Abstract: The present application relates to a method for diagnosing graft intolerance or for assessing the risk of graft intolerance in a patient, the patient having received a liver transplant, comprising the steps of: (i) determining the level of antibodies against protease-activated receptor 2 (PAR2) in a sample from the patient to be diagnosed; (ii) comparing the determined level in the sample to a control level derived from subjects tolerating a liver transplant/grant, preferably not showing graft rejection and/or fibrosis; wherein an increased level in the sample from the patient to be diagnosed as compared to the control level is indicative for the presence or the risk of graft intolerance in the patient. Furthermore, the application provides a method for differential diagnosis of graft rejection and fibrosis. Also provided is a method for assessing the need of a patient for administration of an immunosuppressant.
PAR2 ANTIBODIES FOR DIAGNOSIS OF GRAFT INTOLERANCE OF A LIVER TRANSPLANT

Technical field of the invention

The present invention is in the filed of biology and biochemistry, more particular in the field of immunology as well as diagnostics and therapeutics. Even more particular in the field of diagnosis of graft intolerance after liver transplantation and the need of immunosuppressant for the treatment of graft intolerance.

Background of the invention

Liver transplantation or hepatic transplantation or Living-donor liver transplantation (LDLT) is the replacement of a diseased liver with some or all of a healthy liver from another person (allograft). The most commonly used technique is orthotopic transplantation, in which the native liver is removed and replaced by the donor organ in the same anatomic location as the original liver. Liver transplantation is a viable treatment option for end-stage liver disease and acute liver failure. There has been a rapid increase in the number of living-donor transplants because of the shortage of deceased donors.

A liver transplant will be rejected by the recipient unless rejection is prevented by immunosuppressant agents. A major problem that has remained unsolved is the dependency of the patients on nonspecific life-long immunosuppression (IS) accompanied by complications (Jonas S, Neuhaus R, Junge G, et al. Primary immunosuppression with tacrolimus after liver transplantation: 12-years follow-up. Int Immunopharmacol 2005; 5: 125). Indeed, most of the causes of short and late morbidity following liver transplantation are associated with the immunosuppression (Sood and Testro: Immune monitoring post liver transplant (2014), World J Transplant; 4(1): 30-39).

The immunosuppressive regimens for liver transplants are fairly similar to those of other solid tumors, and a variety of agents are meanwhile available. A common regimen
comprises the administration of corticosteroids plus a calcineurin inhibitor such as tacrolimus or cyclosporin plus a purine antagonist such as mycophenolate mofetil. Many of the causes of short and late morbidity following liver transplantation are associated with immunosuppression or immunosuppressive medications. Current treatment often involves close monitoring different parameters, like liver biochemistry as well as immunosuppressant levels and liver biopsies (Sood and Testo: Immune monitoring post liver transplant (2014), World J Transplant; 4(1): 30-39). However, liver transplantation can often be associated with significant complications including infection and rejection due to immunosuppressant over or under dosing, showing an inadequacy in current immune function monitoring. Many assays have been tested in the research setting to identify possible biomarkers that may be used to predict clinical events such as graft rejection, and therefore allow modification of a patient’s immunosuppressive regimen prior to a clinical event. However, these generally require significant laboratory processing and have had difficulty becoming established in common clinical use outside the research setting. For determining and monitoring the need of immunosuppressant treatment biopsies are still the gold standard. They include surgical treatment accompanied by further risks (Banff Working Group on Liver Allograft Pathology. Importance of liver biopsy findings in immunosuppression management: biopsy monitoring and working criteria for patients with operational tolerance. Liver Transpl. 2012 Oct;18(10):1154-70; doi: 10.1002/lt.23481. Review. PubMed PMID: 22645090). E.g. Fibrosis cannot be diagnosed without biopsies, but the attendant complications include serious hemorrhage, pneumothorax and biliary peritonitis, possibly even death.

Liver transplantation is unique in that the risk of chronic rejection also decreases over time, although the great majority of recipients need to take immunosuppressive medication for the rest of their lives. It is possible to be slowly taken off anti-rejection medication but only in certain cases. It is theorized that the liver may play a yet-unknown role in the maturation of certain cells pertaining to the immune system. Immune-suppression is needed as long as the patient shows graft intolerance. Furthermore, graft intolerance may also reoccur after successful weaning of the immunosuppressant agents. Nevertheless, the liver allograft can often be maintained after transplantation with low levels of immunosuppression and in some cases be withdrawn completely without histological damage from rejection – defined as operational tolerance.
However, until today the reliable diagnose of graft intolerance after liver transplantation is an unmet need. Likewise, it is not yet possible to determine whether a patient is in need of ongoing treatment by an immunosuppressant.

Summary of the Invention

The inventors interestingly found that the presence of autoimmune antibodies against protease-activated receptor 2 (PAR2) indicates graft intolerance of a patient having received a liver transplant. PAR2 antibodies in patients suffering from graft intolerance, like graft rejection or fibrosis, were present and their levels were increased as compared to patients not showing symptoms of graft intolerance.

The present invention relates to a method for diagnosing graft intolerance or for assessing the risk of graft intolerance in a patient, the patient having received a liver transplant, comprising the step of determining the presence or absence of antibodies against protease-activated receptor 2 (PAR2) (anti-PAR2 antibodies) in a sample of the patient, wherein the presence of anti-PAR2 antibodies is indicative for the presence or the risk of graft intolerance in said patient.

The present invention also relates to a method for diagnosing graft intolerance or for assessing the risk of graft intolerance in a patient, the patient having received a liver transplant, comprising the steps of:
(i) determining the level of antibodies against protease-activated receptor 2 (PAR2) in a sample from the patient to be diagnosed,
(ii) comparing the determined level in the sample to a control level derived from subjects tolerating a liver transplant/graft, preferably not showing graft rejection and/or fibrosis;

wherein an increased level in the sample from the patient to be diagnosed as compared to the control level is indicative for the presence or the risk of graft intolerance in the patient.

It has been found by the inventors that levels of PAR2 antibodies in samples of patients having received a liver transplant is higher the higher the risk or presence of severe
symptoms are, e.g. the level of PAR2 antibodies in patients suffering from fibrosis where higher than in patients showing operational tolerance, while the levels in samples of patients with fibrosis themselves where lower than the levels in samples of patients suffering from graft rejection. This shows that by determining the levels of PAR2 antibodies and comparing them to adequate controls differential diagnosis of graft rejection and fibrosis is possible. Hence, the invention also relates to a method for differential diagnosing graft rejection or fibrosis in a patient or assessing the risk of a patient for graft rejection or fibrosis, the patient having received a liver transplant, comprising the steps of

(i) determining the level of antibodies against protease-activated receptor 2 (PAR2) in a sample from the patient to be diagnosed;

(ii) comparing the determined level in the sample to either one, two or all of a first, a second and a third PAR2 antibody control level;

a) wherein the first PAR2 antibody control level is derived from subjects not suffering from graft intolerance, and

b) wherein the second PAR2 antibody control level is derived from subjects suffering from fibrosis;

c) wherein the third PAR2 antibody control level is derived from a subject suffering from graft rejection;

wherein an increased level in the sample from the patient to be diagnosed as compared to the first PAR2 antibody control level is indicative for graft intolerance in the patient or the risk of graft intolerance in said patient; and

wherein an increased level in the sample from the patient to be diagnosed as compared to the second PAR2 antibody control level and/or an equal or increased level as compared to the third PAR2 antibody control level is indicative for graft rejection or indicative for the risk of graft rejection in the patient;

wherein a decreased level in the sample from the patient to be diagnosed as compared to the third PAR2 antibody control level, and an increased level as compared to the first PAR2 antibody control level is indicative for the presence or the risk of fibrosis in said patient; and

wherein an equal level as compared to the second PAR2 antibody control level is indicative for the presence or the risk of fibrosis in the patient.

The skilled person will acknowledge that different controls may be chosen as outlined above for the method for differential diagnosing graft rejection or fibrosis in a patient or assessing
the risk of a patient for graft rejection or fibrosis. He will instantly see that the
differential diagnosis is for example possible by comparing the determined PAR2 antibody
level to a PAR2 antibody control level derived from one ore more subjects suffering from
fibrosis after liver transplantation, wherein an increased level as compared to this control
level is indicative for more severe symptoms than fibrosis, e.g. graft rejection, and wherein
decreased levels as compared to this control are indicative for less severe symptoms than
fibrosis, e.g. operational intolerance. He will also acknowledge that for example decreased
levels as compared to the control level derived from one ore more subjects suffering from
graft rejection are indicative for the presence or risk of acquiring symptoms less severe than
graft rejection, e.g. fibrosis or operational tolerance. Accordingly he will acknowledge that
increased levels as compared to control levels derived from one or more subjects not
suffering from graft intolerance, e.g. having operational tolerance, are indicative for the
presence of graft intolerance, e.g. fibrosis and/or graft rejection.

As outlined herein above, the assessment of the need of a patient for administration of
immunosuppressant agents is still an unmet need. While it is sometimes necessary to
suppress the immune system of a patient having received a liver transplant, the weaning and
complete withdrawal of the immunosuppressant is always desirable, as immunosuppression
itself provokes health risks as e.g. outlined herein above. The inventors now found that the
presence and/or level of PAR2 antibodies in samples of patients having received a liver
transplant gives an indication on the tolerance of the patient for the transplant. This gives a
clear guidance as to whether administration of an immunosuppressant shall be continued or
whether the patient has tolerated the transplant and immunosuppression is no longer needed.
The present invention also relates to method for assessing the need of a patient for
administration of an immunosuppressant, the patient having received a liver transplant,
comprising the steps of:

(i) determining the level of antibodies against protease-activated receptor 2 (PAR2) in a
sample from the patient;

(ii) comparing the determined level in the sample to either one or both of a first, and a
second PAR2 antibody control level;

a) wherein the first PAR2 antibody control level is derived from subjects not
showing graft intolerance, preferably not suffering from graft rejection and/or
fibrosis; and
b) wherein the second PAR2 antibody control level is derived from subjects showing graft intolerance, preferably graft rejection and/or fibrosis;

wherein an increased level in the sample from the patient to be assessed as compared to the first PAR2 antibody control level, and/or an equal level or increased level as compared to the second PAR2 antibody control level is attributed to the need of said patient for the administration of an immunosuppressant;

wherein an decreased level in the sample from the patient to be assessed as compared to the second PAR2 antibody control level, and/or an equal or decreased level as compared to the first PAR2 antibody control level is attributed to the absence of the need of said patient for the administration of an immunosuppressant.

Furthermore, the invention also includes a method of monitoring the need of a patient for administration of an immunosuppressant, the patient having received a liver transplant, comprising the steps of:

(i) determining the level of antibodies against protease-activated receptor 2 (PAR2 antibodies) in a sample from the patient to be monitored, said sample being taken at a first time point; and

(ii) determining the level of PAR2 antibodies in a sample from the patient to be monitored for the need for administration of an immunosuppressant, said sample being taken at at least one further time point, said at least one further time point being after said first time point;

wherein a constant level in-between the samples of the first and the at least one further time point or an decreased level in the sample of the second time point as compared to the sample of the first time point indicates the possibility for onset of weaning of the immunosuppressant and/or continuing the weaning of the immunosuppressant, and

wherein an increased level in the sample of the at least one further time point as compared to the sample of the first time point is contradicts onset and/or continuing of weaning of the immunosuppressant.

This means that increased and/or increasing levels of PAR2 antibodies indicate that the patient does not show operational tolerance and that hence the administration of an immunosuppressant is (further) indicated. The skilled person will instantly acknowledge that by using the methods according to the present invention therapy monitoring is possible
to give guidance as to whether the therapy with an immunosuppressant shall be started, continued or withdrawn. Hence, in a preferred embodiment the method for monitoring is a method for therapy monitoring.

5 The methods according to the present invention as outlined herein in one embodiment comprise the steps of
(a) contacting the sample with protease-activated receptor 2 (PAR2) or an antigenic peptide fragment thereof under conditions allowing for the formation of a complex between PAR2 antibodies and PAR2 or the peptide fragment thereof,
(b) detecting the complex.

These steps are preferably steps of the determination of the level of PAR2 antibodies, preferably the level of the complex is detected.

15 The invention also relates to an immunoassay method for detecting an anti-PAR2 antibody in a sample from a subject, comprising the steps of:
(a) contacting the sample suspected of comprising an anti-PAR2 antibody with protease-activated receptor 2 (PAR2) or an antigenic peptide fragment thereof under conditions allowing for the formation of a complex between the anti-PAR2 antibody with PAR2 or the peptide fragment thereof,
(b) detecting the complex.

The present invention also relates to a kit for the diagnosis and/or monitoring graft intolerance and/or determining the need of administration of an immunosuppressant in a patient having received a liver transplant comprising:
- PAR2 or an antigenic peptide fragment thereof, and standards; and/or
- controls for comparing determined levels of PAR2 antibody in a sample to be tested with control levels in subjects not suffering from graft intolerance and/or subjects suffering from graft intolerance.

30 The inventors found that PAR2 or antigenic peptides are useful for diagnosing of graft intolerance and determining the need of administration of an immunosuppressant in connection with liver transplantations, as they allow detecting PAR2 antibodies in samples
of patients. Hence, the present invention also relates to the use of PAR2 or an antigenic peptide fragment thereof for the diagnosis and/or monitoring graft intolerance and/or determining the need of administration of an immunosuppressant in a patient having received a liver transplant.

It has furthermore be found by the inventors that by using the methods according to the present invention it is possible to predict or monitor the need for the administration of an immunosuppressant in order to prevent adverse effects of graft intolerance. Hence, the inventors provide guidance for the skilled person as to when a patient can undergo weaning or complete withdrawal of an immunosuppressant and as to when immunosuppressant shall be administered. Hence, the invention furthermore relates to an immunosuppressant for use in the treatment of graft intolerance in a patient having received a liver transplant, wherein the immunosuppressant is administered to the subject when the subject exhibits, the presence of PAR2 antibodies, preferably increased sample levels of PAR2 antibodies.

The inventors provide proof of the correlation of the presence of increased levels of PAR2 antibodies and graft intolerance. The skilled person will instantly acknowledge that this clearly indicates a role of these antibodies in the graft intolerance patho-mechanism. Hence, it is desirable to remove these antibodies from the blood of patients thought to suffer from graft intolerance. Hence, the present invention also relates to a method for removal of PAR2 antibodies from isolated blood, comprising the steps of:

a.) determining the presence or absence of a PAR2 antibody in a blood sample from a patient thought to suffer from graft intolerance of a liver transplant;
b.) wherein upon determination of the presence of a PAR2 antibody in said blood sample, PAR2 antibody is removed from isolated blood of said patient.

**Description of the Figures**

**Fig. 1:** Comparison of the median level of anti-PAR2 antibodies (units/ml) in serum samples of patients having received a liver transplant and shows operational tolerance (o-toler... fibrosis or rejection. The p-value is indicated on top. Bars indicate standard error of mean.
**Fig. 2:** Correlation of levels of anti-PAR2 (x-axis) antibodies with levels of anti AT1R (y-axis) antibodies, a recently discovered marker found marker for graft intolerance.

**Fig. 3:** Comparison of median levels of anti-PAR2 antibodies (units/ml) in samples of patients being positive for C4d (pos) or being negative for C4d (neg). Median levels and p-values are given in the figure.

**Fig. 4:** Correlation of levels of anti-PAR2 (x-axis) antibodies with levels of liver function markers: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin and total albumin. The concentration of the respective liver function marker is given on the y-axis and the concentration of the anti-PAR2 antibody on the x-axis (PAR2; in U/ml).

**Fig. 5:** Standard curve of a PAR2 antibody ELISA

**Detailed Description of the Invention**

The term “graft intolerance” refers to any event in the patient which is connected with adverse effects on the liver graft, in particular the liver allograft, such as graft damage. Different symptoms of graft intolerance are known in the art and are often detectable by biopsies (Banff Working Group on Liver Allograft Pathology. Importance of liver biopsy findings in immunosuppression management: biopsy monitoring and working criteria for patients with operational tolerance. Liver Transpl. 2012 Oct;18(10):1154-70; doi: 10.1002/lt.23481. Review. PubMed PMID: 22645090). In a preferred embodiment graft intolerance is selected from the group of graft rejection, e.g. acute cellular rejection, and fibrosis, preferably graft rejection. As will be understood by the skilled person, the absence of graft intolerance is known as operational tolerance. Hence, he will acknowledge that operational tolerance may also be diagnosed by the method according to the present invention, e.g. by comparing the determined level of antibodies against PAR2 in the patient to be diagnosed, comparing the determined level in the sample to a control level derived from subjects showing graft intolerance and/or no operational tolerance, wherein an decreased level in the sample from the patient to be diagnosed as compared to the control level is indicative for operational tolerance. Likewise, determined PAR2 antibody levels may be compared to a control level from a subject showing operational tolerance in addition
or alternatively, wherein an equal PAR2 antibody level in the sample of the patient to be diagnosed, as compared to the control level from the subject showing operational tolerance is indicative for operational tolerance.

5 The terms “graft” and “transplant” are used interchangeably herein.

“PAR2 antibody” and “anti-PAR2 antibody” are used interchangeably herein and refer to antibodies specifically binding the PAR2 protein or fragments thereof, preferably autoimmune antibodies of a patient having received a liver transplant. The anti-PAR2 antibody binds specifically to the PAR2 or in doing so shows specific immuno reactivity when the anti-PAR2 antibody assumes its function in a binding reaction in the presence of a heterogeneous population of PAR2s or fragments thereof, thereby allowing a conclusion whether the PAR2 or another biological structure is present. Under the present conditions of an immunoassay, the above-mentioned anti-PAR2 antibodies will preferably bind to a specific portion of the PAR2, e.g. SEQ ID NO:2, while no significant binding to other proteins present in the sample will take place. In a preferred embodiment the PAR2 antibodies are selected from the group of antibodies consisting of IgG-antibodies and IgM-antibodies, preferably an IgG antibody, e.g. IgG1, IgG2, IgG3 and IgG4. In a certain preferred embodiment total IgG type PAR2 antibodies are to be detected in the methods of the present invention.

“Equal” level in context with the present invention means that the levels differ by not more than ± 10 %, preferably by not more than ± 5 %, more preferably by not more than ± 2 %.“Decreased” or “increased” level in the context of the present invention mean that the levels differ by more than 10 %, preferably by more than 15 %, preferably more than 20 %.

The term „patient“ and “subjects” as used herein refers to a subject having received a liver transplant, preferably the subject is a mammal, in particular human are preferred. The patient in a preferred embodiment has received said liver transplant at a pediatric age, i.e. at an age of less than 18 years, such as at an age of 1 to 17 years, e.g. 16 years or less, 15 years or less, 14 years or less, 13 years or less, 12 years or less, 11 years or less, 10 years or less, 9 years or less, 8 years or less, 7 years or less, 6 years or less, 5 years or less, 4 years or less, 3 years or less, 2 years or less.
The term "graft" as used herein refers to any kind of liver graft/transplant, e.g. a living donor liver transplant or a transplant from a deceased subject. Preferably the graft according to the present invention is a living donor liver transplant (LDLT).

The term "weaning" in context with the present invention refers to the procedure of reducing the burden of immunosuppression. Liver transplantation is unique in that the risk of chronic rejection also decreases over time, although the great majority of recipients need to take immunosuppressive medication for the rest of their lives. It is possible to be slowly taken off anti rejection medication but only in certain cases. Weaning refers to the procedure of reducing the dose and amount of immunosuppressant administered to the patient having received a liver transplant. The desired outcome of such weaning procedure is the total withdrawal from immunosuppressant. The procedure of weaning and the management of the reduction with regards to the exact decrease in dose and amount differs from case to case. However, the present invention, as outlined herein, gives the practitioner a tool to determine whether onset or continuing of weaning is feasible without any damage to the liver transplant.

The control levels as used herein refer to control levels of PAR2 antibodies. It will be readily understood by the skilled person that the control levels from subjects having the desired disease or symptoms as defined in the methods and to which the determined levels are compared to, are not necessarily determined in parallel but may be represented by previously determined levels. Nevertheless, control levels may be determined in parallel. When referring to control levels from subjects having received a graft or transplant, it is preferred that said graft or transplant is a liver graft or transplant as defined herein. The skilled person with the disclosure of the present invention and his knowledge is able to determine such control levels, as will be outlined herein below. Hence, the control levels of the present invention may be previously defined thresholds. Preferred thresholds are also disclosed herein but are not limiting the invention. Furthermore, it will be acknowledged by the skilled person that control levels are, like the levels to be determined in the patient to be diagnosed or treated, determined in samples of the recited subjects having the desired disease or symptoms or being healthy. Preferably, the sample is the same kind of sample as the sample of the person to be diagnosed or to be treated, e.g. when the sample of the latter
is serum, the control levels are preferably determined in serum samples derived from the control subjects. However, as outlined the control levels according to the present invention may also be predetermined control levels which are also integrated into the assay used. For example the assay may be designed in a way to give only positive results for the determination of the PAR2 antibody if the concentration of PAR2 in the sample to be analysed is above a certain level, e.g. increased as compared to the respective control.

As outlined herein, the levels of PAR2 antibodies in samples of the patient to be diagnosed and treated or to be treated are compared with the control groups as defined herein. However, in one embodiment the levels are compared to fixed values, i.e. thresholds under or over which a certain diagnosis, or prognosis of response is given. To this end, unit-standards may be applied. The present inventors set out such standard for the PAR2 antibody using serum samples from patients suffering from systemic sclerosis. Systemic sclerosis patients are known to have high levels of autoimmune antibodies in general.

Hence, the inventors took a serum sample of a systemic sclerosis patient. However, it will be acknowledged by the skilled person that also other samples may be taken to set a different standard, e.g. samples of healthy subjects. Nevertheless, the principle of generating a standard (units) is the same in any case and are exemplified herein using serum samples of systemic sclerosis patients. In the context of the present invention “units/ml”, unless specified otherwise, refers to the concentration of antibodies standardised as exemplified herein. Hence, in one embodiment of the present invention 40 units/ml refers to a dilution of 1:100 of a serum sample of systemic sclerosis patients. The serum sample may be derived from a single patient or of a cohort of a plurality of patients, e.g. a cohort of 200 patients suffering from systemic sclerosis. The present inventors found that the concentration of PAR2 antibodies in samples of systemic sclerosis do not differ by more than about 10 %, showing such standard being reproducible. In one preferred embodiment the standard for the concentrations of the autoimmune antibodies is generated in the following way: a serum sample of a systemic sclerosis patient (or a larger cohort) is diluted (a) 1:100 for standard point 40 Units/ml, (b) 1:200 for standard point 20 Units/ml, (c) 1:400 for standard point 10 Units/ml, (d) 1:800 for standard point 5 Units/ml, (e) 1:1600 for standard point 2.5 Units/ml. These standards are then used for the immunoassay chosen, e.g. ELISA, and then correlated with the respective read-out value, e.g. for ELISA optical density at 450nm/ optical density at 620 nm. A typical standard curve of a PAR2 auto-antibody ELISA is shown in figure 5.
Nevertheless, the skilled person will readily understand that it may also be possible to standardize the levels of PAR2-autoantibodies using different samples, e.g. samples of healthy subjects or patients with graft rejection.

5 It has been found by the inventors that PAR2 antibody levels in samples of patients suffering from graft intolerance, e.g. showing graft rejection or fibrosis, are increased as compared to patients not showing graft intolerance. In other words, patients with operational tolerance of a liver transplant show decrease PAR2 antibody levels as compared to those patients with no operational tolerance. It has also been found that values of about 1.2 times the concentration of the median of PAR2 levels in patients with operational tolerance. Hence, in one embodiment of the method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to the present invention a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 1.2 fold as compared to the control level is indicative for graft intolerance. In a further preferred embodiment a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 1.4 fold as compared to the control level is indicative for graft intolerance, preferably more than 1.5 fold. In a preferred embodiment a level of anti-PAR2 antibodies above the 90th percentile of the levels of patients not showing graft intolerance is indicative for graft intolerance, more preferable above the 95th percentile.

As also outlined herein, levels of auto immune antibodies in samples of patient/subjects may be measured by units/ml (U/ml). In a preferred embodiment of the method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to the present invention a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 18 U/ml is indicative for graft intolerance, preferably a level of more than 20 U/ml, even more preferably of more than 23 U/ml. As outlined herein, the skilled person is aware of methods for determining U/ml of an antibody: However, in a preferred embodiment the units according to the present invention are determined using the unit-stands or unit solutions as outlined in greater detail herein.

The inventors furthermore found that levels of PAR2 antibodies are particularly high in patients showing graft rejection, i.e. most severe symptoms. The median of levels of PAR2 antibodies was more than 2 fold higher in patients showing graft rejection as compared to
subject showing operational tolerance after liver transplantation. Hence, in one embodiment of the method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to the present invention a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 2 fold as compared to the control level is indicative for graft rejection, preferably a level of more than 2.5 fold, even more preferred of more than 3.0 fold, even more preferred of more than 3.5 fold, yet further preferred of more than 3.75 fold. In a preferred embodiment a level of anti-PAR2 antibodies above the 90th percentile of the levels of patients showing operational tolerance is indicative for graft rejection, preferably above the 95th percentile.

In a further embodiment of the method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to the present invention, wherein a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 30 U/ml is indicative for graft rejection, preferably levels of more than 40 U/ml, even more preferred levels of more than 50 U/ml are indicative for graft rejection. As outlined herein, the skilled person is aware of methods for determining U/ml of an antibody: However, in a preferred embodiment the units according to the present invention are determined using the unit-standards or unit solutions as outlined in greater detail herein.

The inventors found that it is also possible to monitor treatment of patients having received a liver transplant with an immunosuppressant by determining the levels of PAR2 antibodies and following the levels during the course of treatment. Increased and/or increasing levels of PAR2 antibodies indicate that the patient does not show operational tolerance and that hence the administration of an immunosuppressant is (further) indicated. The skilled person will instantly acknowledge that by using the methods according to the present invention therapy monitoring is possible to give guidance as to whether the therapy with an immunosuppressant shall be started, continued or withdrawn or if the dose of the immunosuppressant shall be adjusted. A decrease of the levels of PAR2 antibodies over the time indicates the amelioration of the graft intolerance and the need for reducing the dose of immunosuppressant or even the onset of weaning or complete withdrawal from immunosuppressant. An increase of PAR2 antibody level is indicative for the need for administration of an immunosuppressant and if immunosuppressant is already administered, for the need to increase of the dose of said immunosuppressant. Hence, in a preferred
embodiment the method for monitoring is a method for therapy monitoring. The monitoring may also comprise a combination of methods as outlined above, in particular the method for monitoring the need for administration of an immunosuppressant (therapy monitoring) may be a combination of the method for assessing the need of a patient for administration of an immunosuppressant, the patient having received a liver transplant, and the method for monitoring the need of a patient for administration of an immunosuppressant, i.e. in a first step the levels of PAR2 antibodies in a sample of a patient are determined and compared to the respective controls to evaluate whether graft intolerance is present or a risk in the patient. In a second step further development of the levels are monitored by determining the level of PAR2 antibodies in samples of a patient obtained at an at least one further time point. Using a combination of the outlined method allows to monitor further development of a graft intolerance or an operational tolerance, e.g. increased levels in the first step as compared to the respective control indicate graft intolerance and the need for administration of an immunosuppressant, the second steps then monitors the levels of PAR2 antibodies in the sample of the patient throughout the time, i.e. at at least one further time point after the first step. Such combination may for instance be advantageous in a case were the method for diagnosing graft intolerance and/or the method for assessing the need of a patient for administration of an immunosuppressant has revealed that the patient does not show graft intolerance or the need for administration of an immunosuppressant. This indicates the possibility for the onset of weaning or complete withdrawal of the immunosuppressant. In order to further monitor developments within the patient the method of monitoring the need of a patient for administration of an immunosuppressant may be applied, and hence being a method of therapy monitoring or therapy evaluation. If during weaning or after the complete withdrawal the level of PAR2 antibodies in the sample of the patient increases, indication for stop of the weaning procedure is given and/or the (re)administration of the immunosuppressant is indicated. In line with this, a constant or decreasing level of PAR2 antibodies indicates operational tolerance and the continuation of the weaning procedure or the withdrawal.

Herein, the sample of the subject to be diagnosed in which the level of anti-PAR2 antibodies is to be determined is preferably a bodily fluid such as whole blood or lymph or fractions of blood such as serum or plasma. Preferably in the context of the present invention the sample is plasma or serum. The sample according to the present invention is preferably a bodily
fluid of the patient, however, also tissue samples, e.g. of the transplanted liver may be used. In a preferred embodiment the bodily fluid is blood. Further preferred samples are plasma and serum samples, most preferable serum samples.

Where appropriate, the sample may need to be homogenized, or extracted with a solvent prior to use in the present invention in order to obtain a liquid sample. A liquid sample hereby may be a solution or suspension. Liquid samples may be subjected to one or more pre-treatments prior to use in the present invention. Such pre-treatments include, but are not limited to dilution, filtration, centrifugation, concentration, sedimentation, precipitation, and dialysis. Pre-treatments may also include the addition of chemical or biochemical substances to the solution, such as acids, bases, buffers, salts, solvents, reactive dyes, detergents, emulsifiers, chelators.

“Plasma” in the context of the present invention is the virtually cell-free supernatant of blood containing anticoagulant obtained after centrifugation. Exemplary anticoagulants include calcium ion binding compounds such as EDTA or citrate and thrombin inhibitors such as heparinates or hirudin. Cell-free plasma can be obtained by centrifugation of the anticoagulated blood (e.g. citrated, EDTA or heparinized blood) for at least 15 minutes at 2000 to 3000 g.

“Serum” is the liquid fraction of whole blood that is collected after the blood is allowed to clot. When coagulated blood (clotted blood) is centrifuged serum can be obtained as supernatant. It does not contain fibrinogen, although some clotting factors remain.

In the context of the present invention, the levels of the anti-PAR2 antibodies a may be analyzed in a number of fashions well known to a person skilled in the art. For example, each assay result obtained may be compared to a "normal" value, or a value indicating a particular disease or outcome. A particular diagnosis/prognosis may depend upon the comparison of each assay result to such a value, which may be referred to as a diagnostic or prognostic "threshold". In certain embodiments, assays for one or more diagnostic or prognostic indicators are correlated to a condition or disease by merely the presence or absence of the indicator(s) in the assay. For example, an assay can be designed so that a
positive signal only occurs above a particular threshold concentration of interest, and below which concentration the assay provides no signal above background. Hence, the skilled person knows that the methods according to the present invention may be designed to differentiate between the presence and absence of the anti-PAR2 antibodies in the samples, wherein presence means increased level according to the present invention, and absence means non increased levels.

The sensitivity and specificity of a diagnostic and/or prognostic test depends on more than just the analytical "quality" of the test, they also depend on the definition of what constitutes an abnormal result. In practice, Receiver Operating Characteristic curves (ROC curves), are typically calculated by plotting the value of a variable versus its relative frequency in "normal" (i.e. apparently healthy individuals not having fibrosis or graft rejection, e.g. with operational tolerance) and "disease" populations. For any particular marker, a distribution of marker levels for subjects with and without a disease will likely overlap. Under such conditions, a test does not absolutely distinguish normal from disease with 100% accuracy, and the area of overlap indicates where the test cannot distinguish normal from disease. A threshold is selected, below which the test is considered to be abnormal and above which the test is considered to be normal. The area under the ROC curve is a measure of the probability that the perceived measurement will allow correct identification of a condition. ROC curves can be used even when test results don't necessarily give an accurate number. As long as one can rank results, one can create a ROC curve. For example, results of a test on "disease" samples might be ranked according to degree (e.g. 1=low, 2=normal, and 3=high). This ranking can be correlated to results in the "normal" population, and a ROC curve created. These methods are well known in the art. See, e.g., Hanley et al. 1982. *Radiology* 143: 29-36. Preferably, a threshold is selected to provide a ROC curve area of greater than about 0.5, more preferably greater than about 0.7, still more preferably greater than about 0.8, even more preferably greater than about 0.85, and most preferably greater than about 0.9. The term "about" in this context refers to +/- 5% of a given measurement.

The horizontal axis of the ROC curve represents (1-specificity), which increases with the rate of false positives. The vertical axis of the curve represents sensitivity, which increases with the rate of true positives. Thus, for a particular cut-off selected, the value of (1-specificity) may be determined, and a corresponding sensitivity may be obtained. The area
under the ROC curve is a measure of the probability that the measured marker level will allow correct identification of a disease or condition. Thus, the area under the ROC curve can be used to determine the effectiveness of the test.

In other embodiments, a positive likelihood ratio, negative likelihood ratio, odds ratio, or hazard ratio is used as a measure of a test's ability to predict risk or diagnose a disease. In the case of a positive likelihood ratio, a value of 1 indicates that a positive result is equally likely among subjects in both the "diseased" and "control" groups; a value greater than 1 indicates that a positive result is more likely in the diseased group; and a value less than 1 indicates that a positive result is more likely in the control group. In the case of a negative likelihood ratio, a value of 1 indicates that a negative result is equally likely among subjects in both the "diseased" and "control" groups; a value greater than 1 indicates that a negative result is more likely in the test group; and a value less than 1 indicates that a negative result is more likely in the control group.

In the case of an odds ratio, a value of 1 indicates that a positive result is equally likely among subjects in both the "diseased" and "control" groups; a value greater than 1 indicates that a positive result is more likely in the diseased group; and a value less than 1 indicates that a positive result is more likely in the control group.

In the case of a hazard ratio, a value of 1 indicates that the relative risk of an endpoint (e.g., death) is equal in both the "diseased" and "control" groups; a value greater than 1 indicates that the risk is greater in the diseased group; and a value less than 1 indicates that the risk is greater in the control group.

The skilled artisan will understand that associating a diagnostic or prognostic indicator, with a diagnosis or with a prognostic risk of a future clinical outcome is a statistical analysis. For example, a marker level of lower than X may signal that a patient is more likely to suffer from an adverse outcome than patients with a level more than or equal to X, as determined by a level of statistical significance. Additionally, a change in marker concentration from baseline levels may be reflective of patient prognosis, and the degree of change in marker level may be related to the severity of adverse events. Statistical significance is often determined by comparing two or more populations, and determining a confidence interval
and/or a p value. See, e.g., Dowdy and Wearden, *Statistics for Research*, John Wiley & Sons, New York, 1983. Preferred confidence intervals of the invention are 90%, 95%, 97.5%, 98%, 99%, 99.5%, 99.9% and 99.99%, while preferred p values are 0.1, 0.05, 0.025, 0.02, 0.01, 0.005, 0.001, and 0.0001.

Suitable threshold levels for the stratification of subjects into different groups (categories) have to be determined for each particular combination of PAR2-antibodies, disease and/or medication. This can e.g. be done by grouping a reference population of patients according to their level of PAR2-antibodies into certain quantiles, e.g. quartiles, quintiles or even according to suitable percentiles. For each of the quantiles or groups above and below certain percentiles, hazard ratios can be calculated comparing the risk for an adverse outcome, i.e. an “graft intolerance” or a “need for administration of an immunosuppressant”, e.g. in terms of survival rate/mortality, between those patients who have received a certain medication and those who did not, or in terms of presence and absence of graft intolerance in patients. In such a scenario, a hazard ratio (HR) above 1 indicates a higher risk for an adverse outcome for the patients who have received a treatment than for patients who did not. A HR below 1 indicates beneficial effects of a certain treatment in the group of patients. A HR around 1 (e.g. +/- 0.1) indicates no elevated risk but also no benefit from medication for the particular group of patients. By comparison of the HR between certain quantiles of patients with each other and with the HR of the overall population of patients, it is possible to identify those quantiles of patients who have an elevated risk and those who benefit from medication and thereby stratify subjects according to the present invention.

In some cases presence of graft intolerance or need for administration of an immunosuppressant will affect patients with low levels (e.g. in the fifth quintile) of PAR2-antibodies, while in other cases only patients with high levels of PAR2-antibodies will be affected (e.g. in the first quintile). However, with the above explanations, a skilled person is able to identify those groups of patients having graft intolerance, those groups that do need a medication and those groups that do not need the medication. Exemplarily, some combinations of hormones and medications are listed for several diseases in the appended examples. In another embodiment of the invention, the diagnosis, the differential diagnosis, the assessment of the need for a immunosuppressant or the monitoring of the need for a
immunosuppressant are determined by relating the patient’s individual level of marker peptide to certain percentiles (e.g. 90\(^{th}\), 95\(^{th}\) or even 97.5\(^{th}\) percentile) of a healthy population.

Kaplan-Meier estimators may be used for the assessment or prediction of the outcome or risk (e.g. diagnosis, need of immunosuppressant) of a patient.

Furthermore, in the methods of the present invention further parameters of the subject may be considered as well for diagnosis, differential diagnosis, assessment of the need for an immunosuppressant, therapy monitoring etc. Such parameters in a multivariate model may include gender, age, histological evaluation, and other markers. Dependent variables for determining survival may also be time till rejection, time till onset of weaning, time till withdrawal (shorter interval if both events occurred). A Cox-Proportional-Hazard regression predicts the dependent variable based on one or more independent variables. These predictors can either be measures (as e.g. level of a biomarker) or categorical data (as e.g. response to a previous treatment). The skilled person is aware of the fact that diagnostic markers only give a certain degree of sensitivity and specificity, as also outlined herein. He knows that different further parameters might be considered in order to increase both. For example, when detecting levels of a marker indicative for graft intolerance, *inter alia* liver graft intolerance, the skilled person would not diagnose graft intolerance in a subject not having received a graft. Nevertheless, the present invention provides a new and superior marker for diagnosis, differential diagnosis, assessment of the need for an immunosuppressant, therapy monitoring as outlined herein in great detail. In the context of the methods of the invention and particularly the immunoassays of the invention, the presence of one or more further diagnostic markers for graft intolerance is detected in the sample. For example, in a diagnostic method of the present invention levels of anti-AT1-receptor antibodies detected in addition. The detection of anti-AT1-receptor (angiotensin II receptor type 1) antibodies is described for example in EP 07 80 2440. Increased levels of anti-AT1-receptor antibodies in patients having received a liver transplant are likewise indicative for graft intolerance and the need for the administration of an immunosuppressant (Ohe H, Uchida Y, Yoshizawa A, Hirao H, Taniguchi M, Maruya E, Yurugi K, Hishida R, Maekawa T, Uemoto S, Terasaki PI. Association of Anti-Human Leukocyte Antigen and Anti-Angiotensin II Type 1 Receptor Antibodies With Liver Allograft Fibrosis After
In the method of the present invention, the PAR2 antibody is preferably detected in an immunoassay. Suitable immunoassays may be selected from the group of immunoprecipitation, enzyme immunoassay (EIA)), enzyme-linked immunosorbent assays (ELISA), radioimmunoassay (RIA), fluorescent immunoassay, a chemiluminescent assay, an agglutination assay, nephelometric assay, turbidimetric assay, a Western Blot, a competitive immunoassay, a noncompetitive immunoassay, a homogeneous immunoassay a heterogeneous immunoassay, a bioassay and a reporter assay such as a luciferase assay. Preferably herein the immunoassay is an enzyme linked immunosorbent assay (ELISA).

The immunoassays can be homogenous or heterogeneous assays, competitive and non-competitive assays. In a particularly preferred embodiment, the assay is in the form of a sandwich assay, which is a non-competitive immunoassay, wherein the PAR2 antibody (i.e. the “analyte”) to be detected and/or quantified is allowed to bind to an immobilized PAR2 protein or immunogenic peptide fragment thereof and to a secondary antibody. The PAR2 protein or immunogenic fragment thereof (i.e. a peptide), may e.g., be bound to a solid phase, e.g. a bead, a surface of a well or other container, a chip or a strip, and the secondary antibody is an antibody which is labeled, e.g. with a dye, with a radioisotope, or a reactive or catalytically active moiety such as a peroxidase, e.g. horseradish peroxidase. The amount of labeled antibody bound to the analyte is then measured by an appropriate method. The general composition and procedures involved with “sandwich assays” are well-established and known to the skilled person (The Immunoassay Handbook, Ed. David Wild, Elsevier LTD, Oxford: 3rd ed. (May 2005), ISBN-13: 978-0080445267; Hultschig C et al., Curr Opin Chem Biol. 2006 Feb;10(1):4-10. PMID: 16376134, incorporated herein by reference). Sandwich immunoassays can for example be designed as one-step assays or as two-step assays.

In the methods according to the present invention the PAR2 or the peptide fragment thereof used in the methods are in one embodiment immobilized on a surface.
The skilled artisan will understand that for detection of the so bound PAR2 antibody to be detected a secondary antibody may be advantageous as outlined herein. The secondary antibody preferably binds PAR antibodies in a specific manner. The detection of the complex between PAR2 antibodies and the PAR2 protein or peptide fragment is in one embodiment performed using a secondary antibody against the Fc portion of the PAR2 antibody. Furthermore, if it is desirable to determine only a certain type of antibodies, the skilled person is aware of type specific secondary antibodies, e.g. anti IgG antibodies, or even subtype specific antibodies, e.g. IgG1 antibodies or the like. In the context of the present invention the PAR2 antibody may particularly be selected from the group of IgA-antibody, IgG-antibody and IgM-antibody, preferably an IgG antibody, e.g. IgG1, IgG2, IgG3 and IgG4. In one embodiment of the present invention the anti-PAR2 antibody is an IgG-antibody, preferably detecting all subtypes of IgG. As will be understood by the skilled person, the immunoassay gets its specificity to PAR2 antibodies through the use of PAR2 or an immunogenic fragment thereof for the detection. The thereto binding anti-PAR2 antibodies are then detected by a secondary antibody specifically binding to antibodies, e.g. human antibodies, e.g. the Fc-portion as outlined.

The term "secondary antibody" comprises monoclonal and polyclonal antibodies and binding fragments thereof, in particular Fc-fragments as well as so called "single-chain-antibodies" (Bird R. E. et al (1988) Science 242:423-6), chimeric, humanized, in particular CDR-grafted antibodies, and dia or tetrabodies (Holliger P. et al (1993) Proc. Natl. Acad. Sci. U.S.A. 90:6444-8). Also comprised are immunoglobulin like proteins that are selected through techniques including, for example, phage display to specifically bind to the molecule of interest contained in a sample. In this context the term "specific binding" refers to antibodies raised against the molecule of interest or a fragment thereof. An antibody is considered to be specific, if its affinity towards the molecule of interest or the aforementioned fragment thereof is at least 50-fold higher, preferably 100-fold higher, more preferably at least 1000-fold higher than towards other molecules comprised in a sample containing the molecule of interest. It is well known in the art how to make antibodies and to select antibodies with a given specificity.
The detectable label may for example be based on fluorescence or chemiluminescence. The labelling system comprises rare earth cryptates or rare earth chelates in combination with a fluorescence dye or chemiluminescence dye, in particular a dye of the cyanine type. In the context of the present invention, fluorescence based assays comprise the use of dyes, which may for instance be selected from the group comprising FAM (5-or 6-carboxyfluorescein), VIC, NED, Fluorescein, Fluoresceinisothiocyanate (FITC), IRD-700/800, Cyanine dyes, such as CY3, CY5, CY3.5, CY5.5, Cy7, Xanthen, 6-Carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), TET, 6-Carboxy-4',5'-dichloro-2',7'-dimethyfluorescein (JOE), N,N,N',N'-Tetramethyl-6-carboxyrhodamine (TAMRA), 6-Carboxy-X-rhodamine (ROX), 5-Carboxyrhodamine-6G (R6G5), 6-carboxyrhodamine-6G (RG6), Rhodamine, Rhodamine Green, Rhodamine Red, Rhodamine 110, BODIPY dyes, such as BODIPY TMR, Oregon Green, Coumarines such as Umbelliferone, Benzimides, such as Hoechst 33258; Phenanthridines, such as Texas Red, Yakima Yellow, Alexa Fluor, PET, Ethidiumbromide, Acrdinium dyes, Carbazol dyes, Phenoxazine dyes, Porphyrine dyes, Polymethin dyes, and the like.

In the context of the present invention, chemiluminescence based assays comprise the use of dyes, based on the physical principles described for chemiluminescent materials in Kirk-Othmer, Encyclopedia of chemical technology, 4th ed., executive editor, J. I. Kroschwitz; editor, M. Howe-Grant, John Wiley & Sons, 1993, vol. 15, p. 518-562, incorporated herein by reference, including citations on pages 551-562. Preferred chemiluminescent dyes are acridiniumesters.

The “sensitivity” of an assay relates to the proportion of actual positives which are correctly identified as such, i.e. the ability to identify positive results (true positives positive results / number of positives). Hence, the lower the concentrations of the analyte that can be detected with an assay, the more sensitive the immunoassay is. The “specificity” of an assay relates to the proportion of negatives which are correctly identified as such, i.e. the ability to identify negative results (true negatives / negative results). For an antibody the “specificity” is defined as the ability of an individual antigen binding site to react with only one antigenic epitope. The binding behaviour of an antibody can also be characterized in terms of its “affinity” and its “avidity”. The “affinity” of an antibody is a measure for the strength of the reaction between a single antigenic epitope and a single antigen binding site. The “avidity”
of an antibody is a measure for the overall strength of binding between an antigen with many epitopes and multivalent antibodies.

An "immunogenic peptide" or "antigenic peptide" as used herein interchangeably is a portion of a PAR2 protein that is recognized (i.e., specifically bound) by the PAR2 antibodies in the sample of the patient to be detected. Such immunogenic peptides generally comprise at least 5 amino acid residues, more preferably at least 10, and still more preferably at least 20 amino acid residues of PAR2. However, they may also comprise at least 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 or 150 amino acid residues. In a preferred embodiment the immunogenic peptide is at least 112 amino residues in length. In a further preferred embodiment the immunogenic peptide comprises an amino acid sequence being at least 80 % identical to the sequence of SEQ ID NO: 2, preferably at least 85 % identical, more preferably at least 90 % identical, further preferred at least 95 % identical. In yet further preferred embodiments the immunogenic peptide comprises an amino acid sequence being a least 97 % identical to SEQ ID NO: 2. The immunogenic peptide in a very preferred embodiment specifically binds to anti-PAR2 antibodies present (at increased levels) in samples of patients suffering from graft rejection of a liver transplant. It is also preferred that the immunogenic peptide comprises an amino acid sequence according to SEQ ID NO:2.

Protease activated receptor 2 (PAR2) also known as coagulation factor II (thrombin) receptor-like 1 (F2RL1) or G-protein coupled receptor 11 (GPR11) is a protein that in humans is encoded by the F2RL1 gene. PAR2 modulates inflammatory responses and acts as a sensor for proteolytic enzymes generated during infection (Lee SE, Jeong SK, Lee SH (November 2010). "Protease and protease-activated receptor-2 signaling in the pathogenesis of atopic dermatitis". Yonsei Med. J. 51 (6): 808–22). PAR2 is a member of the large family of 7-transmembrane receptors that couple to guanosine-nucleotide-binding proteins. PAR2 is also a member of the protease-activated receptor family. It is activated by trypsin, but not by thrombin. It is activated by proteolytic cleavage of its extracellular amino terminus. The new amino terminus functions as a tethered ligand and activates the receptor. Additionally, these receptors can be activated by exogenous proteases, such as house dust mite protein Der P9 (Sun G, Stacey MA, Schmidt M, Mori L, Mattoli S (July 2001). "Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial
cells". J. Immunol. 167 (2): 1014–21). These receptors can also be activated non-proteolytically, by exogenous peptide sequences that mimic the final amino acids of the tethered ligand.

In the context of the immunoassays of the present invention the "PAR2" may be present in its natural cellular environment and can be used together with the material associated with PAR2 in its natural state as well as in isolated form with respect to its primary, secondary and tertiary structures. The PAR2 is well known to those skilled in the art. The receptor is preferably used in isolated form, i.e. essentially free of other proteins, lipids, carbohydrates or other substances naturally associated with PAR2. "Essentially free of" means that the receptor is at least 75%, preferably at least 85%, more preferably at least 95% and especially preferably at least 99% free of other proteins, lipids, carbohydrates or other substances naturally associated with the receptor.

In connection with the present invention, the naturally occurring receptor as well as all modifications, mutants, immunogenic peptides or derivatives of the PAR2 can be used. It is preferred that these specifically bind to anti-PAR2 antibodies present (at increased levels) in samples of patients suffering from graft rejection of a liver transplant. Similarly, a PAR2 produced by means of recombinant techniques, which includes amino acid modifications, such as inversions, deletions, insertions, additions etc. can be used according to the invention provided that this part of the essential function of the PAR2 is present, namely the capability of binding antibodies. The PAR2 peptide used in the Examples herein had the sequence of SEQ ID NO:2. The PAR2 being used may also comprise exceptional amino acids and/or modifications of such as alkylation, oxidation, thiol-modification, denaturation, oligomerization and the like. The PAR2 can also be synthesized by chemical means. According to the invention the PAR2 particularly can be a protein and/or peptide or a fusion protein, which in addition to other proteins, peptides or fragments thereof, includes the PAR2 as a whole or in part. Using conventional methods, peptides or polypeptides of the PAR2 which have functionally analogs, analogous properties can be determined by those skilled in the art. For example such polypeptides or peptides have at least 50% to 60%, 70% or 80%, preferably 90%, more preferably 95%, and most preferably 98% sequence identity to PAR2, preferably to the sequence of SEQ ID NO:1. As outlined the PAR2 antibodies of the Examples bound to the peptide of SEQ ID NO:2, hence the polypeptides or peptides
comprise an amino acid sequence having at least 50 % to 60 %, 70 % to 80 %, preferably 90 %, more preferably 95 %, and most preferably at least 98 % sequence identity to SEQ ID NO:2.

The determination of percent identity between two sequences is accomplished using the mathematical algorithm of Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90: 5873-5877. Such an algorithm is incorporated into the BLASTN and BLASTP programs of Altschul et al. (1990) J. Mol. Biol. 215: 403-410. BLAST nucleotide searches are performed with the BLASTN program, score = 100, word length = 12, to obtain nucleotide sequences homologous to the EPO variant polypeptide encoding nucleic acids. BLAST protein searches are performed with the BLASTP program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to the EPO variant polypeptide, respectively. To obtain gapped alignments for comparative purposes, Gapped BLAST is utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25: 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs are used.

The term “peptide” or “polypeptide” of an PAR2 used in the present invention, comprises also molecules differing from the original sequence by deletion(s), insertion(s), substitution(s) and/or other modifications well known in the prior art and/or comprising a fragment of the original amino acid molecule, the PAR2 still exhibiting the properties mentioned above, preferable binding to anti-PAR2 antibodies present (at increased levels) in samples of patients suffering from graft rejection of a liver transplant. Such a peptide has preferably at least a length of 100 amino acid residues but may also be shorter, e.g. at least 12, 15, 20 or 25 amino acid residues in length or even longer, e.g. 112 amino acids or longer. Also included are allele variants and modifications. Methods of producing the above changes in the amino acid sequence are well known to those skilled in the art and have been described in the standard textbooks of molecular biology, e.g. Sambrook et al., supra. Those skilled in the art will also be able to determine whether a PAR2, thus, modified still has the properties mentioned above. The amino acid sequence of PAR2 is known. Database entries exist in several well known Databases. When referring to the amino acid sequence of PAR2 any amino acid sequence known is meant, particularly those disclosed in common databases, preferably of human origin. A preferred sequence for PAR2 is SEQ ID NO:1 and SEQ ID NO:2. The PAR2 may be glycosylated in vivo. In the present specification all of the
above illustrated modifications of the PAR2 will be referred to as "functionally analogous peptides or proteins" in brief.

The antibodies to be detected or determined according to the present invention are directed against PAR2. This means that the antibodies specifically bind PAR2. Specific binding of an antibody normally occurs via binding of a binding site of the antigen. The antibodies of the present invention are those specifically binding to PAR2 or immunogenic fragments thereof. This binding may occur via recognition of sequence or structural epitopes. The skilled person is aware of methods of how to determine specific epitopes, e.g. fragments of the antigen PAR2, which are recognized and bound by the antibodies to be determined. Fragments of PAR2 binding to the auto antibodies are called immunogenic or antigenic fragments. Methods for determining fragments of an antigen binding the antibody are described in several publications (e.g. Gershoni, JM; Roitburd-Berman, A; Siman-Tov, DD; Tarnovitski Freund, N; Weiss, Y (2007). "Epitope mapping: The first step in developing epitope-based vaccines". BioDrugs 21 (3): 145–56; Westwood, MR; Hay, FC (2001). Epitope Mapping: a practical approach. Oxford, Oxfordshire: Oxford University Press. ISBN 0-19-963652-4; Flanagan et al. (2011), "Mapping Epitopes with H/D-Ex Mass Spec". Genetic Engineering and Biotechnology news; 31(1); Gaseitsiwe, S.; Valentini, D.; Mahdavifar, S.; Reilly, M.; Ehrnst, A.; Maeurer, M. (2009) "Peptide Microarray-Based Identification of Mycobacterium tuberculosis Epitope Binding to HLA-DRB1*0101, DRB1*1501, and DRB1*0401". Clinical and Vaccine Immunology 17 (1): 168–75; Linnebacher, Michael; Lorenz, Peter; Koy, Cornelia; Jahnke, Annika; Born, Nadine; Steinbeck, Felix; Wollbold, Johannes; Latzkow, Tobias et al. (2012). "Clonality characterization of natural epitope-specific antibodies against the tumor-related antigen topoisomerase IIA by peptide chip and proteome analysis: A pilot study with colorectal carcinoma patient samples" Analytical and Bioanalytical Chemistry 403 (1): 227–38; Cragg, M. S. (2011). "CD20 antibodies: Doing the time warp". Blood 118 (2): 219–20; Banik, Soma S. R.; Doranz, Benjamin J. (2010). "Mapping Complex Antibody Epitopes". Genetic Engineering and Biotechnology News 3 (2): 25–8; and Paes, Cheryl; Ingalls, Jada; Kampani, Karan; Sulli, Chidananda; Kakkar, Esha; Murray, Meredith; Kotelnikov, Valery; Greene, Tiffani A. et al. (2009). "Atomic-Level Mapping of Antibody Epitopes on a GPCR". Journal of the American Chemical Society 131 (20): 6952–4). In context with the present invention anti-PAR2 antibodies are understood as any immunoglobulin specifically
recognizing/binding to PAR2. The antibody in a preferred embodiment binds any PAR2 variant, preferably to any of a sequence selected from the group consisting of SEQ ID NO:1, and SEQ ID NO:2; preferably an PAR2 peptide comprising the sequence of SEQ ID NO:2.

5 The skilled person will understand that controls for comparing the determined levels may be of different nature e.g. depending on the assay used. The kit according to the present invention may for example comprise one or more controls comprising PAR2 antibody at the desired control level. Furthermore, the kit may comprise one or more standard solutions each solution comprising PAR2 antibody at different levels, such standard solutions are particularly preferred in cases were a standard curves are to be applied. Exemplary dilutions and levels for such standard solutions are outlined herein above.

The embodiments set out for the immunoassays apply also to the kit of the invention. The kits of the present invention are meant for the detection of autoimmune antibodies. Hence, in one embodiment they comprise means for the preparation of blood, e.g. for gaining serum or plasma thereof. Furthermore, the kit may comprise control composition and/or standards. The control composition preferably comprises PAR2 antibodies as positive control. Furthermore, the kit may comprise one or a plurality of standard compositions. A standard composition comprises PAR2 antibodies at a defined concentration. As outlined herein, determination of concentration of autoimmune-antibodies may be performed using standard curves. These curves set out which concentration of antibodies in a sample or solution corresponds to what read-out value of the assay used, e.g. optical density or proportion of optical density at different wavelengths (e.g. 450nm/620nm). To this end the kits of the present invention may comprise one or more standard compositions having a defined concentration of PAR2 antibodies, preferably of the kind to be detected in the method. A standard composition of the kits according to the present invention comprise PAR2 antibodies at concentrations selected from the group consisting of 40 units/ml, 20 units/ml, 10 units/ml, 5 units/ml and 2.5 units/ml. In one embodiment the kit comprises five standard compositions with the recited concentration. In another embodiment the kit comprises one standard composition with the highest concentration of the standard curve, e.g. 40 units/ml or 20 units/ml. The other concentrations may be produced at the side of the end user by further dilutions, e.g. in PBS. A dilution buffer may therefore also be comprised in the kits according to the invention.
Such kits can comprise a carrier, package or container that is compartmentalized to receive one or more containers such as vials, tubes, and the like, each of the container(s) comprising one of the separate elements to be used in the method. The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use. In addition, a label can be provided on the container to indicate that the composition is used for a specific therapeutic or non-therapeutic application, and can also indicate directions for either in vivo or in vitro use, such as those described herein. Directions and or other information can also be included on an insert which is included with the kit.

In a preferred embodiment the kit comprises an PAR2-peptide or a functional analog thereof, preferably as defined above and specifically binding to anti-PAR2 antibodies present (at increased levels) in samples of patients suffering from graft rejection of a liver transplant.

The immunological test kit according to the invention comprises the PAR2 or a functional analog thereof or peptides or proteins of analogous function per se. The test kit of the invention comprises at least one complete PAR2 or functionally analogous peptides or proteins of said receptor, optionally bound to a solid phase. Furthermore, the test kit may also comprise buffers, specific conjugate together with an enzyme, wash solution, substrate solution to detect the immune reaction and/or a quenching solution. Using these substances a person skilled in the art will be able to perform, e.g. an ELISA to detect the anti-PAR2 antibodies. The buffers, specific conjugate plus enzyme, wash solution, substrate solution to detect immune reaction and quenching solution are well known to those skilled in the art. For example, it would be sufficient to have the test comprise a freeze-dried PAR2 or peptides or proteins of PAR2 analogous function and to add the buffers and other solutions immediately prior to testing the biological material. However, it is also possible to provide the test kit with the PAR2 or its functionally analogous peptides of proteins bound to a solid phase. To detect the anti-PAR2 antibodies the specific conjugate, wash solution, substrate solution and quenching solution, which can be components of the test kit, have to be added according to a mode well known to those skilled in the art.
In another advantageous embodiment of the invention, it is envisioned that the test kit is a test strip comprising the PAR2 or its functionally analogous peptides or proteins immobilized on a solid phase. For example, the test strip can be immersed in serum or other patient samples and incubated. Using a specific biochemical reaction on the test strip after formation of the PAR2/anti-PAR2 antibody complex, a specific color reaction can be triggered by means of which the anti-PAR2 antibody can be detected.

The test system of the invention permits quantification of the anti-PAR2 antibodies directly in a sample, e.g. in plasma of patients. The detection method according to the invention is time saving and cost effected. Large amounts of the samples can be tested and, owing to the low amount of the equipment required, routine laboratories can be used.

The invention as outlined also relates to an immunosuppressant for use in the treatment of graft intolerance in a patient having received a liver transplant, wherein the immunosuppressant is administered to the subject when the subject exhibits increased sample levels of PAR2 antibodies. Preferably the immunosuppressant is administered to the patient, if the patient has been diagnosed as suffering from graft intolerance by a method according the present invention, or if the need of administration of an immunosuppressant is attributed to the patient by a method according the present invention, or if onset or continuing of weaning is contradicted by the method according to the present invention. The invention also relates to a method of treating a patient having received a liver transplant with an immunosuppressant comprising the step of determining the level of PAR2 antibodies in said patient and comparing the determined level to a first control level of PAR2 antibodies derived from a patient not suffering from graft intolerance and/or a second control level of PAR2 antibodies derived from a patient showing graft intolerance, wherein the patient is treated with the immunosuppressant if the is determined level of said PAR2 antibodies is increased as compared to the first control level and/or decreased as compared to the second control level.

The term “immunosuppressant” or “immunosuppressive drug” or “immunosuppressive agents” or “antirejection medications” are used interchangeably and synonymously in connection with the present invention and are to be understood as any substance,
pharmaceutical composition or the like which that inhibit or prevent activity of the immune system. Immunosuppressive drugs can be classified into five groups; i.e. glucocorticoids, cytostatics, antibodies, drugs acting on immunophilins, and other drugs. The immunosuppressant according to the present invention is hence preferably selected from the group consisting of glucocorticoids, cytostatics, antibodies, drugs acting on immunophilins, and other drugs. Preferably the immunosuppressant is selected from the group consisting of cyclosporine A, tacrolimus, and steroids. Also preferred are combinations of the above listed immunosuppressants, particular preferred combinations are cyclosporine A in combination with steroids, preferably low-dose steroids, and tacrolimus in combination with steroids, preferably low-dose steroids. Preferred steroids are Hydrocortisones.

As outlined above, the invention also relates to a method for removal of PAR2 antibodies from isolated blood. The skilled person will readily acknowledge that the sample in which the presence and/or level of PAR2 antibodies is determined may be the same as the isolated blood from which the PAR2 antibodies are removed. However, in a preferred embodiment the PAR2 antibodies are determined in a blood sample of a patient having received a liver transplant and PAR2 antibodies are than removed from isolated blood of said patient, the isolated blood not being said blood sample. The gist of the invention is the correlation of the presence and/or increased levels of PAR2 antibodies and adverse effects on the liver transplant, most likely through the immune system of the patient. Hence, the methods as outlined herein above, in particular the method for diagnosing graft intolerance, gives the guidance that the immune system is active against the liver transplant. Hence, it is indicated to remove antibodies from the blood of the patient, e.g. by plasmapheresis. Hence, the method for removal of PAR2 antibodies basically relates to two steps, i.e. the determining step and the removal step. It will be acknowledged that the removal step may comprise the removal of more than only PAR2 antibodies, e.g. the removal of a plurality of immunoglobulins. A specific removal of PAR2 antibodies is not necessary. Hence, in one embodiment of the method for removal of PAR2 antibodies the PAR2 antibodies are removed through removal of a plurality of antibodies comprising PAR2 antibodies. Such removal of a plurality of antibodies is known in the art and also referred to as immunoabsorption.
It will be readily understood that the embodiments outlined above shall apply to the invention as a whole and not be limited to a specific method, unless stated otherwise. It will for example be understood the embodiments for PAR2 or the immunogenic peptide shall be applied to every method, kit or the like disclosed herein. The invention is further illustrated by the following non-limiting examples and figures.
SEQ ID NO: 1 (PAR2 protein sequence; preferred epitope underlined):
1   MRSPSAAWLL GAAILLAASL SCSGTIQTGN RSSKGRSLIG KVDGTSHVTG
51  KGVTVETVFS VDEFSASVLT GKLTTVFLPI VYTIVFVVG CLPSNGMALWVF
101 LFRTKKKHPA VIYMANLALA DLLSVIWFPL KIAYHIHGNN WIYGEALCNV
151 LIGFFYGNMY CSILFMTCLS VQRYWVIVNP MGHSRKKANI AIGISLAIWL
201 LILLVTIPLY VVKQTIFIPA LNITTHCHVL PEQLLVGDMP NYFLSLAIGV
251 FLFPAFLTAS AYVLMIIRML SSAMDENSEK KRKRAIKLIV TVLAMYLICF
301 TPSNLLLVVH YFLIKSOGQS HVYALYIVAL CLSTLNSCID PFVYYFVSHD
351 FRDHAKNALL CRSVRTVQGM QVSLTSKKS RKSSSYSSSS TTVKTSY

SEQ ID NO: 2 (Preferred antigenic Peptide of PAR2)

1   LIGKVDGTSV VTGKVTVET VFSVEFVAS VLTGKLTTVF LPIYVTIVFV
15  VGLPSNGMAL WVFLFRKKK HPAVIYMANL ALADLSVIW FPLKIAYHIH
101 GNNWIYGEAL CN
EXAMPLES

Study design and population

The study included pediatric patients who underwent weaning of immunosuppressant after LDLT from June 1990 to December 2010. Serum samples and pathological data were obtained from January 2011 through December 2012. The total amount of patients was 80. 14 of them were in the process of weaning. The rest had completed withdrawal from immunosuppressants with 17 patients having operational tolerance, 30 showing fibrosis and 19 suffering from graft rejection of the liver transplant. The time since transplant varied from 5.1 to 22.5 years with a median of 16.3 years.

Pathology stages were determined by liver biopsies taken at the time of serum collection using Ilhak’s modified score (Adeyi et al.; Importance of liver biopsy findings in immunosuppression management: Biopsy monitoring and working criteria for patients with operational tolerance (OT); Liver Transpl. 2012; PubMed PMID: 22645090).

Patients were treated with cyclosporine A with low-dose steroids, or with tacrolimus with low-dose steroids. Tacrolimus was continuously given intravenously immediately after LDLT. Subsequently, it was given orally starting one day pre-transplantation. The target through level of tacrolimus is 10 to 12 ng/ml for the first two weeks and 5 to 10 ng/ml for the following two months. After discharge, the dose of tacrolimus is determined individually, depending upon each patient’s condition. Steroids are started during LDLT, then tapered gradually and stopped after three months.

Measurement of PAR antibodies in samples:

We measured the anti-PAR2 autoantibody in serum samples using a sandwich ELISA kit (CellTrend GmbH Luckenwalde, Germany). The microtiter 96-well polystyrene plates were coated with human PAR2 fragment of the sequence of SEQ ID NO:2. To maintain the conformational epitopes of the receptor, 1 mM calcium chloride was added to every buffer. Duplicate samples of a 1:100 serum dilution were incubated at 4°C for 2 hours. After washing steps, plates were incubated for 60 minutes with a 1:20.000 dilution of horseradish-
peroxidase-labeled goat anti-human IgG (Jackson, USA) used for detection. In order to obtain a standard curve, plates were incubated with test sera from an anti-EGF autoantibody positive index patient. The ELISA was validated according to the FDA’s “Guidance for industry: Bioanalytical method validation”.

PAR2-auto-antibodies are not commercially available; a serum sample from a patient with a systemic sclerosis is used for the standard curve. A 1:100 dilution of the serum sample of a systemic sclerosis patient is defined as 40 Units PAR2-Receptor-Antibodies. 1:100 to 1:1600 dilutions of systemic sclerosis donors served as a positive control (range 2.5 to 40 Units/ml). To set a standard for the concentrations of the autoimmune antibodies, a standard curve was generated. In detail, a serum sample of systemic sclerosis serum sample was diluted (a) 1:100 for standard point 40 Units/ml, (b) 1:200 for standard point 20 Units/ml, (c) 1:400 for standard point 10 Units/ml, (d) 1:800 for standard point 5 Units/ml, (e) 1:1600 for standard point 2.5 Units/ml. Then the optical density was determined using the kit and method of above. Each standard point was performed in duplicates. Results are shown in Figure 5.

**Example 1:**

Anti-PAR2 antibody levels in serum samples from the patients as mentioned above were measured using the kit and method mentioned above. The levels were determined in units/ml. Fig. 1 shows the mean values for the PAR2 antibody level for patients undergoing weaning, patients with operational tolerance, patients with fibrosis and patients suffering from graft rejection. Patient suffering from graft rejection had significantly increased levels (p≤0.009) of anti-PAR2 antibodies as compared to those showing operational tolerance and patients with fibrosis. Likewise, patients with fibrosis show increased levels of PAR2 antibodies as compared to patients with operational tolerance.

**Example 2:**

The anti-PAR2 antibody levels as determined in Example 1 were compared to anti-AT1R antibody levels in samples of the same patient. Anti-AT1R antibody levels where measured with a commercially available Kit (Celltrend GmbH, Germany). AT1R antibodies are
known as correlating with graft intolerance of liver transplants (Ohe H, Uchida Y, Yoshizawa A, Hirao H, Taniguchi M, Maruya E, Yurugi K, Hishida R, Maekawa T, Uemoto S, Terasaki PI. Association of Anti-Human Leukocyte Antigen and Anti-Angiotensin II Type 1 Receptor Antibodies With Liver Allograft Fibrosis After Immunosuppression Withdrawal. Transplantation. 2014 Jun 9. PubMed PMID: 24914568). The correlation is depicted in Figure 2. As can be derived from the Figure, the increased levels of anti-PAR2 antibodies significantly correlate with increased levels of anti-AT1R antibodies, while the resolution is higher with anti-PAR2 antibodies.

**Example 3:**

Levels of anti-PAR2 antibodies were determined in samples from 43 patients. These 43 patients were selected as they had been tested for complement component 4d (C4d) in liver tissue staining. C4d staining correlates with antibody-mediated rejection of liver allografts (Ali S, Ormsby A, Shah V, Segovia MC, Kantz KL, Skorupski S, Eisenbrey AB, Mahan M, Huang MA. Significance of complement split product C4d in ABO-compatible liver allograft: diagnosing utility in acute antibody mediated rejection. Transpl Immunol. 2012; 26(1):62-9. The levels of anti-PAR2 antibodies in samples of patients having a C4d positive liver allograft stain were significantly higher than in samples of patients being C4d negative in liver allograft tissue.

**Example 4:**

The levels of PAR2 antibodies were correlated with the respective levels of liver function markers, i.e. aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin and total albumin. There is a strong correlation between the liver enzymes AST and ALT and the occurrence of PAR2-Antibodies in the serum of LDLT patients. Furthermore, with decreasing levels of total bilirubin to total albumin the levels of PAR2 antibodies increased. Both, total bilirubin level as well as total albumin level are decreased in chronic liver disease and hence decrease is associated with a loss in liver function, e.g. through graft intolerance. Hence, the results demonstrate the correlation of increased levels of PAR antibodies in patients with graft intolerance.
Summary:

The Results show that the level of PAR2 antibodies in samples of patient having received a liver transplant and suffering from graft intolerance are increased. Furthermore the results show that the PAR2 antibody levels increase with severity of symptoms in a way allowing differential diagnosis.

Certain aspects of the invention

Item 1: A method for diagnosing graft intolerance or for assessing the risk of graft intolerance in a patient, the patient having received a liver transplant, comprising the steps of:

(i) determining the level of antibodies against protease-activated receptor 2 (PAR2) in a sample from the patient to be diagnosed,

(ii) comparing the determined level in the sample to a control level derived from subjects tolerating a liver transplant/graft, preferably not showing graft rejection and/or fibrosis;

wherein an increased level in the sample from the patient to be diagnosed as compared to the control level is indicative for the presence or the risk of graft intolerance in the patient.

Item 2: The method for diagnosing graft intolerance or for assessing the risk of graft intolerance in a patient according to item 1, wherein the graft intolerance is selected from the group consisting of fibrosis and graft rejection.

Item 3: The method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to item 1 or 2, wherein a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 1.2 fold as compared to the control level is indicative for graft intolerance.

Item 4: The method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to any one of items 1 to 3, wherein a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 18 U/ml is
indicative for graft intolerance.

Item 5: The method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to any one of items 1 to 4, wherein a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 2 fold as compared to the control level is indicative for graft rejection.

Item 6: The method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to any one of items 1 to 5, wherein a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 30 U/ml is indicative for graft rejection.

Item 7: A method for differential diagnosing graft rejection or fibrosis in a patient or assessing the risk of a patient for graft rejection or fibrosis, the patient having received a liver transplant, comprising the steps of

(i) determining the level of antibodies against protease-activated receptor 2 (PAR2) in a sample from the patient to be diagnosed;

(ii) comparing the determined level in the sample to either one, two or all of a first, a second and a third PAR2 antibody control level;

a) wherein the first PAR2 antibody control level is derived from subjects not suffering from graft intolerance, and

b) wherein the second PAR2 antibody control level is derived from subjects suffering from fibrosis;

c) wherein the third PAR2 antibody control level is derived from a subject suffering from graft rejection;

wherein an increased level in the sample from the patient to be diagnosed as compared to the first PAR2 antibody control level is indicative for graft intolerance in the patient or the risk of graft intolerance in said patient; and

wherein an increased level in the sample from the patient to be diagnosed as compared to the second PAR2 antibody control level and/or an equal or increased level as compared to the third PAR2 antibody control level is indicative for graft rejection or indicative for the risk of graft rejection in the patient;

wherein an decreased level in the sample from the patient to be diagnosed as
compared to the third PAR2 antibody control level, and an increased level as compared to the first PAR2 antibody control level is indicative for the presence or the risk of fibrosis in said patient; and
wherein an equal level as compared to the second PAR2 antibody control level is indicative for the presence or the risk of fibrosis in the patient.

Item 8: A method for assessing the need of a patient for administration of an immunosuppressant, the patient having received a liver transplant, comprising the steps of:

(i) determining the level of antibodies against protease-activated receptor 2 (PAR2) in a sample from the patient;
(ii) comparing the determined level in the sample to either one or both of a first, and a second PAR2 antibody control level;
   a) wherein the first PAR2 antibody control level is derived from subjects not showing graft intolerance, preferably not suffering from graft rejection and/or fibrosis; and
   b) wherein the second PAR2 antibody control level is derived from subjects showing graft intolerance, preferably graft rejection and/or fibrosis;

wherein an increased level in the sample from the patient to be assessed as compared to the first PAR2 antibody control level, and/or an equal level or increased level as compared to the second PAR2 antibody control level is attributed to the need of said patient for the administration of an immunosuppressant;

wherein an decreased level in the sample from the patient to be assessed as compared to the second PAR2 antibody control level, and/or an equal or decreased level as compared to the first PAR2 antibody control level is attributed to the absence of the need of said patient for the administration of an immunosuppressant.

Item 9: A method for monitoring the need of a patient for administration of an immunosuppressant, the patient having received a liver transplant, comprising the steps of:

(i) determining the level of antibodies against protease-activated receptor 2 (PAR2 antibodies) in a sample from the patient to be monitored, said sample
being taken at a first time point; and
(ii) determining the level of PAR2 antibodies in a sample from the patient to be assessed for the need for administration of an immunosuppressant, said sample being taken at at least one further time point, said at least one further time point being after said first time point;

wherein a constant level in-between the samples of the first and the at least one further time point or an decreased level in the sample of the second time point as compared to the sample of the first time point indicates the possibility for onset of weaning of the immunosuppressant and/or continuing the weaning of the immunosuppressant, and

wherein an increased level in the sample of the at least one further time point as compared to the sample of the first time point is contradicts onset and/or continuing of weaning of the immunosuppressant.

Item 10: The method according to any one of items 1 to 9, wherein the method is performed before or after onset of weaning of an immunosuppressant in said patient, preferably after onset of weaning.

Item 11: The method according to any one of items 8 to 10, wherein the immunosuppressant is selected from the group consisting of glucocorticoids, cytostatics, antibodies, and drugs acting on immunophilins.

Item 12: The method according to any one of items 1 to 11, wherein the PAR2 antibody is detected in an immunoassay.

Item 13: The method according to item 11, wherein the immunoassay is selected from the group of immunoprecipitation, enzyme immunoassay (EIA), radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), fluorescent immunoassay, a chemiluminescent assay, an agglutination assay, nephelometric assay, turbidimetric assay, a Western Blot, a competitive immunoassay, a noncompetitive immunoassay, a homogeneous immunoassay a heterogeneous immunoassay, a bioassay and a reporter assay such as a luciferase assay, and an FACS based immunoassay.
Item 14: The method according to any one of items 1 to 13, wherein the sample is a blood sample, preferably plasma or serum.

5 Item 15: The method according to any one of items 1 to 14, comprising the steps of
(a) contacting the sample with protease-activated receptor 2 (PAR2) or an antigenic peptide fragment thereof under conditions allowing for the formation of a complex between anti-PAR2 antibodies with PAR2 or the peptide fragment thereof,
(b) detecting the complex.

10 Item 16: An immunoassay method for detecting an anti-PAR2 antibody in a sample from a subject, comprising the steps of
(a) contacting the sample suspected of comprising an anti-PAR2 antibody with protease-activated receptor 2 (PAR2) or an antigenic peptide fragment thereof under conditions allowing for the formation of a complex between the anti-PAR2 antibody with PAR2 or the peptide fragment thereof,
(b) detecting the complex.

15 Item 17: The method according to any one of items 12 to 16, wherein the PAR2 or the peptide fragment thereof is immobilized on a surface.

20 Item 18: The method according to any one of items 12 to 17, wherein the complex is detected using a secondary antibody against the Fc portion of the anti-PAR2 antibody.

25 Item 19: The method according to any one of items 12 to 18, wherein the anti-PAR2 antibody is an IgG-antibody and the secondary antibody is an anti-IgG antibody.

30 Item 20: The method according to any one of items 12 to 19, wherein the secondary antibody is labelled with a detectable marker.
Item 21: A kit for the diagnosis and/or monitoring graft intolerance and/or determining the need of administration of an immunosuppressant in a patient having received a liver transplant comprising:
- PAR2 or an antigenic peptide fragment thereof, and standards; and/or
- controls for comparing determined levels of PAR2 antibody in a sample to be tested with control levels in subjects not suffering from graft intolerance and/or subjects suffering from graft intolerance.

Item 22: Use of PAR2 or an antigenic peptide fragment thereof for the diagnosis and/or monitoring graft intolerance and/or determining the need of administration of an immunosuppressant in a patient having received a liver transplant.

Item 23: An immunosuppressant for use in the treatment of graft intolerance in a patient having received a liver transplant, wherein the immunosuppressant is administered to the subject when the subject exhibits increased sample levels of PAR2 antibodies.

Item 24: The immunosuppressant for use according to item 23, wherein the immunosuppressant is administered to the patient, if the patient has been diagnosed as suffering from graft intolerance by a method according to any one of items 1 to 6 and 9 to 14, or if the need of administration of an immunosuppressant is attributed to the patient by a method according to items 7, and 9 to 14, or if onset or continuing of weaning is contradicted by the method according to any of items 8 to 14.

Item 25: The immunosuppressant for use according to item 23 or 24, wherein the immunosuppressant is selected from the group consisting of glucocorticoids, cytostatics, antibodies, and drugs acting on immunophilins.

Item 26: A method for removal of PAR2 antibodies from isolated blood, comprising the steps of:
a.) determining the presence or absence of a PAR2 antibody in a blood sample from a patient thought to suffer from graft intolerance;
b.) wherein upon determination of the presence of a PAR2 antibody in said blood sample, PAR2 antibody is removed from isolated blood of said patient.

Item 27: The method for removal of PAR2 antibodies according to item 26, wherein the method comprises the steps of the method for diagnosing graft intolerance or assessing the risk of graft intolerance according to any one of items 1 to 6 and 10 to 14, and wherein PAR2 antibody is removed from isolated blood of said patient upon indication of the presence or the risk of graft intolerance in said patient.
CLAIMS

1. A method for diagnosing graft intolerance or for assessing the risk of graft intolerance in a patient, the patient having received a liver transplant, comprising the step of determining the presence or absence of antibodies against protease-activated receptor 2 (PAR2) (anti-PAR2 antibodies) in a sample of the patient, wherein the presence of anti-PAR2 antibodies is indicative for the presence or the risk of graft intolerance in said patient.

2. The method for diagnosing graft intolerance or for assessing the risk of graft intolerance in a patient, according to claim 1, comprising the steps of:
   (i) determining the level of antibodies against protease-activated receptor 2 (PAR2) in a sample from the patient to be diagnosed,
   (ii) comparing the determined level in the sample to a control level derived from subjects tolerating a liver transplant/graft, preferably not showing graft rejection and/or fibrosis;
wherein an increased level in the sample from the patient to be diagnosed as compared to the control level is indicative for the presence or the risk of graft intolerance in the patient.

3. The method for diagnosing graft intolerance or for assessing the risk of graft intolerance in a patient according to claim 1 or 2, wherein the graft intolerance is selected from the group consisting of fibrosis and graft rejection.

4. The method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to any one of claims 1 to 3, wherein a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 1.2 fold as compared to the control level is indicative for graft intolerance.

5. The method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to any one of claims 1 to 4, wherein a level of anti-PAR2 antibodies in the
sample of the patient to be diagnosed of more than 18 U/ml is indicative for graft intolerance.

6. The method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to any one of claims 1 to 5, wherein a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 2 fold as compared to the control level is indicative for graft rejection.

7. The method for diagnosing graft intolerance or for assessing the risk of graft intolerance according to any one of claims 1 to 6, wherein a level of anti-PAR2 antibodies in the sample of the patient to be diagnosed of more than 30 U/ml is indicative for graft rejection.

8. A method for differential diagnosing graft rejection or fibrosis in a patient or assessing the risk of a patient for graft rejection or fibrosis, the patient having received a liver transplant, comprising the steps of

   (i) determining the level of antibodies against protease-activated receptor 2 (PAR2) in a sample from the patient to be diagnosed;

   (ii) comparing the determined level in the sample to either one, two or all of a first, a second and a third PAR2 antibody control level;

   a) wherein the first PAR2 antibody control level is derived from subjects not suffering from graft intolerance, and

   b) wherein the second PAR2 antibody control level is derived from subjects suffering from fibrosis;

   c) wherein the third PAR2 antibody control level is derived from a subject suffering from graft rejection;

wherein an increased level in the sample from the patient to be diagnosed as compared to the first PAR2 antibody control level is indicative for graft intolerance in the patient or the risk of graft intolerance in said patient; and

wherein an increased level in the sample from the patient to be diagnosed as compared to the second PAR2 antibody control level and/or an equal or increased level as compared to the third PAR2 antibody control level is indicative for graft rejection or indicative for the risk of graft rejection in the patient;
wherein an decreased level in the sample from the patient to be diagnosed as compared to the third PAR2 antibody control level, and an increased level as compared to the first PAR2 antibody control level is indicative for the presence or the risk of fibrosis in said patient; and
wherein an equal level as compared to the second PAR2 antibody control level is indicative for the presence or the risk of fibrosis in the patient.

9. A method for assessing the need of a patient for administration of an immunosuppressant, the patient having received a liver transplant, comprising the steps of:
   (i) determining the level of antibodies against protease-activated receptor 2 (PAR2) in a sample from the patient;
   (ii) comparing the determined level in the sample to either one or both of a first, and a second PAR2 antibody control level;
      a) wherein the first PAR2 antibody control level is derived from subjects not showing graft intolerance, preferably not suffering from graft rejection and/or fibrosis; and
      b) wherein the second PAR2 antibody control level is derived from subjects showing graft intolerance, preferably graft rejection and/or fibrosis;
wherein an increased level in the sample from the patient to be assessed as compared to the first PAR2 antibody control level, and/or an equal level or increased level as compared to the second PAR2 antibody control level is attributed to the need of said patient for the administration of an immunosuppressant;
wherein an decreased level in the sample from the patient to be assessed as compared to the second PAR2 antibody control level, and/or an equal or decreased level as compared to the first PAR2 antibody control level is attributed to the absence of the need of said patient for the administration of an immunosuppressant.

10. A method for monitoring the need of a patient for administration of an immunosuppressant, the patient having received a liver transplant, comprising the steps of:
   (i) determining the level of antibodies against protease-activated receptor 2 (PAR2 antibodies) in a sample from the patient to be monitored, said sample being
taken at a first time point; and
(ii) determining the level of PAR2 antibodies in a sample from the patient to be assessed for the need for administration of an immunosuppressant, said sample being taken at at least one further time point, said at least one further time point being after said first time point;
wherein a constant level in-between the samples of the first and the at least one further time point or an decreased level in the sample of the second time point as compared to the sample of the first time point indicates the possibility for onset of weaning of the immunosuppressant and/or continuing the weaning of the immunosuppressant, and wherein an increased level in the sample of the at least one further time point as compared to the sample of the first time point is contradicts onset and/or continuing of weaning of the immunosuppressant.

11. The method according to any one of claims 1 to 10, wherein the method is performed before or after onset of weaning of an immunosuppressant in said patient, preferably after onset of weaning.

12. The method according to any one of claims 9 to 11, wherein the immunosuppressant is selected from the group consisting of glucocorticoids, cytostatics, antibodies, and drugs acting on immunophilins.

13. The method according to any one of claims 1 to 12, wherein the PAR2 antibody is detected in an immunoassay.

14. The method according to claim 13, wherein the immunoassay is selected from the group of immunoprecipitation, enzyme immunoassay (EIA)), radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), fluorescent immunoassay, a chemiluminescent assay, an agglutination assay, nephelometric assay, turbidimetric assay, a Western Blot, a competitive immunoassay, a noncompetitive immunoassay, a homogeneous immunoassay a heterogeneous immunoassay, a bioassay and a reporter assay such as a luciferase assay, and an FACS based immunoassay.

15. The method according to any one of claims 1 to 14, wherein the sample is a blood
sample, preferably plasma or serum.

16. The method according to any one of claims 1 to 15, comprising the steps of
   (a) contacting the sample with protease-activated receptor 2 (PAR2) or an antigenic
       peptide fragment thereof under conditions allowing for the formation of a
       complex between anti-PAR2 antibodies with PAR2 or the peptide fragment
       thereof;
   (b) detecting the complex.

17. An immunoassay method for detecting an anti-PAR2 antibody in a sample from a
    subject, comprising the steps of
   (a) contacting the sample suspected of comprising an anti-PAR2 antibody with
       protease-activated receptor 2 (PAR2) or an antigenic peptide fragment thereof
       under conditions allowing for the formation of a complex between the anti-
       PAR2 antibody with PAR2 or the peptide fragment thereof;
   (b) detecting the complex.

18. The method according to any one of claims 13 to 17, wherein the PAR2 or the peptide
    fragment thereof is immobilized on a surface.

19. The method according to anyone of claims 13 to 18, wherein the complex is detected
    using a secondary antibody against the Fc portion of the anti-PAR2 antibody.

20. The method according to claim 19, wherein the anti-PAR2 antibody is an IgG-antibody
    and the secondary antibody is an anti-IgG antibody.

21. The method according to any one of claims 19 to 20, wherein the secondary antibody is
    labelled with a detectable marker.

22. A kit for the diagnosis and/or monitoring graft intolerance and/or determining the need
    of administration of an immunosuppressant in a patient having received a liver
    transplant comprising:
    - PAR2 or an antigenic peptide fragment thereof, and standards; and/or
controls for comparing determined levels of PAR2 antibody in a sample to be tested with control levels in subjects not suffering from graft intolerance and/or subjects suffering from graft intolerance.

23. Use of PAR2 or an antigenic peptide fragment thereof for the diagnosis and/or monitoring graft intolerance and/or determining the need of administration of an immunosuppressant in a patient having received a liver transplant.

24. An immunosuppressant for use in the treatment of graft intolerance in a patient having received a liver transplant, wherein the immunosuppressant is administered to the subject when the subject exhibits presence or increased sample levels of PAR2 antibodies.

25. The immunosuppressant for use according to claim 24, wherein the immunosuppressant is administered to the patient, if the patient has been diagnosed as suffering from graft intolerance by a method according to any one of claims 1 to 7 and 10 to 15, or if the need of administration of an immunosuppressant is attributed to the patient by a method according to claims 9 and 11 to 15, or if onset or continuing of weaning is contradicted by the method according to any of claims 10 to 15.

26. The immunosuppressant for use according to claim 24 or 25, wherein the immunosuppressant is selected from the group consisting of glucocorticoids, cytostatics, antibodies, and drugs acting on immunophilins.

27. A method for removal of PAR2 antibodies from isolated blood, comprising the steps of:
   a.) determining the presence or absence of a PAR2 antibody in a blood sample from a patient thought to suffer from graft intolerance;
   b.) wherein upon determination of the presence of a PAR2 antibody in said blood sample, PAR2 antibody is removed from isolated blood of said patient.

28. The method for removal of PAR2 antibodies according to claim 27, wherein the method comprises the steps of the method for diagnosing graft intolerance or assessing the risk of graft intolerance according to any one of claims 1 to 7 and 11 to
15, and
wherein PAR2 antibody is removed from isolated blood of said patient upon indication of the presence or the risk of graft intolerance in said patient.
Figure 1

![Graph showing PAR2 antibodies (U/ml) with median values indicated.]
Figure 2

Tolerance: $r^2 = 0.028$, $P = 0.55$
Fibrosis: $r^2 = 0.115$, $P = 0.08$
Rejection: $r^2 = 0.695$, $P < 0.0001^*$

Figure 3

$C_4d$  

- neg  
  - Median 21.8 U/ml  
- pos  
  - Median 61.6 U/ml  

anti-PAR2  

(P = 0.05*)
Figure 4

**Anti-PAR2**

![Graphs showing relationships between AST/LALT and PAR2 levels, with statistical significance marked.](image)

![Total bilirubin levels in relation to PAR2 and Anti-PAR2 levels.](image)

Figure 5:

**Standard curve of a PAR2-Antibody ELISA (450nm/620nm)**

![Graph of optical density against PAR2-Ab concentration.](image)
A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/564

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
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<td>WO 2011/015380 A1 (CELLTREND GMBH [DE]; HEIDECHE HARALD [DE]; SCHULZE-FORSTER KAI [DE]) 10 February 2011 (2011-02-10)</td>
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X Further documents are listed in the continuation of Box C.

X See patent family annex.

* Special categories of cited documents:
* "A" document defining the general state of the art which is not considered to be of particular relevance
* "E" earlier application or patent but published on or after the international filing date
* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
* "O" document referring to an oral disclosure, use, exhibition or other means
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"S" document member of the same patent family

X Date of the actual completion of the international search

19 August 2015

Date of mailing of the international search report

18/12/2015

Name and mailing address of the ISA/
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2040
Fax: (+31-70) 340-3016

Authorized officer
Barnas, Christoph
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<td>TANIGUCHI MICHIKO ET AL: &quot;ANTIBODIES AGAINST PROTEASE-ACTIVATED RECEPTORS (PAR) AFTER IMMUNOSUPPRESSION WITHDRAWAL IN PEDIATRIC LIVING-DONOR LIVER TRANSPLANT&quot;, 2014, HUMAN IMMUNOLOGY, VOL. 75, NR. SUPPL. 1, PAGE(S) 43-44, 40TH ANNUAL MEETING OF THE AMERICAN-SOCIETY-FOR-HISTOCOMPATIBILITY-AN D-IMMUNOGENETICS (ASHI); DENVER, CO, USA; OCTOBER 20 -24, 2014, XP055208390, ISSN: 0198-8859(print) the whole document</td>
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INTERNATIONAL SEARCH REPORT

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 8.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of Item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

   1-16, 22-26(completely); 18-21(partially)

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-16, 22-26(completely); 18-21(partially)

A method for diagnosing graft intolerance or for assessing the risk of graft intolerance in a patient, the patient having received a liver transplant, comprising the step of determining the presence or absence of antibodies against protease-activated receptor 2 (PAR2) (anti-PAR2 antibodies) in a sample of the patient, wherein the presence of anti-PAR2 antibodies is indicative for the presence or the risk of graft intolerance in said patient.

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2. claims: 17(completely); 18-21(partially)

An immunoassay method for detecting an anti-PAR2 antibody in a sample from a subject, comprising the steps of (a) contacting the sample suspected of comprising an anti-PAR2 antibody with protease-activated receptor 2 (PAR2) or an antigenic peptide fragment thereof under conditions allowing for the formation of a complex between the anti-PAR2 antibody with PAR2 or the peptide fragment thereof, (b) detecting the complex.

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3. claims: 27, 28

A method for removal of PAR2 antibodies from isolated blood, comprising the steps of: a.) determining the presence or absence of a PAR2 antibody in a blood sample from a patient thought to suffer from graft intolerance; b.) wherein upon determination of the presence of a PAR2 antibody in said blood sample, PAR2 antibody is removed from isolated blood of said patient.

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