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Assessment of Humoral Alloimmunity in Mixed Lymphocyte Reaction

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[Abstract] Humoral alloimmunity remains a significant and unresolved problem that constrains allograft survival. Thus, there is a need for the development of an easy, preferably non-radioactive, and inexpensive protocol for assessing the effect of various drug treatments on humoral alloimmunity. In order to satisfy this demand, we developed such a protocol in which de novo alloantibodies production is induced in one-way mixed lymphocyte reaction (MLR). The amount and capacity of the generated alloantibodies in the supernatant of each one-way MLR is assessed using an antibody-mediated cell dependent cytotoxicity (CDC) assay. The principle of the assay relies on the assessment of cellular survival of resting PBMCs isolated from the same donors, supplemented with the alloantibodies from the one-way MLR supernatant. The lesser is the cellular survival, the higher the production of alloantibodies in one-way MLR and consequently the more potent the humoral alloimmunity.
Keywords: Humoral alloimmunity, Alloantibody, Mixed lymphocyte reaction, Antibody-mediated CDC assay, Antibody-mediated cell dependent cytotoxicity (ADCC), PBMC isolation

[Background] Kidney transplantation is the treatment of choice for patients with end-stage kidney disease. Modern immunosuppression strategies considerably increased the one-year graft survival by decreasing the frequency and the potency of acute cellular-mediated rejection episodes, though long-term graft survival remains problematic mainly due to chronic antibody-mediated rejection, a case without effective treatment (Abramowicz *et al.*, 2018). Thus, there is a need for the development of an easy, preferably non-radioactive, and inexpensive protocol for assessing humoral alloimmunity during various drug interventions, as well as for preclinical evaluation of new medications.

We developed such a protocol in order to test the effect of certain compounds on humoral alloimmunity (Eleftheriadis *et al.*, 2017a, 2017b and 2017c). Although this protocol has been applied in human peripheral blood mononuclear cells (PMBCs), it could also be easily modified for using cells from other species, for instance from different mouse strains.

Our protocol was based on previous studies, supporting that one week is enough for the development of IgM and IgG alloantibodies in one-way mixed lymphocyte reaction (MLR) with a stimulator to responder cell ratio equal to 1:1 (Rumke *et al.*, 1982), along with the well-established antibody-mediated complement-dependent cytotoxicity (CDC) assay (Konishi *et al.*, 2008). Mitomycin C was used to treat the stimulator cell population. Mitomycin C is an anti-tumor antibiotic agent, which acts via the inhibition of DNA and RNA synthesis, rendering the lymphocytes unable to proliferate or differentiate in effector cells. Prolonged treatment with mitomycin-C may also induce apoptosis. The responder PBMCs produced alloantibodies against the mitomycin-C treated stimulator cells.

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To determine humoral alloimmunity, de novo alloantibodies production is induced in one-way MLR. Amount and capacity of generated alloantibodies in the supernatant of each one-way MLR are assessed using an antibody-mediated cell dependent cytotoxicity (CDC) assay. The principle of the assay relies on the assessment of cellular survival of resting PBMCs isolated from the same donors, supplemented with the alloantibodies from the one-way MLR supernatant (Figure 1). Cell survival was measured by performing a well-known cell proliferation assay [Tetrazolium salt 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, XTT cell proliferation assay kit].

We avoided direct cytotoxicity assessment through the non-radioactive lactate dehydrogenase release assay (LDH) mainly because cellular cytotoxicity inevitably occurs in the context of MLRs (Sato *et al.*, 1999) and lactate dehydrogenase is released in the supernatant by dead cells only. Thus, different drug treatments may exert different effects on the MLR mixed cell populations resulting in different amounts of LDH released in the supernatant, a fact that would provide erroneous data in the subsequent antibody-mediated CDC assay.

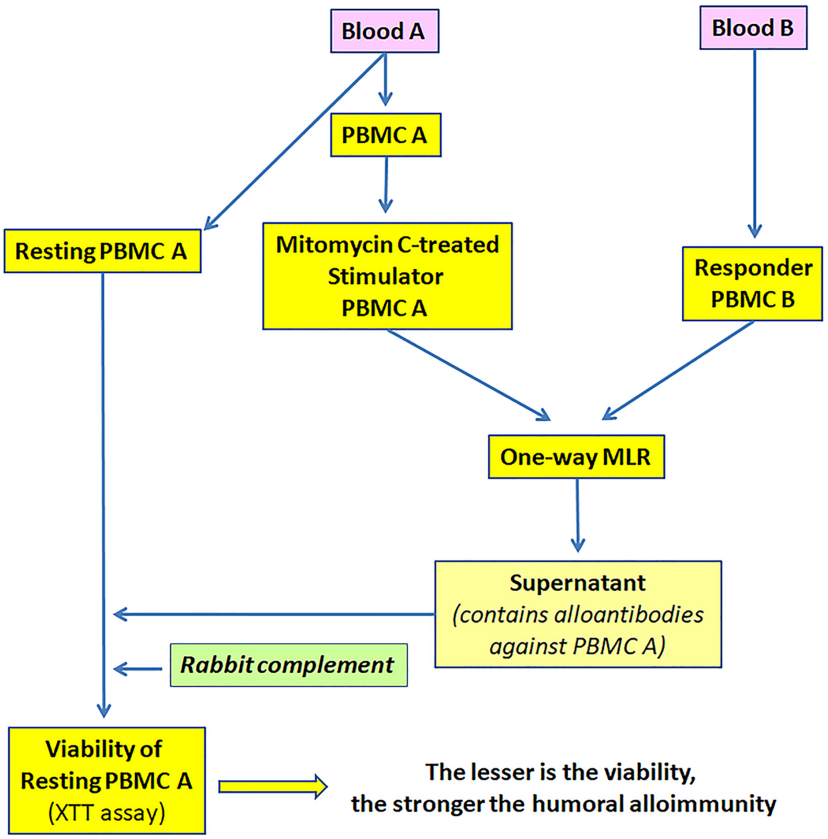

Figure 1. Assessment of humoral alloimmunity in mixed-lymphocyte reaction. The flow-chart depicts the protocol to assess humoral alloimmunity using MLR. In one-way MLR, responder PBMCs produce alloantibodies against the mitomycin-C treated stimulator cells. Blood A and B correspond to blood donors A and B, respectively. Blood B sample will be used to generate the resting cells of that donor that will constitute the responder cells against the mitomycin-C treated stimulator cells of Blood A. Resting cells, or responder cells of Blood A will be used against the

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mitomycin-C treated stimulator cells of another blood donor's sample (*e.g.*, Blood B). Quantitative measurement of alloantibodies in the supernatant of the MLR is assessed by adding this supernatant to resting PBMCs (from the same individual who provided the stimulator PMBCs used for the MLR) using an antibody-mediated CDC assay and measuring cell viability with XTT assay.

Materials and Reagents

1. Filtered P1000 tips DF1000ST (Gilson, catalog number: F171705)
2. Filtered P200 tips DF200ST (Gilson, catalog number: F171503)
3. Filtered P20 tips DF30ST (Gilson, catalog number: F171303)
4. Cover glasses 22 x 22 mm (VWR, catalog number: 631-0125)
5. Serological pipette 10 ml sterile (Greiner Bio-One, catalog number: 607180/10ml)
6. Cell culture 6-well plate, flat bottom, tissue culture treated (Eppendorf, catalog number: 0030720113)
7. Cell culture 24-well plate, flat bottom, tissue culture treated (Eppendorf, catalog number: 0030722116)
8. Cell culture 96-well plate, flat bottom, tissue culture treated (Eppendorf, catalog number: 0030730119)
9. K3-EDTA vacuum blood collection tube (C.D.RICH, catalog number: CDRP007)
10. Conical tubes 15 ml (Corning, catalog number: CLS430790)
11. Conical tubes 50 ml (Corning, catalog number: CLS430828)
12. Pasteur Plastic Pipettes, 3 ml, sterile (Fisher Scientific GmbH, catalog number: 3124975)
13. Microcentrifuge Tube, 1.7 ml, Snap Cap, Clear, sterile (Corning B.V., catalog number: 3621)
14. Sterile blood syringes, 20 ml (Nipro, catalog number: 200812)
15. Low-Tox-H rabbit complement (Cedarlane, catalog number: CL3331)
16. Ficoll Histopaque-1077 (Sigma-Aldrich, Merck Millipore, catalog number: 10771-100ML)
17. Trypan blue solution (Sigma-Aldrich, Merck Millipore, catalog number: T8154-100ML)
18. RPMI 1640 cell culture medium (Sigma-Aldrich, Merck Millipore, catalog number: R7388-100ML)
19. RPMI 1640 cell culture medium without phenol red (Gibco, catalog number: 11835-063 500ML)
20. Fetal bovine serum (Sigma-Aldrich, Merck Millipore, catalog number: F2442-500ML)
    Note: The fetal bovine serum has to be heat inactivated at 55 °C for 1 h.
21. Antibiotic-antimycotic solution 100x (Sigma-Aldrich, Merck Millipore, catalog number: A5955)
22. Dulbecco's phosphate buffer saline (Sigma-Aldrich, Merck Millipore, catalog number: D8537-500ML)
23. XTT cell proliferation assay kit (Trevigen, catalog number: 4891-025-K)
24. Mitomycin C (Sigma-Aldrich, Merck Millipore, catalog number: M0503)

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Equipment

1. Pipetman Starter kit (Gilson, catalog number: F167300)
2. CappTempo motorized pipette controller (Capp, catalog number: T100-B)
3. Cell Counting Chamber Neubauer (Marienfeld, catalog number: 0640010)
4. Safety Cabinet Safeflow 1.2 (BioAir, catalog number: LD60000)
5. CO_2 Incubator (Heraeus, model: BB6220)
6. Inverted Microscope (Zeiss, model: Axiovert 40C)
7. Varispeed Refrigerated Centrifuge (Centurion Scientific, model: K280R)
8. Heated Bath Circulator (Lauda, model: M20)
9. Refrigerator Cell Plate Centrifuge (Eppendorf, model: 5810R)
10. Multimode Plate Reader (Perkin Elmer, model: Enspire)
11. Microcentrifuge (Eppendorf, model: 5415 R)
12. Any Styrofoam box that will serve as an ice bucket

Software

1. The SPSS software (version 13; SPSS Inc, Chicago, IL, USA) (for statistical analysis of the data)

Procedure

A. Blood collection

Notes:

a. To exclude pre-sensitization events, male subjects without blood transfusion history or previous organ transplantation are preferred. Subjects must be healthy and not related.

b. A written informed consent must be obtained from each individual enrolled in the study.

c. The ethics committee of your respected institute/country must formally approve your study, providing also a study protocol number.

d. The whole procedure requires aseptic conditions, the use of sterile reagents and plastic ware is highly recommended.

1. With a sterile blood syringe, draw about 20 ml of blood from each individual and immediately dispense it into a pre-labeled K3-EDTA vacuum blood collection tube (one tube/donor).
2. Invert the tube several times for the blood to mix properly with K3-EDTA and, keep the collection tubes in a cold environment (4 °C, incubation time can be no longer than 2 h) until PBMCs isolation commences.

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B. PBMCs isolation

Notes:

a. *The whole procedure must be performed inside a sterile cabinet (hood).*
b. *Whether you use a swinging bucket centrifuge rotor, or a rotor with a fixed angle will not significantly affect the total numbers of isolated PMBCs.*
c. *During Step B1, layering of blood on the Ficoll Histopaque should be done really gently in order not to disturb the Ficoll layer (Ficoll allows for elimination of dead cells).*
d. *Following these instructions, a proper isolation should yield 1.8-2.3 x 10^6 PBMCs/1 ml of blood of a healthy individual.*
e. *The ratio of blood to Ficoll Histopaque volume should be 1:1.*

1. Dependent on the volume of blood collected, add the same total volume of Ficoll into a 50 ml conical tube per donor. Slowly add the blood of each individual with a plastic Pasteur pipette at the surface of the Ficoll without disturbing it.
2. Immediately centrifuge the tubes containing Ficoll and blood at 400 *x g* for 25 min at room temperature (RT). Make sure that the deceleration speed of the centrifuge machine is 0.
3. Using a sterile plastic Pasteur pipette carefully breach the plasma layer and, without disturbing the Ficoll Histopaque layer, aspirate the white PBMCs layer (“buffy” coat) formed in the interphase between blood plasma and Ficoll Histopaque (Figure 2). Pour the PBMCs into a new 15 ml conical tube.

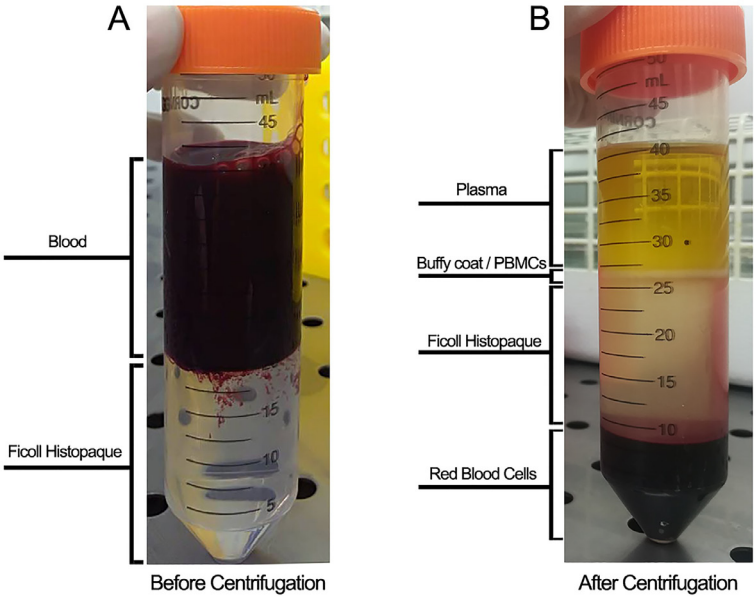

Figure 2. Isolation of PBMCs. Layers of Ficoll Histopaque and whole blood prior to (A) and after (B) centrifugation showing the separation of layers in the conical tube.

4. Add RPMI 1640 cell culture medium without phenol red up to 15 ml for each PBMCs collected.
5. Pellet PBMCs by centrifuging samples at 315 *x g* for 10 min at RT. Revert deceleration speed

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to normal (6 on Centurion Scientific centrifuge).

6. Remove the supernatant, wash cell pellet with 10 ml of RPMI 1640 cell culture medium without phenol red and centrifuge samples at 315 *x g* for 10 min at RT.
7. Remove the supernatant and resuspend each cellular pellet with 2 ml of complete RPMI medium (10% FBS, 1% antibiotic-antimycotic solution) and count them using Neubauer chamber. Mix 90 µl of trypan blue solution and 10 µl of cells into a clean Eppendorf tube, load the Neubauer chamber and begin counting immediately under a microscope at the lowest magnification. Prolonged overstaining with trypan blue solution stains all cells blue. Always count the viable cells that appear as spherical white cells. Use of trypan blue allows for distinction of dead cells from live cells, since dead cells are stained in blue. A detailed protocol for cell counting using a Neubauer chamber was discussed in (Phelan and Lawler, 2001).

C. Assessment of humoral alloimmunity

Notes:

a. As already mentioned, a 7-day incubation period in Step C1f is necessary for the development of IgM and IgG alloantibodies. Any drug treatment should last for the entirety of the period.

b. During Step C3a, seed the appropriate number of cells that you will need to generate enough wells to assess humoral immunity in non-treated cells, treated cells and control cells. We recommend to perform nine to twelve identical experiments, each in triplicate, and the results should refer to the mean of the three measurements.

c. Low-Tox-H rabbit complement is provided as a lyophilized compound. Shortly before the use of Low-Tox-H rabbit complement, reconstitute it with 1 ml of ice-cold distilled water (reconstitute during the 30 min incubation on ice during Step C3f). According to the manufacturer's instructions, the complement must be stored at 4 °C for a maximum of one hour after reconstitution.

d. Have access to an ELISA plate reader capable of reading wavelengths at 490 nm and 630 nm, respectively.

e. Make sure to remove any bubble formed in the XTT 96-well plate before reading the plate with an ELISA plate reader machine. Any foam or bubble will negatively impact OD readings. The tip of a syringe works best for bubble or foam removal.

1. Preparation of one-way MLR
   a. Resuspend 0.5 x 10^6 PBMCs in 500 µl with complete RPMI from each sample and transfer them into a sterile 1.7 ml microcentrifuge tube. Treat them with 50 µg/ml mitomycin C for 30 min at 37 °C. These are the stimulator cells.
   b. Centrifuge cells at 300 *x g* for 5 min at RT, remove supernatant and wash cells with Dulbecco's phosphate buffer saline.
   c. Repeat Step C1b two more times.
   d. Seed 0.5 x 10^6 stimulator cells/well resuspended in 250 µl complete RPMI into a 24-well

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plate.

e. To each well already containing the 0.5 x 10^6 stimulator cells, add 0.5 x 10^6 resting PBMCs (resuspended in 250 µl complete RPMI) from a different donor; these are the responder cells (*e.g.*, stimulator cells from donor A with responder cells from donor B). Together stimulator and responder cells constitute the MLR.

f. Incubate MLR for 7 days at 37 °C in a CO_2 incubator with or without the treatment that is under your investigation. The treatment of choice should be added right at the start of the 7-day incubation period. Culture media must not be changed during the 7-day incubation.

2. Preparation of resting PBMCs

a. In parallel to the preparation of one-way MLR (Steps C1a-C1f), resting PBMCs samples should be prepared. Resuspend 1 x 10^6 PBMCs in 2 ml of complete RPMI and transfer the cells into one well of 6-well plate. Prepare as many wells as needed. These cells are the resting PBMCs.

b. Incubate resting PBMCs for 7 days at 37 °C in a CO_2 incubator, without changing the culture media during the 7-day incubation period.

3. Antibody-mediated complement-dependent cytotoxicity

a. Once the 7-day period is over, count the viable resting PBMCs from each well. Counting of cells from each resting PBMCs sample is laborious. It has to be done for each sample, as it will ensure accurate counting of resting cells, excluding proliferation or apoptotic rates during the 7-day period that may greatly vary between PBMCs populations from different blood donor individuals.

b. Following counting, resuspend 0.5 x 10^5 resting PBMCs into 50 µl of complete RPMI and seed 50 µl of cells/well into a flat bottom 96-well plate. The above resting PBMCs must be derived from the same individual that also provided the stimulator PBMCs of the one-way MLR (as seen in Figure 1, *e.g.*, PBMC A individual). Humoral alloimmunity will be evaluated in the person who provided the responder PBMCs (as seen in Figure 1, *e.g.*, responder PBMC B individual).

c. Once the 7-day period is over, centrifuge each 24-well plate containing the MLR, from Step C1f, on a Cell Plate Centrifuge at 300 *x g* for 10 min at RT.

d. Collect the supernatant from each MLR. The volume of the supernatant should be around 450-500 µl. Please be gentle during handing and pipetting, in order not to accidentally pick any centrifuged cells.

e. Add 50 µl from each MLR supernatant or 50 µl of complete RPMI (control) into the wells of that 96-well plate containing the resting target PBMCs. In addition, in an empty well add 100 µl of complete RPMI that will serve as the blank well for the XTT assay. It is recommended to generate nine to twelve control wells for statistical analysis.

f. Incubate the 96-well plate on ice for 30 min inside the hood.

g. After the 30 min incubation, add 11 µl of rabbit complement Low-Tox-H rabbit complement (10% [v/v], working concentration) in each well (samples, controls, blanks) at a final

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concentration of 10%. Now the total volume in each well should be 111 µl.

h. Incubate the 96-well plate at 37 °C for 2 h in a CO_2 incubator.

i. Immediately before the end of the incubation period, prepare the XTT working solution from the XTT assay kit that will be used to measure cell survival via colorimetry. Briefly and according to the manufacturer's protocol, add 100 µl of the XTT activator to 5 ml of XTT reagent in order to prepare the XTT working solution.

j. Once the 2 h incubation is over (Step C3i), add 50 µl of the XTT working solution to each well of the 96-well plate and mix gently.

k. Return the plate at 37 °C for another hour in the CO_2 incubator.

l. Read the absorbance of your samples using an ELISA Plate Reader set at 490 nm with a reference correction at 630 nm.

m. After subtracting background OD values derived from the blank wells, calculate cell survival in each sample by the equation: Cell survival (%) = (XTT assay OD of the evaluated condition/XTT assay OD of the control) x 100. The lesser the cell survival, the higher the production of alloantibodies in one-way MLR and, consequently the more potent the humoral alloimmunity.

Data analysis

We recommend to perform at least nine to twelve one-way MLRs for each different condition, for instance with or without various tested substances, run in triplicates. Once all cell survival ratios are assessed (Step C3m), you can implement the data into SPSS software. If two conditions are evaluated, paired *t*-test should be used for comparison of means, since each MLR (consisted of PBMCs from two different donors) is unique in experiments with human subjects. For more than two evaluated conditions one-way repeated-measures ANOVA followed by Bonferroni's correction test should be used. If you apply this protocol to two different mouse strains, and you consider that subjects of each strain are genetically matching, unpaired *t*-test and one-way ANOVA followed by the Bonferroni's correction test respectively are the appropriate statistical tests.

An example of an antibody-mediated CDC is depicted in Figure 3, showing inhibition of humoral immunity by the specific proteasome inhibitor CEP18770 at a concentration of 10 nM. Proteasome plays a key role in cellular protein turnover and protein homeostasis. Moreover, it is well known that plasma cells are susceptible to proteasome inhibition since they produce large amounts of antibodies and consequently exhibit high protein turnover. Direct inhibition of the proteasome in plasma cells leads to impaired protein turnover, eliciting the unfolded protein response, which in its turn will induce apoptosis (Walsh *et al.*, 2012). The presented published results render proteasome as an excellent target for the development of new immunosuppressive medications in the field of transplantation (Eleftheriadis *et al.*, 2017a).

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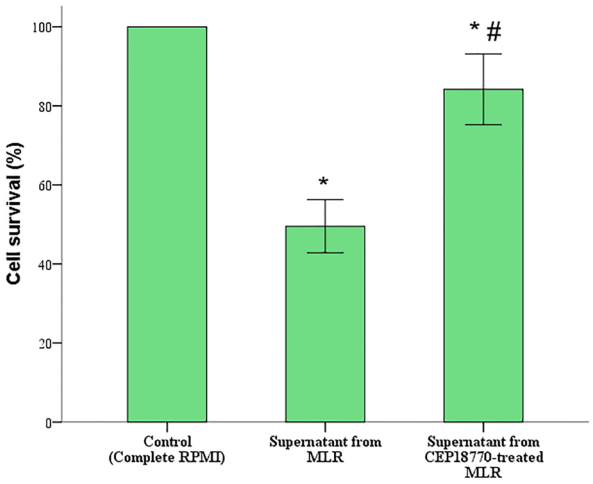

Figure 3. Proteasome inhibitor CEP-18770 efficiently inhibits humoral alloimmunity. One-way MLRs were performed in the presence or not of 10 nM CEP-18770 for a 7-day period. Supernatants were collected and used in an antibody-mediated CDC assay against resting target PBMCs similar to those used as stimulator cells in the respective MLRs. Supernatants derived from CEP-18770-treated MLRs exhibited smaller antibody-mediated CDC than those derived from untreated MLRs. Marks denoted as * and #, indicate a $P < 0.05$ compared between the conditions. Error bars correspond to SD. Twelve such experiments were performed, each in triplicates and the results refer to the mean of the three measurements.

Notes

Inconsistent variability between ODs during XTT assay should not emerge, and triplicate ensures reproducibility of data. We obtained consistent results by performing this protocol many times. Three studies based on this protocol have already been published (Eleftheriadis *et al.*, 2017a, 2017b and 2017c).

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Competing interests

Declaration of interest: None declared.

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Ethics

An informed consent was obtained from each individual enrolled in the study and the Ethics Committee of the University of Thessaly, Faculty of Medicine, Larissa, Greece approved the study protocol (Number of approval: 558/10-2-2017).

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