gene expression profiling with data analysis of the proteomic and gene expression (transcriptomic) data to identify markers of rejection.
- RNA extraction and quality control of the PBMC samples and subsequent shipment for microarray profiling, as well as flow cytometry-based immunoassay profiling and analysis of the samples.
- Translation of identified immune biomarkers to alternative assay platforms.
- Validation of biomarker(s) assays in future independent cohorts.

Key results

Transcriptomic data analysis

RNA preparations from PBMC samples were extracted and quality checked. A total of 573 samples from 241 subjects were available for gene expression profiling using Affymetrix Clariom S human microarrays using standard protocols. Pre-defined categories of high/low patient risk groups and full thickness (FT) vs posterior lamellar (PL) samples were available, in which analyses were performed separately for the high-risk and low-risk subjects.

The high-risk group comprised 296 PBMC transcriptomes of 102 high-risk CTs of whom 11 subjects had been confirmed to have rejected the transplanted cornea. Quality control of the data identified one sample for exclusion, leaving 295 high-risk samples for downstream analysis. Exploratory analysis revealed associations between gene expression profiles and technical study variables, which were corrected for in the normalisation of the data.

Statistical comparisons were performed comparing the gene expression profiles of patients with or without CT rejection using only baseline data as well as between time-points.

No genes appeared to be significantly differentially expressed between the patient groups using recommended statistical thresholds. To facilitate pathway analyses, a relaxed statistical cut-off was used for exploratory purposes. Significant pathways include e.g. metabolism, cardiomyopathy-related pathways and cell senescence.

The low-risk group comprised 277 PBMC transcriptomes of 102 high-risk CTs of whom 11 subjects had been confirmed to have had an episode of acute rejection. All samples passed QC following manual inspection. Exploratory analysis revealed associations between gene expression profiles and technical study variables, which were corrected for in the normalisation of the data.

Using similar comparisons as in the high-risk group, no genes appeared to be significantly differentially expressed between the patient groups using recommended statistical thresholds. To facilitate pathway analyses, a relaxed statistical cut-off was used for exploratory purposes. Significant pathways were primarily linked to metabolism.

Additionally, machine learning methods were utilised to identify potential models with combination of markers. The performance of these models was determined with 10-fold cross-validation based on best-practice approaches within the field. The performance of the models were only marginally improved as compared to a result predicted by chance (~60% correct classification relative to 50% expected).

Proteomics data analysis

Proteomic profiling was performed on plasma samples from the high-risk group of the prospective study using liquid chromatography coupled with label-free mass spectrometry (LC-MS). Raw proteomic high-risk data comprised 296 high-risk patient samples and 39 control samples to give a total of 335 samples. 331 samples passed QC and were retained for downstream analyses from 209 proteins following filtering. Exploratory analysis revealed associations between proteomics profiles and technical study variables, which were corrected for in the normalisation of the data.

Five statistical contrasts were performed in the same way as for the transcriptomic data. No proteins appeared to be significantly differentially expressed between the two patient groups (no rejection and rejection) using recommended statistical thresholds. To facilitate pathway analysis analyses, a relaxed statistical cut-off was used for exploratory purposes. Significant pathways were primarily linked to immune processes and cell-cell stimulus.

Additionally, machine learning methods were utilised to identify potential models using a combination of markers in the same way as for the gene expression data. The performance of the models were the same as would be expected by chance.

The results of these extensive analyses of transcriptomic and proteomic datasets from the VISICORT prospective study were considered not to have identified highly promising candidate predictive or diagnostic biomarkers of acute corneal transplant rejection in either high-risk or low-risk recipients. For this reason, subsequent planned steps including migration of candidate biomarkers assays to clinically compatible platforms and validation of biomarkers in additional patients were not prioritised.

Flow cytometry data analysis

INSERM UMR1064 (NANT) performed immune-profiling of cryopreserved PBMCs from the five VISICORT clinical sites using flow cytometry. As described in the report for WP4, the following panels of monoclonal antibodies were designed, optimized and validated by NANT for this work:

1. **B cell matrix**. Viability, CD19, CD24, CD27, CD38, IgD and IgM
2. **Regulatory CD4 T cells.** Viability, CD3, CD4, CD45RA, CCR7, CD25, CD127, CD45RA, Foxp3 and Helios
3. **Memory CD8 T cells.** Viability, CD3, CD8, CD45RA, CCR7, CD27, CD28, GZMb, PERF, CD57, T-bet and Eomes

4. **Memory CD4 T cells.** Viability, CD3, CD4, CD45RA, CCR7, CD27, CD28, CD38 and HLA-DR

552 cryopreserved PBMC samples from patients enrolled into the **cross-sectional study** were analysed as were **760** samples representing multiple time-points from patients enrolled into the **prospective study**. Data collected with the different panels have been collated to build a unique database of immune-profiling of CT recipients (collaboration with Matilde Karakachov, biostatistician at CHU de Nantes). The flow cytometry database has also been linked with the clinical database to enable the statistical analysis.

The analysis of the samples obtained following clinical visits from patients enrolled in the VISICORT cross-sectional study are summarised in the report for WP4.

The results of analyses of the flow cytometry data from the VISICORT prospective study are still being finalised.

Assay migration & validation

This task was planned to take biomarker panels and immunoassays identified in earlier WPs and, where available, migrate to one or more streamlined platforms with lower cost and better analytical properties. Due to lack of markers predictive of rejection from the earlier results, however, it was not possible to proceed with this task.

Conclusions

Statistical analyses of the transcriptomic and proteomic data for the purpose of identifying markers linked to rejection did not reveal any markers strongly associated with rejection events. For both data types, this was at the same level as one would expect by chance. Machine learning approaches identified signatures (panels of biomarkers) predictive of rejection events in both the transcriptomic and proteomic data, however, neither of these were significantly above what would be expected by chance. These results suggest that corneal transplant rejection is not preceded by detectable changes in blood cell mRNAs and proteins that can be developed into clinically valuable predictive biomarkers.

Flow cytometry analysis was performed to design, optimise and validate the panels of mAbs to capture numerous cell subtypes. The primary focus of analysis to date has been on the cross-sectional study (described in WP4 report with findings being prepared for publication), while the analysis of the samples obtained following clinical visits from patients enrolled in the prospective study are in progress. These may reveal blood immune cell abnormalities that precede acute rejection.

Planned work related to assay migration & validation was not possible to deliver due to lack of promising markers predictive of rejection from the earlier results.

Following on from the results generated as part of this work package, a successful grant application has been awarded to members of the VISICORT team to extend these proteomic studies in the three year project ‘A multi-omics approach to eye tissue characterisation in keratoconus & Fuch’s dystrophy patients’ has been awarded by the National Eye Research Centre of the UK.

1.3.6 WP6 – Immunomodulatory stromal cell therapy clinical translation for human corneal transplantation

One of the specific objectives of VISICORT was to *design and initiate a mechanistically-informed clinical trial of immunomodulatory stromal stem cells (iSSC) to prevent adverse immune responses in high-risk CT recipients*. Addressing this objective was the primary goal of WP6, which was carried out collaboratively by partners, ORB, NUIG, CUB, BIOS and, from month 43 to end-of-project, CRO. In addition, clinical corneal transplant specialists from partners RCSI, AUH, AU, UBR and NANT contributed essential expertise to the development of the clinical trial protocol. The work for this WP spanned pre-clinical (animal model) experimentation, translational research toward development and characterisation of a novel good manufacturing practice (GMP)-compliant allogeneic stromal cell product, clinical trial protocol design and preparation/submission/amendment of a regulatory dossier and ethics committee application.

Background to the Work package

At the time the VISICORT project was conceived, a limited number of pre-clinical reports had been published investigating the immunomodulatory potential of iSSCs also commonly known as mesenchymal stem/stromal cells (MSCs), in corneal transplantation or injury. Different MSC-application strategies (time point of injection, cell number and number of injections, mode of injection, species, licencing, animal model) had been investigated to explore the therapeutic potential of MSC in corneal transplantation (and in other transplant models). Mostly positive results had been reported to date and, while encouraging, there were also reports showing that MSC application is not beneficial in promoting corneal graft survival. Published experimental evidence from VISICORT coordinating partner, NUIG, indicated that intravenous administration of "third-party" allogeneic bone marrow-derived MSCs significantly and safely reduced the occurrence of acute corneal allo-transplant rejection. The induction of $CD4^+$ $Foxp3^+$ regulatory T cells following MSC-injection appeared to be critical for corneal allograft survival although other regulatory cell populations were also described. The available pre-clinical evidence as considered to be strong enough to support the development of a clinical translation pathway for MSC as an "off-the-shelf" treatment protocol with third-party MSC produced under GMP conditions. Initially, it was planned to focus on a novel MSC products – CD362-selected bone marrow MSCs (ORBCEL-M) – developed by partner ORB. However, as described in the report for WP1, animal model results for corneal allo-transplants treated with both human- and animal-derived ORBCEL-M did not provide evidence of efficacy for preventing acute rejection. In contrast, results for allogeneic bone marrow MSCs isolated by plastic adherence from a "third party" animal strain (PA-allo-BM-MSCs) were strongly positive for an anti-rejection, immune modulatory effect. For this reason, the focus for further pre-clinical experimentation and subsequent human translation was changed to PA-allo-BM-MSCs.

The remainder of this report briefly describes the work carried out for WP6 with illustrative examples of the results in four linked domains of research and development: (a) Pre-clinical experimentation in support of a clinical trial concept. (b) Development of a clinical trial protocol. (c) Development and validation of novel a GMP-compliant manufacturing process for human plastic adherent allogeneic bone marrow-derived MSCs (PA-allo-hBM-MSCs). (d) Preparation and submission of a regulatory dossier and ethics application for a first-in-human, phase 1b clinical trial of PA-allo-hBM-MSCs for prevention of acute rejection in high-risk corneal transplant recipients.

Pre-clinical experimentation (lead partner NUIG)

Preclinical experiments were performed using a fully-MHC mismatched rat model of corneal allo-transplantation in which published results from NUIG had shown intravenous injection of MSCs from a "third party" (non-donor/non-recipient strain) at 7 days and 1 day prior to transplantation to significantly increase rejection-free survival of the transplants with evidence of donor-specific immune

modulation. The initial experiments for WP6 were designed to replicate these results with human and rat ORBCEL-M but the results did not support a similar immunomodulatory effects of these cells in the model. Subsequently, results for third-party rat allo-BM-MSCs isolated by plastic adherence were confirmed and a new model was developed in which to test the efficacy of these cells in the setting of increased immunological risk of rejection. This "pre-sensitised" model was associated with rapid rejection compared to the normal risk, fully MHC mismatched model (Figure 11).

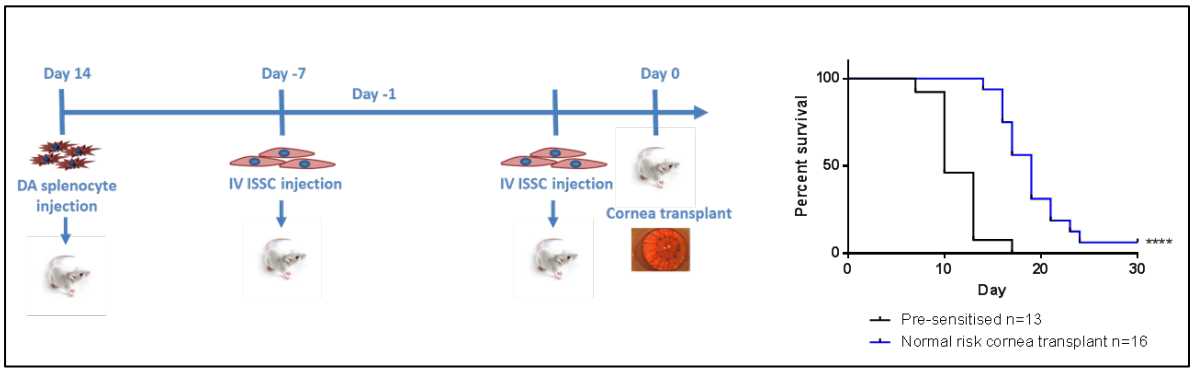


Figure 11: *Left: Protocol for pre-sensitisation corneal transplant recipient rats with corneal donor strain (DA) splenocytes followed by intravenous injection of allogeneic MSCs (IV iSSC injection) 7 days and 1 day before transplantation. Right: Reduced rejection-free survival of corneal transplants in pre-sensitised compared to non-sensitised (normal risk) corneal transplant recipients.*

In a subsequent series of experiments it was shown that: (a) Third party strain PA-allo-BM-MSC injections significantly prolonged the rejection-free survival of pre-sensitised recipients of corneal allotransplants (Figure 12). (b) Mechanisms of preventing acute rejection in presensitised recipients included promotion of regulatory macrophages as well as regulatory T cells. (c) The immune modulatory effects of the cells in the pre-sensitised model remained present following cryopreservation and thawing prior to administration (Figure 12). (d) The anti-rejection effects of the cells were retained when the recipient animals were also treated with the conventional immunosuppressive drug mycophenolate mofetil.

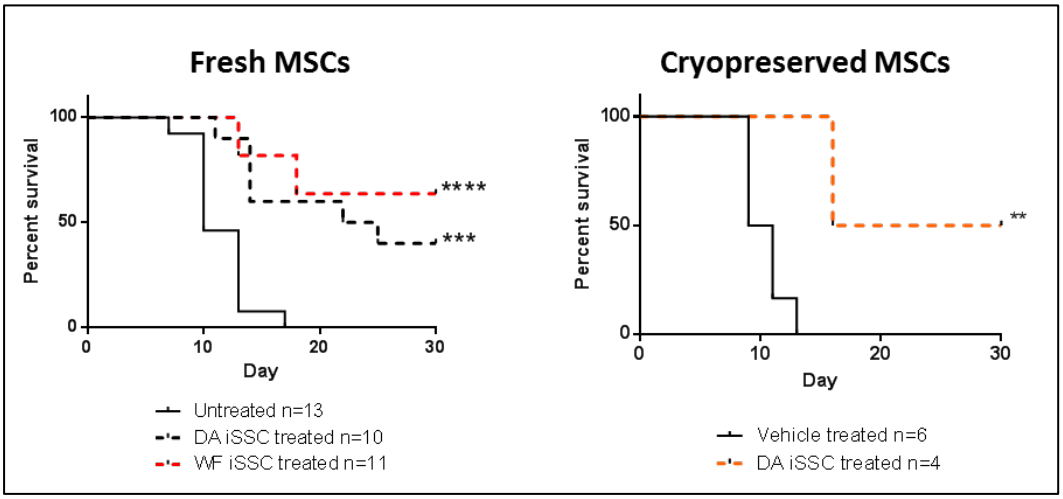


Figure 12: *Left: Rejection-free survival of pre-sensitised rat recipients of fully MHC mismatched corneal allotransplants with vehicle injections (Untreated) or with pre-transplant injections of non-cryopreserved (Fresh) corneal donor strain MSCs (DA iSSC) or third party strain MSCs (WF iSSC). Right: Rejection-free survival of pre-sensitised rat recipients of fully MHC mismatched corneal allotransplants with vehicle injections (Untreated) or with pre-transplant injections of cryopreserved third party strain MSCs (WF iSSC). **, ***, **** = p < 0.01, < 0.001, < 0.0001 compared to vehicle.*

The results of this pre-clinical experimental work, which was published in leading stem cell and immunology journals during the course of the project, were essential for subsequent clinical trial design, selection of a cell manufacturing protocol and justification of the clinical trial dossier to the regulatory authority and ethics committee.

Development of clinical trial protocol (co-lead partners ORB, NUIG, CUB and CRO)

Beginning in Month 24 of the VISICORT project, a working group consisting of partners from ORB, NUIG, CUB, AUH, AU, RCSI, UBR and NANT began to develop the framework for a phase 1b clinical trial based on the emerging pre-clinical experimental results as well as a broad literature review related to MSC therapies and trials related to corneal and other allotransplants. Initial details were agreed at the VISICORT 36 month plenary meeting. With the incorporation of a new partner, CRO, with extensive experience and resources in early-phase clinical trials into the consortium at Month 43, the trial design and planning gained additional momentum and an organisational plan and initial time-lines were agreed (Figure 13).

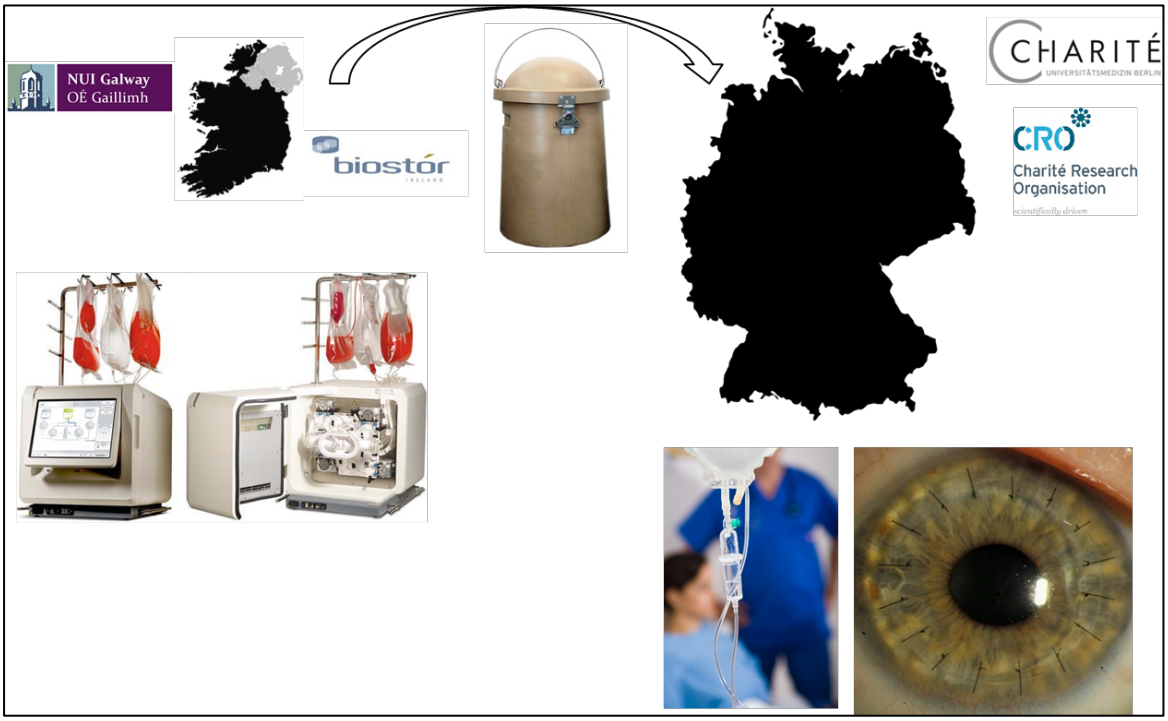


Figure 13: *Diagram of the organisational plan for the VISICORT clinical trial. Cell manufacture using a bioreactor-based protocol is carried out at NUIG, Ireland with cryopreserved cell product transported by dry shipper in collaboration with partner BIOS (Biostór) to Berlin, Germany, where trial procedures including cell infusions are performed in the clinical trial unit of CRO and corneal transplants are performed by the trial principal investigator and Charité University Hospital.*

Over the next 12 months, the clinical trial protocol was fully developed. Among the key details decided upon by the Trial Steering Committee comprised of members from all partners involved were: (a) A focus on patients receiving corneal re-transplants following loss of one or more transplants to the same eye (a common clinical scenario associated with significantly higher risk of transplant loss due to rejection). (b) A dose escalation design consisting of two sequential dose cohorts of 4 patients each with dosing selected based on pre-clinical data as well as dose ranges used for other human MSC clinical trials in the setting of allo-transplantation. (c) A 3 month primary safety and feasibility end-point with extended safety reporting to 1 year post-transplant. (d) A full set of inclusion and exclusion criteria. (e) An extensive risk-benefit analysis. (f) Detailed time-line and protocol for each individual patient and for the full trial (Figure 14).

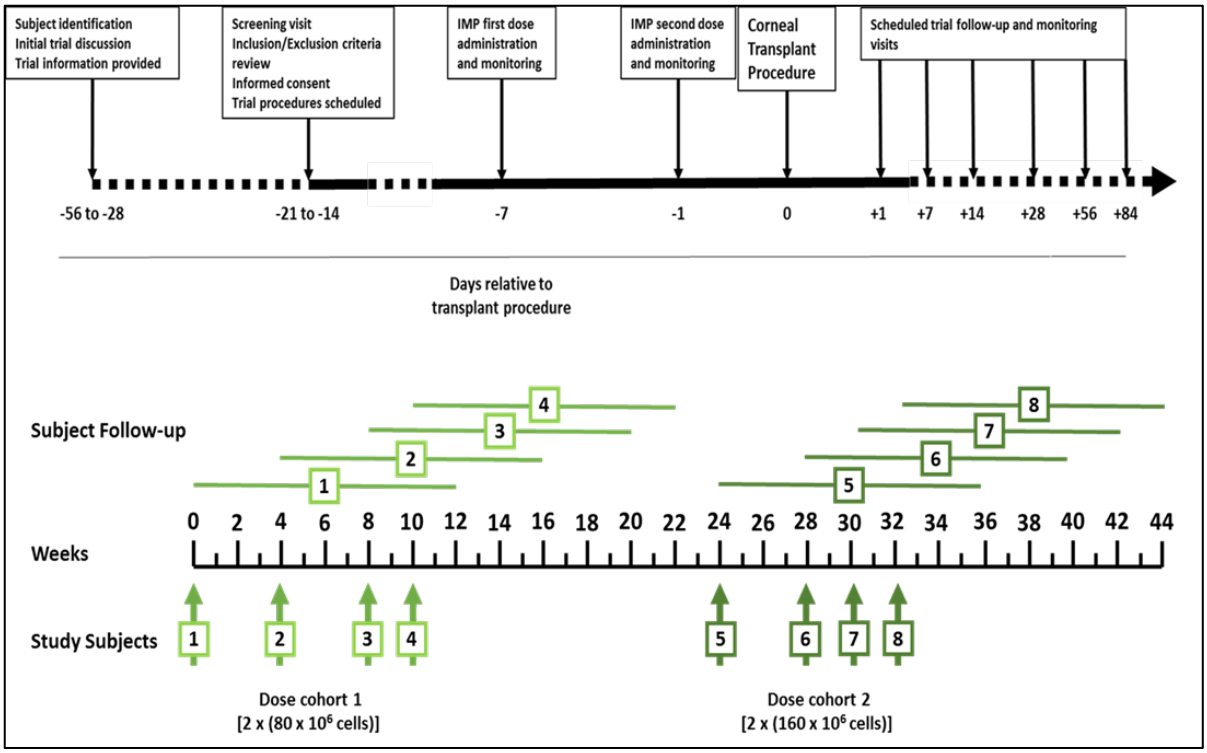


Figure 14: *Upper: VISICORT clinical trial time-line for individual patients (not shown: an extended safety follow-up via clinical outpatient assessment at day 358). Lower: VISICORT clinical trial time-line for the full trial consisting of two dose cohorts of 4 patients each over 44 months.*

The completed trial protocol and accompanying investigator brochure were subsequently incorporated into the regulatory dossier and ethics committee submission by CRO as described below.

Development of a GMP-compliant cell manufacturing protocol (lead partner NUIG)

Once a decision was made to focus on cryopreserved plastic adherent allogeneic BM-MSC for the clinical trial planning, the process of developing a GMP-compliant cell manufacturing protocol was initiated at the Centre for Cell Manufacturing Ireland (CCMI) facility on the NUIG campus. For this, healthy adult bone marrow samples were acquired through an ethically-approved healthy volunteer marrow donor programme coordinated through the NUIG Clinical Research Facility (CRF). Key initial decisions included: (a) The use plastic adherence for primary outgrowth of MSC colonies from fresh marrow samples. (b) The application of a hollow fibre bioreactor system (Terumo Quantum CES) for batch manufacture (Figure 15). (c) The use of small batch human platelet lysate (HPL) as a medium supplement. (d) The use human cryoprecipitate as a bioreactor cartridge coating agent for initial cell adherence within the bioreactor. (e) Cryopreservation of the final cell product in infusion bags containing CryoStor preservative at doses of 80 x 10^6 cell and 160 x 10^6 cells/bag (Figure 15).

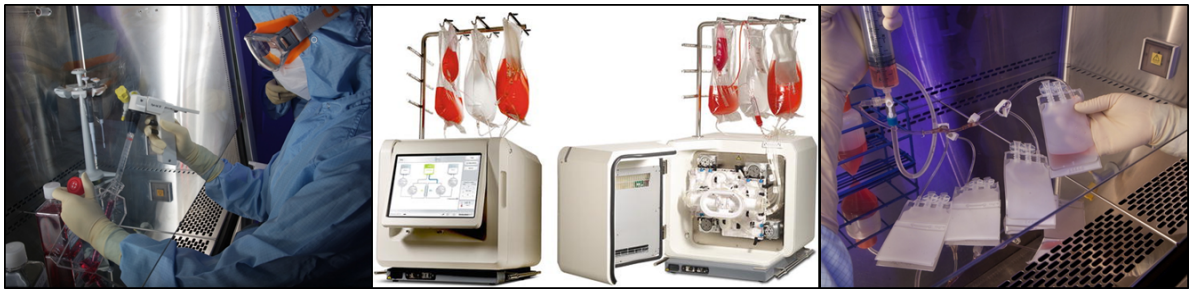


Figure 15: *Left: Primary bone marrow plating into plastic tissue culture flasks. Middle: Terumo Quantum CES used for secondary cell expansion. Right: Filling of cryobags with final cell product.*

The cell manufacturing protocol was successfully validated to meet all pre-determined release criteria and immune modulatory potency of the resulting cells was confirmed in T cell proliferation assays. An Investigational Medicinal Product Dossier (IMPD) was prepared in collaboration with CRO and was submitted along with the clinical trial protocol and investigator brochure for regulatory and ethical approval as described in the next section.

However, between Month 54 and 60 of the project, it became necessary to revise the manufacturing protocol and IMPD for the cell product as a result of two reagents – small batch HPL and human cryoprecipitate becoming unavailable. In response to this, additional laboratory studies and validation manufacturing batches were performed at CCMI between Months 60 and 72 using alternative reagents – large batch, pathogen-reduced HPL (nLiven) as a medium supplement and recombinant human Vitronectin as a bioreactor cartridge coating agent. The results from new validation batches using the new reagents provided the basis for regulatory authority approval of a substantial amendment to the IMPD as described below.

Between months 72 and 84, the cell manufacturing programme for the trial was further delayed by prolonged closures of the CRF and CCMI facilities due to the COVID-19 pandemic and by the need to further revise the manufacturing protocol to improve the cell release step from the bioreactor cartridge at the end of the cell expansion process. This latter problem was overcome through additional laboratory experiments and validation batches performed at CCMI between COVID-19 closures. Ultimately, despite the unavoidable delays, a total of 5 validation batches meeting all pre-defined release criteria and encompassing the original and two revised manufacturing protocols were completed and incorporated into the IMPD. Table 8 summarises the release criteria data for validation batches 1-4. In addition, a cell product stability testing protocol was established by the end of the project.

Table 8: *Summary of release criteria results for 4 validation batches of the VISICORT allo-hBM-MSC manufacturing protocol (Batch 1+2:small batch HPL/cryoprecipitate; Batch 3: pathogen-reduced HPL/vitronectin; Batch 4: pathogen-reduced HPL/vitronectin + prolonged cell release step)*

Batch Number		**Validation Batch 1**	**Validation Batch 2**	**Validation Batch 3**	**Validation Batch 4**
Test	**Specification**				
Cell Number	≥350x10^6	408 x 10^6	530 x 10^6	365.5 x 10^6	389.19 x 10^6
Sterility	No Microbial growth	No Microbial growth	No Microbial growth	No Microbial growth	No Microbial growth
Endotoxin	≤10EU/ml	<5.0EU/ml	<2.5EU/ml	<2.5EU/ml	<2.5EU/ml
Mycoplasma	Negative	Negative	Negative	Negative	Negative

Karyology	No chromosomal abnormalities	Pass 46,XY	Pass 46,XX	Pass 46,XY	Pass 46,XX
CD73+	≥90 %	99.9%	99.9 %	99.9 %	100%
CD90+	≥90%	99.9 %	99.9 %	99.9 %	100%
CD105+	≥90 %	99.1 %	99.7 %	96.8 %	99.2%
CD45+.CD34, CD11b, CD19, HLA-DR	≤ 5 %	0.1%	0.0 %	0.1 %	0.2%

Development and submission of a regulatory dossier and ethics application (lead partner CRO)

Upon joining the consortium, and working in coordination with NUIG, CUB and the full Clinical Trial Steering Committee, CRO prepared the initial and subsequent revised dossier of documents for submission of the trial to the German Regulatory Authority (Paul Ehrlich Institute, PEI) and to the Ethics Committee (EC) of the primary trial site (Charité University Hospital). This process included the coordination of responses to major and minor queries from PEI and the EC within tight deadlines. One minor amendment to the clinical trial protocol was required and approved by the EC. The need for changes to the manufacturing protocol and the resulting delays to validation of those changes at CCMI, as described in the previous section, more significantly impacted the timing of amendment submissions. Nonetheless, all versions of the trial-related documents prepared and submitted by CRO throughout this phase of the project were subsequently approved within the required time-frames. The EudraCT number for the trial is 2018-000890-60. Table 9 summarises all of the relevant primary and amended document submissions and their outcomes.

Table 9: *Summary of primary and amendment documents submitted to date to the institutional Ethics Committee (EC) and regulatory body (PEI) in relation to the VISICORT clinical trial*

Type of submission	Submission Date		Approval Date		Key Supporting documents
	EC	PEI	EC	PEI	
Initial	21 June 2018	27 June 2018	05 Feb 2019	11 Jan 2019	-Protocol V 1.0 + Protocol V2.0 (as answer to conditions) -IB Ed. 1.0 + IB Ed.2.0 (as answer to conditions) -ICF V1.0 + ICF V2.1 (as answer to conditions) -IMPD V1.0 + IMPD V1.1 + IMPD V1.2 (as answer to conditions)
Non-substantial Supportive material for patient recruitment	15 Feb 2019	N/A	28 Feb 2019	N/A	- WebInfo CRO -VISICORT postcard V 1.0

Substantial Protocol version 3.0	26 Jun 2019		05 Jul 2019	18 Jul 2019	-Protocol V3.0 -ICF V2.2
Substantial IMPD update	N/A	07 Apr 2020	N/A	13 May 2020	- IMPD V1.3

CRO also developed extensive trial initiation plans, a number of relevant legal agreements among the key trial partners and a plan for trial-related laboratory monitoring.

Although a screen by the trial principal investigator of patients attending the Charité University Hospital for corneal transplant evaluation confirmed that potentially eligible and willing patients could be identified, it was, ultimately, not possible to initiate the approved clinical trial during the project funding period. During the final months of WP6, NUIG, CRO and CUB worked together to develop an updated, comprehensive trial budget estimate in order to plan toward new funding to complete the trial.

Conclusions

The ambitious plan for WP6 was to complete the translational process for a novel immune modulatory cell therapy for prevention of rejection in high-risk corneal transplant recipients from bench (pre-clinical models of high risk corneal transplantation) to bedside (a completed phase 1b clinical trial documenting safety and feasibility of the cell product). Strongly supportive pre-clinical evidence was generated and the highly challenging intermediate steps of developing and validating a novel allogeneic cell therapy manufacturing process, collaboratively designing a first-of-its-kind clinical trial protocol and preparing a trial dossier that received regulatory and ethical approvals within tight time-frames were all negotiated successfully. Ultimately, despite project extensions that were facilitated by the Commission, delays imposed by challenges beyond our control (withdrawal of key manufacturing reagents and facility closures due to the COVID-19 pandemic) precluded initiating the trial prior to the end of the VISICORT project. However, with all necessary approvals, protocols, facilities and expertise in place, the designed trial is very feasible and, to our knowledge, it remains the only approved clinical trial seeking to translate an extensive pre-clinical literature on MSC therapy in corneal transplantation to clinical practice. The partners are committed to pursuing it through alternative funding mechanisms.

1.3.7 Conclusion

VISICORT set out to provide a new level of insight into the immune responses involved in the rejection of corneal transplants. In pursuit of that aim, it has carried out a series of investigations and has produced a number of significant outcomes.

- VISICORT has collected, managed, and distributed a very large number of tissues samples, and the VISICORT Foundation Biobank, which contains these samples, is one of the project's significant and enduring outcomes. Beyond the life time of the project, it continues to provide researchers with a unique collection of samples, and is already enabling further research by members of the VISICORT team and by other researchers.
- VISICORT has also carried out extensive and detailed multi-omics analysis of these human samples, along with those produced by the work carried out in animal models of clinical rejection. The analysis of these samples has provided an unprecedented level of insight into the processes of transplant rejection, and has produced a number of new methodologies, biomarker panels, and a range of scientific publications.
- By carrying out a detailed clinical variables study, VISICORT has highlighted significant links between clinical variables and transplant outcomes. This information has the potential to inform clinical practice and help to improve patient outcomes.
- Although VISICORT was ultimately unable to complete the planned clinical trial, the pre-clinical work carried out by the project has produced valuable data and had confirmed the efficacy of plastic-adherent mesenchymal stem cells in the animal model. This data, along with the necessary regulatory and ethical submissions and approvals that it has enabled, means that VISICORT has laid the groundwork for a Phase I trial of MSCs for the treatment of adverse immune rejection.

1.4 Impact, dissemination and exploitation

The VISICORT project assembled a multi-disciplinary team, bringing together clinicians and researchers, specialists in transplant and immune medicine, -omics profiling and data analysis, biobanking and logistics, stem cell therapies, and clinical trials. In each of these areas, VISICORT has had an important impact. The work of the project has resulted in 25 scientific papers, to date, with a number more still in progress.

The VISICORT Foundation Biobank (VFB) with profiled data from more than 1,000 corneal transplant patients, collected from five leading European laboratories provides a unique and invaluable resource for researchers and will live on beyond the end of the project, supporting a range of important research.

Through VISICORT, new strategic research collaborations were founded and existing ones strengthened, an additional impact of the EU funding received. The partners have established a Virtual Research Community and developed a Joint Action Plan for Research based on potential avenues for future collaborative research. VISICORT has been instrumental in securing a range of additional funding, including a National Eye Research UK funded PhD, and a collaborative immune profiling study with Epimune Diagnostics GmbH.

Throughout the project, partners engaged in a wide range of dissemination events, from academic conferences and global biobanking events to events aimed at schools and the general public.

VISICORT

IMPROVING THE SUCCESS RATE OF CORNEAL TRANSPLANTS

OUR LEGACY

- Profiled data from over 1000 corneal transplant recipients from 5 leading laboratories to ultimately improve corneal transplant success
- Regulator approved clinical grade cell therapy: Human Allogeneic Bone marrow-derived MSCs
- The VISICORT Foundation Biobank: a sustainable resource of bio-specimens to support future research in eye disease

EDUCATION, CAREERS, TRAINING

Two PhDs and post-doctoral researcher trained and currently employed in academia; Study nurse employed; Promotion of study nurse to advanced nurse practitioner;
Advanced FlowJo and OMIQ for Flow Cytometry data analysis training

NEW KNOWLEDGE, NEW SERVICES

- 25 Publications
- Automated, supervised and unsupervised Analysis of High-dimensional Flow Cytometry Data

FUNDING and RESEARCH EXPLOITATION

- Epimune GmbH collaboration
- National Eye Research Centre UK PhD
- BPI France: POLYCOR N° DO50121115 APP PSPC
- SATT Ouest valorisation
- Fondation Centaure
- Labex IGO
- Health Service Executive (Ireland)
- Novel immunology aspects of VISICORT are important enablers in a current EIC Pathfinder application.

VISICORT has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no. 602470.

1.5 Website and contact details

Prof. Matthew Griffin,

National University of Ireland, Galway,

Galway,

Ireland.

www.visicort.eu