PERSPECTIVE

Strategy to achieve biomarker-driven immunosuppression after solid organ transplantation by an academic-industry partnership within the European BIO-DrIM consortium

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Abstract: Solid organ transplantation has emerged as the “gold standard” therapy for end-stage organ failure as it improves both quality of life and survival. Despite the progress in short-term graft survival, that is closely associated with the impressive reduction of acute rejections within the first year, long-term graft and patient survival remain almost unchanged and unsatisfactory. Incomplete control of chronic allograft injury but particularly the adverse effects of long-term immunosuppression, such as graft toxicity, diabetes, cardiovascular events, infections, and tumours continue to challenge the long-term success. In general, immunosuppression is applied as one-size-fits-all strategy. This can result in over- and under-immunosuppression of patients with low and high allo-responsiveness, respectively. Trial- and -error strategies to minimize or even completely wean of immunosuppression have a high failure rate. Consequently, there is an unmet medical need to develop biomarkers allowing objective risk stratification of transplant patients. To achieve this goal, we engaged in an academic-industrial partnership. The central focus of the European-wide BIO-DrIM consortium (**BIO**marker-**Dr**iven **IM**munosuppression) is the implementation of biomarker-guided strategies for personalizing immunosuppress- ion to improve the long-term outcome and to decrease the adverse effects and costs of chronic immunosuppression in solid organ transplant patients. The concept includes four innovative investigator-driven clinical trials designed by the consortium.

Keywords: BIO-DrIM, personalized immunosuppression, biomarker, solid organ transplantation, stratification into high/low responder patients, Elispot, multiparameter flow cytometry, urinary IP-10, health-economic analyses

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Received: November 14, 2015; **Accepted:** December 30, 2015; **Published Online:** March 18, 2016

Citation: Volk H-D, Banas B, Bemelman F, *et al*. 2016, Strategy to achieve biomarker-driven immunosuppression after solid organ transplantation by an academic-industry partnership within the European BIO-DrIM consortium. *Advances in Precision Medicine*, vol.1(1): 34–47. http://dx.doi.org/10.18063/APM.2016.01.001.

Introduction

Solid organ transplantation (SOT) is well established to date but despite the progress in short-term allograft survival, that is closely associated with the impressive reduction of acute rejections within the first year, long-term graft and patient survival remain almost unchanged and unsatisfactory. Chronic rejection is not well controlled yet. In particular, however, the adverse effects of long-term immunosuppression, such as graft toxicity, diabetes, cardiovascular events, infections, and tumours endure to challenge the long-term success of transplantation. Moreover, multidrug treatment and side effects decrease the patients' adherence to the therapy.

Therefore, the paradigm of immunosuppression in transplant patients is presently shifting from the recent focus on searching for novel drugs to further increase "net"-immunosuppression, to the concept of minimizing long-term immunosuppression "as much as feasible" and "as early as possible". Intensive research is currently on-going to improve the treatment complexity and thus improve the adherence of patients, reduce the burden of side effects and decrease the cost of therapy.

Very few patients (liver > kidney transplantation) can be completely weaned-off immunosuppression; when achieved, this clinical outcome suggests patients are operationally tolerant. At least for liver recipients, there are data to suggest that the longer the period post-transplant, the higher the chance to become immunosuppressive drug-free. A significant proportion of patients (low-responders) can be maintained on immunosuppressive monotherapy (liver > kidney, the longer post-transplantation the more frequent), but others (high-responders) develop accelerated chronic rejection despite maintaining combined standard immunosuppression. These observations were almost completely generated by "trial and error" observational studies and warrant an unmet need to stratify transplanted patients regarding their immunological responsiveness to the allograft and define their individual need of immunosuppression.

As suggested, in order to decrease the adverse effects and costs of long-term combinatory high-dose immunosuppression in allograft recipients, many clinical trials have been conducted in which completely different immunosuppression minimization strategies have been used (mostly steroid withdrawal, conversion from calcineurin inhibitors [CNI] to mTOR inhibitors, CNI avoidance, or early monotherapy by use of new biologicals). In kidney transplant patients, complete weaning was mostly either the result of the physician´s reaction to severe complications (e.g. Post-Transplant Lymphoproliferative Disease, BKV-nephropathy) or compliance issues by the patient. By contrast, the higher incidence of spontaneous operational tolerance and the high regenerative capacity of liver following reversal of on-going rejection allowed targeted drug weaning studies in long-term liver allograft recipients. Despite some very promising results, the outcome is not satisfactory[1,2]. Minimization, even a moderate one, is failing in many patients and complete weaning is only rarely successful (liver > kidney). A disadvantage and major limitation of almost all studies so far has been the absence of patient stratification and follow-up monitoring using biomarkers in order to identify the right patient, time point, and minimization protocol as well as to detect the success or even more the failure, before clinical signs of rejection occur — in other words, clear evidence of personalized immunosuppression is missing.

From all emerging biomarkers, whose implementation might be of benefit for transplant medicine, only a few candidates have reached the methodical and diagnostic level that is suitable for clinical decision-making. With the exception of the detection of humoral sensitization by screening for panel-reactive and donor-specific anti-HLA and non-HLA alloantibodies that indicates a high-risk for SOT recipients, no

any other marker reached the level of a stratification marker that is applied in daily routine. In other words, we are far away from personalized immunosuppression but apply immunosuppression according to one-size-fits-all in two categories only (risk group according to humoral sensitization or medical history of recent graft loss by immunological reasons). However, we know about the presence of distinct individual immune responsiveness and related risk of rejection (Figure 1). There are multiple reasons favouring this situation:

(i) The development, validation, and implementation of a biomarker, in particularly if used for decision-making (companion diagnostics), is a long-lasting and costly procedure. Almost all academic studies performed so far do not jump about the road blocks on the way from marker discovery to an approved test (Figure 2).

(ii) For decades biomarker discovery was mostly focused on the non-invasive detection/prediction of rejection. However with the appearance of the more recent immunosuppressive protocols, rejection is a much lesser clinical problem than before. Search for markers allowing the stratification of patients into high- and low-responders or even operationally tolerant patients only became more popular during the last few years after realizing that many patients are over-immunosuppressed, and on contrary, some develop spontaneous operational tolerance without need for any immunosuppression.

(iii) The pharmaceutical industry did have limited interest (and experienced limited pressure by the health systems) to invest into costly companion diagnostics that may result in splitting of the market for a particular drug.

Methodical and clinical biomarker validations require interdisciplinary multicenter efforts, including academic/industry partnerships. Based on the biomarker discovery and exploratory clinical results of the recent European networks sponsored within the 6th

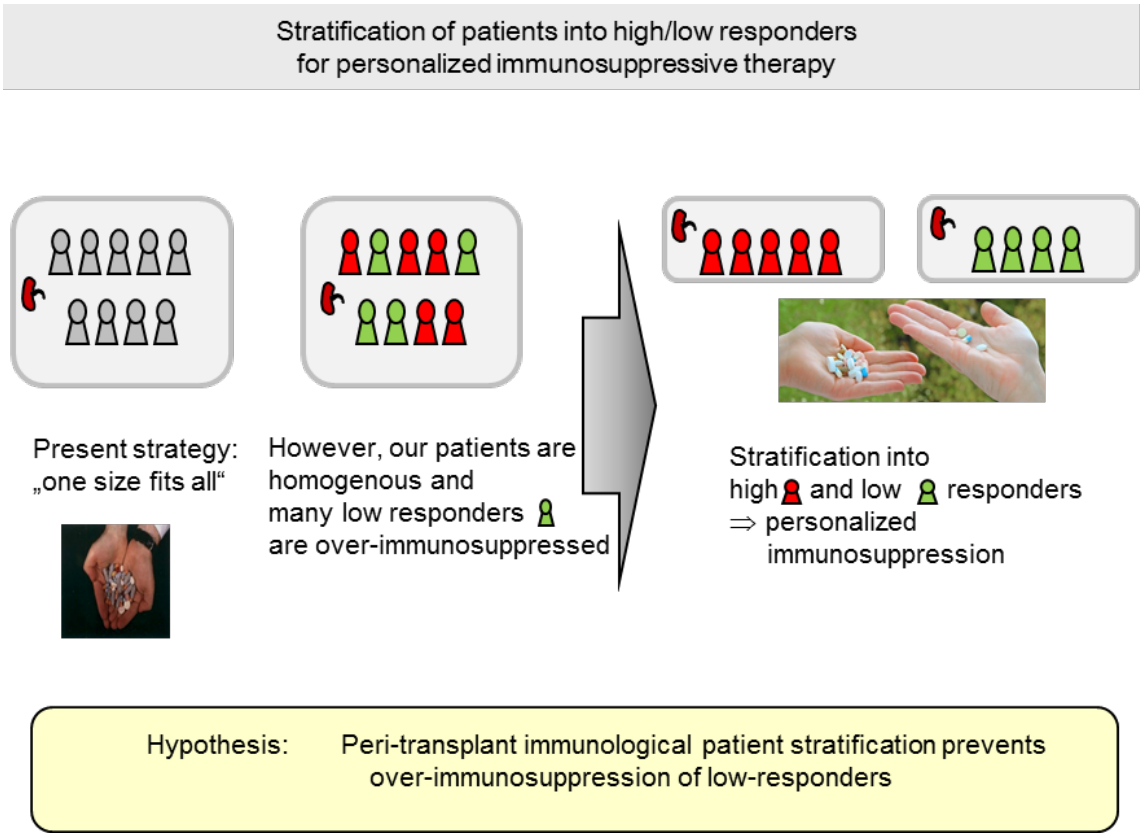

Figure 1. From “one-size-fits-all” to a biomarker-driven strategy after solid organ transplantation.
Presently, patients at enhanced risk for rejection post SOT are stratified by pre-transplant alloantibody screening and medical history only. Generally, patients are treated according to the more or less “one-size-fits all” strategy in those groups (adaption only in case of adverse effects), although trial-and-error minimization studies demonstrated heterogeneity in immune responsiveness among the graft recipients. Perioperative stratification by validated immunological biomarkers might allow safe early drug minimization. Moreover, detection of operationally tolerant patient among stable long-term allograft recipients might allow even safe complete weaning of immunosuppression in a subset of patients.

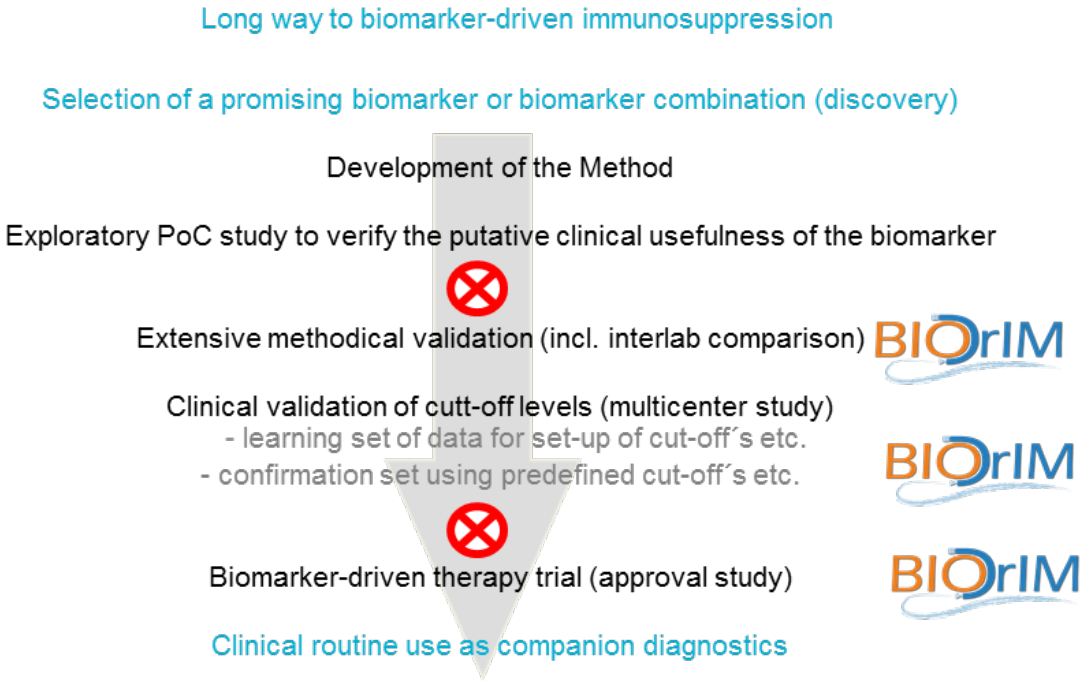

Figure 2. Long-way to biomarker-driven immunosuppression.
Most biomarker studies exploring companion diagnostics for guiding immunosuppression after solid organ transplantation that are published so far, do not reach sufficient levels of methodical and clinical validation (road blocks are indicated as ⊗). BIO-DrIM addresses several milestones on the way to personalized immunosuppression.

framework, "Indices of Tolerance (IOT)" and "Reprogramming the Immune System for the Establishment of Tolerance (RISET)"[3–16], the concept of the "Biomarker-driven Immunosuppression (BIO-DrIM)" consortium has been developed and is sponsored by the 7th framework of the European Commission (www.bio-drim.eu). The academic partners of the consortium are well experienced in performing clinical trials in SOT patients as well as the development, validation, and performance of biomarkers. The early partnering of small and medium-sized enterprises (SME´s) and diagnostic industry with experiences in the development and commercialisation of in vitro diagnostics (IVD) helps to implement standardized test procedures and will allow a fast translation of the results into biomarker product development. The embedding of professional health-economic studies into all the biomarker trials will deliver cost/benefit data that are useful for the discussions following with health insurances following marker approval regarding reimbursement options. Finally, the big pharma partner within the BIO- DrIM consortium supports the performance of challenging clinical trials. The BIO-DrIM consortium has/had to face several challenges on the way to personalized immunosuppression that will be discussed in this paper.

Material and Methods

Structure and Specific Aims of the BIO-DrIM Consortium

The BIO-DrIM (**BIO**marker-**Dr**iven **IM**munosppression) consortium has been founded at 2013 with the support of the 7th framework program of the European Commission. The full title of the project reads: "Personalized minimization of immunosuppression after solid organ transplantation by biomarker-driven stratification of patients to improve long-term outcome and health-economic data of transplantation". BIO-DrIM consists of nine academic partners (Amsterdam, Barcelona, Berlin, Hamburg, London, Nantes, Oxford, Prague, and Regensburg) and six industry partners covering well recognized experiences in clinical management of SOT patients (academic partners), development, validation, and implementation of biomarkers (academic partners), diagnostic test development and marketing (Beckman-Coulter Immunotech, Genome Identification Diagnostics (GenID)/Autoimmune Diagnostics (AID), Milenia Biotec), health economic analyses (Cellogic), drug development and marketing (Teva), and management of consortia (Alta) (Figure 3).

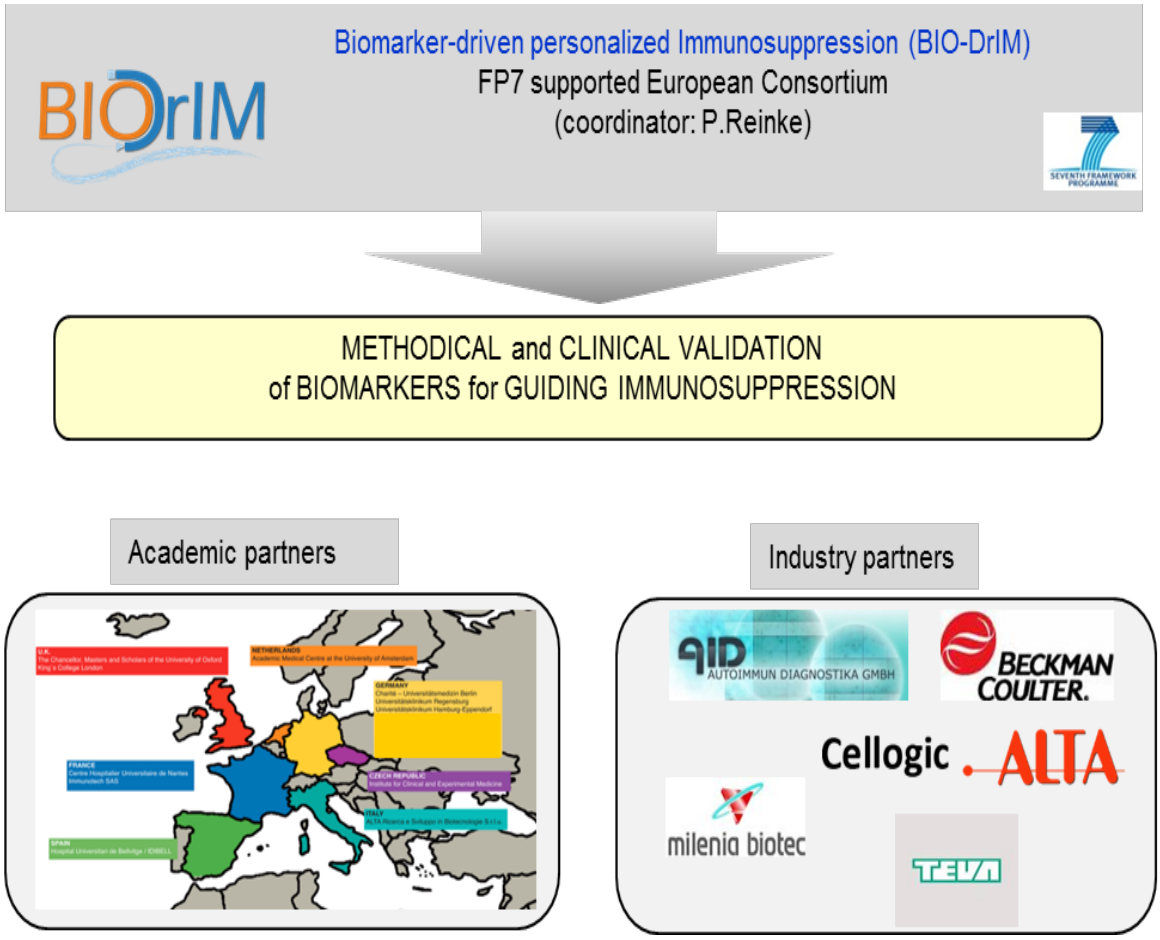

Figure 3. The BIO-DrIM consortium.
The European consortium for "Biomarker-driven Immunosuppression (BIO-DrIM)" (funded by 7th EU-framework) consists of nine academic partners (Amsterdam, Barcelona, Berlin, Hamburg, London, Nantes, Oxford, Prague, and Regensburg) and six industry partners covering well recognized experiences in clinical management of SOT patients (academic partners), development, validation, and implementation of biomarkers (academic partners), diagnostic test development and marketing (Beckman-Coulter Immunotech, Genome Identification Diagnostics (GenID) / Autoimmune Diagnostics (AID), Milenia Biotec), health economic analyses (Cellogic), drug development and marketing (Teva), and management of consortia (Alta).

The program is structured into six work packages (WPs):

- WP1 Targeted complete and partial weaning of immunosuppression in long-term stable liver and kidney transplant patients characterized as low-responders and identified by the recently established tolerance signature biomarker tests
- WP2 Prevention of the high-dose standard immunosuppression in low-responder kidney transplant recipients identified by perioperative patient stratification
- WP3 Increasing the population of kidney transplant patients belonging to the low-responder group by early targeting of recently activated alloreactive effector/memory T cells
- WP4 Biomarker analyses, data management & biostatistics, health-economic analyses, and IVD test development
- WP5 Mechanisms behind successful minimizing immunosuppression explored in preclinical studies
- WP6 Dissemination, training and other activities

BIO-DrIM aims to implement biomarker-guided strategies for personalizing immunosuppression in order to improve the long-term outcome and to decrease the adverse effects and costs of chronic immunosuppression in SOT patients. The concept includes four innovative investigator-initiated clinical trials (IITs) designed by the consortium. The expected results of the BIO-DrIM project will be:

(i) Targeted complete/partial weaning of standard immunosuppression in long-term stable liver and kidney transplanted patients identified as "operationally

tolerant" by recently developed biomarker panels

⇨ Stratification into tolerant vs non-tolerant patients for safe weaning-off immunosuppression

(ii) Prevention of high-dose standard immunosuppression in low-responder kidney transplant recipients by perioperative biomarker-driven stratification

⇨ Stratification into low- vs high-responder patients for guided immunosuppression

(iii) Shifting high-responder into low-responder kidney transplant patients who might be suitable for early minimization by the recently explored selective targeting of alloreactive effector/memory T cells

⇨ Using tolerance/rejection biomarker monitoring as surrogate markers

(iv) Implementing new biomarker candidates supporting personalized immunosuppression within the clinical trials

⇨ Biomarker discovery and PoC analyses

(v) Analysing the health-economic impact of biomarker-guided immunosuppression

⇨ The interesting exploitable potential

(vi) Studying the mechanisms behind successful weaning (regulation/effector balance)

⇨ The mechanisms of success vs. failure of minimizing immunosuppression

(vii) Disseminating the results and developing commercialization by partnering with SME/industry

⇨ Translation from clinical research to the broad implementation into clinical practice

Biomarker Tests

The biomarker platforms of BIO-DrIM consist of the following methods selected from previous work:

(i) Elispot platform: Interferon-gamma (IFN-gamma) Elispot-Assay for detecting donor-reactive memory/effector T cells[11,12,17]

⇨ Decision-making parameter (candidate for companion diagnostics)

(ii) Real-time RT-PCR platform: Gene panel expression analysis to identify molecular tolerance (and rejection) signatures[4–10,18,19]

⇨ Decision-making parameter (candidate for companion diagnostics)

(iii) Multiparameter flowcytometry platform: Several 10-color panels for the characterization of circulating immune cell subsets[20–22]

⇨ Exploratory test to detect patients at risk (high-responder) and to monitor therapy response

(iv) Ligand assay platform: Multiplex and Singleplex Elisa-based tests for quantification of cytokines in blood and urine[23,24]

⇨ Exploratory test to detect graft injury and to monitor minimization strategies

The tests are performed either in a core laboratory (gene expression, urinary cytokine levels) or in the on-site laboratories of different clinical centres under the guidance and control of a central core laboratory (all others).

Additionally, new biomarker tests/test platforms (e.g. T-cell receptor DNA and whole genome RNA expression by Next-Generation Sequencing, new multiplex ligand assay platforms) are explored in accompanying analyses by the on-site laboratories at different centres[25–31].

Results

Design of the Clinical Trials

The BIO-DrIM consortium will perform several clinical trials for the clinical validation of biomarkers within WPs 1–3. Two of them will use worldwide for the first time biomarkers as “decision makers” (companion diagnostics) in randomized multicentre studies after SOT.

LIFT — Molecular tolerance signature for guiding immunosuppression withdrawal in stable long-term liver transplant patients (biomarker-driven multicentre intervention study)

In contrast to other organs, following liver transplantation significant number of patients can stop immunosuppression without undergoing rejection. This phenomenon is known as operational tolerance. Several studies of medically-supervised immunosuppression withdrawal have been performed after liver transplantation. Until recently, however, it had been impossible to discriminate between recipients who could safely wean their drugs and those who could not. As a result, a large proportion of liver patients enrolled in immunosuppression withdrawal studies experienced rejection episodes. While the rejection episodes occurring within these closely monitored trials can be easily reversed in the large majority of cases, a small risk of irreversible graft damage remains. As a consequence, immunosuppression withdrawal remains an experimental procedure only performed in selected liver transplant units across the world. This includes efforts of the preliminary work of members of the BIO-DrIM consortium that formed the basis for this new trial described here. Thus, in the withdrawal trial recently completed by Sanchez-Fueyo *et al.*, of 98

selected recipients undergoing weaning, 42% achieved successful immunosuppression withdrawal[18].

The success rate was greatly influenced by how long after transplant was weaning attempted. For patients between 3 and 6 years post-transplant at the time of initiating weaning, only 12% achieved successful drug withdrawal (and all of them were >50 years of age). In contrast, patients 6–11 years post-transplant experienced a success rate of 38%, and in those >11 years post-transplant, the success rate was 80%, regardless of recipient age[32]. In contrast, a recent trial on early weaning during the 1st year failed completely despite using a "pro-tolerogenic" protocol[33]. To identify the state of operational tolerance and predict the development of rejection following withdrawal, Sanchez-Fueyo performed several gene expression studies for discovering and validating a biomarker signature[4]. Whole genome gene expression studies in peripheral blood comparing successfully vs unsuccessfully weaned liver transplant recipients revealed a particular molecular tolerance signature[3]. However, this peripheral blood profile of successfully weaned patients could not be confirmed as predictive for successful withdrawal if analysed in patients before initiating weaning in a prospective trial. Obviously, the presence of immunosuppression in operational tolerant patients before weaning has a significant impact on the gene expression pattern in peripheral blood, overlapping with the tolerance signature. Remarkably, however, the analysis of gene expression in liver biopsies collected before initiating of weaning revealed a robust tolerance signature predicting successful withdrawal[34].

A specific combination of gene expression markers in allograft biopsies was highly accurate in predicting drug withdrawal outcome. Interestingly, the signature was more associated with the iron metabolism as directly with immune markers[34]. Iron metabolism has, however, indirect impact on immune reactivity[35].

This novel technology could constitute the basis of a diagnostic test for detecting tolerance capable of identifying operationally tolerant recipients before an attempt at immunosuppression withdrawal is made. Such a test would radically change the long-term management of liver transplant recipients and would have a great beneficial impact in the well-being and quality of life of liver. Before this novel strategy can be applied to routine clinical practice, however, it needs to be validated within a large clinical prospective multicentre study. This is addressed in the ongoing "Prospective randomised biomarker-based trial to assess the risk-benefit ratio of a biomarker-guided immunosuppression withdrawal strategy in liver transplantation" (LIFT) supported by BIO-DrIM and MRC/NHS (PI: A. Sanchez-Fueyo, KCL).

The hypothesis of the current study is that the use of an *in vitro* transcriptional test of operational tolerance to stratify liver transplant recipients undergoing immunosuppression withdrawal results in an accurate identification of tolerant recipients and reduces the rate of rejection. To demonstrate this hypothesis, biomarker-based stratification must be non-inferior with respect to the number of successfully weaned patients, and superior with respect to the proportion of rejection episodes.

The main objective of the current study is to determine whether this novel molecular test of tolerance can be employed to optimise immunosuppression withdrawal protocol so that only operationally tolerant recipients are weaned-off drugs and the risk of rejection can be substantially reduced. In order to do so, liver transplant recipients who are 3 or more years post-transplant and who are eligible for drug withdrawal will undergo a liver biopsy to rule out the presence of occult rejection and to conduct the molecular test of tolerance. Patients will then be randomly allocated to two different strategies of gradual immunosuppression withdrawal. In the first group of patients, immunosuppression withdrawal will be performed in all recipients independent on the biomarker signature. In the second group, only those patients with a positive diagnostic test of tolerance will be weaned off immunosuppression, while patients with a negative tolerance signature will be kept on maintenance immunosuppression. By comparing the outcome of the two strategies we will be able to evaluate the clinical utility of the diagnostic test. In patients with a negative diagnostic test and regulated on immunosuppressive medication the test will be repeated at the end of the study to assess their phenotype has changed over time. Additional experiments will be performed to gain a precise understanding on the mechanisms responsible for the development of transplantation tolerance.

A total of 156 liver recipients will be enrolled in several European Liver Transplant Units. Patients will be enrolled over 18 months, and immunosuppressive drugs will be discontinued over 6–9 months. Patients will be followed-up for 3 additional years after complete drug withdrawal and the total study duration will

be 70 months.

*Cellimin — a prospective donor-specific **Cell**ular alloresponse assessment for **i**mmunosuppression **mi**-**n**imization in de novo renal transplantation (biomarker-driven multicentre intervention study)*

The current immunosuppressive therapy mainly consists from the combination of three to four immunosuppressant agents. Minimizing immunosuppression, e.g. monotherapy, as early as possible without losing control of acute/chronic rejections would be already of great benefit and could reduce adverse effects and costs. However, this is only possible in a minority of patients as yet. Therefore, a precise evaluation of the anti-donor alloimmune responsiveness in order to identify patients likely to accept the graft with no or very low immunosuppression would be of great value. One of possible approaches is the tacrolimus (TAC) monotherapy avoiding corticosteroids and anti-proliferative agents (mycophenolate mofetil — MMF), which may lead to substantial reduction of the immunosuppressive load and improve the cardiovascular risk profile. Several studies on TAC monotherapy were already published in the area of kidney transplantation. Although most of them reported relatively positive results with monotherapy, biopsy-proven acute rejection (BPAR) rates were significantly higher as compared to standard of care immunosuppression, despite using relatively high TAC trough levels which also negatively impacted to the 6/12-month allograft function. Other attempts for TAC monotherapy have been done in non-randomized, single centre pilot studies, especially using T-cell depleting agents such as alemtuzumab with rather contradictory and inconclusive results.

The assessment of the immunologic risk is exclusively based on the detection of preformed circulating alloantibodies, with the assumption that humoral allosensitization also illustrates the allospecific T-cell effector/memory immune response. This is of great importance, as it is well known that cellular memory may occur without humoral activation and that alloreactive cellular responses are key players in initiating and mediating allograft rejection[11]. In fact, with the current accurate screening of humoral sensitization, rates of antibody-mediated rejection (ABMR) have significantly been reduced but T-cell mediated acute and chronic rejection (TCMR) is still observed after renal transplantation, especially among patients not receiving standard CNI-based at least triple drug immunosuppression. A noteworthy point is that, in the last years, attempts trying to monitor the T-cell alloimmune response have been done in kidney transplant patients. Among the most robust functional assays measuring T-cell alloreactivity, the IFN-F enzyme-linked immunosorbent spot (Elispot) assay has been shown in multiple reports to be capable of accurately assess the frequency of alloreactive circulating memory/effector T-cells with donor-antigen or panel reactivity, both before and after transplantation, discriminating patients with increased risk for TCMR and worse graft function evolution, even in absence of humoral allosensitization. Furthermore, our preliminary data of a very recent PoC-trial showed that randomization of kidney transplant patients within the first two days post-transplantation by applying the IFN-gamma Elispot may allow safe personalization of the immunosuppression by using an early CNI-free protocol in renal transplant recipients identified as low-responder[37].

Therefore, pre-transplant assessment of anti-donor memory/effector T-cell alloresponse using the IFN-gamma Elispot may help to accurately discriminate patients that may safely benefit from receiving low-dose immunosuppression based on induction therapy with basiliximab (anti-CD25 monoclonal antibody) and low-dose TAC monotherapy, from others that should stay on higher immunosuppression such as the current gold-standard regimen sustained on basiliximab induction and TAC/MMF/steroid triple-drug maintenance therapy. Thus the randomized phase II trial called “Cellimin” will test the hypothesis that using the IFN-F to assess donor-specific memory/effector T-cell alloreactivity, the biomarker-driven low-dose immunosuppression will be non-inferior with respect to biopsy-proven TCMR/ABMR rate and graft function at 12 months post-transplantation, compared to patients receiving a standard-of-care immunosuppressive therapy (Figure 4).

First kidney transplant recipients that provide consent to participate in the study will be evaluated peri-operatively for their anti-donor T-cell alloresponse using the IFN-gamma Elispot. Patients with frequencies of alloreactive T cells above the defined cut-off (high-responder) will receive stand-of-care immunosuppression; patients identified as low-responder (below the defined cut-off) will be randomised 1:1 into two groups:

(i) Standard of care: the patients will be treated by standard-of-care immunosuppressive regimen based on TAC (achieving 4–8 ng/ml trough levels), MMF (1 g

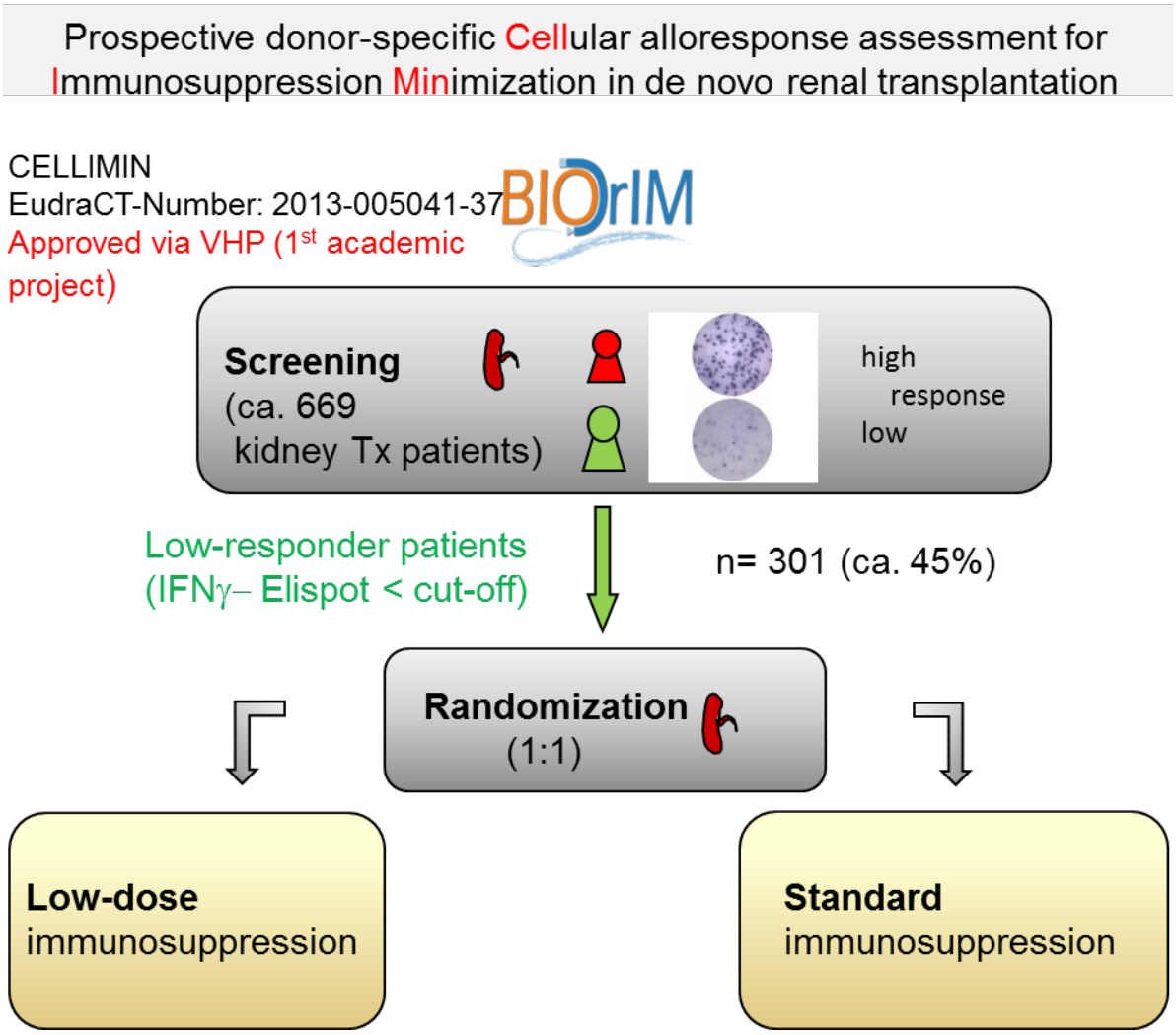

Figure 4. The Cellimin Trial Design.
The multicenter, randomized Cellimin trial is designed to verify the usefulness of the IFN FNial for stratification of kidney transplant patients into high/low responder according to their frequency of donor-reactive memory/effector-T cells. Low responder patients defined to have <25 reactive spots/300,000 PBMC will be identified by perioperative Elispot and randomized 1:1 into two groups receiving either standard triple-drug regimen or minimized therapy (monotherapy) with the aim to demonstrate non-inferiority if minimization is guided by this biomarker. High-responder patients will get standard therapy as well.

bid) and steroids (according to KDIGO guidelines).

(ii) Biomarker based minimization strategy: the low responders will be treated following a "low" immunosuppression regimen (based on TAC monotherapy to achieve 8–10 ng/ml trough levels during the first 4 weeks after transplantation and 4–8 ng/ml thereafter, MMF (1 g bid) during the first 7 days post-transplant and stopped thereafter) and steroids (tapering until discontinuation on month 2 post-transplant)

All patients will receive 2 doses of Basiliximab (day 0 and day 4 after transplantation). In addition to the decision-making Elispot, several other biomarkers will be analysed during follow-up in order to see whether low/high-responder stratification can be further improved and to understand better the mechanisms behind and stability of low/high response.

To get the approval for all European clinical centres involved in the BIO-DrIM study by the regulatory authorities of the respective countries, we applied for the very recently implemented Voluntary Harmonization Process (VHP) for multicentre trials in Europe. The European-wide multicenter study, Cellimin, got the VHP approval for our knowledge as the first Investigator-Initiated Multicenter Trial (IIT). The trial was initiated very recently.

Remini — a multicenter open-label single-arm Simon's two-stage phase II clinical trial aiming to provide evidence for efficacy and safety of the novel in-

*duction combinatory **re**gimen with rATG and infliximab to reach earl **mini**misation of immunosuppression after renal allotransplantation and a go/no go rule for further clinical development (From a biomarker with negative predictive value to a new target)*

The data from several groups, including those from members of the BIO-DrIM consortium, demonstrated an association between high frequencies of donor-reactive memory/effector T cells as sign of T-cell sensitization already before transplantation and the incidence of acute rejections and poor 1-year graft function[11,12,17]. Recent data in an experimental rat kidney transplant model revealed that induced T-cell sensitization by pre-transplant adoptive transfer of donor-reactive memory/effector T cells up to levels comparable to patients at high risk (high-responder) required high-dose CNI-based immunosuppression for preserving long-term graft function[36]. The biomarker studies suggest that selective targeting of activated donor-specific memory/effector T cells might be a new strategy to switch high- into low-responders (from a biomarker to a target) — but how might this be feasible?

Acute rejection rate in low-risk kidney transplant patients treated with quadruple immunosuppression based on basiliximab induction, tacrolimus, mycophenolate mofetil and steroids in Symphony or OSAKA trials doesn´t exceed 20%. Long term immunosuppression, however, is not only costly, but leads also to many undesirable side-effects. Minimization of immunosuppression as early as possible and further reduction of acute rejection incidence is a goal of transplant research. Rabbit antithymocyte globulin (rATG, Thymoglobulin) is a polyclonal antibody approved for prevention of acute rejection in kidney transplantation. rATG has been shown to be effective tool to decrease T (and B) cell populations for several months and thus allowing safe reduction/minimization of other immunosuppressive drugs. Reducing clonal size of alloreactive T cells by rATG followed by low-dose IS revealed promising results but only in some patients. An alternative for reducing clonal size was the anti-CD52 monoclonal antibody, alemtuzumab. Unfortunately, this antibody is not available longer for transplant patients as Sanofi after purchasing Genzyme, took it from the market by business reasons.

The disadvantage of this protocol is the alloantigen-driven lymphopenia-induced proliferation of remaining donor-reactive memory/effector T cells escaping from deletion in high responder patients. Unselective depletion/targeting of almost all memory/effector T cells also attack protective memory against pathogens and increase the risk of infections. Therefore, increasing the pool of low-responders by specifically targeting donor-reactive effector/memory T cells would have a big advantage. In a pilot trial (Viklicky O *et al*., submitted[19]) we demonstrated that a novel induction protocol based on the combination of clonal size reduction (by low-dose alemtuzumab) and selective targeting of very recently (re)activated (allospecific) effector/memory T cells (expressing temporarily mTNF) as well as acute inflammation by the anti-TNF monoclonal antibody, infliximab, allows safe monotherapy (low-dose tacrolimus) as early as after day 3 post-transplantation in all kidney transplant patients, even in patients with high frequencies of donor-specific IFN-gamma Elispot + cells before transplantation. The 5-year data revealed excellent graft function and histology (almost no signs of chronic rejection). This data allows the formulation of the hypothesis that after using the new induction protocol, the proportion of non-responders will increase and the incidence of acute rejection will remain low in tacrolimus monotherapy treated patients. Besides clinical observations, several biomarker analysis using different BIO-DrIM immune monitoring platforms are being planned to underline expected results, particularly the Elispot, molecular tolerance signature, and urinary IP-10 levels.

Primary endpoint will be the evaluation of clinical response to the new protocol determined by the absence of the following outcomes up to 12 months post-transplantation: acute rejection, graft loss or poor graft function defined as eGFR<40 ml/min. Unfortunately, the design of the study based on the promising pilot trial data had to be adapted to using rATG for initial reduction of clonal size as alemtuzumab is not available longer as suggested above. This change might be associated with some risk to reproduce the promising data seen following alemtuzumab induction together with the anti-TNF approach. Therefore, the Remini trial is designed as Simon`s two-stage trial with interims analysis after 64 patients before enrolling all 161 patients.

Implementation of the Biomarker Test Analyses

After selecting the biomarker portfolio for the clinical validation trials based on preliminary work and proof-of-concept trials, the consortium decided whether the

tests will be performed at a central core lab or under supervision of a core lab at the onsite labs. The complexity of the method, the preanalytic prerequisites, and the time window in which the results have to be provided, determined this decision.

Most critical was the standardization and implementation of the decision-making markers — the gene expression profile in liver biopsies and the IFN-gamma Elispot in the peripheral blood in order to stratify the transplant patients according to their molecular tolerance signature into tolerant/non-tolerant liver transplant recipients and their pre-transplant frequency of donor-reactive memory/effector T cells into low/high responder kidney transplant recipients, respectively.

Molecular Tolerance Signature for Guiding Immunosuppression Withdrawal in Stable Long-term Liver Transplant Patients

For the immunosuppression withdrawal study in stable long-term liver transplant patients the biomarker gene expression analyses by RT-PCR and microarray can be performed by a central core lab that process and analyse the samples collected from all clinical centres as the delivery of data within few weeks is sufficient to guide the recommendation of stepwise weaning until complete withdrawal. Although this strategy reduces the challenges of interlab comparability, such as different equipment, procedures, technicians etc., extensive standardization, including preanalytics, intra-/inter-assay and inter-operator variability, had to be ensured. After ensuring these criteria, the clinical trial could be very recently started after we got the approval by the regulatory authorities few weeks ago.

Identification of High/low Responder Renal Allograft Recipients by Quantifying the Pre-transplant Donor-specific Memory/effector T-cell Response

In contrast, an onsite monitoring in each clinical centre is required for the perioperative stratification into high-responder/low-responder kidney transplant patients to identify the low-responder patients and to randomize the low-responder patients into the groups of standard or minimized immunosuppression, as the data has to be available within 48 hrs post-transplantation. This required a dissemination of the test system (Elispot) to each clinical study centre lab and interlab comparisons to validate the robustness and correctness of the assay. In close collaboration between the core lab at Berlin, the study centre laboratories, and the provider of the Elispot system/test (GenID/AID), we were able to implement after 1.5 years intense work a well validated assay fulfilling the criteria required[38]. Most importantly, interlab comparisons confirmed correct categorization of almost all patients into high/low-responder according to the predefined cut-off of IFN FNdefined T-effector/memory cells/300,000 PBMC (Figure 5).

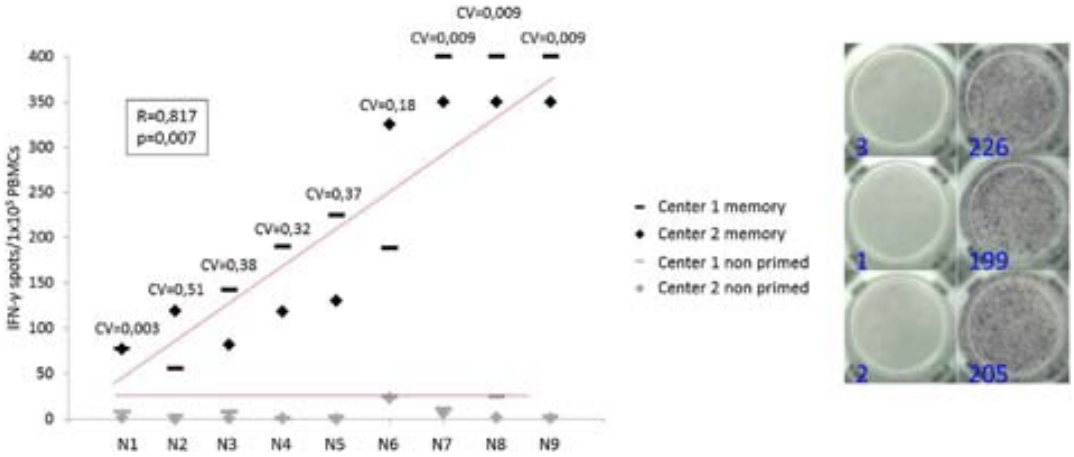

Figure 5. The interlab methodical validation of the IFN FNthodic assay.

Extensive work was performed to standardize the decision-making Elispot. After implementation and training, several interlab comparisons were performed. The data shown here, exemplary demonstrate the correct categorization of all patients by two centers (Barcelona and Berlin) into high/low-responder groups. In the double-blinded interlab comparison, nine samples were categorized by both centers into low responder (non-primed) patients, two of them even near the cut-off level of 25 spots. The remaining nine patients were categorized as high-responders (memory) by both centers with levels between 50 and 400 spots. The data showed very high correlation between the two centers ($R>0.8$; $p<0.01$). Moreover, the variation (cv) was low in intralab and interlab comparison.

The right panel shows exemplary the images for one low responder and one high-responder patient in triplets.

As the precision of the test is a key factor for the successful outcome of the clinical trial, the interlab comparison will be repeated every year.

Exploratory Biomarker Studies

In addition to the two decision-making tests described above, the consortium will analyse several parameters either in central core laboratories or in on-site laboratories in order to explore the values of further biomarkers for stratification, safety, monitoring of therapy response etc. The collaboration with partners from the diagnostic industry will further improve the quality of the test performance, particularly for multi-parameter flow cytometry and quantification of urinary IP-10 levels. Recently, we established in collaboration with industry several 10-color flow cytometry panels for quantifying different immune cell subsets in whole blood[20]. These panels were improved further for the BIO-DrIM studies and adapted to the recent Duraclone^{TM} technology allowing long-lasting storage and easy-to-handle procedure, and delivers even lower variability (Streitz *et al.*, in preparation[20]).

One additional goal is the identification of a molecular tolerance signature in long-term stable kidney transplant patients in order to allow safe minimization of immunosuppression in operational tolerant patients, like in liver transplant patients. In several independent studies, a particular B-cell signature was most prominently and robustly associated with stable drug-free kidney transplant recipients[5–10]. However, it is not clear whether this signature seen in stable drug-free patients weaned-off immunosuppression because of non-compliance or medical indication (mostly lymphoma), can be also seen in operationally tolerant patients who are still on immunosuppression. In contrast to liver, operational tolerance is a rather rare event after kidney transplantation and targeted weaning is ethically difficult because of the lower regenerative potency of renal allografts following undesired rejection. Therefore, another BIO-DrIM study of WP1 focuses on the evaluation of this signature in many long-term renal allograft recipients kept on different immunosuppressive schemes to get a better impression on the incidence of this signature and the impact of distinct drugs. Moreover, in a small multicentre study based in France, carefully selected long-term stable kidney transplant patients will be step-by-step weaned-off immunosuppression under intense clinical control and biomarker monitoring. Both trials are on-going.

Discussion

The aim of the consortium is to develop biomarker-driven immunosuppression for SOT patients in order to change the presently used one-size-fits all strategy to a more personalized approach. Based on extensive preliminary work of both academic and industry partners of the consortium, ready-to-go biomarker tests using different technology platforms were selected and clinical studies were designed for their validation. Two BIO-DrIM studies will validate for the first time decision-making parameters for patient stratification in randomized multicentre studies.

The following major challenges had to be faced during the start-up phase:

(i) Implementation of a functional biomarker (IFN-gamma Elispot) at different study sites at the high performance quality that it is required for a decision-making parameter,

(ii) Underestimation of the personal and financial resources needed for the preparation and performance of such complex multicentre IITs. The new VHP tool has significant advances but is also very challenging for an academic consortium consisting of several legal entities because of the short time lines for responses to comments predefined by the regulatory authorities,

(iii) Limited interest by the majority of big pharma to implement biomarker-driven personalized therapies after marketing approval of a specific drug for the whole patient population; and unexpected changes in the marketing strategy of big pharma with major impact on our consortium structure, the study design, and study population (need or re-designing Cellimin and Remini trials because of strategy changes by big pharma)

(iv) Different reimbursement systems within Europe challenge the health-economic analyses.

Nevertheless, the consortium made significant progress since its formation about two years ago. All but studies are carried out and results can be expected soon. It could be a big step forward on the way to personalized immunosuppression after SOT in order to improve the cost/benefit and side effect/efficacy ratio.

Conclusion

The implementation of biomarker-driven immunosuppression is challenging and requires a multicentre academic/industry partnership. The BIO-DrIM con-

sortium might be a road model for such an approach. It includes joint activities for the development, validation, implementation, and analysis of biomarkers; the design, getting of approval, and performance of clinical studies, accompanied by health-economic analyses (see accompanying paper). To implement the biomarkers into daily routine, next steps after successful clinical trials have to be the registration as IVD by FDA and EMA. The regulatory rules are still quite different between Europe and US. Scientific advice meetings with both agencies in 2016 will help to define the next steps on the road more in detail.

Author Contributions

HDV: writing manuscript, supervising biomarker tests; BB, FB, MK, BN, IRJMB: help in designing clinical trial; OB and JG: WP2 co-leaders, design Cellimin study; SB: WP1c leader, design weaning study after kidney transplantation, MH-F: WP1b leader, biomarker analyses in long-term kidney transplant patients; IR-M: biometry, study designs, AS-F: WP1a leader, design LIFT study, liver tolerance signature; BS: WP4 co-leader, flowcytometry; OV: WP3 leader, design Remini study; CC and KW: experimental work; PR: coordinator BIO-DrIM consortium, manuscript writing, design Cellimin and Remini studies.

Conflict of Interest and Funding

No conflict of interest was reported by all authors contributed. The BIO-DrIM consortium acknowledges the support by the 7th framework program of the European Commission and the additional funding by the industry partners of the consortium.

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