

**RESEARCH SERVICES AGREEMENT**

**THE UNIVERSITY OF FOGGIA - DEPARTMENT OF MEDICAL AND SURGICAL SCIENCES** with registered offices at Biomedical Research Center "E. Altomare", Via Luigi Pinto no. 1, 71122 Foggia, Italy, Fiscal Code 94045260711, Vat no. IT03016180717, here represented by the Director of the Department of Medical and Surgical Sciences, Prof. Gaetano Serviddio born in Bari (BA) on 22/08/1971 Fiscal Code SRVGTN71M22A662L (the "**University**")

**INVIZIUS LIMITED**, a company incorporated in Scotland under the Companies Acts (Company Number SC586903) and having its offices at Biocity Scotland, Bo'Ness, Motherwell, Lanarkshire, Scotland, ML1 5UH (the "**Company**")

**3 DEFINITIONS**

In this Offer, unless the context otherwise requires, the undernoted terms in Column 1 shall have the meanings allocated to them in Column 2, namely:

The Agreement	means this Offer and the Schedule and the acceptance thereof by the Company.
The Contract Price	means the price of the Research Services as detailed in Part 3 of the Schedule.
Commencement Date	means 1 <sup>st</sup> November 2020, notwithstanding the date or dates hereof.
Materials	means any material provided by the Company for use within the Research Services.
Period	means three (3) months commencing on the Commencement Date.
Research Services	means the Research Services more particularly described in Part 1 of the Schedule
Schedule	means the schedule in 3 parts attached to and forming part of this Agreement
Supervisor	means Prof. Giuseppe Castellano (or his successor as appointed pursuant to Clause 1.2 of Part 1 of the Schedule).

**4 ACCEPTANCE:**

- 4.1 Return of one copy of this Agreement (bearing an original signature) shall constitute the Company's acceptance of this Offer.

**Signed for and on behalf of the University as follows:**

Name: Gaetano Serviddio

Designation: Director of the Department of

Medical and Surgical Sciences

DocuSigned by:  
*Gaetano Serviddio*  
1EF613C8CE1648D...

**Signature**

Date: 10.22.2020

Place: Foggia

**Acceptance for and on behalf of the Company as follows:**

Name: Dr Magnus Nicolson

Designation: COO

DocuSigned by:  
*Magnus Nicolson*  
3B17E9115B2B450...

**Signature**

Date: 10/22/2020

Place Balfon

**THIS IS THE SCHEDULE REFERRED TO IN THE FOREGOING OFFER****PART 1****1 RESEARCH SERVICES**

1.1 The University shall use reasonable endeavours to supply the Research Services during the Period.

1.2 The Research Services shall be directed by the Supervisor or such other suitably qualified person as appointed by the University.

**2 PAYMENT**

2.1 The Company hereby agrees to make the payments specified in Part 3 of the Schedule to the University together with any VAT or other applicable sales tax in respect thereof. Such payments shall be due within (30) thirty days of the date of the invoice(s) from the University.

2.2 If, during the Period, the Company seeks to vary the Research Services or such variation becomes desirable due to the nature of results obtained, adjustment of the payments specified in Part 3 of the Schedule should be mutually agreed and incorporated into the final account to be submitted to the Company for payment.

2.3 In the event of the Company failing to make timeous payment, the University may either cease to provide the Research Services forthwith until payment is made in full or, at its option, treat this Agreement as repudiated.

2.4 Notwithstanding the terms of Clause 2.3, the Company shall pay interest on such payments from the due date until the actual date of payment at the rate of five per centum per annum over the base lending rate of The Royal Bank of Scotland plc from time to time. Such interest shall accrue on a daily basis and be compounded quarterly.

**3 CONFIDENTIALITY**

3.1 Subject to Clause 3.3 below, both parties shall be obliged to keep strictly confidential and not to make use of howsoever or to disclose to any third party any information relating to the affairs of the other or the existence or content of this Agreement or otherwise arising out of the provision of the

Research Services which may reasonably be regarded as or which it ought to regard as confidential without the prior written consent of the other.

3.2 Information shall not be considered to be confidential if: (a) it is and can be shown to be already known to the receiving party at the date of disclosure; (b) it subsequently becomes lawfully available to the receiving party; (c) it is published in a patent specification or is otherwise in the public domain other than through default of the receiving party; or, (d) it is required to be disclosed by law.

3.3 The University shall be free to use information relating to the Research Services for academic purposes, including publication, subject to safeguarding the commercial interests of the Company which shall receive a copy of proposed publications for comment within a thirty (30) day period prior to submission for publication.

**4 INTELLECTUAL PROPERTY**

4.1 For the avoidance of doubt, all background information and intellectual property rights ("**background IPR**") in existence prior to the Commencement Date shall remain the property of the party introducing such background IPR.

4.2 Subject to receipt of the full Contract Price by the University, all patentable inventions or other intellectual property rights relating to any new product, technique or invention arising from the provision of the Research Services by the University (the "**Results**") shall become and be viewed as the sole property of the Company.

4.3 The Company grants the University a royalty-free, non-exclusive licence to use the Results for non-commercial activities such as teaching and scientific or clinical research.

**5 MATERIALS**

During the term of this Agreement it may be necessary for the Company to provide the

University with various proprietary Materials for which the following terms will apply:

5.1 Materials will be provided solely for use in the Project, in the University's laboratories only. The University undertakes that any Materials provided will be used only by the Supervisor and such persons under the direct supervision of the Supervisor as are required to perform the Research Services. The Materials will not be provided to any other scientist or institution (public or private) without prior written permission from the Company.

5.2 On termination of the Agreement the University will discontinue use of the Materials and at the direction of the Company any remaining Materials will be returned to the Company or destroyed, and destruction certified by the University.

5.3 All experimental work within the Project and any destruction of Materials pursuant to Clause 5.3 above will be carried out in accordance with all applicable local, national and international legislation relating to the safe handling, use and disposal of potentially hazardous materials.

## 6 ASSIGNATION

6.1 The Company shall not, without the prior written consent of the University, assign, transfer, charge, sub-contract or deal in any other manner with all or any of its rights or obligations under this Agreement.

## 7 LIABILITY

7.1 The University makes no representation or warranty that advice given by its employees or agents is accurate, conform to contract or free from defects, latent or patent; nor does it warrant that the use of the Results or any information provided in connection with the provision of the Research Services will provide the desired objective or not result in infringement of third party rights and the University does not accept any responsibility whatsoever for infringement of such rights.

7.2 The University accepts no liability for any use which may be made by the Company of any work carried out under or pursuant to this Agreement, or of the Results, nor for any reliance which may be placed by the Company on such work or Results, nor for

advice or information given in connection with them.

7.3 The Company shall indemnify and keep indemnified the University, its employees or agents against all claims, actions, losses, damages, costs and expenses which may be brought against or incurred or suffered by the University, its employees or agents as a result of the Company's use of the Results or any materials, works or information received from the University, its employees or agents pursuant to the terms of this Agreement.

7.4 Subject always to the terms of this Agreement, neither party will use the other party's name or logo in any publicity, advertising or press release without the prior written approval of the other party.

## 8 NO AGENCY OR PARTNERSHIP

This Agreement shall not be taken as creating or evidencing any agency or partnership between the University, on the one hand, and the Company on the other.

## 9 NOTICES

Any notice, request or consent under this Agreement shall be in writing and shall be sufficiently served if sent by email. For the University this means all correspondence should be sent to Manuela del Prete [manuela.delprete@unifg.it](mailto:manuela.delprete@unifg.it) and for the Company all correspondence, including invoices should be sent to [mnicolson@invizius.com](mailto:mnicolson@invizius.com).

## 10 TERMINATION

10.1 Either party shall have the right to terminate this Agreement forthwith by notice in writing to the other in the event of any material or persistent breach by the other party of any of its duties or obligations hereunder or in the event of failure by the other party to remedy to the reasonable satisfaction of the party serving the notice any breach of any of its duties or obligations hereunder within thirty (30) days following written notice requiring it to do so.

10.2 In the event of the liquidation or receivership of the Company or in the event of an administrator being appointed to the Company, the University shall be entitled to terminate this Agreement forthwith.

10.3 Termination of this Agreement shall not affect the acquired rights and obligations of either party under this Agreement.

10.4 On the termination of this Agreement, the Company shall pay the University for all work done prior to termination.

## 11 APPLICABLE LAWS

11.1 The parties shall comply with all applicable laws, including but not limited to: (1) all applicable anti-bribery and anti-corruption laws, statutes, regulations and codes from time to time in force including but not limited to the Bribery Act 2010; (2) all applicable anti-facilitation of tax evasion laws, statutes, regulations and codes from time to time in force including but not limited to the Criminal Finances Act 2017; (3) all applicable anti-slavery and human trafficking laws, statutes, regulations and codes from time to time in force including but not limited to the Modern Slavery Act 2015; and (4) the requirements of all legislation and regulatory requirements in force from time to time relating to the use of personal data, including, without limitation (i) the General Data Protection Regulation ((EU) 2016/679) (GDPR) and any national implementing laws, regulations and secondary legislation, for so long as the GDPR is effective in the UK; and (ii) any successor legislation to the Data Protection Act 2018 and the GDPR, in particular, any legislation enacted following the UK leaving the EU.

## 12 GENERAL

12.1 This Agreement constitutes the whole agreement between the parties and supersedes all previous agreements between the parties relating to its subject matter.

12.2 If and in so far as any part or provision of the Agreement is or becomes void or unenforceable it shall be deemed not to be or never to have been or formed a part of the Agreement and the remaining provisions of the Agreement shall continue in full force and effect.

12.3 Neither party shall be liable to the other for loss or damages arising from prevention or delay in performance of this Agreement where same is a result of force majeure, provided immediate written notice is sent by the party so prevented or delayed.

12.4 Any variation to the Agreement shall only be effective if in writing and signed by both parties.

12.5 The failure on the part of either party to exercise or enforce any right conferred upon it under this Agreement shall not be deemed to be a waiver of any such right or operate to bar the exercise or enforcement thereof at any time or times thereafter.

## 13 GOVERNING LAW

This Agreement shall be governed by and construed in accordance with the laws of Scotland and the parties hereby submit to the exclusive jurisdiction of the Scottish Courts.

## PART 2

### THE RESEARCH SERVICES

#### Modulation of Complement activation in Peritoneal Dialysis (PD)

**Authors:**

Giuseppe Castellano, MD, PhD; Associate Professor

**Key Words:**

Peritoneal fibrosis, epithelial-to-mesenchymal transition, Complement inhibition.

**Background:**

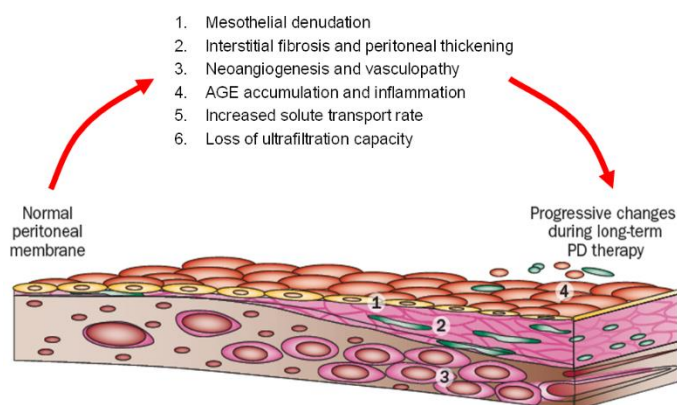
Peritoneal dialysis (PD) is the leading form of home-based dialysis therapy and is a well-established alternative to hemodialysis (HD) for the dialytic treatment of end-stage renal disease (1). Compared to HD, the PD is associated to a good preservation of residual renal function, lower infectious risk and higher satisfaction rates. In contrast with HD, in the PD no synthetic membrane is used and the peritoneum of the patients acts as a semi-permeable membrane allowing diffusion between the dialysis fluid and the circulation. The peritoneal membrane is lined with a monolayer of mesothelial cells that have some characteristics of epithelial cells, act as a permeability barrier, and secrete various substances involved in the regulation of peritoneal permeability and local host defense (2). The osmotic gradient during PD is based on high glucose levels in the dialysate.

However, this use of glucose is also responsible for the incompatibility reaction and for the side effects of PD. Indeed, the chronic exposure to dialysate leads to tissue remodeling of this layer resulting in peritoneal fibrosis. This progressive fibrosis forms a major limitation for chronic PD treatment. Another common complication in PD is peritonitis. Patients who develop peritonitis can have irreversible peritoneum damage, PD failure and significant morbidity or even mortality. For this reason, avoiding PD failure due to peritonitis or fibrosis remains a challenge for nephrologists. However, the non-physiological composition of conventional glucose-based solutions, including the high concentrations of glucose, glucose degradation products (GDPs) and lactate, the high osmolality, and the low pH, is harmful for the peritoneal membrane and also has detrimental systemic effects in the patient. The negative local effects, which can in part be attributed to the composition of the solutions, include impairment of mesothelial cell viability, adverse effects on leukocyte recruitment, accelerated angiogenesis and vascular proliferation, and low-grade inflammation, which lead to epithelial-to-mesenchymal transition, fibrosis and thickening of the peritoneal membrane(3). In addition, the high concentration of glucose and GDPs promotes the formation of advanced glycation end products (AGEs), which are deposited within the peritoneal membrane (4). These changes alter the dialytic properties of the peritoneal membrane and lead to increased peritoneal small solute transport rate (called “high transport” or, more appropriately, “fast transport”) and reduced osmotic conductance (that is, a reduced capacity of each unit of the osmotic agent to generate one unit of peritoneal ultrafiltration). Moreover, this functional decline of the peritoneum may be accelerated by recurrent or severe episodes of peritonitis or hemoperitoneum (5). In particular, Ultrafiltration failure (UFF) is a major clinical problem in peritoneal dialysis (PD), because it accounts up to 20 percent of patients undergoing continuous ambulatory peritoneal dialysis and it occurs

especially in long-term patients. UFF is diagnosed when net ultrafiltration (UF) is <400 ml after a 4-hour dwell with 3.86%/4.25% glucose peritoneal dialysis fluids (PDF), in the absence of catheter malfunction, fluid leaks or extensive intraperitoneal adhesions. If normal hydration is not restored, PD patients with UFF are at higher risk for fluid overload, left ventricular hypertrophy and cardiovascular accidents. Moreover, UFF is the consequence of changes to peritoneal membrane function over time, which pose a risk for the rarer but life-threatening condition of encapsulating peritoneal sclerosis (EPS).

Glucose has a dose-dependent inhibitory effect on mesothelial cell proliferation that is mediated by oxidative

**Figure 1. Peritoneal membrane injury during long-term PD**



stress (6). Mitochondrial DNA damage also occurs after exposure of human peritoneal mesothelial cells (HPMCs) to glucose (7). The local toxicity of glucose is, in part, related to a variety of GDPs that are formed in PD solutions and may cause mesothelial cell injury (8). GDP-induced mesothelial cell injury and apoptosis lead to intracellular hydrogen peroxide production and free radical formation, which are inciting events for peritoneal membrane inflammation and injury (9). Moreover, GDPs induce

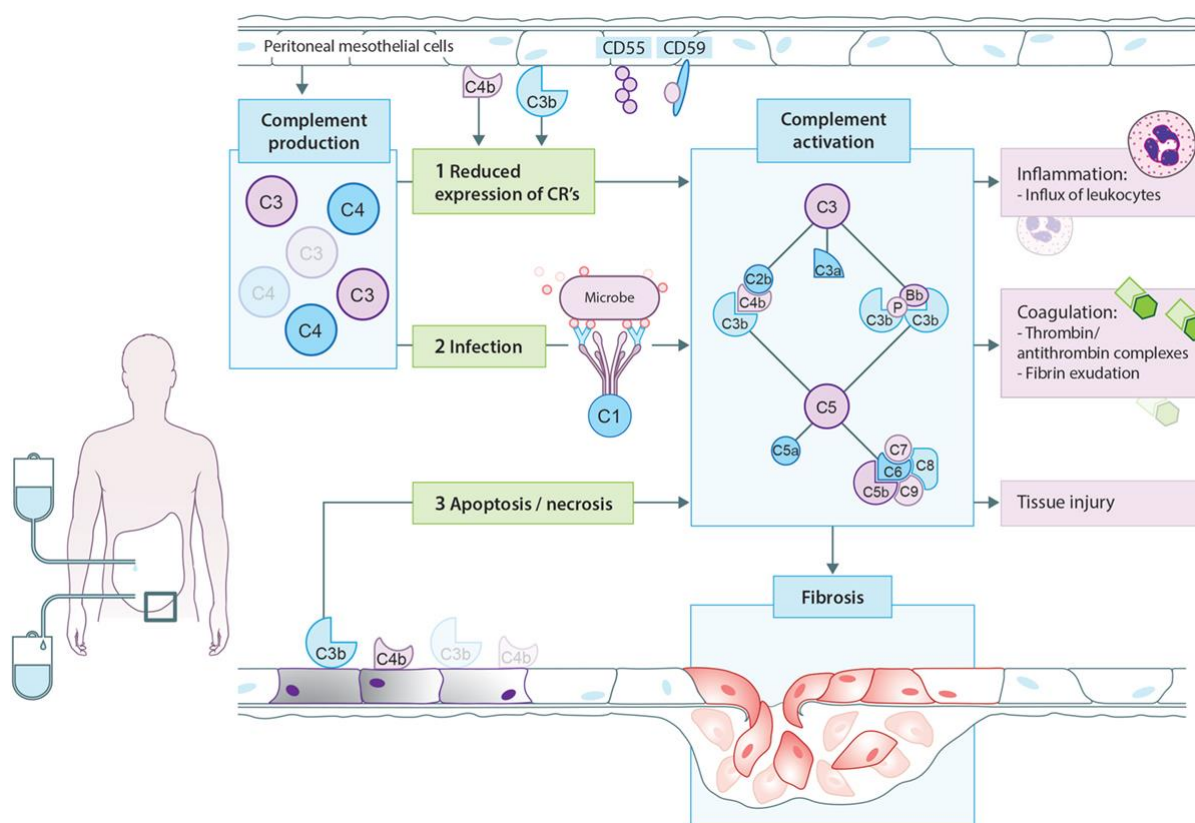
production of the profibrotic cytokine Transforming Growth Factor (TGF)- $\beta$ , and stimulate Vascular Endothelial Growth Factor (VEGF) synthesis in HPMCs (10–12). TGF- $\beta$  and VEGF are thought to have key roles in the progressive peritoneal membrane fibrosis and vascular proliferation that is seen with long-term PD (13). Glucose and GDPs promote the formation of AGEs, which increase vascular permeability by disruption of the vascular basement membrane as a result of protein cross-linking to basement membrane components or by the activation of the receptor for AGEs (RAGE) on endothelial cells. The changes in the peritoneal membrane, induced in part by high levels of glucose and GDPs, initiate a cascade of events that lead to progressive peritoneal membrane injury (Figure 1).

During peritoneal dialysis (PD), the peritoneum is exposed to bioincompatible dialysis fluids, which causes progressive fibrosis and angiogenesis and, ultimately, ultrafiltration failure. However, in parallel with fibrosis, the peritoneum also displays increases in capillary number (angiogenesis) and vasculopathy in response to PD. Nowadays, there is emerging evidence pointing to peritoneal microvasculature as the main factor responsible for increased solute transport and ultrafiltration failure. However, the pathophysiologic mechanisms involved in starting and maintaining peritoneal fibrosis and angiogenesis remain elusive. Peritoneal stromal fibroblasts have been considered (for many years) the cell type mainly involved in structural and functional alterations of the peritoneum; whereas mesothelial cells have been considered mere victims of peritoneal injury caused by PD. Recently, ex vivo cultures of effluent-derived mesothelial cells, in conjunction with immunohistochemical analysis of peritoneal biopsies from PD patients, have identified mesothelial cells as culprits, at least in part, in peritoneal membrane deterioration. Recent findings suggest new peritoneal myofibroblastic cells may arise from local conversion of mesothelial cells by epithelial-to-mesenchymal transition during the repair responses that take place in PD. The transdifferentiated mesothelial cells may retain a permanent mesenchymal state, as long as initiating stimuli persist, and contribute to PD-

induced fibrosis and angiogenesis, and hence to membrane failure (14). Local production of VEGF during PD appears to play a central role in the processes leading to peritoneal neo-angiogenesis and functional decline.

Interestingly, complement activation has been demonstrated in patients with PD; mesothelial cells can produce complement factors that contribute to local complement activation. Complement components C4, C3, Factor B, Factor D, Factor H and C9 have been demonstrated in dialysate of healthy PD patients by proteomic approach. In addition, the process of PD therapy reduces expression of membrane CRegs complement regulators (as CD46, CD55 and CD59) on peritoneal mesothelial cells and triggers local complement activation (15). However, to further confirm an aberrant local complement activation, sC5b-9 was also shown in the peritoneal fluid. Thus, could be possible to speculate that PD fluid leads to the loss or dysfunction of CD59 on mesothelial cells in the peritoneal cavity, making the mesothelial cells more vulnerable for complement activation. Additionally, complement production and activation can be further stimulated by PD solution containing glucose.

We recently showed that Complement activation is pivotal in driving several profibrotic response at cellular and organ level (16); Complement products can drive this process by inducing trans-differentiation of cells towards a myofibroblast phenotype such as in Endothelial to mesenchymal transition (17) and pericyte to mesenchymal transition (18). Finally, Complement can induce accelerate cellular senescence regulating the WNT pathways and suppressing Klotho, the principal anti-ageing factor(19,20).



**Figure 2: Proposed model for complement activation in peritoneal dialysis (PD).** In PD patients, mesothelial cells produce and secrete different complement factors. One of the proposed mechanisms of complement activation in PD patients is that PD therapy decreases the expression of complement regulators such as CD55 and CD59 on the peritoneal mesothelium, leading to local complement activation. In addition, cellular debris as a result of direct peritoneal damage by bioincompatible PD fluids as well as antibodies

against microorganisms could contribute to local complement activation during PD. Complement activation will result in the formation of anaphylatoxins (C3a, C5a), opsonin (C3b, iC3b), and the membrane attack complex (C5b-9). First, complement activation leads to the influx of leukocytes, predominantly neutrophils. Second, complement activation increased the production of thrombin anti-thrombin complexes and fibrin exudation on the surface of the injured peritoneum. Altogether, these events indicate the activation of the coagulation system. Third, complement activation during PD leads to direct damage of the peritoneum. Moreover, recent evidence suggests that complement activation promotes the progression to fibrosis after tissue injury. In PD, complement activation could stimulate mesothelial cells to undergo epithelial-to-mesenchymal transition, resulting in the accumulation of myofibroblasts and consequently peritoneal fibrosis (21). Therefore, it might be possible to hypothesize that interfering with Complement activation in the context PD might interfere with fibrotic processing and preserve peritoneal membrane by inflammation and senescence process.

### ***Aims:***

1. investigate the pattern of Complement activation during PD in vitro and ex-vivo
2. investigate the effects of different PD solutions on Complement production and activation in mesothelial cells
3. investigate the effect of *H-Guard protein* in inhibiting Complement systems thereby protecting mesothelial cells from activation

### ***Materials and Methods:***

#### **Cell Culture HMC Culture**

The Immortalized Human Mesothelial Cells-SV40 (HMC) will be purchased from Applied Biological materials (ABM [www.abmgoods.com](http://www.abmgoods.com)); these cells are isolated from the human mesentery mesothelium and immortalized with recombinant lentiviruses carrying SV40 large T antigen. Cells will be maintained in Prigrow I medium (cat. # TM001, [www.abmgoods.com](http://www.abmgoods.com)) supplemented with 10% FBS, 1% Pen/Strep, according to supplier indications. Cells will grow on 25 cm<sup>2</sup> or 75 cm<sup>2</sup> extracellular matrix (ECM)-coated flasks at 37 °C in a humidified incubator and a 5% CO<sub>2</sub> atmosphere. Culture medium will be changed every 2-3 days and cells will be subculture when they became around 80% confluent. ECM and ECM-coated flasks will be purchased from ABMgood.

#### **Isolation of HMC from PD effluent**

Effluent-derived HMC isolated from clinically stable PD patients using a method described hereafter. Briefly, peritoneal dialysates from clinically stable patients will be drained and processed immediately. Bags will be hanged for 3-4 hours in an incubator at 4°C to facilitate the accumulation of floating cells at the bottom of the bags. The supernatant will be then carefully removed by vacuum with a sterile pipette, leaving approximately 200 mL of sediment. The cells will be transferred to four 50-mL tubes, centrifuged at 1200 rpm for 10 minutes, and washed twice with phosphate-buffered saline (PBS). The cell pellets will be suspended in 5-7 mL of culture medium, counted in a Neubauer chamber, seeded in 25-cm<sup>2</sup> tissue culture flasks, and incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells will be cultured in RPMI 1640 medium containing 26 mM of bicarbonate and supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml),

streptomycin (100 µg/ml), L-glutamine/L-alanine (2 mM), hydrocortisone (0.4 µg/ml), transferrin (0.5 µg/ml), and insulin (0.5 µg/ml) and incubated at 37°C in a humidified atmosphere with 5% carbon dioxide (CO<sub>2</sub>). After confluence will be reached, the morphological features of cells in confluent cultures will be assessed. Confluent HPMC cultures from PD effluents will show one of two major phenotypes, epithelial-like (epithelioid) or non-epithelioid, which are expected to remain stable for two to three cell passages. While epithelioid cells have a cobblestone appearance and share with normal HPMC expression epithelial markers E-cadherin and cytokeratins, even if at lower level, non-epithelioid cells have a spindle-like fibroblastic shape, and are in advanced steps of the Epithelial-to-Mesenchymal phenotype process (late EMT).

For our study we will use different PD Solution which are glucose-based:

- Physioneal 40 (Baxter Healthcare Corporation, Deerfield, IL, USA); pH=7.4, bicarbonate and lactate-buffered;
- Dianeal PD4 (Baxter Healthcare Corporation, Deerfield, IL, USA); pH= 5-6.5, lactate-buffered;
- Others

In this context, we will investigate the effects of **H-Guard protein** in inhibiting Complement activation in presence of different PD solutions, thereby protecting mesothelial cells from activation

#### **FACS ANALYSIS ON CELLS FROM PD EFFLUENT**

The expression of CD55, CD46 and CD59 will be assessed by monoclonal anti-human antibodies (Hycult), labeled with secondary antibody Alexa Fluor 488 (Thermo Fisher Scientific).

For antibodies that recognize an intracellular epitope permeabilization will be performed by fixation and permeabilization with IntraPrep Kit (Instrumentation Laboratory, Bedford, MA, USA) before continuing with conjugated antibody staining. Finally, cells were washed twice and resuspended in FACS buffer for acquisition.

At the end of in vitro stimulations, the cells were washed twice with PBS and removed with PBS-EDTA 2 mM and trypsin × 0.001. For surface staining, cells were resuspended in flow cytometry [fluorescence-activated cell sorting (FACS)] buffer (PBS pH 7.2, 0.2% bovine serum albumin, and 0.02% sodium azide) and incubated with FcR blocking reagent (Miltenyi Biotec) for 10 min at room temperature. After blocking incubation, surface markers were added for 20 min at 4°C. The unconjugated primary antibody was added for 25 min at 4°C, and then cells were washed and labeled with secondary antibody for 25 min at 4°C. Then, cells were washed with the FACS buffer and resuspended in each tube with 500 µl of FACS buffer for FACS analysis.

Data were obtained by using an FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed with Kaluza software (Beckman Coulter, Brea, CA, USA). The area of positivity was determined by using an isotype-matched mAb, and in total, 104 events for each sample were acquired. A total of 3 independent experiments were performed.

#### ***In vitro model of Epithelial-to-Mesenchyme Transition (EMT)***

EMT will be assessed with confocal microscopy, real time PCR and western blotting, as described hereafter. For confocal analysis, cells will be fixed for 20 minutes in 3% formaldehyde in PBS or ice-cold methanol, permeabilized in 0.2% Triton X-100/PBS for 5 minutes, and blocked with 2% BSA for 20 minutes. Afterward cell will be stained with appropriate primary antibodies (E-cadherin, pan-cytokeratin, tubulin, α-SMA, vimentin,

Snail,  $\beta$ 1-integrin and I-CAM1), and counterstained with secondary antibodies (conjugated to Alexa-647, -488 and -546) and TOPRO-3 (nuclear staining). Confocal images will be acquired using a Leica SP5 spectral confocal microscope.

For Real-time PCR, total RNA will be extracted from HPMC using RNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. One microgram of total RNA (tRNA) will be reverse-transcribed using MuLV reverse transcriptase (Roche, Germany) and oligo(dT)/random hexamer primers (10:1). Quantitative real-time polymerase chain reaction (PCR) will be performed using the ABI Prism 7500 Real Time PCR system (Applied biosystems, Darmstadt, Germany).

For western-blot analysis, HPMC samples will be homogenized in appropriate cell lysis buffer containing proteinase inhibitors and centrifuged at 13,000 rpm for 15 min. After determination of protein concentration, the extracts will be subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE) under reducing conditions after boiling at 100°C for 5 min in buffer plus  $\beta$ -mercaptoethanol. Thirty micrograms of protein will be loaded into gels, separated by SDS-PAGE gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (millipore). After blocking for 1 h in Tris-buffered saline with Tween-20 containing 5% non-fat dry milk, membranes will be incubated with appropriate primary antibodies (1:1,000) at 4°C overnight. After being washed three times for 15 min with TBST, membranes will be incubated with horseradish-peroxidase-conjugated secondary antibody (1:2,000) for 1 h at room temperature and washed again three times. The immunoblots will be visualized by enhanced chemiluminescence and exposed to X-ray films. Protein bands were quantified densitometrically;  $\beta$ -actin will serve as protein-loading control.

### **Study of Complement activation**

The involvement of Complement system in our project will be investigated by different approaches:

- Measurement of Complement components and active fragments in PD effluents or culture supernatants by ELISA or multiplex ELISA, in particular we will assess C3a, C5a, sCD59 levels, Factor D, Factor H, Factor B, and soluble CD59
- Analysis of synthesis of Complement components by RT-PCR in Mesothelial cells under different conditions, in particular we will assess the expression of membrane complement regulators, CD46, CD55 and CD59
- Analysis of Complement inhibitors and Complement depositions on the surface of mesothelial cells under different conditions by FACS (i.e for membrane complement regulators as CD46, CD55 and CD59.
- Analysis of Complement activation by Wieslab test

Importantly, all the analysis will be conducted in presence/absence of H-Guard protein added to different PD solutions

### **Statistical analysis**

All data will be show as means $\pm$ standard deviation (SD) of at least three independent experiments performed in duplicate. Differences between conditions will be tested for statistical significance by one-way analysis of variance (ANOVA) and Kruskal-Wallis test, with pairwise multiple comparisons or Student's t test, as appropriate. A P value<0.05 will be considered statistically significant.

***Main Expected Results and Impact:***

This project aims to explore the possibility to modulate Complement activation in the contest of PD. We hypothesize that Complement inhibition might prevent or reverse epithelial-to-mesenchymal transition of mesothelial cells or its pernicious effects. This approach is expected to delay and/or prevent the cellular and histological changes of peritoneal membrane during PD treatment.

All experimental data of the present project aims to improve the clinical management of patients undergoing PD and to development of new solutions for PD ultimately targeted to prevent the fibrosis associated with PD.

***Significance and Relevance for National Health System (SSN):***

PD is a safe and an effective treatment modality in the integrated care approach to patients with end stage renal disease (ESRD) and it's the most important home dialysis treatment. Possible prevention of PD-related peritoneal membrane deterioration (as by Complement inhibition) may improve the management of PD and prolong its duration. This data may support the expansion of home dialysis with a significant cost saving for National Health System and an improvement in quality of life for ESRD patients.

## REFERENCES

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**PART 3****CONTRACT PRICE**

In consideration of the Research Services, University of Foggia shall invoice the Company as follows:

<b>Invoicing Schedule</b>	<b>Amount (exclusive of VAT and any applicable taxes)</b>
Upon Execution of Research Agreement	50% of Project Price: Euro 15,000
Upon Completion of Project and Provision of Final Project Report	50% of Project Price: Euro 15,000

Payment will be made by the Company within thirty (30) days of receipt of invoice.