HOSPITAL UNIVERSITARIO MARQUÉS DE VALDECILLA
UNIVERSIDAD DE CANTABRIA

# Intracellular ATP Production By CD4 T Cells

## Comparison of Different Conditions of Cells Sources And Clinical Utility In Renal Transplantation

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# Introduction

The immune system has two different ways to defend from foreign agents: humoral immunity and cell-mediated immunity (CMI). B lymphocytes are responsible of the humoral immunity by the production of antibodies. The acquire immune response is mediated by T lymphocytes. These cells regulate CMI together with the innate immune response, which is mediated by monocytes.

Transplantation is the process of taking cells, tissues or organs, called a graft, from one individual (donor) to place it in another individual (recipient). There is a classification according to the kind of graft:

- Autologous graft (autograft): Graft transplanted from one individual to the same individual.
- Syngeneic graft: Graft transplanted from one individual to another individual genetically identical.
- Allogeneic graft (allograft): Graft transplanted from one individual to another of the same specie but genetically different.
- Xenogeneic graft (xenograft): Graft transplanted from one individual to another of different species.

The allograft rejection is mediated by CD4+ T cells mostly. The main reason why transplantation induces such strong immune response is the high variability of the T cells able to respond to mismatched major histocompatibility complex (MHC) molecules. MHC molecules are responsible for almost all strong rejection reactions. There is a high level of combinations for specific recognition of alloantigens or alloantigenic peptides by T cells and also there is cross-reactivity of T cells with MCH complexes of the graft, which has the alloantigens. The rejection can occur even between siblings with identical MCH molecules. In this case, the rejection is because T cells recognise other polymorphic non-MHC molecules called minor histocompatibility antigens (miH).

There are fundamentally two different ways to the T cells to recognise the allogeneic MCH molecules of the graft. The direct presentation occurs when an intact MHC molecule is displayed by donor antigen-presenting cells (APCs) in the graft. In this case, the MHC molecule

of the graft has a similarity with the MHC molecules of the recipient, and it's directly recognised by T cells of the recipient. In the indirect presentation, the MHC of the donor is processed by recipient APCs. Recipient APCs present derived peptides from allogeneic MHC associated with self MHC molecules. In indirect presentation foreign MHC molecules are handled like a microbial protein antigen and the mechanism of processing is the same.

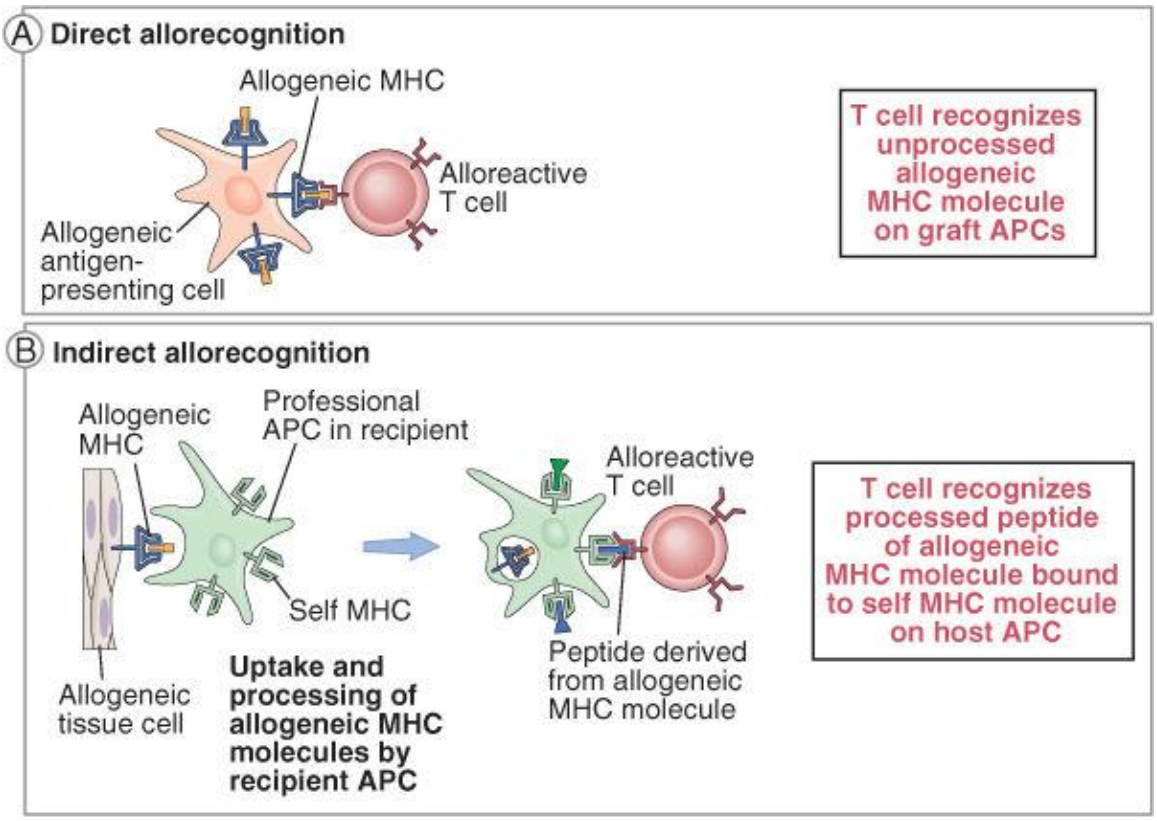


**Figure 1:** Summary of the direct allorecognition (A) and indirect allorecognition (B). In direct allorecognition the allogeneic MHC is not processed as it occurs in the indirect allorecognition.

Normal T cells receptors (TCR) are selected to recognise a self MHC molecule plus foreign peptide. In the direct allorecognition there is a cross reaction of a normal TCR with MHC plus allogeneic peptide. Normally, TCRs are selected to be self MHC-restricted, but they can recognize foreign MHC because same TCR may recognize self-allogeneic MHC molecules and MHC-foreign peptide complexes. The reason is because a foreign MHC is enough similar to self MHC plus foreign peptide to be recognised by T cells.

In the indirect alloantigen presentation, on the other hand, recipient APCs may process allogeneic MHC molecules. The self MHC of APCs plus peptides of foreign MHC molecules

are recognised by T cells like conventional foreign protein antigens. The foreign MHC differs enough from self MHC to be processed as any foreign protein antigen. This is an example of cross-presentation, in which are produced APCs (dendritic cells, usually) with self MHC plus peptides of foreign MHC molecules. The foreign MHC will be processed by host APCs by endosomal vesicular pathway (as a consequence of phagocytosis) and they will be allorecognised by CD4+ T cells. The MHC involve in this process are class II MHC. If the MHC molecules are from class I, APCs will be recognised indirectly by CD8+ T cells.

The rejection of allograft is a consequence of activation of alloreactive T cells in vivo. This activation requires presentation of alloantigens of the donor. This presentation may be direct (by donor-derived APCs in the graft) or indirect (by host APCs plus alloantigens of the graft). Furthermore, organs may contain donor APCs, such as dendritic cells, with donor MHCs molecules. These cells can migrate to regional lymph nodes and are recognised by T cells of the recipient (direct pathway). Graft alloantigens can migrate to lymph node and been captured by and presented by recipient APCs or dendritic cells can also migrate to the graft (indirect pathway). Both pathways can activate T cells of recipient. This activate T cells can migrate to the graft and cause graft rejection, mediated by alloreactive CD4+ helper T cells and CD8+ T cells.

There is a graft damage that resembles delayed-type hypersensitivity (DTH) mediated by CD4+ helper T cells. Activated CD4+ T cells produce cytokines that damage the graft. On the other hand, activated CD8+ T cells differentiate to CTLs, recognise class I MHC in the nucleated cells in the graft and kill them. If CD8+ T cells are produced by the indirect pathway they can't kill foreign cells directly because they are MHC-restricted. Probably, DTH mediated by CD4+ effector T cells is the principal mechanism of rejection when alloreactive T cells are stimulated by the indirect pathway. Host APCs plus alloantigens that are in the graft are recognised by CD4+ effector T cells that also infiltrate in the graft. There is an unclear relation between direct and indirect pathways in graft rejection. There are theories that explain in acute allograft rejection CD8+ CTLs induced by direct recognition of alloantigens are the most important cells. On the other hand, in chronic rejection CD4+ effector T cells induced by indirect pathway will be more important.

There are three mechanisms of allograft rejection (Figure 2):

- Hiperacute rejection: Starts within minutes to hours after the surgery. Is characterized by thrombotic occlusion of the graft vasculature. It's caused by pre-existing antibodies in the host circulation that bind to donor endothelial antigens. Nowadays with the previous studies of patients is almost impossible to occur this kind of rejection.
- Acute rejection: Could start after the first week of transplantation. Is mediated by T cells and antibodies that cause a vascular and parenchymal injury. Activated T cells cause injury on the graft by cell lysis, production of cytokines and recruitment of activate inflammatory cells. Both CD4+ T cells and CD8+ cells may contribute in the acute rejection. There are evidence of CD4 T cells are sufficient to cause acute rejection.
- Chronic rejection: May occur long time after the transplantation. Is characterized by vascular abnormalities with loss of the graft function. Chronic rejection is less understood due its complexity. The fibrosis may be due as result of cytokines that stimulate fibroblast. There is also a proliferation of intimal smooth muscle cells that cause arterial occlusion following necrosis.

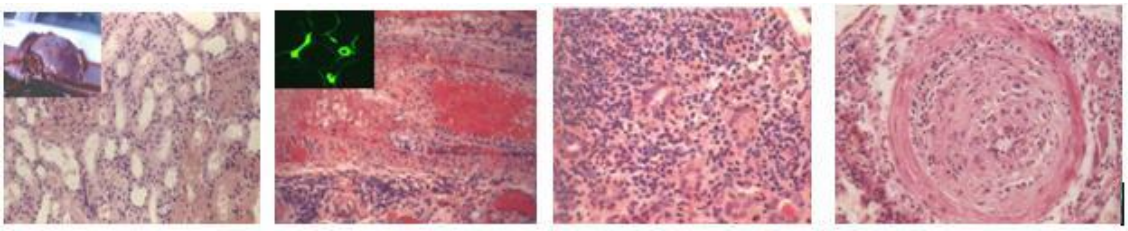

**Figure 2:** Histological samples or graft in different kind of rejection process. Left shows hiperacute rejection. The two pictures in the centre show two manifestations of acute rejection. Right shows chronic rejection.

To avoid organ rejection as much as can be possible, is mandatory to administrate immunosuppressive drugs that inhibit or kill T lymphocytes. The success of transplantation is linked to the development of immunosuppression and the immunosuppressive drugs. In absence of immunosuppressive drugs there will be a rejection of the organ. The alloantigen of the graft will be recognised and the immune system will attack it. This can be avoided by using immunosuppressive drugs. The immunosuppression is a chronic treatment to prevent the rejection of the organ. This therapy to get this affects the patient and the graft itself.

There is a lot of combination of drugs, and it's not easy to choose the better one. The complexity of the immune system and its variability complicate the prediction of the response of the graft. Because of this, the treatment must be personalized to each patient. The administration of drugs is done accord to the clinical, histological and pharmacokinetic data. In the beginning, the only way to control the immunosuppression was by pharmacokinetics. The pharmacokinetic studies the concentration of drug in the body. This method is very useful, but the actual knowledge of the immune system allows us to develop therapies more personalized. Now we can use pharmacodynamics and pharmacokinetics data. This additional information increases the variability to the monitoring of immunosuppression, but permits us to develop more specific and personalized treatment. There are different techniques to monitor the immunosuppression of the patient by pharmacokinetics and by pharmacodynamics data.

The pharmacokinetic study what do the organism with the drug, which are the absorption, distribution, metabolization and excretion of the drug. This can be measure by the concentration in blood. The reason to monitor the concentration of the drug is because the therapeutic window is very narrow. The difference between the beneficial dose and the adverse dose is very small. The therapeutic window depends on the patient, his gender, age, kind of graft, other drugs, etc. So, we have to monitor the immunosuppression of the patient and look for new strategies to get a correct therapy.

The monitoring of immune therapy is based in the blood concentration of the drug/s to maintain it in the therapeutic window. The aim is to avoid an excessive immunosuppression (because the patient could develop an infection or a tumour) or an insufficient immunosuppression (because it could cause a rejection of the graft) in most of the recipients. However, the relationship between blood concentration of the drug and its immunosuppression level is too weak and variable. Furthermore, it depends on the method used to measure the concentration of the drug and the standard ranges for a population are not very useful.

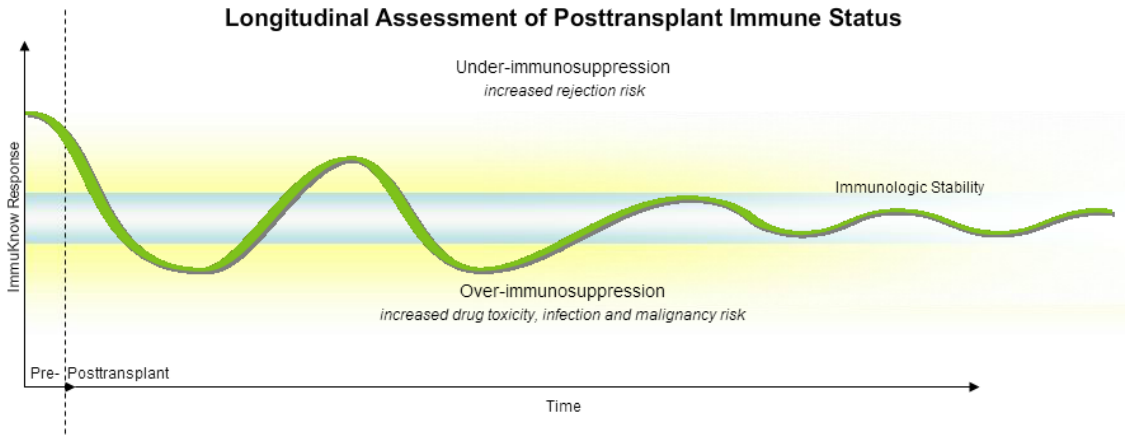


**Figure 3:** Evolution of the immunosuppressive treatmet to reach the immunologic stability and keep the graft without negative efects. The aim is reach the optimal immunosuppresion without over or under immunosupression.

Recently there are other strategies to monitor the immunosuppression by analysing specific biomarkers of the immunomodulation effect of immunosuppressed drugs on T cells against the graft. This is pharmacodynamics monitoring. Pharmacodynamics is based on the biological effect of the drug in its target, that is, the pharmacodynamics if focused in the immune response, studies how the drug affects the organism. The combination of pharmacodynamics and pharmacokinetic pretends to reach the optimal concentration of the drug without toxic levels. The pharmacodynamics also value the individual variability, because not all the immunosuppressive drugs acts equal in all patients.

The pharmacodynamics looks for biomarkers to monitor de immunosuppression of the patients. Biomarkers of immunity reflecting the state of immunosuppression are demonstrating to be of clinical utility in human solid organ transplantation. Different markers and assays have been proposed, most of them being difficult to use in clinics. Among them, the measurement of intracellular ATP production in vitro after polyclonal stimulus of peripheral blood lymphocytes using phytohemagglutinin (PHA) with a commercial kit, ImmuKnow®, has been very recently proposed. There are previous studies that show that the intracellular ATP production of CD4+ T cells doesn't depend on gender, age or race.

The ImmuKnow®-the Cylex® Immune Cell Function Assay is an easy and rapid kit to detect cell-mediated immunity (CMI) by measuring the concentration of adenosine triphosphate (ATP) from CD4+ cells following stimulation. This kit was approved in 2010 by the Food and Drug Administration (FDA) as a valid method to measure the intracellular production of ATP of

CD4+ lymphocytes. It has proved its value as a biomarker to monitor immunosuppression of transplanted patients.

This technique is based on unspecific polyclonal stimuli for lymphocytes with phytohemagglutinin (PHA) in a culture plate for 15-18 hours. The CD4+ T cells are isolated cells with magnetic beads. The ATP is released by the lysis reactant and measured by a luminic reaction with luciferin/luciferase. Concentration is measured in a luminometer at 562 nm. CD4+ lymphocytes are involved in the immune response of rejection and infection. Because of this intracellular ATP production is been considered as a possible biomarker, as long as it reflects the level of activation of CD4+ T cells. It has been proved in other studies that intracellular ATP production has a good correlation with other biomarkers proposed for PD monitoring of T-cell activation, such as measurement of T-cell proliferation.

$$\text{Luciferin} + \text{ATP} + \text{O}_2 \xrightarrow{Mg^{2+},\ Luciferase} \text{AMP} + \text{Pyrophosphate} + \text{CO}_2 + \text{Light}$$

The ImmuKnow® uses L-PHA to stimulate the proliferation of T cells. The PHA is a lecithin derived from *Phaseolus vulgaris*. This protein has two different forms: H-PHA and L-PHA. H-PHA is a potent haemagglutinating that can cause the agglutination of lymphocyte-red blood (RBC) cell. Due this propriety, it's also a potent mitogen in presence of (RBC) cell and can induce lymphocyte transformation. On the other hand, L-PHA is an excellent leucoagglutinin and a poor haemagglutinin and the one really important in this work.

L-PHA is a potent mitogen capable of leucoagglutination. It has not been affected by RBC due its poor capability of haemagglutination. PHA changes the permeability of the cells, causing an entry of nutrients in the cell. This induces the synthesis of macromolecules and ends with cell division. *In vitro* PHA cause an increment of the synthesis of RNA and then of DNA after 24 hours in culture. The cell division occurs after 48 hours. Also, PHA induces the production of IL-2 in lymphocytes, a grow lymphocyte factor needed for PHA action. The cell division is produced by induction of Hox genes, from Hoxb1 to Hoxb9 in 3'-5' sense.

ImmuKnow® allows detecting intracellular production of ATP between 1 and 1000 ng/ml. The standard ranges used to value the immunosuppression are reflected in the next table:

| | ATP concentration (ng/ml) | |
|---|---|---|
| Response | Adults | Children |
| Low response | ≤ 255 | ≤ 175 |
| Moderate response | 266-524 | 176-395 |
| High response | ≥ 525 | ≥ 396 |

**Table 1:** Summary of interpretation of results obtained by ImmuKnow® technique according to intracellular ATP production (ng/ml). This is a qualitative assay. The results don't quantify the level of immunosuppression directly.

These are artificial ranges as result of extensive studies. The clinical history of each patient has to be taken into account. Because of this, one single assay can't be trusted; it has to be performed periodically throughout the treatment.

ImmuKnow® only requires 250 µl of fresh whole blood collected in sodium heparin and permit obtain results in just 24 hours. These characteristics and the low laboratory equipment requirements allow using it at hospitals together with other techniques of monitorization. Also, it allows to the clinician immediate results of the status of the patient to considerate clinical decisions faster than other methods.

Previous studies show there is a significant reduction of ATP production in 1 day old blood samples from transplanted patients; despite of manufacture instructions indicate the test is valid within 30 hours. The intracellular ATP production in old samples is lower than in fresh samples, so it can lead a bad diagnostic of the immune state of patients and suggest there is a low immune function level than reality. Due a low immune function indicate there is a risk of infection, we could reduce incorrectly the load of immunosuppressive drugs of the patient and cause a risk of rejection of the graft.

To use whole blood instead PBMC has some advantages. Mostly the load of immunosuppressive drugs is in the erythrocyte. The selective isolation of some cell types could affect the analysis, like in the Ficoll technique. In ImmuKnow® technique there is a loss of erythrocyte, and that can affect the interactions between cells. Also, whole blood looks like much more to *in vivo* process. Other advantage is whole blood assay are much faster than lymphocyte isolation methods. Furthermore whole blood techniques require less amount of blood and less manipulation of the sample.

The predictive capacity of ImmuKnow® has not been totally confirmed yet. The most uncertain characteristic is its capacity to predict rejection. On the other hand, some studies have been proved ImmuKnow® is a good method to predict infection due a low immune response of CD4+. This low activity is reflected in the intracellular ATP production. In contrast to this, there are other studies that indicate the ImmuKnow® can't predict rejection or infection.

The studies show there isn't any correlation between the intracellular ATP production and the drug concentration in blood. This makes intracellular ATP production a good biomarker. Intracellular ATP production, as other biomarkers, correlates better with immunosuppressive treatment response of the patient than immunosuppressive drug blood concentration.

The present work analyses the predictive capacity of ImmuKnow® assay and try to optimize it for other cell sources. In a laboratory, due the logistics of a laboratory itself, the samples cannot always be processed on time. For this reason is always interesting to look for alternatives for the stabilised techniques. One of the aims of this work is to look for an optimization for the ImmuKnow® commercial kit to measure intracellular ATP production in different conditions of storage. Also it has been investigate an alternative kind of samples using PBMC and frozen PBMC. To perform PBMC in frozen cells could be useful to make retrospective works.

# Aims

The ImmuKnow® uses complete fresh blood to measure the intracellular ATP production. The technique cannot be doing in samples older than 30 hours after the extraction, as manufacturer instructions indicate.

Complete blood samples are too labile. The techniques doing in complete blood samples must be processed quickly before they decay. This sometimes complicates the laboratory work when the number of samples to analyse is high.

This work is based in the research of kinds of storage that permit analyse the samples at any time without lose its predictive capacity.

The ImmuKnow® test value the intracellular production of ATP in CD4+ T cells. Another source of T cells in a laboratory is with Ficoll technique by gradient density centrifugation to get PBMC. The PBMC obtained by this method can be frozen and storage for a long time without decay, and they are a great source of information. For this reason, in this work we analyse de efficiency of ImmuKnow® in PBMC and the results will be compared with the results in fresh blood. If the ImmuKnow® test is valid for PBMC, the intracellular ATP of frozen cells storage in the laboratory could be used to make retrospective works.

Furthermore, ImmuKnow® has been tested in a subset of renal transplant patients suffering of biopsy-proven acute rejection to evaluate its predictive capacity for rejection. The technique has been performance at the moment of biopsy after the suspicion of rejection. The intracellular production of ATP was also performed 10 and 30 days post-biopsy to monitor the immune state of the patients.

# Methodology

Samples

This work has been made with blood collected in heparin sodium from 6 healthy adult donors. Two aliquots from each donor were done: fresh complete blood and fresh PBMCs. Thus, each kind of sample was tested in two different storage conditions. As result, ImmuKnow® has been performance in six conditions for each donor:

- Whole blood: Fresh blood, fresh blood after been stored 24 hours at room temperature, fresh blood after been stored 24 hours at 4ºC.
- PBMC: Fresh PBMC, PBMC after been stored 24 hours at 4ºC and PBMC after one week frozen

In addition, the assay was run for 5 whole blood samples from renal transplant recipients of Hospital Universitario Marques de Valdecilla under suspicious of renal rejection at three time-point controls: At the moment of biopsy, 10 days post-biopsy and 30 days post-biopsy.

Peripheral Blood Mononuclear Cells (PBMC)

The PBMC have been obtain by Ficoll-Hypaque density centrifugation in a table centrifuge (H-103N, Kokusan) at 2500 rpm, 30 minutes, room temperature, without break. Relation between Ficoll and blood is 1:3. The cells have been collected in R10 medium. The ImmuKnow® was performance with fresh PBMC, 24 hours stored at 4ºC and after 1 week frozen.

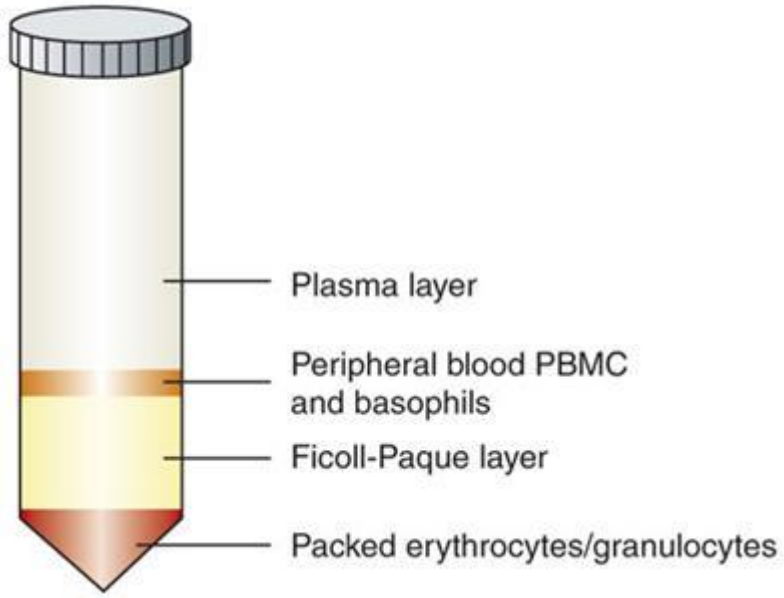


**Figure 4:** Result of whole blood centrifugation with Ficoll-Paque technique. The technique was made using 1:3 of Ficoll:whole blood.

Reactive used

R10 medium using to collect and storage PBMC contains: RPMI medium with fetal bovine serum (FBS), L-Glutamine, penicillin-streptomycin, non-essential amminoacids, pyruvate and sodium bicarbonate. The medium was sterilized by filtration.

Freezing of PBMC

PBMC were frozen in FBS-RPMI 1:1 medium with 10% Dimethil Sulphoxide (DMSO) using CoolCell® Alcohol-Free Cell Freezing Containers at -80ºC (Alcohol-free controlled-rate -1ºC/minute cell freezing containers).


**Figure 5:** Container to freeze PBMC in cryotubes slowly, named Coolcell®.

ImmuKnow®-the Cylex® Immune Cell Function Assay

The ImmuKnow® has been done following the manufacturer instructions in the whole blood samples. This technique has three parts:

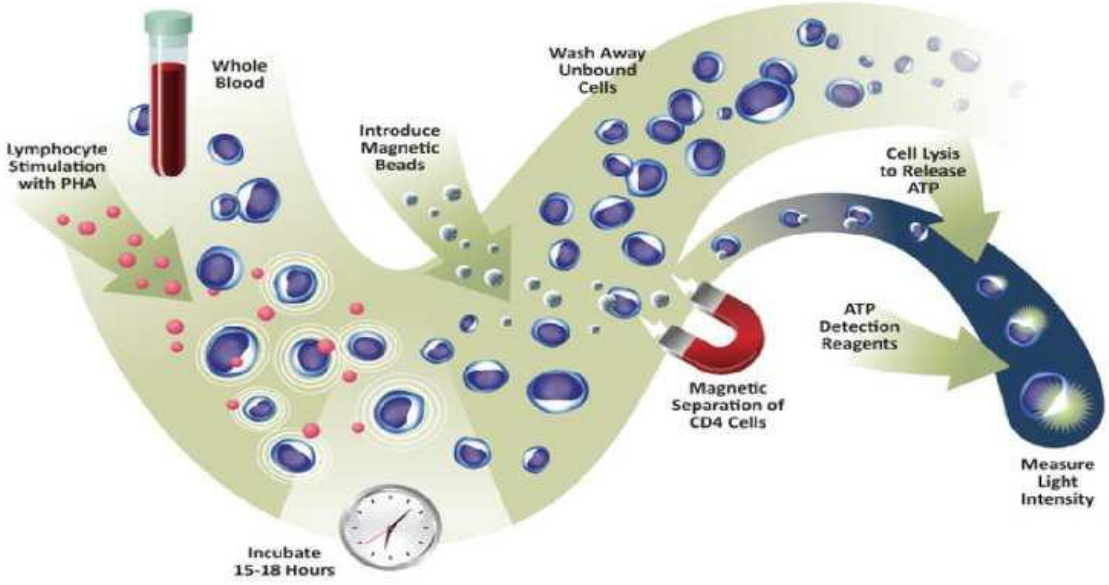


**Figure 6:** Summary of ImmuKnow® technique.

First part: Stimulation of samples. Whole blood was dilute 1:4 with the solvent of the kit. Four wells have 25 µl of that same solvent and another four wells have 25 µl of PHA. Each well has 100 µl of the dilute blood. The assay was adapted to perform it with PBMC. There was a concentration of $25x10^4$ cells/well. This is based on the estimation that the concentration of PBMC in blood is about $1x10^6$ cells/ml. To adjust the concentration, PBMC were counted using a Neubauer chamber. Whole blood and PBMC were incubated with PHA 15-18 hours at 37ºC and 5% $CO_2$ atmosphere.

Second part: Releasing of ATP. 50 µl of Dynabeads® CD4+ were added to each well, following two incubations of 15 minutes. The plate was shaking 15 seconds after each incubation. CD4+ cells were isolated with a magnetic plate. Once the cells are sticking to a wall of the well, the content was eliminating without touching the Dynabeads®. Isolate CD4+ T cells were washed three times with the wash buffer to eliminate residual cells. Then 200 µl of lysis solution was added to each well.

Third part: 50 µl of each well are transferred with a multichannel pipette to the lecture plate. After that, 150 µl of luminic reactant are added to the wells with a multichannel pipette. Lecture of the luminic reaction of the plate is performance after 3 to 10 minutes in GloRunner Microplate Luminometer (Turner BioSystems). Data is analyzed by GlowRunner ImmuKnow® Data Analysis Calculator™.

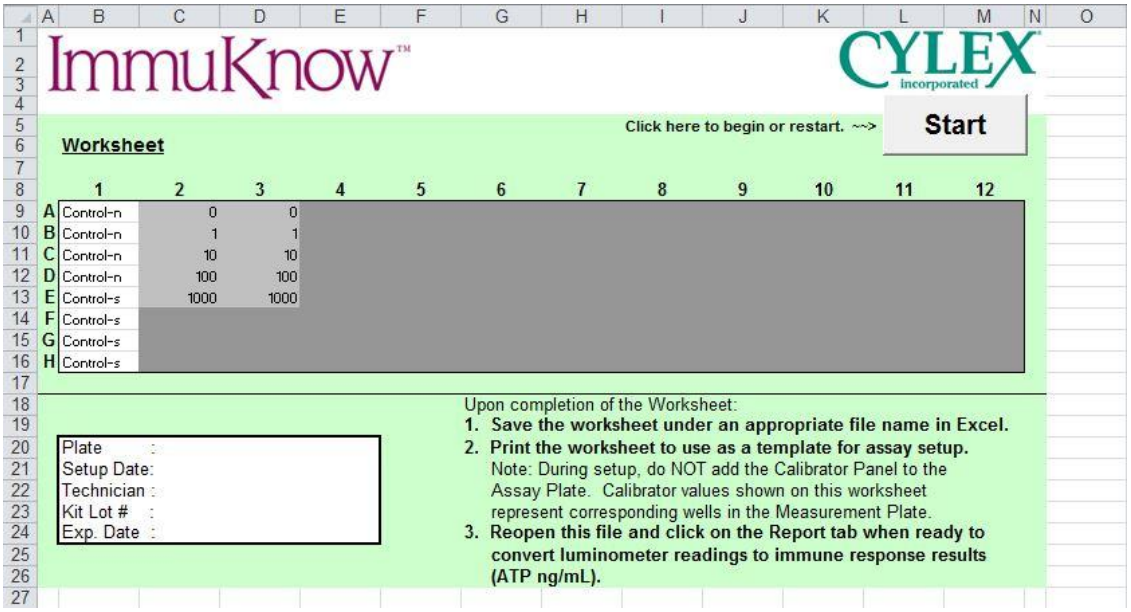


**Figure 7:** Analysis program of the plate lecture ImmuKnow®. It has to be specified and identified each sample.

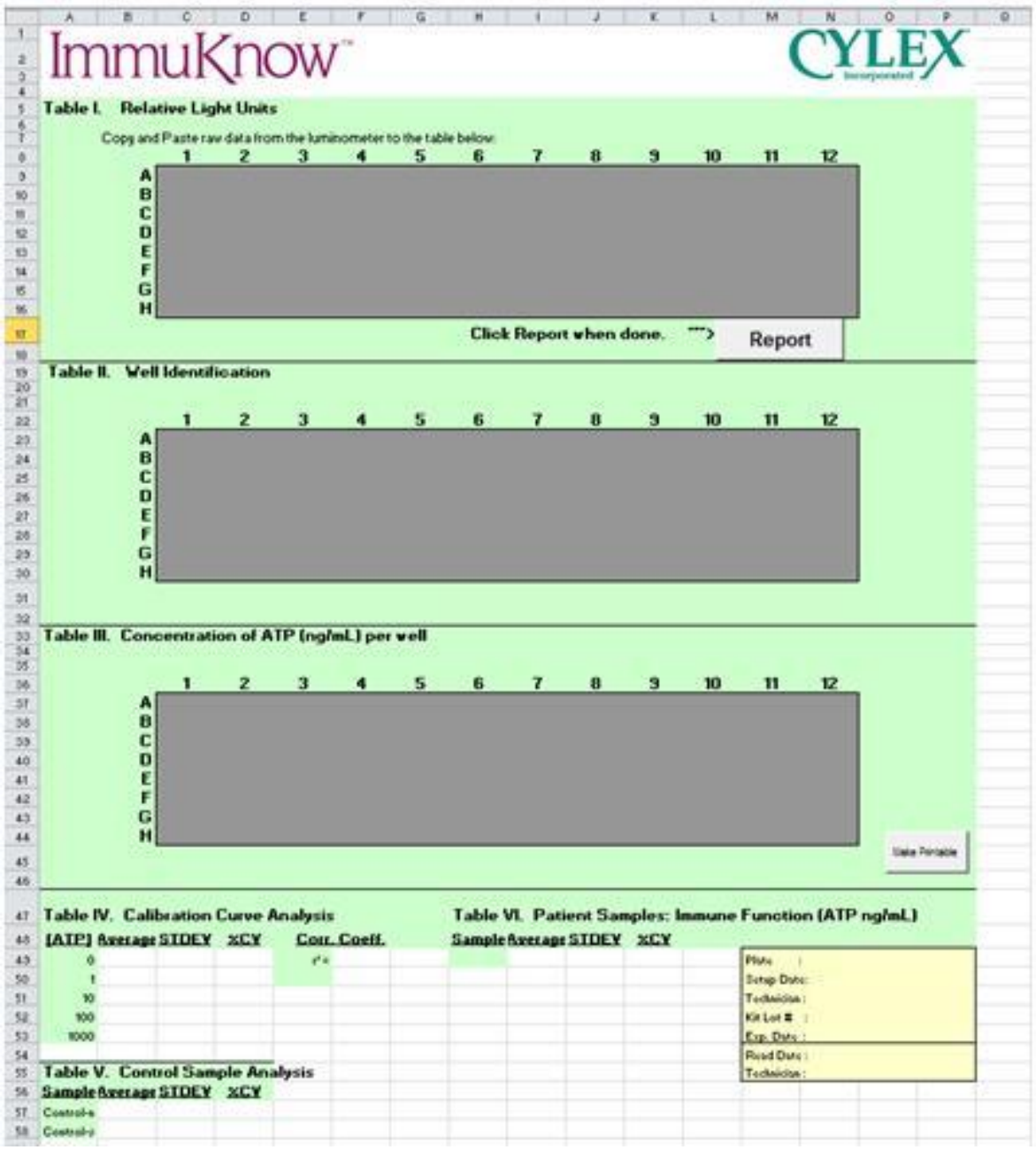


**Figure 8:** Analysis report of the ImmuKnow®. There are three tables for relative data of luminometer, identification of the wells and concentration of ATP (ng/ml) per well respectively. Also there is an average of concentration of each sample with its standard deviation.

Statistical analysis

Data have been analyzed using GraphPad Prisms 5.03 software (San Diego, CA). The graphics were also made with GraphPad Prisms software. Medians were compared using Mann-Whitney and Wilcoxon test.

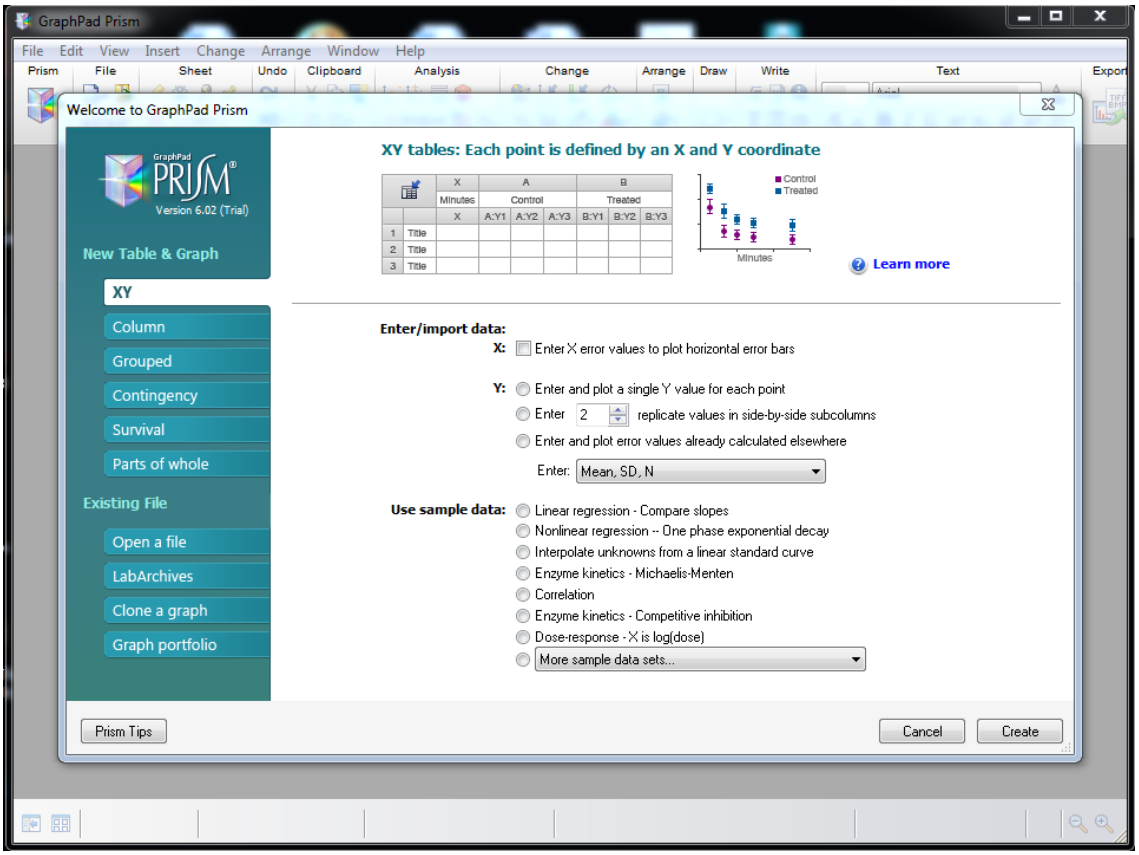


**Figure 9:** GraphPad Prisms 5.03 software (San Diego, CA) appearance.

# Results

INTRACELLULAR ATP PRODUCTION IN DIFFERENT CONDITIONS

| | Whole blood | | | | | |
|---|---|---|---|---|---|---|
| | Fresh blood | | 24 hours RT | | 24 hours 4ºC | |
| Donors | W/O | W | W/O | W | W/O | W |
| 1 | 27,18 | 271,92 | 24,67 | 354,42 | 25,69 | 174,39 |
| 2 | 23,68 | 316,72 | 2,73 | 383,25 | 23,20 | 323,24 |
| 3 | 13,05 | 192,51 | 10,99 | 204,26 | 7,89 | 135,18 |
| 4 | 2,62 | 414,68 | 9,91 | 262,86 | 12,79 | 166,43 |
| 5 | 14,10 | 242,17 | 6,60 | 173,33 | 15,76 | 91,83 |
| 6 | 17,31 | 241,45 | 14,96 | 242,03 | 20,26 | 185,82 |

**Table 2:** Results of intracellular production of ATP (measured in ng/ml) of 6 adult healthy donors in whole blood at different conditions of storage. RT: Room temperature. W/O: Without stimuli. W: With stimuli.

| | Median | Range |
|---|---|---|
| Fresh blood | 257.05 | [192.51 - 414.68] |
| 24 hours RT | 252.45 | [173.33 - 383.25] |
| 24 hours 4ºC | 170.41 | [91.83 - 323.23] |

**Table 3:** Summary of medians and ranges of whole blood of 6 adult healthy donors in different storage conditions (measured in ng/ml).

| | PBMC | | | | | |
|---|---|---|---|---|---|---|
| | Fresh PBMC | | 24 hours 4ºC | | 1 week frozen | |
| Donors | W/O | W | W/O | W | W/O | W |
| 1 | 18,85 | 43,97 | 14,44 | 33,71 | 22,20 | 10,46 |
| 2 | 12,97 | 45,19 | 13,90 | 40,85 | 11,02 | 25,34 |
| 3 | 2,32 | 19,01 | 1,37 | 3,05 | 15,86 | 32,97 |
| 4 | 19,12 | 54,57 | 13,84 | 44,40 | 8,97 | 26,61 |
| 5 | 3,00 | 10,14 | 10,47 | 29,03 | 8,06 | 32,02 |
| 6 | 11,36 | 50,56 | 14,14 | 40,18 | 11,33 | 28,74 |

**Table 4:** Results of intracellular production of ATP (measured in ng/ml) of 6 adult healthy donors in PBMC at different conditions of storage. W/O: Without stimuli. W: With stimuli.

| | Median | Range |
|---|---|---|
| Fresh PBMC | 44.58 | [10.14 - 54.57] |
| 24 hours 4ºC | 36.94 | [3.05 - 44.40] |
| 1 week frozen | 27.68 | [10.46 - 32.97] |

**Table 5:** Summary of medians and ranges of PBMC of 6 adult healthy donors in different storage conditions (measured in ng/ml).

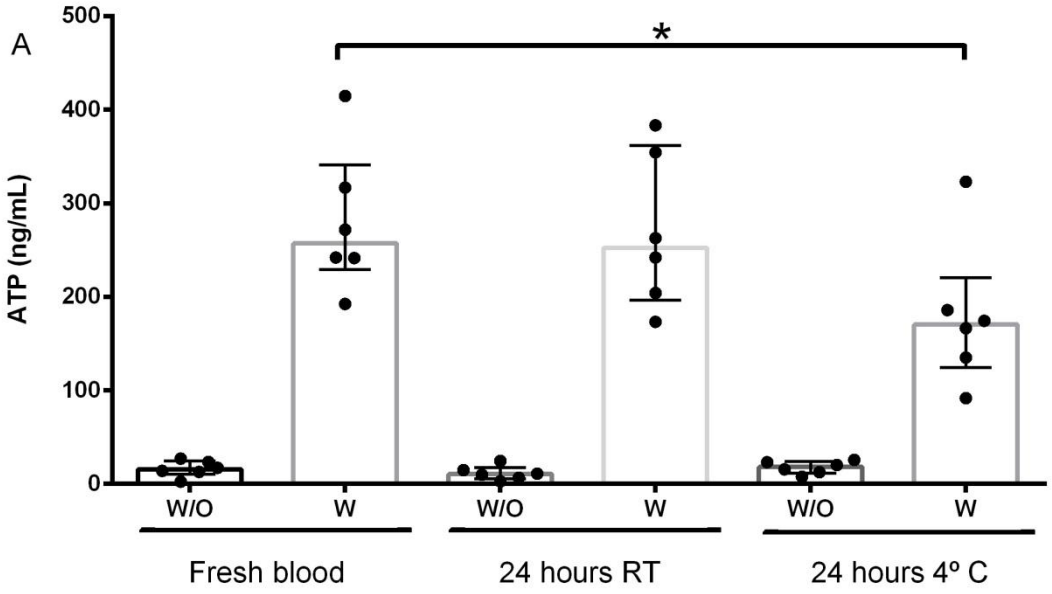


**Figure 10:** Intracellular ATP concentration of lymphocytes analyzed in whole blood at different conditions. Samples were processed under three different conditions, fresh blood (bars on left hand-side), stored at room temperature (RT) (bars on center) and at 4ºC (bars on right hand-side). The medians in columns and interquartilic range are depicted, medians were compared using U-Mann Whitney test ($p<0.05$ was considered significant). W/O: Without stimuli. W: With stimuli.

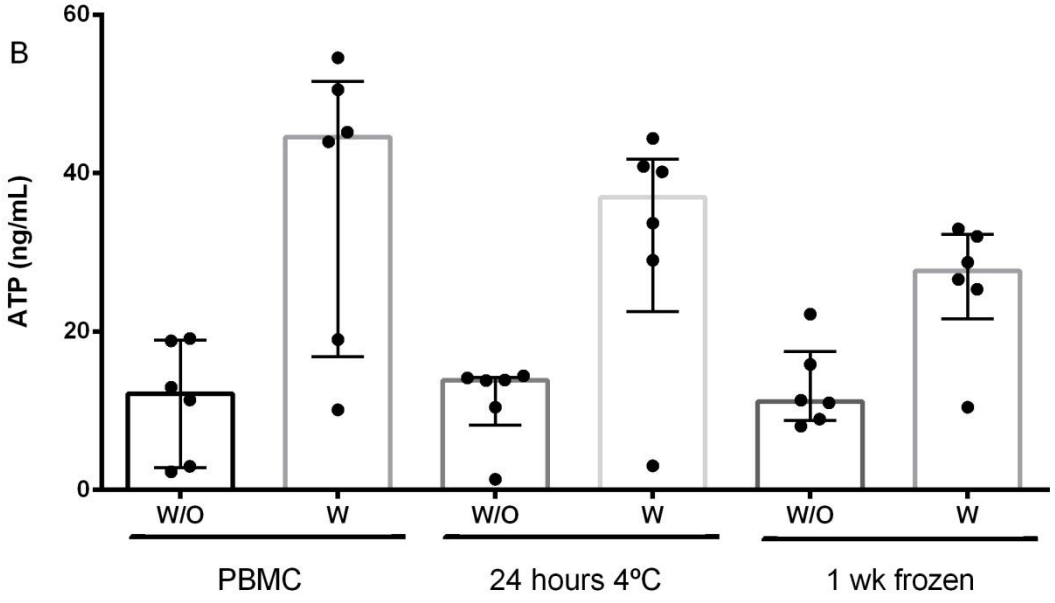

**Figure 11:** Intracellular ATP concentration of lymphocytes analyzed in PBMC at different conditions. PBMC processed under three different conditions were tested, fresh PBMC (bars on left hand-side), stored at 4ºC (bars on the center) and 1 week (wk) frozen (bars on right hand-side). The medians in columns and interquartile range are depicted, medians were compared using U-Mann Whitney test ($p<0.05$ was considered significant). W/O: Without stimuli. W: With stimuli.

Whole blood from 6 adult healthy controls was processed in three different conditions of storage in order to assess the reproducibility of the test (ImmuKnow®-the Cylex®). The medians of intracellular ATP production are (shown in table 3): Fresh blood was 257.05 ng/ml, 24h RT stored blood was 252.45 ng/ml and 24h 4ºC stored blood was 170.41 ng/ml. No differences in ATP production in fresh blood and room temperature-stored blood was observed nevertheless a decrease in ATP production after 4ºC storage was demonstrated ($p<0.05$ was considerate significant).

In the laboratory another source of lymphocytes is performed after isolation upon Ficoll to get PBMC. The next step was to compare the results of ATP production of fresh whole blood and PBMC. The median of ATP production in fresh whole blood was 257.05 ng/ml whereas in PBMC only achieved 44.58 ng/ml (17% of stimulation in relation with fresh whole blood). The intracellular ATP production in 24h 4ºC stored PBMC and in PBMC after 1 week frozen were 36.94 ng/ml and 27.68 ng/ml respectively. No statistical differences were observed in intracellular ATP production in any condition ($p<0.05$ was considerate significant).

INTRACELLULAR ATP PRODUCTION IN PATIENT UNDER SUSPICIOUS OF RENAL REJECTION

| Renal transplantation patients | | | | | |
|---|---|---|---|---|---|
| Biopsy | | 10 days post-biopsy | | 30 days post-biopsy | |
| W/O | W | W/O | W | W/O | W |
| 15,08 | 169,95 | 4,37 | 297,33 | 2,85 | 219,55 |
| 13,23 | 336,19 | 44,39 | 334,07 | 3,66 | 502,26 |
| 8,09 | 546,39 | 73,16 | 485,38 | 23,93 | 709,29 |
| 1,00 | 355,94 | 1,02 | 127,58 | 30,97 | 283,89 |
| 4,14 | 491,08 | 9,89 | 302,60 | 8,71 | 174,04 |

**Table 6:** Results of intracellular production of ATP of 5 renal transplantation patients in whole blood at different times of the suspicious of rejection. W/O: Without stimuli. W: With stimuli.

| | Median | Range |
|---|---|---|
| Biopsy | 355.94 | [169.95 - 546.39] |
| 10 days post-biopsy | 302.60 | [127.58 - 485.38] |
| 30 days post-biopsy | 283.89 | [174.04 - 709.29] |

**Table 7:** Summary of medians and ranges of whole blood of 5 patients under suspicious of acute renal rejection at different point control.

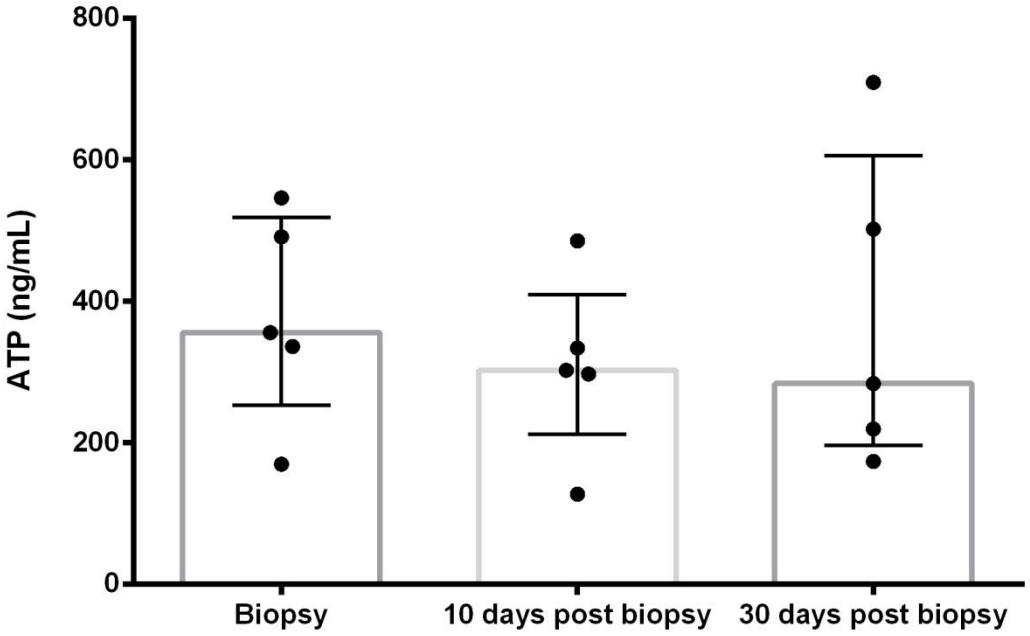


**Figure 12:** Intracellular ATP concentration in renal transplant patients. Levels of ATP production were measured at the time of biopsy (left), 10 days (center) and 30 days post biopsy (right). No differences in medians were observed at any time point. The medians in columns and interquartile range are depicted, medians were compared using U-Mann Wilcoxon test ($p<0.05$ was considered significant).

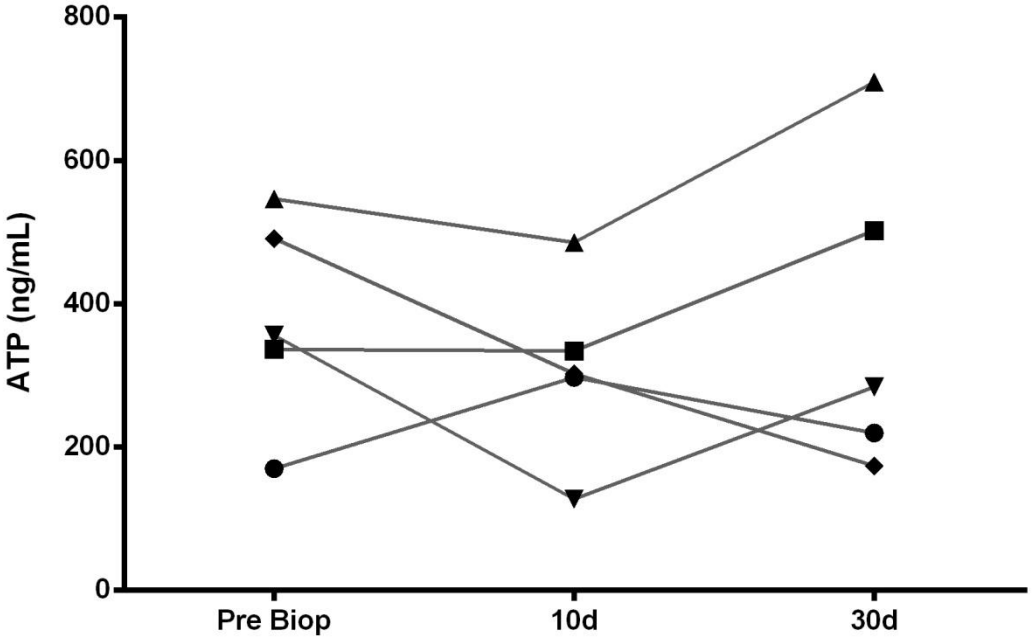

**Figure 13:** Monitorization of each patient thought time at three time-point control (each patient depicted with different symbols: ▲,▼,■,●, ◆): At biopsy, 10 days and 30 days post-biopsy.

Five samples from renal transplant patients were monitored due suspicious of renal rejection. . Results are shown in table 5. Samples were processed at the time of biopsy, 10 and 30 days post biopsy. A decrease in the median of ATP production was observed from the moment of the biopsy (355.94 ng/ml), 10 days post-biopsy (302.60 ng/ml) and 30 days post-biopsy (283.89 ng/ml) but these differences were not significantly statically ($p<0.05$ was considerate significant).

There was made a comparison of the evolution of each patient under suspicious of acute rejection since biopsy up to 30 days. There is a slightly reduction of intracellular ATP concentration at 10 days after biopsy. Also there is a trend to show an increment of ATP concentration at 30 days respect the 10 days point-control, and even greater than biopsy-time in some cases. Nevertheless one of the samples shows a constant decrease of ATP concentration on each point-control.

# Discussion

There is a trend to obtain worse results as the samples become cooler. Intracellular production of ATP decrease significantly in the whole blood samples 24 hours stored at 4ºC in relation to fresh blood. Probably this significant decrease were also found between 24 hours RT storage blood and 24 hours 4ºC storage blood with major number of samples, due the p-value was close to 0.05. In PBMC the intracellular production of ATP also decreases in samples 24ºC stored at 4ºC and decreases even more in frozen samples, but not significantly in any case.

These results could be due to a decay of the efficiency of the CD4 on the surface of lymphocytes. This would difficult the isolation of CD4+ cells in the development of the technique and will be reflected as a decrease in the intracellular ATP production due a lower number of CD4+ cells that have to be. It also could be due cell death or necrosis, but previous studies show there isn't any reduction of the CD4 cells after been storage. There is a small but significant reduction on the expression of CD4 during the storage. Nevertheless this slightly decrease doesn't explain the significant reduction of intracellular ATP production in fresh blood in relation to storage blood.

In PBMC the descent of the concentration could be due to the Ficoll technique used to isolate them, but it can't be, because this kind of isolation is also made to other techniques that require intact CD4 on the surface of lymphocytes. Another possible explanation is that the number of cells put on each well ($2.5x10^4$ cells) is too low or the PHA concentration used from the commercial kit was also too low. There were performance parallel experiments with more cells ($5x10^4$ cells/well) and a high PHA concentration (5 ng/ml) not showed in this work, but the intracellular ATP production obtained was also too low, in the range of the results showed on this work.

The concentration used in the experiment is based on the supposition there are one million PBMC for one milliliter of whole blood. Due in one step of ImmuKnow® technique there is a dilution 1:4 of blood with the diluyent of the kit, the supposed final number of cells on each well is $2.5x10^4$ cells. This was made to establish an equal number of cells on all the experiments, but it is not really necessary, because the technique normalize the result of intracellular ATP production independent of the number of cells. The number of cells per well is independent because there is a constant number of isolated cells by the magnetic beads, i. e.

the concentration of magnetic beads is always lower than the number of cells per well, so the number of cells isolated is always the same. Furthermore, there will be need to perform more complementary experiments with different cell concentration and PHA concentration to obtain more accuracy results.

One possible explanation for the failure to intracellular ATP production in PBMC is due the amounts of red blood cells (RBC). There is a study that indicates the production of IL-2 by CD8- and CD8+ is significantly lower in PBMC that in whole blood. PHA needs IL-2 to induce proliferation, and if the concentration of IL-2 is too low, could be an explanation of why the ImmuKnow® doesn't work on PBMC. Also, RBC interferes in the inhibition of lymphocytes with immunosuppressive drugs as tacrolimus.

However, even with this supposition, there will be needed a more extensive study to determinate if it is possible to establish a new standard of intracellular ATP production using PBMC. Maybe it could be possible by adding additional IL-2 to stimulate the lymphocytes. If there will be found an optimal protocol to PBMC, the test could be adapted to frozen PBMC. This would be useful to perform retrospective studies with frozen cells.

Another possibility could be to performance ImmuKnow® with PBMC isolated with a different technique, to prove if the decrease of the ATP concentration is due to de isolation method.

Data of patients with suspicious of acute rejection of the organ don't show statistics differences at any point of the experiment, but there is a slightly reduction of the production of intracellular ATP at 10 days. This could be explained due to an increase of immunosuppressive drugs load for acute rejection treatment at the moment of admission. But after that, there is a slightly increase of ATP concentration at 30 days, probably due to a decrease of immunosuppressive drugs after the crisis was solved. However, the number of patients are too low (n = 5). With more samples probably there will be significant results.

At the moment, ImmuKnow® technique cannot be used as a unique and absolute method to monitor immunosuppression but it's a good complement to other techniques.

# Conclusions

ImmuKnow® is only reliable with fresh blood and comparable after storage 24 hours at room temperature. The intracellular ATP production in blood 24 hours stored at 4ºC suffers a statistical significant descendant. There isn't any statistical significant differences between intracellular production of ATP in blood 24 stored at 4ºC and fresh blood.

Intracellullar ATP production measured by ImmuKnow® in PBMC can't be compared with the established values for whole blood due the low concentration obtained. PBMC stored at 4ºC and 1 week frozen show a slightly but not statistically significant descent compared to fresh PBMC.

There is a decrease on the level of intracellular ATP production in renal transplantation after diagnosis of rejection that could be explained due to an increase of immunosuppressive load for acute rejection treatment. However this slightly decrease is not statistically significant.

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