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Impact of tacrolimus on interferon gamma ELISpot assay results for the assessment of T-cell immunity: Proof-of-concept

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Abstract

SOT patients require immunosuppressors to avoid graft rejection. Therapeutic drug monitoring is insufficient to find the optimal balance with immunosuppression. The evaluation of cell-mediated immunity by enzyme-linked immunospot (ELISpot) assay enumerating interferon-gamma (IFN-γ) is increasingly use. ELISpot assays are performed on peripheral blood mononuclear cells (PBMC) isolated from blood and brought into contact with specific peptides in an immunosuppressor-free environment. This study aims to determine the *in vitro* diffusion of tacrolimus in PBMC and to assess whether prior *in vitro* incubation of PBMC with tacrolimus modifies the IFN-γ ELISpot results when assessing the T-cell immune response. PBMC from healthy volunteers were obtained. Tacrolimus was added to the ELISpot wells at increasing concentration and quantification was obtained using liquid chromatography mass spectrometry. Results showed that the *in vitro* PBMC diffusion rate of tacrolimus was measured at 32%. A decrease in T-cell reactivity occurred with increasing tacrolimus concentration. The intra-PBMC concentration of tacrolimus able to inhibit 50% of T-cell reactivity was 163 pg/10^6 PBMC, which is in the range of the *in vivo* intra-PBMC concentration in SOT recipients. T-cell functional assessment using ELISpot in patients treated with immunosuppressors may require the addition of immunosuppressors *in vitro* to better reflect the *in vivo* situation.

Keywords

tacrolimus, cell-mediated immunity, immunosupressor diffusion rate, peripheral blood mononuclear cells, interferon-gamma ELISpot assay

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Highlights

- IFN-γ ELISpot assay can assess immunosuppression in solid organ transplant recipients.
- This *in vitro* assessment was performed in an immunosuppressor-free environment.
- *In vitro* diffusion of tacrolimus in lymphocytes was assessed at 32%.
- Incubation of lymphocytes with tacrolimus modifies the IFN-γ ELISpot assay results.
- *In vitro* intra-PBMC concentration of tacrolimus inhibiting 50% of T-cell reactivity is in the range of intra-PBMC concentration *in vivo*.

Introduction

Solid organ transplant (SOT) recipients require an immunosuppressive regimen to avoid graft

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rejection. The main challenge in monitoring these patients is to find the optimal balance between over-immunosuppression, with the increased risk of infection and cancer, and under-immunosuppression, with the higher risk of graft rejection. Therapeutic drug monitoring is the cornerstone of exposure monitoring for calcineurin inhibitors. However, tacrolimus trough concentration monitoring remains insufficient.[1,2] Depending on the study, 8%–35% of kidney recipients presented with cytomegalovirus (CMV) replication, leading to CMV disease, retinitis, or graft rejection.[3] Polyomavirus BK virus infections, leading to interstitial nephropathy, are also common in kidney transplant patients even when tacrolimus concentrations are repeatedly within the therapeutic ranges.[4]

New biomarkers and tools are therefore needed to assess the level of immunity. The humoral adaptative immune response evaluated by serology is currently the only marker available for immune risk stratification, which is insufficient to predict infections, reactivations, or associated diseases such as CMV. However, the T-cell immune response is known to be important in controlling viral infections,[5] and the serological approach may underestimate the humoral immune response, because it excludes the whole memory B-cell pool. An assessment of virus-specific memory T and B cells in transplant recipients may be relevant. Pharmacodynamic approaches and monitoring of virus-specific T-cell-mediated immunity responses have been extensively studied, especially interferon gamma (IFN-γ) release assays (QuantiFERON®)[6] and enzyme-linked immunosorbent spot assays (ELISpot).[7] The use of IFN-γ ELISpot assay for SOT recipients is well documented with various applications,[8–11] particularly assessing T-cell responses to viral pathogens and providing further information beyond serology. IFN-γ ELISpot assay can be used to predict the T-cell response to CMV in the pre-transplant setting and to better stratify the risk of CMV reactivations and infections,[7,12] thus guiding the prevention strategy.[13] In the early post-transplantation period, Lee et al. concluded that cell-mediated immunity monitoring at 1-month post-transplantation using pp-65 and IE-1-specific ELISpots can be used to identify patients at risk of CMV reactivation[10] and to guide the duration of valganciclovir prophylaxis.[14,15] For the management of BK virus infection, ELISpot may be useful to determine the cessation of tapering immunosuppression, which is currently the only validated strategy to treat this infection,[16–18] In addition, several studies have pointed to the value of ELISpot assays to assess the level of protection against SARS-CoV-2 in SOT recipients. Some authors demonstrated that immunocompromised patients could have an effective T-cell response against SARS-CoV-2 despite the absence of seroconversion.[19,20] Moreover, the lack of cellular response is associated with a higher risk of severe SARS-CoV-2.[21]

Finally, viral T-cell-mediated immunity response assessments are not the only application of ELISpot assays. They can also be used to investigate the recipient alloimmune cellular response against donor antigens and to analyze virus-specific IgG-secreting memory B cell using B-cell ELISpot. This may not only improve the evaluation of rejection risk[9,22–25] but also guide the most suitable immunosuppressive regimen based on the ELISpot results.[26]

Despite arguments in favor of ELISpot assays, there is a theoretical limit to the interpretation of these tests in SOT recipients. ELISpot requires the separation of peripheral blood mononuclear cells (PBMC) from blood. Thus, the *in vitro* activation of T-lymphocytes occurs in an experimental environment that is free from immunosuppressive drugs, which does not reflect the *in vivo* T-cell environment. Furthermore, the pre-analytic steps for ELISpot (whole blood sampling, PBMC extraction, and freeze/thaw of aliquots) are time-consuming, meaning that the immunosuppressant activity may have disappeared at the time of the assay. Indeed, calcineurin activity returns at baseline level approximately 6–8 h after administration.[27–29]

This unique and innovative study has investigated the impact of tacrolimus on IFN-γ ELISpot assays. Our first objective was to determine the diffusion of tacrolimus in PBMC. The second and final objective was to assess whether prior *in vitro* incubation of PBMC with tacrolimus modified the IFN-γ ELISpot assay results when assessing the T-cell immune response.

Material and methods

Sample collection

For this preliminary retrospective study, whole blood samples from 10 healthy volunteers were

obtained from the French Blood Bank (EFS), between November and May 2023. In brief, healthy volunteers were selected by the EFS, could not be receiving any immunosuppressive drug and were aged 18 years and above. PBMC were isolated by density gradient centrifugation of blood samples using the Ficoll gradient method. They were stored at −80°C with fetal bovin serum and 10% DMSO and then used in batches.

Determination of intra-PBMC tacrolimus diffusion rate

After thawing, viable PBMC were counted and then re-suspended in complete medium (e.g. RPMI with fetal bovine serum, penicillin/streptavidin, and L-glutamine) to obtain a concentration of 1.10^6 PBMC/mL. Then, 1 million PBMC were incubated for 1 h (37°C, 5% CO_2) with increasing amounts of tacrolimus (Prograf®): 0.0, 80.0, 300.0, 500.0, 800.0, and finally, 1000.0 pg/mL. After incubation, cell suspensions were centrifuged for 4 min at 2800 g, and the supernatant was removed and stored. Inspired by the experiments of Tron et al.,[30] cell pellets were re-suspended with 1 mL methanol, and PBMC were counted. Methanol was evaporated using nitrogen flow before resuspending dry residue with 50 µL methanol. Then, 150 µL deproteinization solution (containing MeOH/0.2 M $ZnSO_4$, 80/20 + 0.1% acetic acid + 13C-D4-tacrolimus = internal standard) was added to each aliquot. After being vortexed and centrifuged (12,000 rpm, 10 min), 100 µL supernatant was transferred into micro-insert polypropylene HPLC vials.

Intra-PBMC and supernatant tacrolimus concentrations were assed using an API 5500 QTRAP® tandem mass spectrometer (Sciex). The injection volume was 50 µL. Chromatographic and mass spectrometric conditions were previously described by Chavant et al.[31]

To overcome the matrix effect, the calibration curve for tacrolimus dosing was prepared in a suspension of PBMC from a healthy donor (i.e. PBMC that had never previously been in contact with tacrolimus) prepared. First, a dry range of tacrolimus was produced from 0 to 2 pg/L, then resuspended in 1 mL methanol and 10^6 PBMC from a healthy donor. After evaporation, dry residue was resuspended as described above. This protocol was already described by Lemaitre et al.[32]

Experiments to evaluate the tacrolimus diffusion rate in PBMC were carried out three times on three different days.

IFN-γ ELISpot assay in a medium containing tacrolimus

IFN-y ELISpot assay was performed according to Mabtech® recommendations. The Human IFN-γ ELISpot Pro® kit was used.

Tacrolimus was added to the wells throughout the incubation period at the following quantities: 0.0, 20.0, 40.0, 80.0, 300.0, 600.0, and 1000.0 pg/10^6 PBMC. Each condition was tested in triplicate.

PBMC obtained from the healthy volunteer were diluted in complete medium (e.g. RPMI with fetal bovine serum, penicillin/streptavidin, and L-glutamine) to obtain a concentration of 5.10^6 PBMC/mL. According to the manufacturer's protocol, 50 µL suspension cells were distributed into each well of the ELISpot white MSIP plate, pre-coated with anti-human IFN-γ antibody (mAb 1-D1K). Each well contained 250,000 PBMC and tacrolimus was added. After 2 h of incubation at 37°C and with gentle agitation, the anti-CD3 monoclonal antibody used as a positive control for cytokine production was added to each well (dilution at 1:1000 in the well, Mabtech®), except in the three wells of negative control. Anti-CD3 antibody can induce a nonspecific activation of all T-lymphocytes with polyclonal expansion. Negative control wells contained only cells with complete medium. After 22 h of incubation at 37°C/5% CO_2, cells were removed by washing the plates five times with phosphate-buffered saline. Then, 100 µL alkaline phosphatase anti-IFN-γ antibody was added (1:1000 dilution, 7-B6-1, ALP; Mabtech®) and incubated for 2 h at room temperature. The ELISpot plate was washed a further five times with phosphate-buffered saline. The revelation step occurred for 5–10 min with the addition of 100 µL alkaline phosphatase substrate (bromochlorylindolophosphate/tetrazolium nitroblue-plus). A colorimetric reaction revealed IFN-γ-producing cells. The resulting spots were counted semi-automatically with an ELISpot reader, Bioreader 7000 (Bio-Rad®). Each spot represented an anti-CD3-activated T-cell that secreted IFN-γ. Results were expressed as the number of spots in the CD3 well minus the number of spots in the negative control wells.

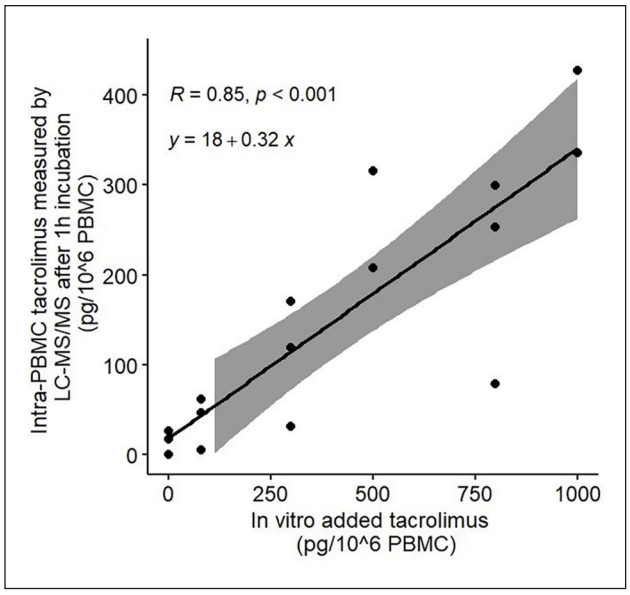

Figure 1. Correlation between the *in vitro* added tacrolimus concentration and the intra-PBMC tacrolimus concentration measured by LC-MS/MS after 1-h incubation, in pg/10^6 PBMC (Pearson correlation coefficient of correlation $R = 0.85$, $p < 0.001$).
One million PBMC were incubated with increasing amounts of tacrolimus: 0.0, 80.0, 300.0, 500.0, 800.0, and 1000.0 pg/10^6 PBMC. Each condition was tested in triplicate (Data missing for one replicate at 500.0 and 1000.0 pg/10^6).

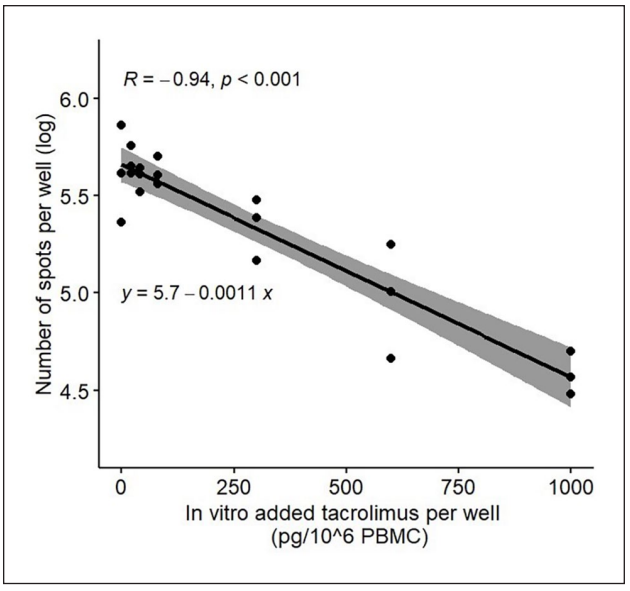

Figure 2. Dose-response curve of T-cell immune reactivity after PBMC stimulation with anti-CD3 antibody measured by IFN-γ ELISpot assay according to the tacrolimus concentration in the well.
The immune response is depicted by the number of spots obtained in each well (Pearson correlation coefficient $R = -0.94$, $p < 0.001$).

The number of spots according to the tacrolimus concentration added to the well was evaluated.

Statistical analysis

Continuous data were presented as medians (10th–90th percentiles) and qualitative data as numbers (percentages). Bivariate correlation analyses were completed using the Pearson correlation test for non-parametric variables, and linear regressions were tested. *R* corresponds to the correlation coefficient. The statistical significance level was defined as $p < 0.05$. Statistical analyses were performed using *R* statistical software® (R Core Team, 2018).

Results

Intra-PBMC diffusion rate of tacrolimus

As shown in Figure 1, a positive correlation was found between the tacrolimus concentration added *in vitro* and the intra-PBMC tacrolimus concentration obtained by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) after 1-h incubation ($R = 0.85$; $p < 0.005$). The linear regression equation between intra-PBMC tacrolimus (TACintra-PBMC) and *in vitro*-added tacrolimus (TACadded) was as follows: TACintra-PBMC (pg/10^6 PBMC) = 0.32 × TACadded (pg/10^6 PBMC) + 18. The *in vitro* diffusion of tacrolimus in PBMC was measured at 32%.

This experiment allowed us to determine the concentration range of tacrolimus required in the wells of ELISpot assays (*in vitro* model) to obtain intra-PBMC concentrations of tacrolimus as close as possible to the intra-PBMC concentrations observed *in vivo*.

Impact of prior PBMC incubation with tacrolimus on T-cell reactivity assessed by ELISpot

Considering the first tests used to determine the diffusion rate of tacrolimus in PBMC, the same ratio of tacrolimus quantity/number of PBMC was used to assess the influence of tacrolimus on the ELISpot results.

The dose-response curve shown in Figure 2 represents the number of IFN-γ-producing T-cells after stimulation with anti-CD3 (in terms of the number of spots) according to the tacrolimus concentration added to the well. The increase in

tacrolimus concentration added to the ELISpot wells results in a decrease in IFN-γ producing T-cells (in terms of the number of spots) after nonspecific stimulation by anti-CD3. The tacrolimus concentration inhibiting 50% of the IFN-γ T-cell response (IC50-TAC_{tot}) was 454 pg/10^6 PBMC.

The first experiments confirmed that the diffusion rate of tacrolimus in PBMC was 32% (Figure 1), allowing us to deduce the intra-PBMC concentration of tacrolimus able to inhibit 50% of the T-cell response. A concentration of 163 pg/10^6 PBMC of intra-PBMC of tacrolimus was thus calculated: $IC50\text{-}TAC_{PBMC} = 0.32 \times IC50\text{-}TAC_{tot} + 18$ with $IC50\text{-}TAC_{tot} = 454$ pg/10^6 PBMC.

Discussion

This is the first study to investigate the impact of tacrolimus on ELIspot. For this purpose, it first determined the diffusion of tacrolimus in PBMC and then assessed whether the prior *in vitro* incubation of PBMC with tacrolimus modified the IFN-γ ELISpot results in terms of the T-cell immune response.

The incubation time of tacrolimus with PBMC was chosen based on the literature on the pharmacokinetics and pharmacodynamics of tacrolimus. The maximum intra-PBMC concentration of tacrolimus occurs 1–2 h after *in vivo* administration in patients according to Fontova et al. and van Merendonk et al.,[29,33] and after 1 h of incubation in the previously mentioned *in vitro* model of Tron et al.[30] Therefore, an incubation time of 1 h was used in this experiment to evaluate the diffusion rate of tacrolimus in PBMC. Regarding pharmacodynamic data, the maximum inhibition of calcineurin activity is reached 2 h after tacrolimus administration.[29] For this reason, PBMC were incubated with tacrolimus 2 h prior to immune activation with anti-CD3 antibody.

These analytical conditions allowed us to confirm that the *in vitro* diffusion rate of tacrolimus in PBMC (in culture medium) is around 32% (Figure 1). Establishing the relationship between the *in vitro*-added tacrolimus concentration and the intra-PBMC tacrolimus concentration obtained after incubation was not straightforward. In whole blood, 80%–85% of tacrolimus binds to erythrocytes, 10%–15% binds to plasma proteins, and only 1% of the drug (free form) can enter PBMC. This very small fraction represents the active part of the drug. These *in vivo* intra-PBMC concentrations of tacrolimus are around 30–60 pg/10^6 PBMC for the residual concentration and around 60–120 pg/10^6 PBMC for the maximum concentration. In the transplant setting, these concentrations vary according to the transplanted organ and the time from transplantation.[29,34–36] Moreover, the *in vitro* diffusion rate of tacrolimus in PBMC is poorly documented. To the best of our knowledge, only one study assessed this relationship between the tacrolimus concentration added *in vitro* to a suspension of PBMC and the intra-PBMC concentration of tacrolimus obtained after incubation. The authors found a diffusion rate of around 20% performed on PBMC from liver transplant patients.[30] The diffusion rate of 32% obtained in this work is thus consistent with their study.

This diffusion rate allowed us to estimate the tacrolimus concentration to be added to a suspension of PBMC in order to obtain *in vitro* intra-PBMC concentrations of tacrolimus as close as possible to the intra-PBMC concentrations observed *in vivo* in SOT recipients. Thus, a range of tacrolimus concentrations between 20.0 and 1000.0 pg/10^6 PBMC was tested. The reactivity of T-cells (expressed in the number of spots) to the nonspecific anti-CD3 stimulation decreased with the increasing tacrolimus concentration. The tacrolimus concentration inhibiting 50% of the IFN-γ T-cells response (IC50-TAC_{tot}) was 454 pg/10^6 PBMC, corresponding to a tacrolimus intra-PBMC concentration of 145 pg/10^6 PBMC. This intra-PBMC concentration of tacrolimus able to inhibit 50% of the T-cell reactivity *in vitro* was close to the maximum tacrolimus concentration observed *in vivo* in SOT patients.[29,34,36]

These preliminary results lead to an important question regarding the interpretation of IFN-γ ELISpot assays in SOT recipients. In fact, most patients for whom an ELISpot test will be prescribed were treated by tacrolimus. Our study suggests that ELISpot response should depend on the tacrolimus concentration used *in vitro*. When ELISpot assays are performed, PBMC are isolated and removed from unbound tacrolimus present in the immunocompromised patient's plasma. PBMC were thus no longer subject to the tacrolimus action. The specific response of IFN-γ T-cells when performing the ELISpot assay without the prior addition of immunosuppressants may be overestimated compared with T-cell reactivity in vivo when T-cells are subjected to the immunosuppressant effect. If

PBMC are not incubated with tacrolimus prior to the ELISpot assay, there is a risk of a false-positive result (i.e. overestimation of the T-cell immune response) and to discontinue prophylaxis in a patient who may be at risk of CMV reactivation. In fact, when ELISpot-T IFN-γ assay is positive, it's recommended to stop CMV prophylaxis treatment in kidney transplanted patients.[37,38] As an example, Bestard et al. recommended the discontinuation of cytomegalovirus prophylaxis when the CMV-specific IFN-γ ELISpot assay is positive in the post-transplantation setting.[7] This might explain some of the CMV reactivations after the discontinuation of valganciclovir prophylaxis in SOT patients. Without immunosuppressive agents, the patient's T-cells may secret IFN-γ after stimulation in the ELISpot assay, whereas they may not be able to react against CMV or other activation peptides *in vivo* under immunosuppressive pressure. This study suggests that testing cell-mediated immunity in immunocompromised patients treated with tacrolimus requires the prior incubation of PBMC with tacrolimus concentrations equivalent to those found *in vivo* or with the patient's plasma. To try to confirm these initial data, we are developing an assay incorporating the patient's own plasma in the ELISpot evaluation. In brief, we will compare the results of an ELISpot in which the PBMC are activated in complete medium (classical) and an ELISpot performed with the plasma of a patient treated with tacrolimus (investigational).

The same questions should be asked with respect to other immunosuppressants, which may differ from tacrolimus in terms of their pharmacokinetics or their impact on the ELISpot assay.

One of the strengths of our study lies in its two-step methodology. The first step investigated the diffusion rate of tacrolimus into the lymphocytes *in vitro*. The second step explored the ELISpot-based functional response of T-lymphocytes in an increasing range of tacrolimus concentrations. This second step established a concentration-dose response curve, which enabled us to estimate the intra-PBMC tacrolimus concentration that has an impact on the ELISpot results.

The main limitation of this study is the reproducibility and repeatability of the LC-MS/MS method at the lowest concentrations of tacrolimus ($CV < 15\%$).[31] It should be noted that this variability was taken into account by performing tests in triplicate and using a calibration matrix similar to the samples (PBMC in the same culture medium and undergoing the same treatment) in order to ensure the same treatment between samples and the calibration points. For the ELISpot assays, Bestard et al. showed that this test can be accurately performed using the same protocol with reproducibility and repeatability exceeding 80%.[39] As this was a preliminary study, without inferential statistics, there was no point in a design based on sample size calculation. It is also important to note that this proof of concept was carried out *in vitro* and didn't consider the *in vivo* environment (e.g. possible binding of tacrolimus to proteins, a compartmental distribution or the biliary elimination of the drug).

Conclusion

Our *in-vitro* experiment assessed the diffusion rate of tacrolimus in PBMC obtained from healthy volunteers. This diffusion rate was eventually measured around 32%. An *in vitro* dose-response curve of anti-CD3 stimulated T-cell activity (measured by IFN-γ ELISpot assay) according to tacrolimus concentration was obtained. The intra-PBMC concentration of tacrolimus able to inhibit 50% of the response *in vitro* was close to the maximum of tacrolimus intracellular concentrations observed *in vivo* in SOT recipients, around 163 pg/10^6 PBMC. These results suggest that performing an ELISpot assay on the PBMC of SOT recipients may require the *in vitro* addition of the patient's usual immunosuppressant to limit false positivity. Further studies are required to assess the effect of other drug adjuncts on anti-viral IFN-γ ELISpot assays.

Abbreviations

CMV:	cytomegalovirus
DMSO:	Dimethyl sulfoxide
ELISpot:	enzyme-linked immunospot
IC50:	concentration of tacrolimus able to inhibit 50% of the response
IFN:	interferon
LC-MS/MS:	liquid chromatography followed by tandem mass spectrometry
PBMC:	peripheral blood mononuclear cells
SOT:	solid organ transplant
γ:	gamma

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Author contributions

Statement detailing the contributions of each person:
Aurélie Truffot: realization of manipulations, statistics, project requests and drafting
Jules Weinhard: realization of manipulations and help for statistics
Pauline Dessaud: realization of manipulations
Patrice Morand: proofreading
Lionel Rostaing: proofreading
Françoise Stanke-Labesque: project advice and proofreading
Xavier Fonrose: patient recruitment, project advice and proofreading
Raphaële Germi: project advice and proofreading
Thomas Jouve: project advice and proofreading

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Experimental ethics

Samples from healthy volunteers were obtained from the French Blood Establishment. Consent are obtained in the time of donation. MARCHEPIED study is non-interventional, monocentric, retrospective study involving data and samples from human participants has been carried out in Grenoble University Hospital according to French current regulation. The investigator (Dr Aurélie TRUFFOT, PharmD/MD) have signed a commitment to comply with Reference Methodology n°004 issued by French Authorities (CNIL). Subjects and samples enrolled in this study come from the EFS collection, which has all the applicable regulatory authorizations. The EFS is responsible for informing patients, at the time of blood donation, of the possibility of reusing leftovers from the donation for scientific research projects, through of an information and consent form presented to the patient prior to blood donation. This flow of samples is governed by a contract between the EFS and its partners. A contract has been drawn up between the EFS and the Grenoble Alpes University Hospital. The raw data supporting the conclusions of this article will be made available by the authors within respect of General Data Protection Regulation, without undue reservation.

Informed consent

Written informed consent was obtained from all subjects before the study.

Trial registration

Not applicable.

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