The challenge of quality assessment and regional perfusion to increase donor organ utilisation

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Part III

Chapter 8

Summary, Future Perspectives and Conclusion

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Summary

Solid organ transplantation has been shown to be one of the most impressive achievements in clinical medicine in the past 50 years. However, transplantation medicine has also become the victim of its own success. With better management of complications, improved results and life-sustaining outcomes for many end stage organ diseases, more patients are eligible for this modality of treatment. Unfortunately, the persistent donor shortage has remained the Achilles heel in transplantation with a widening gap between demand and supply of suitable donor organs. As a response to narrow this gap, clinicians have expanded the deceased donor pool and are nowadays accepting organs from older and so called 'higher risk' donors. The results from these donors are acceptable but this donor type is associated with a significantly lower organ recovery rate and increased posttransplant complications rate and/or poorer function when compared to standard criteria donors.[1-9] Due to uncertainty about the quality and viability of these donor organs at time of offering, higher risk donor organs are often declined and discarded. This raises the question whether this 'underutilisation' of older and higher risk donor organs is actually justified as it might unnecessarily reduce the size of the potential donor pool.

In this thesis we have focused on how the organ utilisation rate may be increased. In the first part of this thesis, we have studied the clinical risk assessment of donor organs with acute kidney injury (AKI) and have investigated how to improve assessment of donor organs by identifying clinically relevant biomarkers or using a novel method to quantify the relative amount of cell death. Biomarkers can be used to assess organ viability and may help clinicians at the time of offering whether to accept or decline a donor organ for transplantation.

In the second part of this thesis, we have focused on optimisation of organ preservation strategies to protect vulnerable grafts by potentially reducing uncertainty about quality and thus increasing organ utilisation.

In the Introduction in **Chapter 1**, background information on the topics is provided that is relevant for the work included in this thesis.

Next, **Chapter 2** focuses on the transplantation of kidneys from donors with acute kidney injury (AKI), defined as an abrupt deterioration of kidney function, classified by the AKIN criteria. As it is current practice in many European national and/or international organ-sharing systems to not accept donor kidneys with AKI, based on the perception that these kidneys are not compatible with successful transplantation, we investigated whether discard of these donor kidneys is justified. In a retrospective

analysis of robust data obtained from the National Transplant Registry in the United Kingdom (UK) hosted by NHS Blood and Transplant, all deceased donors (n = 11,649) where at least one kidney was offered for transplantation between 1st January 2003 and 31st December 2013 were included. The results showed that kidneys were more likely to be declined due to higher AKIN stages. In fact, a total of 30.4% of kidneys with AKI were finally declined or discarded. Especially when AKIN stage 3 was present, the odds for non-use due to presumed inferior function posttransplant were 20 times higher compared to kidneys without AKI. As regards initial graft function in transplanted kidneys, we observed an increase in delayed graft function (DGF) and primary non-function (PNF) rates in kidneys with higher AKIN stages. Further evaluation of long-term posttransplant outcomes, however, showed that graft survival (GS) was only 2% lower when using a first adult kidney-only transplant from donors with AKI versus those without AKI (GS at 1 year 89% versus 91%, p = 0.02; OR 1.20 (95% CI: 1.03 - 1.41)). Although this small reduction in graft survival was statistically significant, the clinical relevance remains questionable given the fact that the 20% increased chance of graft failure due to AKI is significantly lower than the increased risk of graft failure when extending the time on dialysis beyond 1-2 years prior to kidney transplantation.[10] In our analysis, 1667 out of 1,869 recipients (89%) that received a donor kidney with AKI still had a functioning graft at 1 year. These observations suggest that kidneys with AKI stage 1 or 2 should not be discarded as they allow comparable outcomes and significantly contribute to enlarging the donor pool. As regards kidneys from donors with AKI stage 3, however, more caution is advised.

To aim for optimal posttransplant outcomes, assessment of quality and viability of the graft in question is essential. Donor organs from DCD donors are often declined and discarded due to the uncertainty about their quality. Currently, a more sophisticated and objective method to assess the quality and viability of donor organs prior to transplantation is lacking. Therefore, in the next part of this thesis we decided to focus on a more molecular approach by investigating several biomarkers that could be of value to the clinician by offering better information about the graft in question.

In **Chapter 3** we performed an explorative study to identify a molecular signature predicting primary non-function (PNF) in the kidney. PNF - defined as initial non function and permanent absence of function of the kidney graft from time of transplantation - is a devastating event for the recipient. Recipients must return to the waiting list and (re)start with dialysis hoping for a possible re-transplantation in an often-distant future. The possibility to predict PNF would be a major game changer as it could prevent graft failure and allow better donor-recipient matching and organ utilisation. The Quality in Organ Donation (QUOD) biobank, a national consortium

in the United Kingdom, was used for the collection of deceased donor urine and donor blood samples. Three different groups, composed of DCD donors where both kidneys resulted in either a PNF (group 1), DGF (group 2) or immediate function (IF) (group 3) were selected, and donor urine and donor blood plasma from these donors was analysed. When using label free quantitative proteomic analysis 3,955 and 914 proteins were identified in deceased donor urine and plasma, respectively. This study showed that 87 of these proteins were simultaneously enriched in both urine and plasma and 29 were significantly upregulated in urine or in plasma ($p < 0.05$) from donors whose kidneys manifested with symmetrical PNF. The top three proteins (Gelsolin; GSN, IGFBP3; Insulin-like growth factor-binding protein 3, IGF2R; Insulin-like growth factor 2 receptor) that had the highest significance in our primary analysis with LC-MS/MS did not show statistical significance when a the less sensitive validation enzyme-linked immunosorbent assay (ELISA) was used. Considering that the LC-MS/MS technique is a more sensitive and specific method for both quantitative and qualitative assessment of proteins, the identification of these 29 proteins is a first step towards recognition of a predictive protein profile. However, further validation and more in-depth pathway analysis of this proteomic data is required before clinical implementation of these proteins as a potential predictive molecular signature for incident PNF can be used.

In higher risk kidney grafts, dynamic organ preservation strategies are more frequently used. During dynamic machine preservation the perfusate is continuously pumped through the organ which allows for reconditioning and viability assessment of the donor organ. The goal of the study described in **Chapter 4** was to investigate the role of Flavin Mononucleotide (FMN), a cofactor for the mitochondrial membrane NADH:ubiquinone oxidoreductase enzyme (complex I), as a tool to monitor the quality of clinical kidney grafts during hypothermic (oxygenated) machine perfusion. FMN has been reported to dissociate from complex I following ischaemia/ischaemia-reperfusion induced mitochondrial injury. Therefore, some studies have described FMN as a helpful and clinically relevant biomarker of ischaemic injury in liver grafts. To test its role in clinically kidney transplantation, we used all perfusate samples from the paired randomised controlled COPE-COMPARE trial, comparing HMP with oxygenation ($HMPO_2$) versus standard HMP. This study showed that the fluorescence intensity (excitation 450 nM; emission 500 - 600 nM) increased over time during machine perfusion in both groups, however, no correlation with posttransplant outcomes was found. More importantly, we observed that the fluorescence intensity was not due to an increased presence of FMN as the ultimate sensitive test of targeted liquid chromatography mass spectrometry (LC-MS/MS) demonstrated that there was no FMN present in the samples. These observations implied that FMN could

not be confirmed as a relevant predicting biomarker of kidney graft function after transplantation.

Regarding the pancreas, a β-cell specific unmethylated Insulin (*INS*)-DNA marker has been described in studies focusing on the early development of Type 1 Diabetes (T1D) as well as in patients undergoing total pancreatectomy and islet auto-transplantation. With progression of the T1D disease, or transplantation of islets, β-cell death is known to occur and unmethylated insulin (*INS*)-DNA will be released in the bloodstream. This marker can then be detected to inform the clinician on the progression of the disease or the amount of ß-cell death during transplantation. As the concentration of unmethylated *INS*-DNA is often extremely low, digital polymerase chain reaction (PCR) is frequently used. This is then combined with a sodium-bisulfite conversion method that chemically converts an unmethylated cytosine into uracil. The usefulness of the chemical conversion method, however, depends on the completeness of the chemical conversion as partial bisulfite conversion may lead to misinterpretation. Therefore, this method remains a relatively time-consuming technique with a 12 - 16 hour incubation period.

In **Chapter 5** we described a novel improved method for quantification of the unmethylated *INS*-DNA marker using a methylation sensitive restriction enzyme (MSRE) and digital PCR. This method allows for rapid and specific quantification of β-cells by calculating the fraction of unmethylated *INS*-DNA. Validation of this assay was performed in cell line models and further evaluation of the assay showed a significant correlation between the purity of islets and unmethylated *INS*-DNA ($R2 = 0.8318$, $p < 0.0001$). This technique appears to be highly relevant and may be of value to reliably determine the quality of endocrine pancreatic tissue (i.e., during islet isolation, islet culture and subsequent islet transplantation).

In addition to ex-situ machine perfusion strategies, such as hypothermic machine perfusion which is described in **Chapter 4**, to date in-situ regional perfusion techniques are emerging. In **Chapter 6** and **Chapter 7** the novel in-situ machine perfusion technique of abdominal normothermic regional perfusion (aNRP) in donation after circulatory death (DCD) donors is investigated.

Chapter 6 provides a review of the recent literature on the added value of aNRP when compared to local standard perfusion techniques. In this systematic literature search, 24 studies were identified that evaluated aNRP that can be used to better assess abdominal donor organs and improve function and outcomes of these organs. Results showed unanimously that this technique is feasible and safe in both uncontrolled and controlled DCD donors (uDCD and cDCD, respectively). Several studies found

lower complications and increased survival rates.[11,12] However, it should be noted that heterogeneity among the data sets in the respective articles was considerable. Some of the differences in protocols were inevitable as management of donor organ retrieval differ across several countries (e.g., the possibility to administer heparin or start cannulation before the declaration of death, the length of obligated no-touch period or the use of different preservation strategies of grafts during the cold ischaemia time). In addition, the lack of uniformity of reporting of definitions and outcome measures was observed. These findings implicate that, to be able to quantify the success of aNRP amongst other organ preservation techniques, consensus on different aNRP protocols and uniform definitions are needed.

The results obtained in **Chapter 6** confirmed that aNRP is a feasible and safe technique to use, however, uniform reported outcome measures are lacking. In this context, we tried to systematically evaluate current protocols on liver viability assessment. In **Chapter 7** an overview is given of the current protocols used in liver viability assessment during aNRP and how this correlates with clinical outcomes. Fifteen studies were included in this analysis using aNRP in controlled or uncontrolled DCD donors. All studies used a combination of criteria (i.e., macro- or microscopic assessment, ALT or lactate levels in perfusate) to assess the viability of the liver and suitability for transplantation. This review also showed that macroscopic assessment was the main determinator of acceptance for transplantation in controlled DCD donors whereas the more objective criteria of ALT levels in perfusate was the main determinator in uncontrolled DCD donors. The organ utilisation rate was 16% in uDCD and 64% in cDCD. In terms of clinical outcomes, PNF occurred more frequently in uDCD compared to cDCD grafts (13% versus 3%, respectively). Additionally, the overall 1-year graft and patient survival was lower in uDCD than in cDCD (75% and 82% versus 91% and 93%, respectively). However, no differences in the incidence of non-anastomotic strictures (NAS) were seen. These observations suggest that current assessment of ALT levels in the perfusate may overestimate the suitability for transplantation of uDCD livers as the incidence of PNF is unacceptably high when considering the modest organ utilisation rate of 16%. For cDCD donors, however, the macroscopic assessment criteria of especially hepatic steatosis may underestimate the suitability for transplantation of cDCD livers as the quality of aNRP grafts resembles that of DBD liver grafts, although the organ utilisation rate is lower than in DBD donors.

Future perspectives

Due to the increasing number of patients on the waiting list for transplantation and the persistent donor organ shortage an optimised and maximal utilisation of potential donor organs is mandatory. The studies in this thesis provide further detail and suggestions towards better assessment of abdominal donor organ quality and include organ preservation strategies to increase the organ utilisation rate.

Assessment of abdominal donor organs

To accurately assess the sufficient quality and viability of a donor organ prior to transplantation is not possible yet, however, by using enhanced assessment strategies and tools better insight and more adequate prediction of transplantability appears within reach. The holy grail in organ transplantation to identify and successfully validate a single or more likely a combined set of molecular markers capturing the (future) function of a graft in all its complexity and predicting successful outcome in the recipient has not be realised. However, and rather hopeful, novel bioanalytical technologies including transcriptomics and proteomics have been developed and are able to identify individual genes and/or complete set of proteins as described in **Chapter 3**. Similarly, as a barcode contains more information than just one single number, the identification of a molecular signature using proteomics may have such diagnostic potential. Adequate clinical validation of these marker profiles, however, has remained a major bottleneck in the development of clinically relevant molecular signatures. Integrated databases in combination with large high-quality bioresources of samples and tissues (e.g., using the UK QUOD Biobank) may facilitate and hopefully speed up the necessary clinical validation process.

Another important diagnostic potential may be the detection of circulating cell-free DNA (cfDNA) in so-called 'liquid' biopsies. cfDNA, or its fragments, is released from dying cells and can be detected in blood or perfusate. It was first reported by Lo et al.[13] that donor-derived cfDNA is present in the plasma of transplanted recipients and can be used as a detection marker for transplant rejection. In this context, an emerging innovative technology in the field of cfDNA is the additional value of the epigenetic process of DNA methylation. Each cell type in the human body is characterised by a unique DNA methylation pattern. Tissue-specific methylation or even cell-specific methylation, as described in **Chapter 5**, can be used to identify multiple methylation markers for each cell type of interest. Therefore, it could be important and relevant to create an extensive atlas of human tissue methylomes, thus contributing to the detection and development of certain methylation biomarkers. Caution may be advised, however, when interpreting cfDNA signals based on a single methylation marker alone, as biological variability of tissues may be present. Simultaneously

testing multiple differentially methylated sites in a specific cell type or tissue will result in higher specificity and may offer the potential to form an ideal 'identifier barcode'. It appears that further studies involving multiplex cfDNA methylation assays that are tissue- or cell-specific are needed. High throughput screening and validation of integrated data and sample analysis sets could significantly accelerate the process towards novel viability scores combining clinical risk factors with biological profile markers.

Machine perfusion

Following many decades when the main interest in transplantation focused on graft rejection and more effective immunosuppressive regimen, currently, organ preservation is being revisited as a key phase in the cascade of events in transplantation when bridging from donor to recipient. When accepting higher risk and older deceased donor organs, optimised organ preservation strategies are required to maintain quality of the organ from the moment that it is removed from the donor until transplantation in the recipient. Dynamic preservation techniques using machine perfusion either in-situ (in the donor) or ex-situ (after retrieval) have been developed. Successful machine perfusion, either (oxygenated) hypothermic or normothermic, has been shown to be safe and feasible and is now considered a better strategy to enhance preservation, especially of those higher risk donor organs. During machine perfusion - either in-situ or ex-situ - the organ is continuously perfused with a special preservation fluid. This dynamic perfusion allows for real-time sampling and assessment of the donor organ prior to implantation in the recipient.[14-21] Also, organs initially considered not to be transplantable and discarded organs can now be placed 'on the pump' for a better assessment or even repair, making them transplantable.[15,22,23] Many reported preservation parameters or injury markers that have been published are often based on small cohorts or datasets and a thorough and systematic analysis or validation is lacking. An important aspect that emerges from this thesis is that, when analysing samples using fluorescence spectroscopy for biomarkers (e.g., FMN), one should be aware that the fluorescent signal obtained may not be an accurate predictor of the biomarker as many fluorescent compounds will fluoresce in the same region. To confirm the presence of a potentially relevant marker, we consider it necessary to use a highly sensitive LC-MS/MS method as a powerful analytical tool that will help to validate the results and prevent disappointment or incorrect assumptions (**Chapter 4**).

Consensus on the choice of injury markers and relevant endpoints regarding the quality of the graft should be a key element in future improvement of assessment of a donor organ (**Chapter 7**).[24] To date, no validated viability criteria or markers are available for liver, kidney or pancreas, and protocols of viability assessment may vary

significantly.[25] As regards in-situ perfusion using aNRP, international efforts are already underway to standardise nomenclature and agreement on a set of recommendations.

Despite great enthusiasm in the surgical transplant community and promising data, the benefit of aNRP has not been compared yet in clinical trials with better perfusion techniques than just simple static cold storage. Although in some countries aNRP is considered to be standard of care, more evidence is needed and a randomised controlled trial should be performed to evaluate the impact of this in-situ organ preservation method as a stand-alone followed by simple SCS versus either end-$HMPO_2$, end-NMP, continuous $HMPO_2$/NMP or a combination of those in organ transplantation of abdominal cDCD donor organs.

It can be expected that transplant logistics will become more challenging, as in the future more organs will be transplanted to more surgically and/or immunologically complex patients. Machine perfusion may also add logistical benefits as it offers the possibility to extend the preservation time until transplantation and facilitates more accurate and efficient theatre planning. Also, the use of Assessment and Recovery Centres (ARCs) that are in a geographically distinct area to assess dubious organs that are perceived to suboptimal or injured organs requiring repair will increase efficiency and efficacy of the donation and transplantation process whilst enhancing organ utilisation.[26] Organ transplantation can be transformed to a semi-elective procedure by reducing logistical issues. The information on the cost of various strategies of machine perfusion is still limited. Continuous (oxygenated and non-oxygenated) hypothermic machine perfusion has been shown to be cost-effective over static cold storage in kidney transplantation due to a reduction of DGF and the need of posttransplant dialysis with less graft failure and better graft survival. The cost-utility analyses for NMP strategies, including devices, disposables, perfusion solution as well as monitoring analyses should be evaluated per organ programme and country (with different healthcare systems/policies) following appropriate clinical trials that will objectively focus on short- and long-term outcomes and/or improved organ utilisation.

Another exciting but challenging topic to further advance is the resuscitation, repair, or even regeneration of the donor organ during machine perfusion. Ex-situ machine perfusion (HMP or NMP) offers a platform to apply therapeutic or immunomodulatory strategies. Several strategies are currently investigated in (pre)clinical research models that concern: (i) cell therapies, the application of stem and progenitor cells with the potential to suppress immunogenicity these multipotent candidate cells can help to repair injured tissue [27,28]; (ii) drug therapies, the administration of (immunomodulatory) pharmacological agents aiming to enhance organ viability or

assess drug safety or toxicity of anti-inflammatory or defatting substances/agents [29-31]; (iii) targeted therapies, with for example nanoparticles to target the endothelium and tubular epithelial cells [32,33] and (iv) gene therapies, to modify expression and either inhibit or stimulate pathways that lead to an improved condition of the perfused organ.[34,35] With more physiological temperatures normothermic conditions might constitute an ideal platform that creates a near-physiological environment where the organ in question is fully metabolically active and therefore targeted intervention may be best controlled and become most successful.

Conclusions

This thesis has studied several modalities how to increase the organ utilisation rate. The results in this thesis indicate that the acceptance of kidneys with acute kidney injury stage 1 or 2 will significantly contribute to the donor pool as AKI kidneys have comparable outcomes and should therefore not be discarded. Clinically relevant biomarkers such as cell-free unmethylated-*INS* DNA, FMN, GSN, IGFBP3 and IGF2R were identified or explored in the first part of this thesis and may, if analysed and/or validated thoroughly, contribute to a better assessment of organ viability supporting the justified decision whether to accept or decline the donor organ.

The second part of this thesis describes different aspects of the organ preservation technique of abdominal normothermic regional perfusion (aNRP). This relatively new machine perfusion technique has been shown to be feasible and safe, however, consensus regarding assessment parameters during perfusion, protocols and outcome measurements is still lacking. Despite of an inspiring surgical enthusiasm and keen interest to accept this modality as a new standard, a randomised clinical trial is still required and entirely ethically justifiable in order to scientifically demonstrate superiority of this method for each individual abdominal organ comparing it to other successful (ex-situ) preservation and perfusion strategies. If aNRP can be shown to obtain better post transplantation outcomes whilst increasing organ utilisation, it may be the least complex and most cost-effective strategy in organ preservation. On the other hand, aNRP will only be used in DCD donors. As such, uncertainty regarding the quality of higher risk organs from DBD donors will still be evaluated ex-situ during cold and/or warm machine perfusion with the potential to repair or even regenerate injured organs and making them 'transplantable' again.

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