Thank you very much. First of all I’d like to thank the organisers for inviting me. I’d like to summarise some of the data from the recent European network.
and I think it’s clear that there’s a shift in the therapy paradigm or after transplantation. During the 90s we increased and introduced new immunosuppressive drugs to improve the results and the results are quite good, we have less acute rejections, we have better 1 year graft survival despite using more and more marginal organs but we have to say we have no significant improvement of long-term results and many adverse effects by the chronic immunosuppression and of course high costs.

Slide 3

Therefore, the new strategy is to minimize the long-term immunosuppression as much as possible or as much as necessary or as little as possible and that needs really to take into account the individual immune response profile and to get a personalised immunosuppression.
On the other hand, of course, there is one aim to increase the proportion of patients who are suitable for minimizing because of course we have so-called high responders and low responders. Some you can define simply by clinical points like β-transplantation, HLA antibodies and so on but even the so-called low risk you have higher and lower risk and that is only possible to define by immune response profiling. Therefore, of course, there’s the idea to find new tools to increase the proportion of patients which can be partially or even totally weaned. The maximum, of course, as Jean Paul just explained is to go to complete weaning.

That needs a monitoring as a risk of over-immunosuppression and the monitoring of successful tolerance induction because if you use new induction protocols which support regulation of course at the beginning you can also induce over-immunosuppression. Therefore, there is a clear need for biomarker guided immunosuppression. That was one of the aims of the recent network.

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The problem is there’s a long way to a biomarker guided immunosuppression because first of course the easiest way is to select promising candidates but here’s the first hurdle that we have really stable methods which can be reproduced in different labs at different time points and here we have to filter many of the tests published because the criteria of the methodological validation is not sufficient.

The next step of course is you need proof of concept studies to verify the productive clinical usefulness and then you need really to be extensive. If you have a promising candidate you need extensive methodical validation by interlap comparison and so on and that was part of RISET and we now really have progressed for example from the 80s as Jean Paul just suggested. Now we are able to measure it at different places in a blinded way with a variation of less than 20% that means we are now on the way that we can really compare the data.

Finally of course, and most importantly the clinical validation, learning set, confirmation set and finally to biomarker guided immunosuppression trials which is the next step and has been planned for one year by Jean Paul but --.
There's clear need for multiple cross-platform tests, need for high-quality standardization, need for multicentre clinical validation and therefore, we need really the collaboration between the centres and that is on a good way and therefore, it is very helpful to have international networks like RISET, IOT or ITN.

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The RISET was one work package. You see it's quite an international collaboration work package for the diagnostic tests

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and we had some core labs, Miltenyi for microarray and TcLand for the T cell receptor analysers, Leiden for the HLA antibodies, Brussels, Amsterdam, Berlin and of course all the onsite labs in the clinics, very professional labs particularly in Nantes or Barcelona and so on. Based on the data we had and on the quality of the methodology validation we selected so-called core tests which have promising preliminary and methodically well validated data, consensus selection of the network and of course all the problems of how much material can be supplied and we divided them into obligatory and optional tests. Then we have of course, in-house tests, our own research projects, exploration of new markers and time/material-sensitive things which are not able to be transported to a core lab. Finally a central data management for biostatistics.

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### What was the rationale for selecting the RISET biomarker portfolio?

1. **Safety markers**
   - Rationale: to detect overimmunosuppression by novel approaches
   - HLA-DR+ monocytes (Berlin)
   - Anti-viral (EBV/GMV) memory T-cell response (Berlin)
   - Viral load (EBV/GMV/BKV) (Berlin)
I’ll show you a couple of reviews on these markers we are using. The first were the safety markers to detect over-immunosuppression by any novel approaches. One is HLA-DR expression of monocytes, other ones are anti-viral memory T cell response and viral load and I’ll explain that to you shortly because many of you are probably not familiar with the HLA-DR. It’s a very simple parameter.

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The blood monocytes circulate about 1 day in the blood and then they come from the bone marrow and then they go through the tissue, differentiate into macrophages and express in the human cysteine in contrast to the mouse cysteine or rat cysteine almost all monocytes express HLA-DR at different expression levels and are very tightly regulated. We have a lot of negative regulators like stress hormones, IL-10 on the other hand proliferative IFNγ and GM-CSF. Of course immunosuppression down regulates by two ways first a direct effect like cortisol on HLA-DR expression – inhibits T cells to produce IFNY, GM-CSF therefore resulting in a decrease of monocytic class II.

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If you have a dramatic decrease to less than 8000 molecules, normally you have 20,000-35,000 molecules per cell in the peripheral blood and in the immunosuppressed patient between 15,000-25,000 and if you have a dramatic decrease, then you have evidence for so-called immune paralysis which is associated with very high risk of infection particularly bacterial and fungal infection. For example, I’ll show you one example from a recent cell therapy trial with the mesenchymal stem cells. You see immediately after giving the cells, you have a dramatic drop in the class II expression for a couple of days but fortunately – patients went to the immune paralysis but therefore, it is important to know if you use it all new immunosuppressive drugs, new antibodies in the phase I trials.

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<td>ii. alloreactive memory T cells (IFNγ ELISPOT) (Berlin/Barcelona)</td>
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<td>iii. alloreactive CTL precursor frequency (CTLD) (Leiden)</td>
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<td>iv. alloreactive T-cell activation by gene expression (Brussels)</td>
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Ok another couple of data look for the donor-specific alloresponsiveness you just ask for high sensitive alloantibody tests including LUMINEX and so on, alloreactive memory T cells by ELISPOT, alloreactive CTL precursor frequencies and alloreactive T cell activation.

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What was the rationale for selecting the RISET biomarker portfolio?

1. Safety markers
   - rationale: to detect overimmunosuppression by novel approaches
     i. HLA-DR+ monocytes (Berlin)
     ii. anti-viral (EBV/CMV) memory T-cell response (Berlin)
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2. Donor-specific alloresponsiveness
   - rationale: to detect humoral and cellular allostimulation
     i. high sensitivity alloantibody tests (Leiden)
     ii. alloreactive memory T cells (IFN; ELISPOT) (Berlin/Barcelona)
     iii. alloreactive CTL precursor frequency (CTLP) (Leiden)
     iv. alloreactive T-cell activation by gene expression (Brussels)

3. Intrgraft inflammation (kidney)
   - rationale: to detect intrarenal inflammation
     X i. urinary IP-10 levels (Berlin)

Finally whereas the first two tests are the most robust for the daily work, the next one only used for the kidney is a local measurement of the inflammatory reaction by urinary IP-10 levels. You have now a bedside test, within a few minutes you can see how much inflammation you have in the kidney.

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Urinary IP-10 => early non-invasive marker of intrgraft inflammation

- subclinical immunological attack
- rise of serum creatinine
- non-invasive immune monitoring for detecting "subclinical" processes
  => urinary IP-10=CXCL-10 (IFN; induced)
Of course, the idea is to detect the inflamed graft whatever the reason is, by subclinical immunological attack, by rejection or whatever and if you have a rise of serum creatinine that means you have inflammation of the graft and mostly associated with graft injury. To detect it by subclinical processes IP-10 is a very reasonable parameter.

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What was the rationale for selecting the RISET biomarker portfolio?

4. T-cell receptor repertoire
   rationale: to detect (allo)antigen-driven oligoclonal expansions
   i. T-cell landscape (TcLand)

The next was a T-cell receptor repertoire by TcLand, the rationale is to detect antigen driven oligoclonal expansions that means if you have an immunopathologic process like rejection, you expect a bias and that is indeed the case.

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That means we have a normal polyclonal repertoire that means each T cell is recognized as a different thing. If you have an antigen driven process like alloantigen, then you have a clonal expansion like the rat clones here which you can see in the profile and if you have a target therapy, you can see a normalization of the oligoclonal T cell receptor repertoire but I will to come back to this point.

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<td>5. Immune pre-activation / silence level</td>
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<td>rationale: to measure immune activation / tolerance balance (qRT-PCR)</td>
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<td>i. Tog-1 (tolerance-ass-gene-1) (Berlin/Oxford =&gt; Berlin)</td>
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<td>ii. FoxP3/alpha-1,2-Mannosidase (Berlin/Oxford =&gt; Berlin)</td>
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<tr>
<td>iii. TORID (tolerance-related and induced transcript) (Nantes =&gt; Berlin)</td>
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<td>6. Gene Expression Profiling</td>
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<td>rationale: to detect novel molecular signatures ass. with tolerance</td>
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<td>i. microarrays RISET (Miteny)</td>
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Last but not least we have two sets of tests: one for detecting immune pre-activation and immune silence, there are a couple of genes which came from animal experience in a collaboration from --- from our lab and – from Oxford and --- help to detect and also the one from --- but it looks like the most promising and FOXP3 and last but not least of course the gene expression profiling which was more – to look for – as told to you by Jean Paul before.

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Ok the question we addressed by the biomarkers is clear. First it must be general aspects, it must be stable, it must be easy to reproduce, material collection feasible and cost-effective.

Then of course we have specific aspects, the detection of the patient subgroup suitable for weaning or minimizing immunosuppression is the first aim by measuring the level of immune response profile or even by tolerance signature.
The first category of studies was long-term stable kidney or liver transplant patients, identification of operational tolerant patients based on the – work of Jean Paul’s group for the kidney and Alberto Sanchez-Fueyo from Barcelona for the liver.

The idea is clear if you have two groups the patients who are stable and drug free as defined just before and stable on immunosuppression or chronic rejection you have 3 different groups and you can look for differential expression. Indeed there’s a clear discrimination by biomarkers and molecular signature, functional tests and flow cytometry. I won’t go through the details because Jean Paul has done it extensively and that’s why we have several different results.
Interestingly for example the B cell expansion was really confirmed independently in different trials from the Nantes group, from the European network including some Nantes patients and of course, ITN in a blinded cross-over study.

The next step is, is it possible to identify the tolerant patients if they are still on immunosuppression? That’s the most challenging one because you want to see it before. One study we performed in RISET led by Alberto on the liver trial because we looked for the biomarker profile and looked at whether it was possible to categorise the patients before. Unfortunately, in the peripheral blood it failed. It was not as good as in the post weaning strategy.
Fortunately, it looks very promising by liver biopsies as suggested by Jean Paul that means that in the peripheral blood it was not as good with a marker identified here to predict. We will see how it is working for the kidney and we hope with the new network we are performing now in the Nantes --- we hope can really predict that is -- prediction. The molecular signature is clear-- kidney and interestingly as suggested by Jean Paul already, it’s a completely different pattern in kidney and liver transplant patients, the same as in the RISET data comparing liver and kidney.

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Category II  Minimizing immunosuppression as early as possible--
identification of low-responder patients

As drug-free is rarely possible - Who would be happy with less IS ?
Personalized minimizing IS based on low-responder signatures

So the next category of patients was to minimise because we have also performed two tolerance trials one – based on a cell therapy inducing regulatory T cells and failed, the total weaning failed but interestingly all patients were on monotherapy and that means there’s a success that you can go on minimisation.

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Another trial immediately after liver transplant in Brussels also failed to induce tolerance that means abuse in the first years is very difficult to induce tolerance after liver transplantation in contrast to year 5 or 10. That means therefore we focused on a second point to minimise immunosuppression as early as possible by helping to identify the low responder patients. That means it’s clear the idea.

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At the moment we are doing try and error.

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Some patients are weaned and all patients are minimised, some are successful and some develop rejection or even if you have no rejection you see it now in the Zeus trial after 2 years you see it’s different and some patients have a poor function. Therefore it’s important to identify the low responders that are really suitable for minimising as early as possible.

There are several data now for example for the Zeus trial we could see that before starting with a switch from CNI to non-CNI after 5 months if you take a molecular profiling you see a clear different between patients later developing rejection or poor function versus the other one.
That means for the future it should be possible to categorise the patients better. A couple of markers: T cell alloresponse, urinary IP-10 at a particular molecular signature and a couple of papers will come out hopefully very soon.

The next point is to ask is it possible much earlier? That means immediately after transplantation and that means if you could identify peritransplanted not only 5 months later that would be fine to start with completely CNI free protocols immediately after transplantation.
The question is how is that possible? The hypothesis is clear, the individual immune response profile discriminates between high and low responders already before transplantation.

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How can you measure it? The alloreactivity is a mixture, it’s not only a donor specific response because we have a complex system which influences alloreactivity as a sum.

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Allosensitisation by the actual graft, preformed allospecific memory by cross-reactivity against infection or whatever or a previous transplantation, bystander activation is a very important point which is not simulated in the animal models because antiviral T cells can help alloresponse and intragraft inflammation particularly using marginal organs and the pre-injury of the graft as well.

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The question is how can we measure it? There is overall activation markers of adaptive immunity unfortunately I have no time to go through that but a quite promising one, for example Toag-1 expression and of course allospecific reactivity measured by ELISpot. You see I’ve put in these 3 categories patients who have pre-transplant very, very high levels of donor specific memory whatever the reason is mostly unspecific cross-reactivity. Patients that have a couple of hundred – cells – thousand and already develop very, very severe rejection, poor 1
year graft function. Then you have an intermediate category which develops a high frequency of rejection, poor 1 year graft function and then you have low.

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The question is what is the difference between humans and rats or humans and mice?

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The problem is normally in the mouse you have almost no memory T cells because we have very clean living conditions and therefore our cysteine is not very predictive. But if we introduce in the rat and the mouse system and in this case the rat donor reactive memory cells to the same level as in the patient, you see intermediate and high depending on the time you give the cells, you can simulate the human situation and the answer is clear if you compare
now the rat cysteine with the patient situation, it’s clear if you have low donor reactive memory T cells you need no CNI.

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If you have intermediate, you need low dose CNI or rapamycin is working. If you have high donor memory, you need really high dose permanent CNI.

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In this case the question was clear that if you have low donor memory in patients, it should be possible to prevent any CNI treatment from the beginning. That was the rationale for the study performed – in Barcelona to identify peritransplant low responders by the ELISPOT and allows safe CNI avoidance.
The results are quite promising.

that was the principle scheme of the study in Barcelona and a short ATG induction and then performing in parallel the ELISpot you need two days for the ELISpot you have the results and day 3 you can make based on the results a decision on whether the donor specifically – patients over a threshold that goes to the traditional CNI arm if not it goes to the mTOR arm from the beginning.
A very short summary CNI avoidance after pre-selection is safe. You have a low rejection rate, you have an overall good graft function months 6, 12, and now also 24 is available. You have a low infection rate.

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Based on this data we can also now say we can use this biomarker in our research for the discovery of novel targets of the immunosuppression and one is of course donor specific cells are the bad guys and limiting the pool size of poor responders.

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In other words if you target donor specific memory T cells are the bad guys and limiting the pool size of low responders. In other words if you target donor specific memory, you should increase the pool of low responders to have a higher proportion of patients which are suitable for minimised immunosuppression.

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The question is how can we do it? We started in the RISET and we continue now in the follow up project with 3 different approaches. One was the so-called Mregs that are regulatory macrophages from Kiel/Regensburg group, Faendrich/Hutchinson and it looks very promising. You cannot reach tolerance but you can obviously reduce a number of memory/effector cells and I’ll come back to this point.
A second one is a combination of two antibodies---that was a collaboration between Prague Vicklicky and Reinke from Berlin targeting specific activated effector cells not a complete depletion because that might be dangerous and finally as use of regulatory T cells and we have formed now a new European network ‘the one study’ another leadership of Regensburg to perform this.

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For this if you use such kind of new trials you need of course markers for detection of over-immunosuppression and that pathogen control is not targeted

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Detection of ‘over-immunosuppression’ if novel IS protocols are used—saving pathogen control

Safety markers

- rationale: to detect overimmunosuppression by novel approaches

- HLA-DR+ monocytes (fungal/bacterial control)

- anti-viral (EBV/CMV) memory T-cell response

- viral load (EBV/CMV/BKV)

both novel protocols targeting activated memory/effector T cells and supporting regulation

Macs: Kiel/Regensburg

targeting T effmem by mAb combination: Prague/Berlin

did not harm pathogen control
and we use the safety markers I told you and the message is quite easy. The protocols we have used so far, the Mregs and the combination of two monoclonals both novel protocols are supporting regulation and did not harm the pathogen control. The most important message we have clear safety which can help you to go forward in the direction.

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Questions addressed to biomarker research

- general aspects:
  - stable and reproducible
  - material collection feasible
  - cost effective

- specific aspects:
  - detection of patient subgroup suitable for weaning or minimizing IS
    - individual immune response profile (activation level)
    - tolerance signature
  - discovery of novel targets of immunosuppression
    - up-regulation of markers associated with failure of weaning
    - detection of „over-immunosuppression“ in case of novel IS protocols
    - pathogen control (safety)
  - monitoring success failure of novel protocols

Finally, of course it’s important to monitor the success/failure of such kinds of novel protocols, are they able to increase the low response immune profile?

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I’ll show you two examples for example with the Mregs which were used in the living donor situation you give it at day 7 before you come with a therapy and quite interestingly the two
patients who had a quite high number of memory T cells in the biased T cell receptor repertoire measured by the TcLand if you look a couple of days after giving the donor macrophages you have complete normalisation of the profile indicating a disappearance of the pre-activated memory cells. Interestingly it was a completely successful monotherapy in one and a half years.

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Another interesting marker I had no time to explain it before, Toag-1 is a marker of general silence alloactivation in the peripheral blood measured by PCR and what you see is that the patients from the Kiel had very high activation that means they have a down regulation of Toag, much lower than healthy controls or low responders and giving the donor derived macrophages a couple of days later, one week, all patients recovered to normal or even supernormal levels of silence indicating that the Tregs reach what we wanted, a silence of the immune system.

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Last but not least a summary, I think operational tolerance and low responsiveness are associated with a particular biomarker signature and retrospective analysis and the biomarker selection/standardisation is quite good now. The clinical biomarker validation retrospective is fine, prospective is on-going because it is very sensitive as Jean Paul explained and therefore several groups including the Nantes group first again validate the biomarkers that we can really use it for a guidance to see who is suitable or not. Second the increase of pool size for low responders seems to be possible by novel approaches targeting effector cells, Mregs, Tregs and proof of concept trials were done and now the control phase trials for the new network are in preparation.

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Thank you very much.
Thank you!

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