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Study of Th17 cells in patients under peritoneal dialysis

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Resumo

A Doença Renal Crónica é uma doença complexa, cada vez mais prevalente no mundo, que se define por danos irreversíveis na estrutura e função do rim. Uma das terapias mais usadas é a diálise peritoneal, que permite preservar parte da função renal. No entanto, o seu uso a longo prazo leva à deterioração do peritoneu, sendo mediada por processos inflamatórios e respostas imunes. De entre estes fatores, as células Th17 e a IL-17A parecem assumir um papel de destaque na patogénese da doença.

Deste modo, este trabalho teve como objetivo quantificar as populações de linfócitos totais, linfócitos T, linfócitos T $CD4^+$ e $CD8^+$, Th17 e Th1 no sangue periférico e no efluente peritoneal de 26 doentes a realizar diálise peritoneal e comparar com o sangue periférico de um grupo controlo, constituído por 10 indivíduos saudáveis. Também foi estudada a concentração local de IL-17A no soro e no efluente peritoneal.

As principais diferenças foram observadas nas frequências de células Th17 e Th1, significativamente mais baixas nos doentes em diálise peritoneal do que no grupo controlo. Também foi observado que a frequência de células Th1 era mais elevada no efluente peritoneal do que no sangue. Relativamente à concentração de IL-17A, esta era significativamente mais elevada no efluente peritoneal, quando comparado com o soro.

Apesar de os níveis de IL-17A não seguirem a tendência da maioria da literatura, os resultados parecem sugerir que um processo inflamatório se está a desenvolver no peritoneu destes doentes, que precisa de ser melhor caraterizado, de forma a clarificar qual a ação efetiva desta citocina na patologia.

Palavras-chave

Doença Renal Crónica;Diálise Peritoneal;Células Th17;IL-17A

Resumo Alargado

A Doença Renal Crónica é uma doença heterogénea, caraterizada pela deterioração gradual e permanente da função e morfologia do rim. Atualmente, a diálise peritoneal é uma das terapias de reposição da função renal mais usada, já que alia a preservação da função renal residual com a manutenção da autonomia do doente. No entanto, o seu uso a longo prazo leva a mudanças a nível molecular, que afetam maioritariamente a membrana peritoneal, podendo inclusive colocar em causa a viabilidade da técnica. Estas alterações são mediadas por processos inflamatórios, existindo evidências da envolvência do sistema imunitário, nomeadamente através das células Th17 e da principal citocina por elas secretadas, IL-17A, na patogénese desta doença.

Assim, o principal objetivo deste trabalho foi a quantificação das populações de linfócitos totais, linfócitos T, linfócitos T $CD4^+$ e $CD8^+$, Th17 e Th1 no sangue periférico e no efluente peritoneal de doentes a realizar diálise peritoneal e comparar com o sangue periférico de um grupo de voluntários saudáveis. Além disto, também foi estudada a concentração local de IL-17A no soro e no efluente peritoneal dos doentes em diálise peritoneal.

Foram recrutados 26 doentes a realizar diálise peritoneal na ULS Castelo Branco, EPE-Hospital Amato Lusitano e 10 indivíduos saudáveis, que constituíram o grupo controlo, tendo sido recolhidas amostras de sangue periférico. No caso dos doentes em diálise peritoneal, também foram recolhidas amostras de efluente peritoneal. Numa primeira fase, as amostras recolhidas (sangue periférico e efluente peritoneal) foram estimuladas. Em seguida, foi efetuada uma marcação de membrana e, após um protocolo de permeabilização, uma marcação intracitoplasmática, tendo as amostras sido imediatamente adquiridas e analisadas por citometria de fluxo. Posteriormente, a concentração de IL-17A foi determinada recorrendo a um ensaio ELISA, que permitiu a deteção e quantificação da IL-17A tanto no soro como no efluente peritoneal.

Relativamente aos resultados obtidos, as principais diferenças foram observadas no valor absoluto de todas as subpopulações mencionadas anteriormente, nas frequências de células Th17 e Th1 e nos rácios de células T $CD4^+/CD8^+$ e Th1/Th17, que eram significativamente mais baixos nos doentes em diálise peritoneal do que no grupo controlo. Adicionalmente, comparando o sangue e o efluente peritoneal dos doentes em diálise peritoneal, foi observado que a frequência de células Th1 e os valores absolutos de todas as subpopulações em estudo eram, respetivamente, mais elevada e mais baixos, no

efluente peritoneal. Já a concentração de IL-17A, era semelhante no soro dos doentes e dos controlos. No entanto, quando comparado o soro e o efluente peritoneal dos doentes em diálise peritoneal, a concentração estava significativamente mais elevada no último. Para uma análise mais detalhada, a amostra dos doentes foi subdividida de acordo com o tipo de diálise que realizam, a ocorrência (ou não) de episódios de peritonite desde que iniciaram o tratamento e o número de anos a realizar diálise. Os resultados obtidos foram semelhantes aos anteriores, tendo a frequência de células Th17 permanecido sempre mais elevada no efluente peritoneal do que no sangue e, comparativamente, a mesma situação foi observada no caso da concentração de IL-17A no soro e no efluente peritoneal.

No efluente peritoneal, o aumento da frequência de células Th17 é acompanhada de uma evidente produção local de IL-17A, fundamentada pela elevada concentração desta citocina neste tipo de amostra. De uma forma geral, estes resultados sugerem que um processo inflamatório, que necessita de ser clarificado, está a ocorrer no peritoneu dos doentes em diálise peritoneal. A literatura disponível no contexto humano, que relaciona a diálise peritoneal com a IL-17A, é diminuta e por vezes discordante com os resultados obtidos neste trabalho. No caso do sangue periférico, tem sido reportado um aumento desta subpopulação e consequentemente da citocina secretada. Contudo, para além da amostra ser inferior à aqui considerada, as caraterísticas inerentes ao tratamento de diálise não foram especificadas. Pelo contrário, no caso do efluente peritoneal, os resultados obtidos neste trabalho parecem ser concordantes com os anteriormente observados. No entanto, convém referir que essa amostra considerava não só doentes com um episódio de peritonite ativo, como também aqueles que realizavam diálise há mais de três anos. Ainda assim, os resultados aqui descritos são relevantes e demonstram bem a heterogeneidade patente entre os doentes a realizar diálise peritoneal.

No futuro, será necessário não só investigar de forma abrangente as restantes células imunes que secretam IL-17A no peritoneu e a sua relação com as outras subpopulações, bem como determinar o real impacto das diferentes soluções de diálise na cavidade peritoneal. Para além disto, o uso da IL-17A como agente terapêutico, se provada a sua eficácia no contexto da doença renal, pode abrir as portas à implementação global da diálise peritoneal.

Abstract

Chronic Kidney Disease is a complex disease, with increasing prevalence in the world, and is defined by irreversible damage in the kidney structure and function. One of the most used treatments is peritoneal dialysis, that allows to preserve part of the renal function. However, in the long-term, it leads to peritoneal damage, that seems to be modulated by inflammatory processes and immune responses. Among these mediators, Th17 cells and IL-17A seem to play an important role in the pathogenesis of the disease.

Therefore, this study aimed to quantify the populations of total lymphocytes, T cells, $CD4^+$ and $CD8^+$ T cells, Th17 and Th1 cells in peripheral blood and peritoneal effluent from 26 patients undergoing peritoneal dialysis and compare with peripheral blood from a control group, consisting of 10 healthy volunteers. The local IL-17A concentration in the serum and peritoneal effluent was also studied.

The main differences were found in the frequencies of Th17 and Th1 cells, that were significantly lower in patients on peritoneal dialysis than in the control group. It was also observed that the frequency of Th1 cells was higher in the peritoneal effluent than in the blood. IL-17A concentration was significantly higher in the peritoneal effluent when compared to the serum.

Although IL-17A levels do not follow the same pattern as some previous research, the results seem to suggest that in the patient's peritoneum, an inflammatory process is emerging, and further clarification is needed regarding the mechanisms of action of this cytokine in the pathology.

Keywords

Chronic Renal Disease;Peritoneal Dialysis;Th17 cells;IL-17A

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACR	Albumin to creatinine ratio
AKI	Acute kidney injury
APC	Allophycocyanin
APD	Automated peritoneal dialysis
CAPD	Continuous ambulatory peritoneal dialysis
CCL	Chemokine C-C motif ligand
CVC	Cardiovascular diseases
CKD	Chronic kidney disease
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GFR	Glomerular filtration rate
IFN	Interferon
IL	Interleukin
PB	Peripheral blood
PD	Peritoneal dialysis
PE	Phycoerythrin
PerCP	Peridinin chlorophyll-A protein
PMA	Phorbol 12-myristate-13-acetate
PDE	Peritoneal dialysis effluent
RORγt	Retinoid related orphan receptor γt
RT	Room temperature
SD	Standard deviation
SSC	Side scatter
STAT3	Signal transducer and activator of transcription 3
TGF	Transforming growth factor
Th	T-helper
TNF	Tumor necrosis factor
5PL	Five-parameter logistic

I. Introduction

I. Introduction

1. Chronic Kidney Disease

Chronic kidney disease (CKD) is a complex and heterogeneous disease, and is increasingly becoming a global public health concern, affecting approximately 11 to 13% of the world's population, especially those in developed countries [1–3]. It is universally defined as the presence of irreversible abnormalities in the kidney structure and function, lasting for more than three months [4]. These processes seem to be modulated by inflammation and immune responses [5] and ultimately will lead to renal failure. Clinically, it is characterized by a glomerular filtration rate (GFR) of less than 60 mL/min/1.73 m^2, that defines the volume of plasma filtered by the glomeruli per unit of time. Additionally, it can also be characterized by one or more markers of kidney impairment, frequently identified by the presence of proteinuria and albuminuria [4].

The disease is divided in six stages, based on the severity of the kidney damage, that inversely correlates with the GFR measurements, as shown in **Table 1**. The stratification also relies on the values of albuminuria, establishing three categories, A1 to A3, ranging from an albumin to creatinine ratio (ACR) of less than 3 mg/mmol to more than 30 mg/mmol [4]. In the beginning, the disease is relatively silent, which means that the patients only start to manifest symptoms when it progresses to advanced stages. When dealing with those cases, unfortunately, a poor outcome is expected, since patients have fewer clinical opportunities to change the course of the disease [6].

Table 1. Staging of CKD based on GFR measurements

GFR category	GFR *(ml/min/1.73 m^2)*	Terms
G1	≤90	Normal or high
G2	60-89	Mildly decreased
G3a	45-59	Mildly to moderately decreased
G3b	30-44	Moderately to severely decreased
G4	15-29	Severely decreased
G5	<15	Kidney Failure

Adapted from [4].

1.1. Management and risk factors

Given the disease's heterogeneity and complexity, which is reflected in the fact that each patient presents a specific pattern of renal decline, the strategies applied in the management of CKD need to be carried out individually. The main approaches should,

however, be always based on slowing down the progression and minimizing the additional damage to the kidney, as well as preventing the development of other comorbidities, that may accelerate even more the deterioration of the kidney [6].

There are several risk factors that can adversely impact the development and progression of CDK. While some variables are modifiable and treatable, like lifestyle-related or systemic and metabolic disorders (including diabetes, hypertension and obesity) [7,8], others like age, gender, ethnicity [9] and genetic susceptibility [10] cannot be changed. Indeed, age and gender seem to be relatively significant, because the prevalence of CDK increases with age [1] and, although women show a higher prevalence and incidence of the disease, men are more likely to progress to the later stages of it [11].

Diabetes and hypertension constitute the most common primary causes of CKD [12]. It is known that the disease is closely associated with the development of cardiovascular diseases (CVC), since most people are diagnosed with both pathologies [13]. Moreover, besides accelerating the development of CVD, CKD is also an independent risk factor for the onset of cardiovascular events [14]. In fact, although the disease may lead to irreversible damage and, in worst cases, to death, patients with CKD are more likely to die from cardiovascular complications before reaching an end-stage phase [15]. Nevertheless, the behavior of the kidney itself must also be considered, as it may play a more active role than expected in the declining of the renal function [16].

1.2. Treatment strategies: basic principles of peritoneal dialysis

Currently, there is no cure for CKD, so the available treatment options relay on both non-pharmacologic and pharmacologic approaches, meant to target, to a certain extent, the modifiable risk factors [6,9]. However, as the disease evolves to an end-stage phase, patients will need renal replacement therapy [17], that will only partially replace the lost kidney function. The most effective and that may grant, in the long-term, a potential full recovery is kidney transplant, but nowadays, dialysis therapy, whether hemodialysis or peritoneal dialysis (PD), also offers reasonable results.

Based on the removal of water and toxins, both dialysis technics aim to restore the electrolyte balance and artificially perform the function of the damaged kidney. While in hemodialysis this removal is achieved across a synthetic membrane and involves the passage of blood through an extracorporeal circuit, in PD, the exchange of water and solutes occurs between the blood in the peritoneal capillaries and a solution, known as the dialysate, that is instilled into the peritoneal cavity. This means that the internal

lining of the peritoneum, characterized by its large surface and dense vascularization, has the necessary features to be used as a natural semipermeable dialysis membrane [18,19].

Essentially what happens is that the dialysate is firstly instilled into the peritoneal cavity and a certain amount of time is given for it to dwell in the abdominal cavity. During this period, three transport processes will happen simultaneously. On the one hand, solute transfer occurs as a bidirectional process, in which, solutes like urea and creatinine diffuse from the blood into the dialysate, while others like glucose move in the opposite direction. On the other hand, fluid removal depends on the intraperitoneal instillation of a hypertonic high glucose solution that is able of creating an osmotic gradient, allowing the ultrafiltration of fluid from the capillaries. Thus, the volume of ultrafiltration will depend not only on the concentration of glucose (or other osmotic agent) in the dialysate, but also on the dwelling time of the fluid in the cavity, as well as the physiological characteristics of each patient. After this, the used dialysate is drained, and the cycle can start over [18–20]. The transport of water and solutes across the vascular endothelium of the peritoneal membrane is based on the three-pore model, that proposes that this exchange is possible due to the presence of three pores of different sizes in the endothelial cell layer: large, that are responsible for the convective transport of macromolecules; small, that account for the majority of the solute transport and water removal; and aquaporin, that are associated with the transport of water only [21,22] (**Figure 1**).

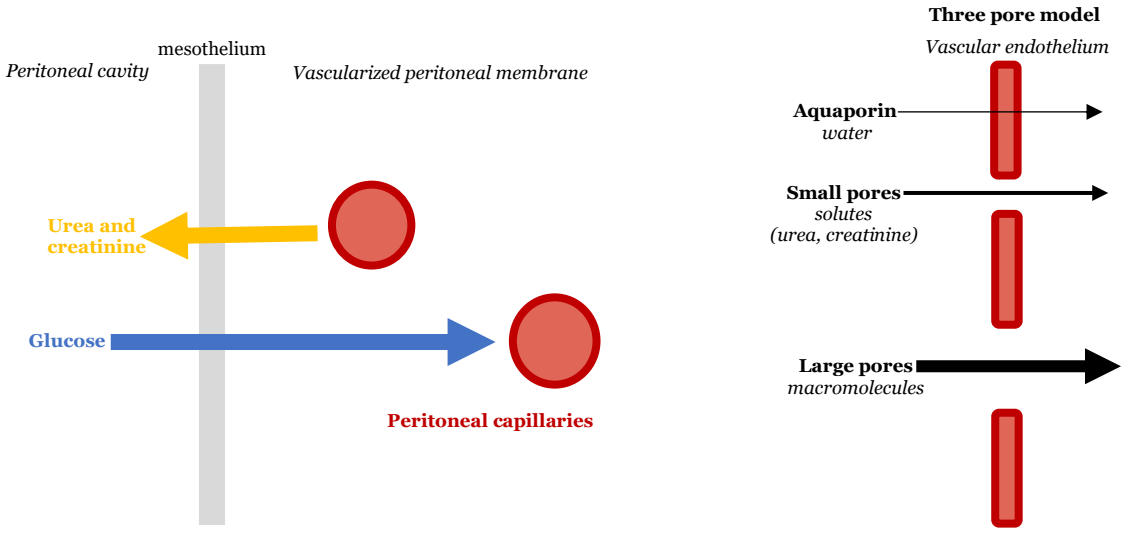

Figure 1. Solute and water transport across the peritoneal membrane. During PD exchange, the dialysate is instilled into the peritoneal cavity and then is drained. In between these two moments (dwell time), water and solutes will be transported across the membrane through diffusion, convection, and ultrafiltration. This mechanism is explained by the three-pore model and the relative abundance of three pores of varying sizes. Adapted from [18] and [23].

The dialysis process may be performed through manual exchanges throughout the day, in which the peritoneal cavity is always filled with a PD solution (continuous ambulatory PD, CAPD), or , mechanically, with the help of an automatic cycler (automated PD, APD), that will perform several exchanges in a certain period of time [19,20].

Given this and compared to hemodialysis, the best advantage of PD is the fact that allows the patient to be more independent, because the therapy can be delivered by the patient itself or a caregiver, which, at the same time, also makes them fully responsible for compliance with the treatment [20]. In fact, approximately 11% of the patients undergoing dialysis globally are submitted to PD [24]. Furthermore, it provides cost savings, with patients experiencing a superior quality of life [25,26], higher survival rate and a more favorable profile towards preservation of residual renal function [27].

1.3. CDK and PD-related complications

Over time, some changes are expected to occur in the peritoneal membrane [28–30]. The chronic exposure to commercial PD solutions, that are unphysiological, traditionally containing an acidic pH and high concentrations of glucose and lactic acid [31,32], will eventually lead to some deleterious processes affecting the function and morphology of the peritoneal membrane. These alterations, accounting for peritoneal dysfunction, remain as one of the most common complications among PD patients. Although PD patients can experience these changes at any time, it was reported that a decline in the ultrafiltration rate was likely to occur two to four years after starting PD [33].

Another major problem that affects these patients is peritonitis, a serious infectious process, that may even compromise the maintenance of the technique [34]. Nevertheless, they may also experience non-infectious complications, related, for example, to a catheter dysfunction or to the development of a metabolic disorder [20,35].

The peritoneal membrane failure will also be the result of molecular changes [36,37], characterized by the increased production of proinflammatory mediators, proangiogenic and profibrotic factors [38,39], that, respectively, lead to the recruitment of inflammatory cells and to changes in the peritoneal vasculature (angiogenesis) and membrane (fibrosis) [40] (**Figure 2**). Due to its complexity, this inflammatory process is poorly understood and needs further clarification.

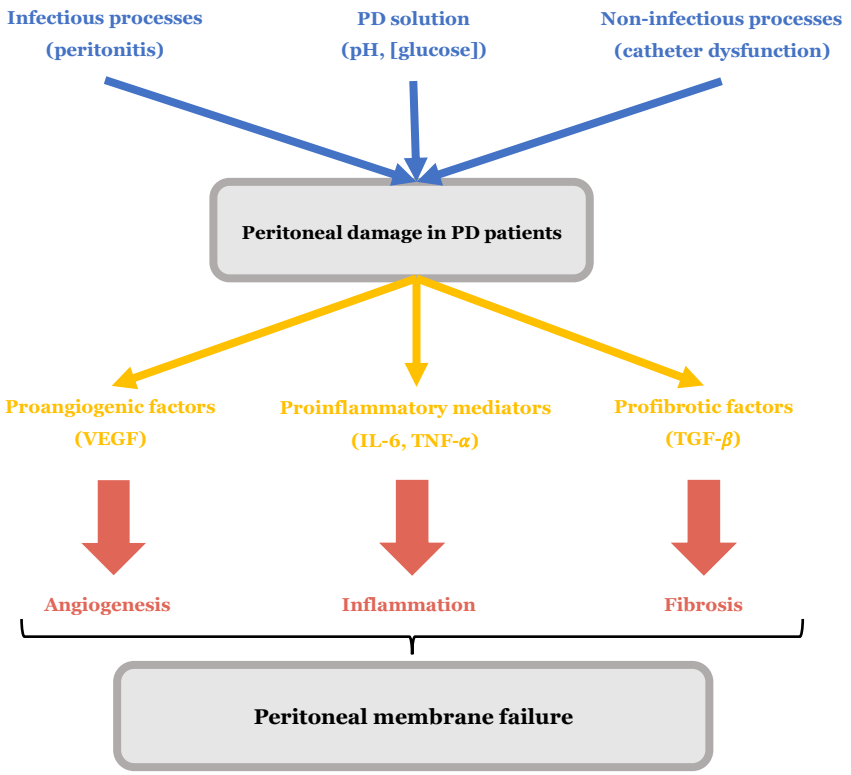

Figure 2. Processes involved in the peritoneal membrane damage in PD. As a result of the exposure to PD solutions, non-infectious and infectious processes, the peritoneal membrane will suffer several molecular changes, mediated by inflammation, angiogenesis and fibrosis. The perpetuation of these will ultimately lead to the failure of the membrane, and subsequently, to the failure of the PD technique. Adapted from [41] and [42].

2. Pathogenesis of CKD: aspects of immune dysfunction

Unlike acute kidney injury (AKI), where the loss of epithelial cells is followed by their regeneration and complete kidney functional recovery (when treated appropriately) [43], CKD is characterized by persistent inflammation and is said to occur because of an initial trigger, like abnormalities during the kidney development, that are normally mediated by inflammation or immune responses. This will, however, be perpetuated by a process of hyperfiltration and hypertrophy of the remaining nephrons, eventually resulting, contrary to AKI, in the irreversible loss of those cells. These processes appear to be caused by cytokines, grown factors and hormones, and lead to changes in the glomerular structure and function, that eventually decrease renal function and predispose to the development of fibrosis and scarring (glomerulosclerosis) [44,45].

Regardless of the mechanism that led to the kidney failure in the first place, several reports have shed a light on the importance of the immune responses in the pathogenesis of a number of chronic inflammatory diseases, like renal and PD-related. As a result of the impairment of both innate and adaptative immune responses [46], CKD patients are at high risk of developing several infectious processes [47] and malignancies [48]. The accumulation of uremic toxins, normally excreted by a functioning kidney, and

cytokines, leads to a vicious cycle in which immune cells are continuously being activated, producing more cytokines and reactive oxygen species, that contribute further to the tissue damage and inflammation [49]. Thus, PD patients, besides showing signals of significant immune activation, they are usually characterized by an altered T-helper (Th) 1/ Th2 balance, that favors a Th2 immune response [50,51]. Likewise, it is also common to observe an impaired monocyte function in these patients, resulting in inadequate antigen presentation, that eventually leads to weaker memory cells and deficient antibody production [52]. Additionally, they are also known to develop defective neutrophils, with a lower capacity of phagocytosis [53], as well as elevated levels of proinflammatory cytokines, that inversely correlate with the decline of GFR [54].

Among these inflammatory mediators, interleukin (IL)-17A, as an effector cytokine, and Th17 cells, seem to stand out, since several studies have suggested that an increased frequency of Th17 cells, as well as an increase in the IL-17A expression, are common features of chronic inflammatory illnesses like renal diseases [55].

2.1. IL-17A: general characteristics

IL-17A is considered a pleiotropic cytokine, since it can provoke a wide variability of responses, depending on the cell type and the underlying pathological condition. It is known to play an important role in inflammation and autoimmunity [56]. However, when its signaling becomes inappropriate or when acting together with other proinflammatory molecules (like tumor necrosis factor (TNF) and IL-1-β), it may exacerbate tissue damage due to its strong proinflammatory proprieties. The discovery of this cytokine led to the foundation of the IL-17 family of cytokines, that comprises six members, from A to F [57]. IL-17A binds primarily to IL-17RA, requiring, however, complex formation with IL-17RC for an efficient binding, enabling the activation of several downstream intracellular signals [58,59].

Biopsies from end-stage renal disease patients showed that mast cells, NKT cells, neutrophils, and innate lymphoid cells, produced these cytokine [60]. Still, Th17 and γδ T cells [61], are considered to be the main producers of IL-17A.

2.2. IL-17A-producing cells: Th17 cells

Naïve $CD4^+$ T cells can differentiate into distinct Th effector subpopulations, after committing to a specific cytokine profile, that will be associated with a distinct pathological response. To differentiate from naïve cells, Th17 cells are dependent on the expression of the transforming growth factor (TGF)-β and proinflammatory cytokines, like IL-6. This process is also mediated by the activation of the transcription factors

signal transducer and activator of transcription 3 (STAT3) and retinoid related orphan receptor γt (RORγt) [62,63]. Besides IL-17A, these cells also secrete IL-17F, IL-21, IL-22, IL-23, TNF-α and chemokine C-C motif ligand (CCL)-20 [60,64].

Th17 cells play a critical role in the protection against extracellular pathogens, which are unusual targets of Th1 and Th2 cells [65]. Like IL-17A, these cells also participate in several autoimmune, inflammatory, and chronic diseases. Nevertheless, the mechanisms through which they contribute to those processes are not fully understood, especially in the context of renal diseases.

2.3. Role of IL-17A in CKD patients under PD

As for other diseases, the use of experimental models has been fundamental for better understanding what happens to the peritoneum during long-term exposure to PD fluids. In recent years, with the help of some animal models, several studies have reported, as previously said, that inflammation mediated by IL-17A leads to peritoneal damage [66–68]. Accordingly, in an experimental peritoneal fibrosis model, the immune response was activated and characterized by the peritoneal infiltration of Th17 cells and by the expression of Th17-related cytokines, like IL-17A [66]. Supporting these results is the fact that the neutralization of IL-17A, either with antibodies or by using an IL-17A deficient mice, has shown to be protective when developing experimental renal diseases [69,70]. Likewise, in a murine chronic PD fluid exposure model, researchers identified the infiltration of Th17 and γδ T cells as a plausible explanation for the increased peritoneal IL-17A levels [66,68]. On another perspective, an experimental study has shown that the *in vivo* delivery of Th17 cells resulted in the acquisition of some biological hallmarks associated with renal diseases [71]. Regarding PD patients, similar results were observed: not only IL-17A was found in the peritoneum, but it also correlated with therapy duration and the extend of inflammation and fibrosis in the peritoneal membrane [66].

Although most of the published results corroborate the harmful role played by Th17 cells, it is also truth that some studies have reported protective effects of IL-17A, namely its inhibition aggravated the renal injury in an experimental model [72]. This means that a Th17 cells balance is essential to achieve an immune homeostasis.

Recalling **Figure 2**, it seems that IL-17A is intimately involved in initiating and further perpetuating the mechanisms that lead to the peritoneal membrane failure. A chronic exposure to a PD solution will, firstly, trigger the recruitment of inflammatory cells, among which are Th17 cells and other IL-17A-producing cells. The presence of IL-17A will amplify the inflammatory response, since other proinflammatory mediators will be

released. At the same time, the mesothelium cell layer will lose its integrity and the submesothelial zone will become thicker. Thereby, IL-17A is thought to stimulate the development of fibrosis and angiogenesis, and other deleterious processes that compromise the adequate function of the peritoneal membrane [41,60].

Since the mechanisms that induce IL-17A activity in the peritoneum are poorly understood, it is important to acquire more knowledge regarding the behavior of this cytokine and, consequently, of one of its main producers (Th17 cells), especially in the human context.

II. Aims of the study

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Given the suggested active role played by Th17 cells and IL-17A in the development and progression of inflammation and other deleterious processes in PD patients, the main objective of this study was to assess the frequency of Th17 subpopulation, and the IL-17A secretion in a group of PD patients and in healthy individuals, as a control group.

To address this objective, the following specific aims were outlined:

- To quantify the frequency and absolute number of total lymphocytes, T cells, the $CD4^+$ and $CD8^+$ T subsets, Th1 and Th17 cells in peripheral blood (PB) and peritoneal dialysis effluent (PDE) samples of CKD patients under PD, collected at the same time, alongside PB samples of healthy donors;

- To determine the expression levels of IL-17A in the serum and effluent of PD patients, as well as in the serum of the control group.

III. Materials and methods

III. Materials and methods

1. Patients and healthy controls

Twenty-six PD patients were recruited from the Nephrology Service of ULS Castelo Branco, EPE-Hospital Amato Lusitano. All patients followed in the unit and undergoing PD were selected. Ten healthy, age and sex-matched volunteers were included as the control group. They had no medical history of hypertension, diabetes or immune-related disorders, as well as any ongoing inflammatory process, as accessed by the measurement of serum C-reactive protein levels (CRP).

Concerning the PD patients, as part of routine laboratory testing, both PD and PDE samples are analyzed. In this case, both samples were collected at the same time by nurses from the Nephrology Service of ULS Castelo Branco, EPE-Hospital Amato Lusitano. PB samples were collected in a heparin tube, for the flow cytometry analysis, and in a serum-gel tube, for the ELISA assay. The latter was centrifugated at 3000 rpm for 10 min and the serum was frozen at -20ºC. Regarding the PDE samples, approximately 20 mL of sample were collected in sterilized containers, no specific preservation was required. To concentrate the number of cells, 12 mL of sample were centrifugated at 2000 rpm for 5 min before the following protocol. The remaining of the samples was frozen at -20ºC to use later in the ELISA assay. Since the collection of peritoneal fluid samples is an invasive process, only PB samples from the control group were collected and analyzed.

Absolute blood and peritoneal effluent cell counts were calculated using a Yumizen H2500 (Horiba, Kyoto, Japan) hematological cell analyzer.

All participants (PD patients and volunteers) provided written informed consent and the study was approved by the Ethics Committee of ULS Castelo Branco, EPE-Hospital Amato Lusitano (**Appendix 1**).

2. Reagents and antibodies

Cell Activation Cocktail (with Brefeldin A) and LEGEND MAX™ Human IL-17A ELISA Kit were purchased to BioLegend (San Diego, CA, USA), RPMI-1640 medium was obtained from Gibco (Paisley, Scotland, UK). Cytofix/Cytoperm™ Fixation/ Permeabilization Kit was from BD Biosciences (San Jose, CA, USA).

Regarding the antibodies, CD3-Allophycocyanin (APC, clone OKT3, isotype IgG2a, κ), CD4-Peridinin chlorophyll-A protein/Cyanine5.5 (PerCP, clone RPA-T4, isotype IgG1, κ) and Interferon (IFN)-γ-Fluorescein isothiocyanate (FITC, clone 4S.B3, isotype IgG1, κ) were purchased to BioLegend (San Diego, CA, USA), while IL-17A-Phycoerythrin (PE, clone N49, isotype IgG1, κ) was from BD Pharmingen (San Diego, CA, USA).

3. Cell activation

After being collected, PB and PDE samples were transported to CICS-UBI, where the following procedure took place. To measure the intracellular IL-17A levels, the samples had to, firstly, be activated with a Cell Activation Cocktail (with Brefeldin A), containing an optimized concentration of phorbol 12-myristate-13-acetate (PMA), ionomycin and Brefeldin A, that is a protein transport inhibitor. A total of 500 µL of PB and PDE samples were diluted in 500 µL of RPMI-1640 medium, in the presence of 1 µL of the referred cocktail and incubated at 37ºC in a sterile environment with a 5% CO_2 humid atmosphere for 4h.

4. Peripheral blood and peritoneal dialysis effluent T cell subsets phenotyping

After the activation period, the next part of the experiment took place at the Clinical Pathology Service of Centro Hospitalar Cova da Beira. Firstly, the samples were aliquoted in two tubes (500 µL of PB sample/tube and 200 µL of PDE sample/tube), one of them being the control (without antibodies). For staining intracellular cytokines, Cytofix/Cytoperm™ Fixation/ Permeabilization Kit, a permeabilization and fixation protocol, was followed. According to the manufacturer's instructions, the samples were initially stained for the surface antigens with the mouse anti-human monoclonal antibodies (5 µL of anti-CD3 and anti-CD4 for the PB samples and 2,5 µL of anti-CD3 and anti-CD4 for the PDE samples), incubated for 15 min in the dark at room temperature (RT), washed with 2 mL of PBS and centrifuged at 2000 rpm for 5 min. The cell pellet was then resuspended in 500 µL of Fixation/ Permeabilization solution, incubated for 20 min in the dark at RT and centrifugated at 2000 rpm for 5 min. A washing step was performed next with 2 mL of BD Perm/Wash™ buffer (diluted 1:10 in distilled H_2O), followed by a 10 min incubation period in the dark at RT and centrifugation at 2000 rpm for 5 min. For the intracellular staining, 5 µL of anti-IFN-γ and 20 µL of anti-IL17A (PB samples) and 2,5 µL of anti-IFN-γ and 10 µL of anti-IL17a (PDE samples) were added to the cell pellet and incubated for 30 min in the dark at RT. Another washing step was performed, after which the cell pellet was resuspended in less than 500 µL of PBS and samples were immediately acquired in a FACSCalibur™ (BD

Biosciences, San Jose, CA, USA) flow cytometer. For each sample and whenever possible, $1x10^4$ events were acquired and analyzed using Infinicyt™ software (version 1.8, Cytognos SL, Salamanca, Spain).

To quantify the T cell subsets, firstly, the total lymphocytes population was identified based on forward (FSC) and side (SSC) scatter properties. Within this cell population, T cells were identified based on the expression of CD3 and subsequently the $CD4^+$ T cells, according to the expression of CD4. $CD8^+$ T cells were identified by exclusion, as $CD4^-$ $CD3^+$ T cells. Considering the expression of IL-17A and IFN-γ, Th17 and Th1 cells were identified, respectively, as $CD4^+$ $IL\text{-}17A^+$ $IFN\text{-}\gamma^-$ and $CD4^+$ $IL\text{-}17A^-$ $IFN\text{-}\gamma^+$ [73]. **Figure 3** exemplifies a strategy of flow cytometry analysis to identify T cells subpopulations.

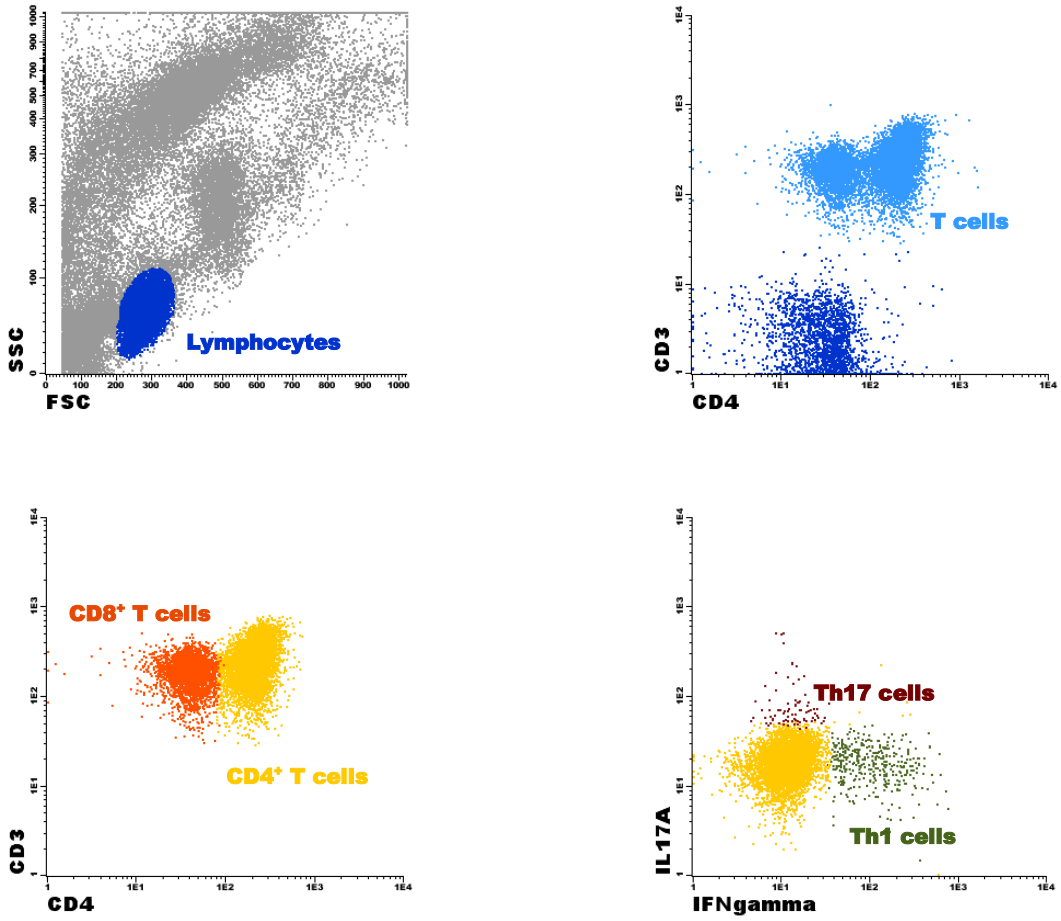

Figure 3. Flow cytometry analysis strategy. Within the total lymphocytes' population and based on the expression of CD3, T cells can be identified and, subsequently, $CD4^+$ and $CD8^+$ T cells. Th17 and Th1 cells were then identified in the $CD4^+$ T cells compartment, based on the expression of IL-17A and IFN-γ, respectively.

5. ELISA assay

To measure the expression levels of IL-17A in the serum and effluent samples, LEGEND MAX™ Human IL-17A ELISA Kit, an enzyme-linked immunosorbent assay (ELISA) was used. This kit is based on sandwich ELISA, and the plate was pre-coated with a capture antibody. Briefly, the plate was firstly washed four times with Wash Buffer (diluted 1:20 in deionized water) and then 50 µL of Assay Buffer A were added to each well. After this, 50 µL of either standard dilutions or serum and effluent samples from PD patients were added to the appropriate wells, followed by an incubation period of 2h, at RT, while shaking at 200 rpm. To remove unbound material, a washing step was performed, after which 100 µL of Human IL-17A Detection Antibody were added and the plate was incubated for 1h, at RT, while shaking at 200 rpm. Next, after another washing step, 100 µL of the enzyme Avidin-HRP D solution were added and the plate was again incubated for 30 min, under the same conditions. In order to detect the formation of the antigen-antibody complex, 100 µL of Substrate Solution F were added, followed by an incubation period of 30 min in the dark. Wells containing human IL-17A should turn blue after this step, with a color intensity proportional to its concentration. To stop the reaction, 100 µL of Stop Solution were added and the color changed from blue to yellow. The absorbance was immediately read at 450 nm on a plate reader (Quilaban, Lisbon, Portugal) and a standard curve was generated from the standard dilutions data using a five-parameter logistic (5PL) regression (available at: https://www.arigobio.com/elisa-analysis).

6. Statistical analysis

Data are expressed as mean ± standard deviation (SD). To determine the significance of the differences between PB samples of PD patients and controls, since all variables were normally distributed, the independent samples t-test or one-way ANOVA were used, depending on whether the PD patients were divided into one or two groups, respectively. Regarding the differences between PB and PDE samples of PD patients, the normality of all variables was maintained, so the differences were accessed by the paired samples t-test. Concerning the ELISA assay results, none of the variables was normally distributed, so when considering serum samples of PD patients and controls, Mann-Whitney or Kruskal-Wallis tests were used. The differences between serum and effluent samples of PD patients, on the other hand, were determined using Wilcoxon-Signed rank test. Statistical analysis was performed using SPSS software (version 28.0.1.0, IBM, Armonk, NY, USA) and GraphPad Prism software (version 9.0.0, GraphPad Software, San Diego, CA, USA). A p-value of $p<0.05$ was considered statistically significant.

IV. Results

IV. Results

1. General characteristics of the subjects

Table 2 summarizes the demographic and clinical characteristics of PD patients and controls included in this study. Concerning the control group, only data regarding the age, gender, blood leukocyte counts, CRP and serum creatinine were available. In both groups, the male gender represented more than 60% of the studied population. Besides this, it was possible to observe that PD patients had a significantly higher mean age than the control group (p=0.005). Likewise, and since it was one of the inclusion criteria for the control group, PD patients also had a significantly higher CRP value (p<0.001). Lastly, as expected, due to the direct correlation between CKD and creatinine levels, PD patients had significantly higher serum creatinine values than the control group (p<0.001). No significant differences were observed concerning the blood leukocyte counts.

Regarding the PD patients, all of them are submitted to an additional supplementation, consisting of folic acid and B-complex vitamins. Besides this, it is important to note that eleven patients are at an even higher risk, because they are obese. Another relevant risk factor is smoking and, among the studied patients, four have a history of being smokers: three have quitted in the meantime, while one is still an active smoker.

The data shown reflects PD patients' heterogeneity that, subsequently, influences their disease management and course. Therefore, when speaking about their medication, for example, each patient has a specific prescription dedicated to their medical profile and comorbidities. Likewise, no reference is made regarding the composition of the PD solutions because each patient is given a specific formulation, suitable to their disease evolution.

Table 2. Demographic and clinical characteristics of PD patients and controls

	PD patients *n=26*	**Controls** *n=10*
Age (*years*)	**59.7±16.3**	43±11
Male/Female [n(%)]	18/8 (69/31%)	6/4 (60/40%)
BMI (kg/m^2)	28±5.3	NA
CKD primary cause [n(%)]		
ADPKD	1 (4%)	NA
Chronic glomerulonephritis	2 (8%)	NA
Diabetic nephropathy	7 (27%)	NA
FSGS	1 (4%)	NA
Hypertensive nephrosclerosis	5 (19%)	NA
IgA nephropathy	4 (15%)	NA
IgG/κ MGUS	1 (4%)	NA
Unknown etiologies	5 (19%)	NA
CKD diagnosis (*years*)	9.4±7.3	NA
PD duration (*months*)	29.9±22.1	NA
PD modality [n(%)]		
CAPD	15 (58%)	NA
APD	5 (19%)	NA
APD/CAPD Plus	6 (23%)	NA
PDE dwelling time (*hours*)	8±4.7	NA
Episodes of peritonitis since starting PD [n(%)]	2.2±1.6	NA
Comorbidities [n(%)]		
Diabetes mellitus	1 (4%)	NA
Dyslipidemia	11 (42%)	NA
Heart diseases (cardiac insufficiency, atrial fibrillation, coronary disease or ischemic cardiomyopathy)	10 (38%)	NA
Hypertension	22 (85%)	NA
Hyperuricemia	3 (12%)	NA
Medicine use [n(%)]		
Corticosteroids	3 (12%)	NA
Iron	24 (92%)	NA
Vitamin D	17 (65%)	NA
Systolic blood pressure (*mmHg*)	131.3±16.2	NA
Diastolic blood pressure (*mmHg*)	74.5±9.5	NA
Laboratory parameters		
Blood leukocyte counts ($x10^3$ *cells/μL)*	6.8±1.7	8.4±2.6
CRP (*mg/L*)	**4.4±5.1**	0.4±0.3
Serum creatinine (*mg/L*)	**7.9±2.9**	0.9±0.1
PDE leukocyte counts (*cells/μL*)	12.5±16	NA

Values are expressed as mean±SD or number (n); *p* values were determined by independent samples *t*-test; statistically significant differences ($p<0.05$) are identified in bold; PD, peritoneal dialysis; BMI, body mass index; NA, not applicable; CKD, chronic kidney disease; ADPKD, autosomal dominant polycystic kidney disease; FSGS, focal segmental glomerulosclerosis; IgA, immunoglobulin A; IgG/κ MGUS, immunoglobulin G/κ monoclonal gammopathy of undetermined significance; CAPD, continuous ambulatory peritoneal dialysis; APD, automated peritoneal dialysis; PDE, peritoneal dialysis effluent; CRP, C-reactive protein.

2. T cell subsets in PD patients and controls

With this study, we intend to determine the frequency and absolute value of peripheral blood T cell subsets, especially in the Th17 cells subset, in PD patients and controls. Besides this, since PDE samples from PD patients were available, it was also possible to simultaneously compare what is happening in circulation and in the target tissue (peritoneal membrane) in PD patients.

To achieve that, as previously described in the Materials and Methods section, firstly, PB samples from all participants and PDE samples from PD patients were staining with the appropriate antibodies and then analyzed by flow cytometry. Therefore, PB samples from PD patients were compared with PB samples from the control group, as well as with their corresponding PDE samples.

When comparing PB samples from PD patients and the control group, no significant differences were observed regarding the frequencies of total lymphocytes, T cells, $CD4^+$ and $CD8^+$ T cells. However, the absolute values of the abovementioned subpopulations were significantly decreased in PD patients (p=0.019, p=0.005, p<0.001, p=0.027, respectively). As expected, among T cells, $CD4^+$ T cells were the most represented subpopulation, as compared to $CD8^+$ T cells, in both groups. The difference between these two subpopulations heightens in the control group, meaning that the $CD4^+/CD8^+$ T cells ratio in PD patients was significantly decreased (p=0.023). Concerning Th17 and Th1 subsets, the frequencies and absolute values of both were significantly decreased in PD patients (p=0.014 and p<0.001, respectively for the frequency; p<0.001 for both variables, regarding the absolute value) and, subsequently, the Th1/Th17 cells ratio in PD patients followed the same pattern (p=0.009) (**Table 3**).

Regarding PB and PDE samples from PD patients, no significant differences were again observed regarding the frequencies of total lymphocytes, T cells, $CD4^+$ and $CD8^+$ T cells, but their absolute values were significantly decreased in the effluent (p<0.001 for all variables, excepted Th17 cells, in which p=0.003). Moreover, the frequency and absolute value of Th1 cells were, respectively, significantly increased (p<0.001) and decreased (p=0.002) in the effluent. Despite this, it is relevant to note that the frequencies of most cell subsets expanded in the effluent, as opposed to the blood (only the frequencies of total lymphocytes and $CD4^+$ T cells did not follow this pattern) (**Table 3**).

Table 3. Frequency and absolute values of total lymphocytes, T cells, $CD4^+$ and $CD8^+$ T, Th17 and Th1 cells in PD patients and controls

	PD patients *n=26; Blood (PB)*		**PD patients** *n=14; Effluent (PDE)*		**Controls** *n=10; Blood (PB)*	
	%	cells/μL	%	cells/μL	%	cells/μL
total lymphocytes	25.2±10.5	**[a]1398.6±434.9**	19.2±16.1	**[b]1.1±0.9**	21.5±5.7	2520±1161.2
T cells	62±12.2	**[a]822.2±285.7**	71.4±16	**[b]0.8±0.7**	68.9±7.6	1712.4±770.4
$CD4^+$	62.9±12.2	**[a]559.3±269.6**	59.6±14.7	**[b]0.3±0.2**	69.3±6.9	952.9±208.6
Th17	**[a]2±0.9**	**[a]11.6±8.4**	3.7±2.3	**[b]0.1±0.2**	2.6±0.5	32.5±13.3
Th1	**[a,b]10.7±6.7**	**[a]55.6±33.5**	43.5±17.8	**[b]0.2±0.3**	25.6±7.6	293.6±124.6
Th1/Th17	**[a]5.8±4.4**		6.6±2.4		9.2±2.5	
$CD8^+$	37.1±12.2	**[a]283.3±112.3**	42.5±13.1	**[b]0.4±0.4**	30.7±6.9	544.1±309.8
$CD4^+/CD8^+$	**[a]1.7±0.7**		1.4±0.6		2.4±0.8	

Results are expressed as mean±SD; *p* values were determined by independent samples *t*-test; statistically significant differences ($p<0.05$) between [a]PD patients (PB) vs Controls (PB) and [b]PD patients (PB) vs PD patients (PDE) and are identified in bold.

Having in mind the PD patients' heterogeneity, based on the characteristics mentioned before (**Table 2**) and the high clinical relevance of some of those, we next investigated the data subdividing the patients in groups. The stratification criteria was, therefore, directly associated with PD: PD modality, peritonitis and PD duration. As previously referred, firstly, PB samples from both PD patients and the control group were compared and then PB and PDE samples from PD patients.

2.1. Peripheral blood T cell subsets in PD patients and controls

a) PD modality

PD patients were subdivided into being submitted to CAPD or APD, with the latter group also including patients submitted to APD/CAPD Plus, since both modalities are alike.

In general, the results were similar to the ones previously observed. The main differences were found in the frequency and absolute value of T cells, that were, respectively, significantly decreased in CAPD patients ($p=0.042$, when compared with APD patients) and in both CAPD and APD patients ($p=0.012$ and $p=0.016$, when compared with the control group). Besides this, the frequency and absolute value of Th1 cells were also significantly decreased in both CAPD and APD patients, as compared to the control group ($p<0.001$ for both the frequency and absolute value, regarding both CAPD and APD patients). Concerning the absolute values, significant differences were found in total lymphocytes and Th17 cells, that were, respectively, significantly decreased in APD patients ($p=0.027$) and in CAPD patients ($p=0.001$), when compared with the control group. The same pattern was also observed regarding the absolute value of $CD4^+$ T in both CAPD and APD patients ($p=0.007$ and $p=0.009$, respectively) (**Table 4**).

Table 4. Frequency and absolute values of total lymphocytes, T cells, $CD4^+$ and $CD8^+$ T, Th17 and Th1 cells in CAPD patients, APD patients and controls

	CAPD patients *n=15*		**APD patients** *n=11*		**Controls** *n=10*	
	%	cells/μL	%	cells/μL	%	cells/μL
total lymphocytes	20.8±5.7	1693.6±757.7	24.2±8.7	**[b]1336±392.2**	21.5±5.7	2520±1161.2
T cells	**[a]60.4±17.1**	**[b]802.4±287.9**	72.8±4.1	**[b]845.9±296.6**	68.9±7.6	1712.4±770.4
$CD4^+$	61.8±9.8	**[b]562±263.8**	64.4±15.3	**[b]555.9±291.3**	69.3±6.9	952.9±208.6
Th17	2±1	**[b]10.1±4.7**	2±0.9	19.5±19.1	2.6±0.5	32.5±13.3
Th1	**[b]10.7±7.1**	**[b]53.8±35.8**	**[b]10.6±6.5**	**[b]53.4±13.7**	25.6±7.6	293.6±124.6
Th1/Th17	5.8±4.3		5.8±4.7		9.2±2.5	
$CD8^+$	38.2±9.8	277.6±88.9	35.6±15.3	290.1±140.3	30.7±6.9	544.1±309.8
$CD4^+/CD8^+$	1.8±0.8		1.6±0.7		2.4±0.8	

Results are expressed as mean±SD; *p* values were determined by one-way ANOVA; statistically significant differences ($p<0.05$) were found between [a]CAPD patients vs APD patients and [b]CAPD or APD patients vs Controls and are identified in bold.

b) Peritonitis

In this case, the groups were formed based on whether, since starting PD, the patients had (P patients) or not (~P patients) at least one episode of peritonitis.

Once again, the results were quite similar, except for the frequency and absolute value of Th17 cells, that were significantly decreased in ~P patients, as compared to the control group ($p=0.041$ and $p<0.001$, respectively). The frequency and absolute value of Th1 cells in both P and ~P patients were also significantly decreased, as compared to the control group ($p<0.001$ for both the frequency and absolute value, regarding both P and ~P patients). Besides this, while the $CD4^+/CD8^+$ T cells ratio in both P and ~P patients was significantly decreased ($p=0.037$ for P patients and $p=0.048$ for ~P patients, as compared to the control group), the Th1/Th17 cells ratio was significantly decreased in P patients ($p=0.003$, when compared with ~P patients and $p<0.001$, as compared to the control group). Moreover, the absolute values of total lymphocytes and $CD4^+$ T cells were significantly decreased, respectively, in P patients ($p=0.032$) and in ~P patients ($p=0.011$), when compared with the control group. The absolute values of T cells in both P and ~P patients also followed the same pattern ($p=0.023$ and $p=0.014$, respectively) (**Table 5**).

Table 5. Frequency and absolute values of total lymphocytes, T cells, $CD4^+$ and $CD8^+$ T, Th17 and Th1 cells in P patients, ~P patients and controls

	P patients *n=9*		**~P patients** *n=17*		**Controls** *n=10*	
	%	cells/μL	%	cells/μL	%	cells/μL
total lymphocytes	26.6±10	**[b]137±330.3**	21.6±7	1616.5±734.8	21.5±5.7	2520±1161.2
T cells	70.5±6.1	**[b]879.8±321.7**	60±17.5	**[b]835.1±236.3**	68.9±7.6	1712.4±7704
$CD4^+$	64.8±15	731.7±455.2	63.4±8.9	**[b]537.4±252.9**	69.3±6.9	952.9±208.6
Th17	2.1±1	27±23.3	**[b]1.9±0.9**	**[b]8.6±3.9**	2.6±0.5	32.5±13.3
Th1	**[b]9.8±8.1**	**[b]54.2±41.8**	**[b]11.2±5.9**	**[b]56.4±29.5**	25.6±7.6	293.6±124.6
Th1/Th17	**[a,b]1.8±0.8**		7.8±5.9		9.2±2.5	
$CD8^+$	35.2±15	270.3±128.3	36.6±8.9	304.2±95.2	30.7±6.9	544.1±309.8
$CD4^+/CD8^+$	**[b]1.5±0.6**		**[b]1.7±0.7**		2.4±0.8	

Results are expressed as mean±SD; *p* values were determined by one-way ANOVA; statistically significant differences ($p<0.05$) were found between and [a]P patients vs ~P patients and [b]P or ~P patients vs Controls and are identified in bold.

c) PD duration

Lastly, PD patients were divided considering how many years they have been undergoing PD: less than two years (-2PD) or two or more years (+2PD).

Significant differences were only not observed in the frequency of Th17 cells, but its absolute value was significantly different between the three groups. Regarding total lymphocytes and T cells subpopulations, while their frequencies were significantly decreased in -2PD patients ($p=0.043$ and $p=0.030$, respectively, as compared to +2PD patients), their absolute values were significantly decreased in both -2PD and +2PD patients ($p=0.040$ and $p=0.007$, respectively for -2PD patients; $p=0.050$ and $p=0.024$, respectively for +2PD patients), as compared to the control group. A similar tendency was found in the frequency of $CD4^+$ T cells in -2PD patients, when compared with +2PD patients ($p=0.014$) and the control group ($p=0.004$). Considering the absolute values of $CD4^+$ T, significant differences were observed in the three groups. On the contrary, the frequency of $CD8^+$ T cells was significantly increased in -2PD patients, as compared with +2PD patients ($p=0.014$) and the control group ($p=0.004$). This means that the $CD4^+/CD8^+$ T cells ratio was significantly decreased in -2PD patients, when compared with the control group ($p=0.002$). Moreover, a statistically significant decrease was observed concerning the frequency and absolute value of Th1 cells in both -2PD and +2PD patients, as compared to the control group ($p<0.001$ for both the frequency and absolute value, regarding both -2PD and +2PD patients) (**Table 6**).

Table 6. Frequency and absolute values of total lymphocytes, T cells, $CD4^+$ and $CD8^+$ T, Th17 and Th1 cells in -2PD patients, +2PD patients and controls

	-2PD patients *n=11*		**+2PD patients** *n=15*		**Controls** *n=10*	
	%	cells/μL	%	cells/μL	%	cells/μL
total lymphocytes	**[a]18.5±5.4**	**[b]1394±546.9**	26.3±9	**[b]1496.3±72.1**	21.5±5.7	2520±1161.2
T cells	**[a]55.8±16.9**	**[b]717.2±247.4**	71.8±7.5	**[b]909.7±295.6**	68.9±7.6	1712.4±770.4
$CD4^+$	**[a,b]56.7±11**	**[a,b]417.8±196.5**	67.3±5.6	**[b]668.2±273.4**	69.3±6.9	952.9±208.6
Th17	2±0.9	**[a,b]7.7±3.1**	2±0.9	**[b]16.9±12.6**	2.6±0.5	32.5±13.3
Th1	**[b]11.8±8.1**	**[b]39.7±27.7**	**[b]9.9±5.7**	**[b]60.8±27.7**	25.6±7.6	293.6±124.6
Th1/Th17	5.6±3.9		6±4.8		9.2±2.5	
$CD8^+$	**[a,b]43.3±11.1**	299.5±117.3	32.7±5.6	294.6±281	30.7±6.9	544.1±309.8
$CD4^+/CD8^+$	**[b]1.3±0.5**		2±0.7		2.4±0.8	

Results are expressed as mean±SD; *p* values were determined by one-way ANOVA; statistically significant differences ($p<0.05$) were found between [a]-2PD patients vs +2PD patients and [b]-2PD or +2PD patients vs Controls and are identified in bold.

Through this analysis, it can be inferred that, regardless of the way PD patients were subdivided, the frequency of Th1 cells in PD patients was always significantly inferior to the one observed for the control group. Among T cells, $CD4^+$ T cells prevailed over $CD8^+$ T cells as the most represented subset in both PD patients and the control group. The frequency of Th17 cells did not change considerably when PD patients were differently grouped. Lastly, it is also relevant to mention that the absolute values of all subpopulations were always significantly inferior in the disease groups, as compared to the values observed in the control group.

2.2. Peripheral blood and peritoneal dialysis effluent T cell subsets in PD patients

a) PD modality

Regarding the CAPD patients, the frequency and absolute value of Th1 cells were, respectively, significantly increased ($p<0.001$) and decreased ($p=0.003$) in the effluent. Consequently, the Th1/Th17 cells ratio in the effluent followed the same pattern ($p=0.036$). The absolute values of the remaining subsets were also significantly decreased in the effluent ($p<0.001$ for all variables, except Th17 cells, in which $p=0.002$) (**Table 7A**).

Concerning the APD patients, the frequency and absolute value of total lymphocytes were significantly decreased in the effluent, when compared with the blood ($p=0.001$ and $p=0.011$, respectively). Significant differences were again found among the absolute

values of T cells, $CD4^+$ and $CD8^+$ T cells and Th1 cells in the effluent, as compared to the blood (p=0.004, p=0.004, p=0.011 and p=0.007, respectively) (**Table 7B**).

Table 7. Frequency and absolute values of total lymphocytes, T cells, $CD4^+$ and $CD8^+$ T, Th17 and Th1 cells in (A) CAPD patients and in (B) APD patients

	A)				**B)**			
	CAPD patients *n=15; Blood (PB)*		**CAPD patients** *n=8; Effluent (PDE)*		**APD patients** *n=11; Blood (PB)*		**APD patients** *n=6; Effluent (PDE)*	
	%	cells/μL	%	cells/μL	%	cells/μL	%	cells/μL
total lymphocytes	20.8±5.7	1693.6±757.7	23.8±18.5	**[a]3.5±4.1**	24.2±8.7	1336±392.2	**[b]10.9±4.8**	**[b]0.8±1**
T cells	60.4±17.1	802.4±287.9	70.9±18.7	**[a]2.8±3.5**	72.8±4.1	845.9±296.6	72.3±11.6	**[b]0.6±0.7**
$CD4^+$	61.8±9.8	562±263.8	63.7±4	**[a]3.6±4.6**	64.4±15.3	555.9±291.3	51±9.3	**[b]0.2±0.3**
Th17	2±1	10.1±4.7	3±2.2	**[a]0.1±0.2**	2±0.9	19.5±19.1	5.3±2	0.02±0.02
Th1	**[a]10.7±7.1**	53.8±35.8	45.6±15.4	**[a]1.4±1.8**	10.6±6.5	53.4±13.7	39.8±23.1	**[b]0.1±0.1**
Th1/Th17	**[a]5.8±4.3**		29.3±27.6		5.8±4.7		6.3±3.4	
$CD8^+$	38.2±9.8	277.6±88.9	36.3±4	**[a]0.7±0.7**	35.6±15.3	290.1±140.3	49±9.3	**[b]0.3±0.4**
$CD4^+/CD8^+$	1.8±0.8		1.8±0.3		1.6±0.7		0.9±0.2	

Results are expressed as mean±SD; p values were determined by paired samples t-test; statistically significant differences (p<0.05) were found between [a]CAPD Blood vs CAPD Effluent and [b]APD Blood vs APD Effluent and are identified in bold.

b) Peritonitis

When considering the patients that had an episode of peritonitis since starting PD (P patients), the frequency and absolute value of total lymphocytes were significantly decreased in the effluent, as compared to the blood (p=0.013 and p=0.002, respectively). Additionally, while the frequencies of T and Th1 cells were significantly increased in the effluent (p=0.031 and p=0.009, respectively), their absolute values were significantly decreased (p=0.005 and p=0.047, respectively). The absolute values of $CD4^+$ and $CD8^+$ T cells in the effluent also followed the same pattern (p=0.026 and p=0.009, respectively) (**Table 8A**).

As for the ~P patients group, the frequency and absolute value of total lymphocytes were significantly decreased in the effluent (p=0.014 and p=0.001, respectively). Additionally, the frequency and absolute value of Th1 cells were, respectively, significantly increased (p=0.009) and decreased (p=0.003) in the effluent. The absolute values of the remaining subpopulations were again significantly decreased in the effluent, as compared to the blood (p≥0.001 for all variables, excepted Th17 cells, in which p=0.017) (**Table 8B**).

Table 8. Frequency and absolute values of total lymphocytes, T cells, $CD4^+$ and $CD8^+$ T, Th17 and Th1 cells in (A) P patients and in (B) ~P patients

	A)				**B)**			
	P patients *n=9; Blood (PB)*		**P patients** *n=7; Effluent (PDE)*		**~P patients** *n=17; Blood (PB)*		**~P patients** *n=7; Effluent (PDE)*	
	%	cells/μL	%	cells/μL	%	cells/μL	%	cells/μL
total lymphocytes	26.6±10	1370±330.3	**[a]13.3±3.1**	**[a]1.2±0.8**	21.6±7	1616.5±734.8	**[b]7.1±1.4**	**[b]1.1.±0.9**
T cells	**[a]70.5±6.1**	879.8±321.7	83.6±5.7	**[a]3±3.8**	60±17.5	835.1±236.3	70.4±11.5	**[b]0.7±0.7**
$CD4^+$	64.8±15	731.7±455.2	58±20.8	**[a]2.2±3.2**	63.4±8.9	537.4±252.9	62.9±3	**[b]0.5±0.5**
Th17	2.1±1	27±23.3	2.7±1.3	0.02±0.03	1.9±0.9	8.6±3.9	4.1±2.7	**[b]0.1±0.2**
Th1	**[a]9.8±8.1**	54.2±41.8	39.6±21.4	**[a]1.2±1.7**	**[b]11.2±5.9**	56.4±29.5	47.4±14	**[b]0.2±0.3**
Th1/Th17	1.8±0.8		5.7±3.3		7.8±5.9		7.5±0.5	
$CD8^+$	35.2±15	270.3±128.3	46.7±18.3	**[a]0.8±0.7**	36.6±8.9	304.2±95.2	37.1±3	**[b]0.3±0.3**
$CD4^+/CD8^+$	1.5±0.6		1.5±1.3		1.7±0.7		1.6±0.3	

Results are expressed as mean±SD; *p* values were determined by paired samples *t*-test; statistically significant differences ($p<0.05$) were found between [a]P Blood vs P Effluent and [b]~P Blood vs ~P Effluent and are identified in bold.

c) PD duration

Considering -2PD patients, the frequencies and absolute values of both total lymphocytes and Th1 cells followed the pattern previously observed in ~P patients ($p=0.011$ and $p<0.001$ for the frequencies, respectively; $p=0.002$ and $p=0.021$, for the absolute values, respectively). Likewise, the absolute values of the T cells, $CD4^+$ and $CD8^+$ T and Th17 cells were also significantly decreased in the effluent, as compared to the blood ($p=0.002$, $p=0.006$, $p=0.005$ and $p=0.002$, respectively) (**Table 9A**).

Regarding +2PD patients, the frequency and absolute value of Th1 cells were, respectively, significantly increased ($p=0.037$) and decreased ($p=0.009$) in the effluent. The absolute values of total lymphocytes, T cells, $CD4^+$ and $CD8^+$ T cells were again significantly decreased in the effluent, as compared to the blood ($p=0.010$, $p<0.001$, $p<0.001$ and $p=0.002$, respectively) (**Table 9B**).

Given the results, in this case, contrary to the previously mentioned, the stratification of PD patients seems to impact, to a certain extent, the frequencies of the studied subpopulations. It should, however, be taken into consideration that the PDE samples presented a low cellularity, which made their analysis quite tricky. Nevertheless, it is relevant to highlight the fact that the frequency of Th1 cells substantially expanded in the effluent, as compared to the blood.

Table 9. Frequency and absolute values of total lymphocytes, T cells, $CD4^+$ and $CD8^+$ T, Th17 and Th1 cells in (A) -2PD patients and in (B) +2PD patients

	A)				B)			
	-2PD patients *n=11; Blood (PB)*		**-2PD patients** *n=7; Effluent (PDE)*		**+2PD patients** *n=15; Blood (PB)*		**+2PD patients** *n=7; Effluent (PDE)*	
	%	cells/μL	%	cells/μL	%	cells/μL	%	cells/μL
total lymphocytes	18.5±5.4	1394±546.9	**[a]8.7±2.4**	**[b]1.3±0.6**	26.3±9	1496.3±72.1	15.4±9.6	**[b]1±1**
T cells	55.8±16.9	717.2±247.4	70.3±19.9	**[b]3.1±3.7**	71.8±7.5	909.7±295.6	72.5±12.5	**[b]0.7±0.8**
$CD4^+$	56.7±11	417.8±196.5	61.5±20.2	**[b]2.3±3.2**	67.3±5.6	668.2±273.4	57.7±7.2	**[b]0.4±0.5**
Th17	2±0.9	7.7±3.1	4±2.7	**[b]0.1±0.1**	2±0.9	16.9±12.6	3.3±2.1	0.01±0.01
Th1	**[a]11.8±8.1**	39.7±27.7	54.4±5.2	**[b]1.2±1.7**	**[b]9.9±5.7**	60.8±27.7	39.9±21.6	**[b]0.2±0.3**
Th1/Th17	5.6±3.9		6.9±1		6±4.8		6.3±3.5	
$CD8^+$	43.3±11.1	299.5±117.3	42.7±18.6	**[b]0.7±0.7**	32.7±5.6	294.6±281	42.3±7.2	**[b]0.3±0.3**
$CD4^+/CD8^+$	1.3±0.5		1.8±1.2		2±0.7		1.4±0.3	

Results are expressed as mean±SD; *p* values were determined by paired samples *t*-test; statistically significant differences ($p<0.05$) were found between [a]-2PD Blood vs -2PD Effluent and [b]+2PD Blood vs +2PD Effluent and are identified in bold.

3. Serum and peritoneal dialysis effluent IL-17A levels in PD patients

In order to compare the expression levels of IL-17A in the serum and in the effluent of PD patients, an ELISA assay was performed and the concentration of IL-17A was quantified. As previously stated, only serum samples from the control group were analyzed.

When considering PD patients in general, a significant increase in the concentration of IL-17A in the effluent was found, as opposed to the levels observed in the serum ($p=0.039$). No significant differences were detected when compared with the serum of the control group (**Figure 4A**).

Likewise, when considering both CAPD and APD patients, no differences were found regarding the serums of the disease and control groups. However, in the case of APD patients, the concentration of IL-17A was significantly increased in the effluent, when compared to the serum ($p=0.028$) (**Figure 4B**).

Regarding the PD patients that had or not an episode of peritonitis, no statistically significant differences were found in concentration of IL-17A in the serum and the effluent of either the disease or the control groups (**Figure 4C**).

In the case of -2PD or +2PD patients, the same pattern was followed concerning the serums of the disease and the control groups. Still, in patients undergoing PD for two or more years (+2PD), the concentration of IL-17A was significantly increased in the effluent (p=0.028) (**Figure 4D**).

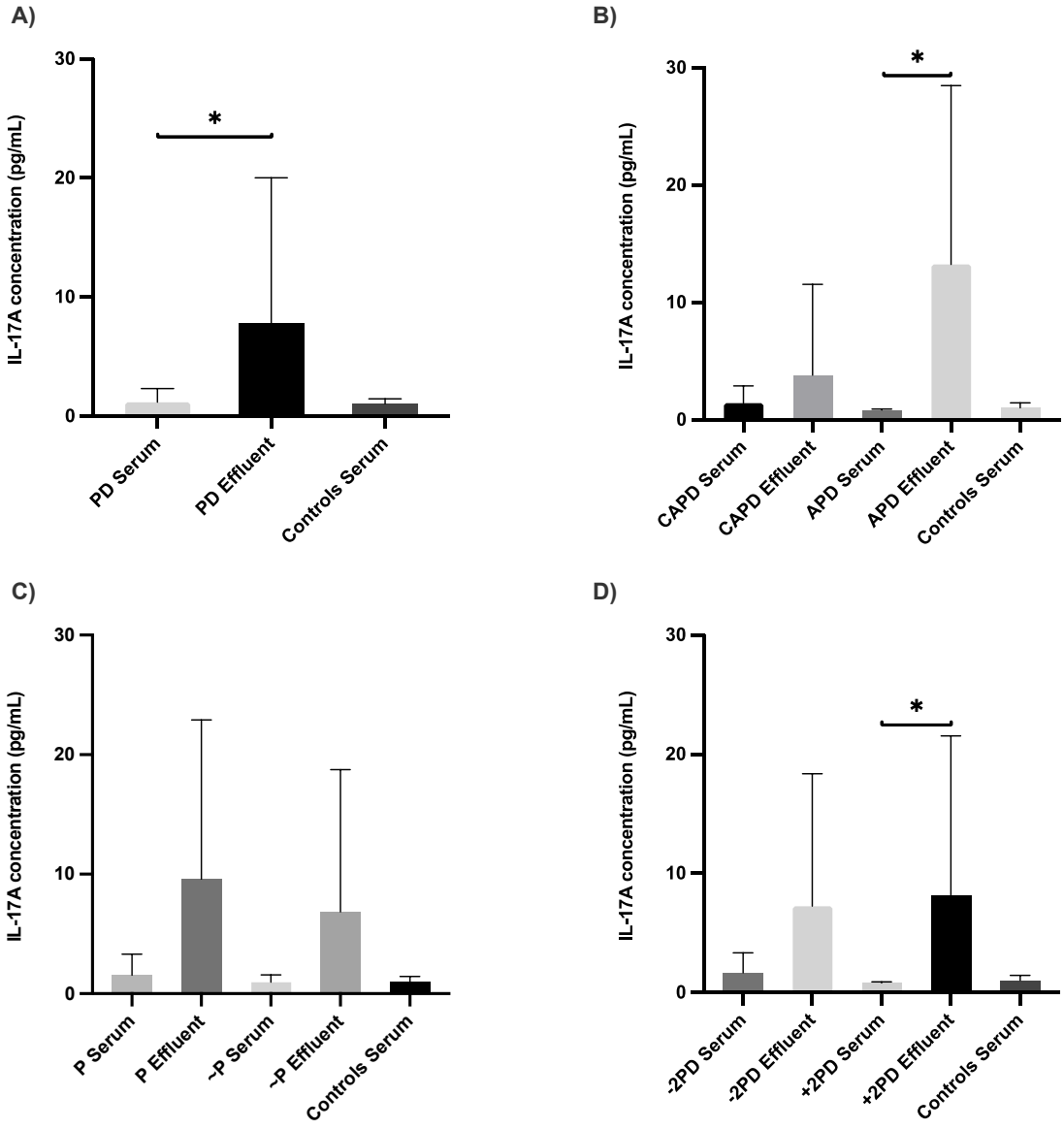

Figure 4. Serum and effluent IL-17A levels. (A) PD patients (in general) vs controls; (B) CAPD and APD patients vs controls; (C) P and ~P patients vs controls; (D) -2PD and +2PD patients vs controls. Bars represent the mean±SD; p values were determined either by Wilcoxon-Signed rank test or Kruskal-Wallis test; statistically significant differences (p<0.05) are identified with *.

Finally, we also observed that, for the majority of the analyzed samples, the concentration of IL-17A expanded from the serum to the effluent, meaning that this cytokine seems to be more produced in the peritoneal cavity. This tendency appeared to be maintained whether PD patients were subdivided or not (**Figure 5**).

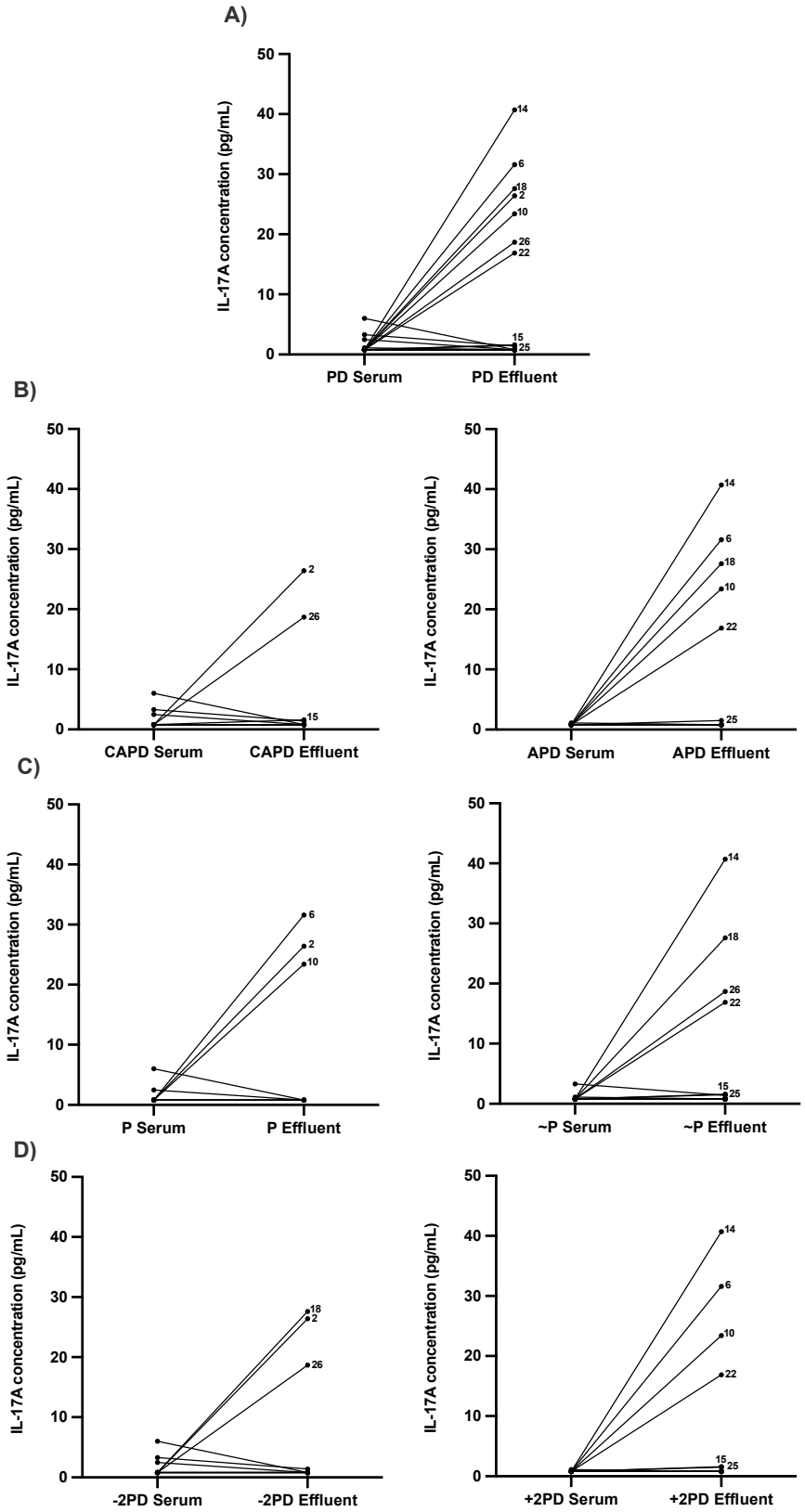

Figure 5. Paired comparison of IL-17A concentration in the serum and effluent. (A) PD patients (in general); (B) CAPD and APD patients; (C) P and ~P patients; (D) -2PD and +2PD patients.

V. Discussion

V. Discussion

Since their discovery, Th17 cells, and its main secreted cytokine, IL-17A, have been on the spotlight as relevant mediators in the pathogenesis of several chronic inflammatory diseases, including renal diseases [55,74–76]. Surprisingly, the first evidence of the potential role played by IL-17A in renal inflammation was reported before the functional significance of Th17 cells was discovered [77,78]. Nevertheless, experimental data has suggested that both immune players seem to be involved in the additional damage and deterioration observed during long-term PD treatment [66,69,79,80].

Regarding the human disease, the available information relating both PD and IL-17A is still sparse and diffuse, needing further clarification. Comparatively, when speaking about hemodialysis patients, it has been observed that when diagnosed with cardiovascular pathologies, they seem to experience elevated circulating IL-17A levels [81]. By contrast, when comparing pre-dialysis and hemodialysis patients, no significant differences were observed in Th17 cell subset [82]. Additionally, in nondiabetic chronic hemodialysis patients, Th17 cells differentiation was related to the serum phosphate level, nutritional status, and the duration of dialysis [83]. Therefore, the specific purpose of this study was to contribute to the clarification of this field through the analysis of peripheral blood and peritoneal dialysis effluent T cell subsets, as well as serum and effluent levels of IL-17A in PD patients and compare them with a control group.

Comparing PB samples from the complete group of PD patients and the control group, the main differences were observed in the frequencies of Th17 and Th1 cells and in the $CD4^+/CD8^+$ T and Th1/Th17 cells ratios, that were significantly decreased in PD patients. Additionally, serum IL-17A levels were similar in both PD patients and controls. Regarding PD patients' blood and effluent, only the frequency of Th1 cells was found to be significantly decreased in the blood, while IL-17A levels were found to be significantly higher in the effluent compared to the serum.

These results appear to differ from previously published data, namely regarding Th17 cells, since most authors reported an increase of this subset and, subsequently, an increase in IL-17A levels (in peripheral blood and serum, respectively) in CKD patients and renal transplant recipients [84–86]. However, only one of those reports [84] considers CKD patients undergoing PD, but no context is given regarding the characteristics of the treatment. Given the fact that the few research made on human context appear to follow the same strategy, a more in-depth analysis of the obtained data benefits from a

discussion based on the stratification made earlier: PD modality, episodes of peritonitis since starting PD and PD duration.

Choosing between CAPD or APD does not depend solely on clinical parameters or disease adequacy, but rather psychosocial aspects about the patients' lives need to be taken into consideration, meaning that it is a quite complex process, implying several variables [87]. Regarding CAPD patients, earlier studies reported an increase in the cytotoxic T cell subset in the peritoneum, that appeared to protect the peritoneal cavity against potential infections [88,89]. However, they were characterized by a defective defense capacity, since their peritoneal total lymphocyte number was reduced [90]. Still, over time, the composition of cell subpopulations in both peripheral blood and peritoneal cavity is expected to reach similar values [91]. More recently, it was observed that, regardless of the peritoneal dialysis fluid CAPD patients were using, the number of IFN-γ-producing cells (Th1 subset) was significantly reduced in peripheral blood (as compared to controls), but significantly increased in the peritoneal effluent (when cells were stimulated, compared to their unstimulated counterparts) [92].

In our study, the frequency of Th1 cells in CAPD patients was significantly reduced in peripheral blood, when compared with the control group. However, no conclusions can be taken regarding the effluent because our samples were all stimulated before analysis. To our knowledge, no published papers so far have specifically highlight APD patients' case. We did not find major significant differences between the two groups, but it is important to note that in the automated dialysis process, the effluent samples are more diluted, meaning that the data might not be as reliable as one would expect. Regarding Th17 cells, its frequency in peripheral blood was similar in both CAPD and APD patients, but in the effluent, it was higher in the latter group. Effluent IL-17A concentration in APD patients also followed the same pattern, as compared to the serum.

There is no doubt that peritonitis still constitutes one of the major causes of morbidity and mortality among PD patients [93]. Several studies have suggested that following episodes of acute peritonitis, the number of total lymphocytes expanded slightly, but no correlation seemed to exist between that value and the incidence rate of infection [88]. Besides this, it has been reported that the presence of a Th1-like immune response in the peritoneal effluent was associated with a lower risk of developing peritonitis, since these cells produce high amounts of IFN-γ and, consequently, increase the killing capacity of peritoneal cells [94,95]. Likewise, effluent IL-17A levels, typically low in uninfected patients, may increase during an episode of peritonitis and even be secreted by cells other than Th17 cells [96]. Moreover, it has also been suggested that the local production of IL-17A was

part of a protective early immune response to the infection and its expression pattern may predict the patients' outcome [97].

In our study, PD patients who had at least one episode of peritonitis had a lower frequency of Th1 cells, both in the blood and effluent, compared to the patients that never had any episode. Regarding IL-17A, although patients with a history of peritonitis have higher serum and effluent IL-17 levels, no conclusion should be drawn, since, at the time of sample collection, none of them had a known infection. However, this finding is interesting, because it suggests that serum and effluent IL-17A levels may be a potential biomarker for the recurrence of peritonitis. Considering Th17 cells, while its frequency was similar in both the blood and effluent of patients who had an episode of peritonitis, in patients that never experienced an episode of peritonitis it was lower in the blood and higher in the effluent.

PD patients are continuously exposed to numerous risks. However, when they are committed to the therapy in the long-term, the real danger seems to truly settle, due to the chronic exposure to bio-incompatible PD solutions and to the decline in ultrafiltration capacity [33,98]. To better characterize the immunological profile of the patients in the course of PD, different time stamps have been studied and, interestingly, the results have been similar. For example, Ohashi et al. observed a similar tendency in the frequency of total lymphocytes, $CD4^+$ and $CD8^+$ T cells in the effluent, six and eighteen months after starting the treatment [99]. Likewise, the same pattern was reported by Caprara et al. after one month of therapy [100], as well as by another study, after six months of therapy [101]. Rodrigues-Díez et al. probably had the most complete approach to this subject, conducting two separated procedures, in animal and human context, suggesting that IL-17A could be a good therapeutic target to preserve the peritoneal membrane integrity in PD patients. It reported, for the first time, that IL-17A was present in the effluent and peritoneal biopsies of PD patients, and higher concentrations were detected in long-term PD patients (more than three years on PD) [66].

In our study, the cut-off was made at less than two years and two or more years, mainly because the health professionals that follow the patients included in this study considered that, from this time point on, the PD-related inflammatory changes will have already taken place (mean PD duration=29 months). Contrary to previously reported, here, the frequencies of total lymphocytes, T and $CD4^+$ T cells were significantly decreased in patients submitted to PD for less than two years, whereas the frequency of $CD8^+$ T cells was significantly increased in the same group. Interestingly, when speaking about IL-17A, the frequency of Th17 cells in both the blood and effluent of the groups was

similar, but the local IL-17A production in the effluent seems to be significantly expanded in patients undergoing PD for two or more years.

Due to the importance of IL-17 in the PD context, we wanted to understand how IL-17A levels might change between people considered to be in a healthy condition and PD patients, whose immunological state is impaired. Despite the way PD patients were subdivided, the frequency of Th17 cells in peripheral blood did not change greatly between groups, but it was considerably higher in the effluent. The local IL-17A production also did not differ in the serum but was again higher in the effluent. From **Figure 5**, it can be suggested two distinct clusters of patients, that may share a specific immunological alteration, which we were unable to identify. Further clarification is needed, as it may explain other differences observed.

Besides an influx of Th17 cells to the peritoneal cavity, locally, Th17 cells and probably other cells, are producing IL-17A, seemly confirming that an inflammatory process is taking place. The nature of this process would, however, need, a more detailed approach considering both immune and renal parameters, to correlate the physiological changes. What happens in endometriosis patients, for example, could serve as an example, since higher IL-17A levels have been found in both blood and peritoneal effluent and are associated with the severity of the disease [102].

Overall, our study is well designed, but has some limitations. The fact that many of the clinical characteristics of the control group (for example, the BMI values) were not available, may have influenced the analysis of the obtained results. Besides this, PDE samples were quite difficult to work with, due to the variability of the obtained results, especially regarding the absolute values, considerably lower than the ones found in the blood. Since PD patients have this sample readily available and it provides an essential knowledge about what is happening inside the peritoneal cavity, future investigations need to further study this samples. This means that a specific methodology regarding, for example, the way PDE samples are collected and the minimal number of cells within the fluid, need to be considered. Still, one of the valuing points of our research is the fact we were able to analyze and compare both PB and PDE samples from PD patients, which allowed us to delineate a distinctive immune profile of these patients and acquire more knowledge regarding their condition.

Although the three stratification approaches are valid, we consider that it would be useful to further investigate PD patients considering their peritonitis history, since the obtained results are intriguing. Perhaps, in the future, it would be relevant to keep a record of their

immunological profile before, during and after an episode of peritonitis, to better understand the immunological changes that occurred and the way IL-17A may have interfered.

Aside from this, future research also needs to include the study of a boarder spectrum of cells and other factors. For example, we observed that Th1 cells were the primary altered subset, meaning that it is relevant to investigate how this subset relates to Th17 cells. Actually, it has been reported that an initial Th17-mediated kidney damage might be followed by a Th1-mediated illness later on [71]. Likewise, Treg/Th17 balance seems to be of interest since the predominance of Th17 cells over Treg is said to lead to fibrosis development and peritoneal damage [103].

VI. Conclusion

VI. Conclusion

In the present work, it was possible to observe that the frequency of Th17 cells in the blood was significantly decreased in PD patients, as compared to the control group, but expanded (although not significantly) in the effluent of PD patients, as compared to their blood values. In addition, the local IL-17A production, although found in both serum and effluent of PD patients, was more significant in the latter.

The data observed here, although not always in line with previously published research, does seem to suggest that IL-17A contributes to the damage and inflammation associated with PD patients. However, the exact mechanisms behind its action and the way its function may influence the peritoneum physiology still needs to be further investigated and, subsequently, correlated with clinical outcomes. Since Th17 cells are not the only cells capable of producing IL-17A, in the future, it would also be necessary to explore those other cell subsets and their relationship with other immune cells. Additionally, one of the key issues that also needs to be addressed is the real impact PD solutions have in the course of the disease and how they can affect and contribute even more to the expected deterioration of the peritoneal membrane.

Due to its pleiotropic character and proinflammatory action, IL-17A seems to have potential as a therapeutic target in several diseases, like psoriasis. Its clinical application in the context of renal disorders, especially PD-related, still requires further clarification. PD therapy is a double edge sword: despite improving the quality of life of CKD patients, its side effects negatively impact and contribute furthermore to the deterioration of their health. If the efficacy of IL-17A as a therapeutic target is proven, it could be a game-changer, easing the burden of people undergoing PD, and possibly leading to the global implementation of the technique.

VII. References

VII. References

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VIII. Appendix

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Appendix 1

ULS
Unidade Local de Saúde
de Castelo Branco, EPE

Documento: Submissão de pedido de autorização para a realização do estudo - Diálise peritoneal: relação entre os níveis sanguíneos e no efluente peritoneal de Lth17 e no perfil tensional (dipping vs nom dipping) – um estudo observacional.

Assunto: Diálise peritoneal: relação entre os níveis sanguíneos e no efluente peritoneal de Lth17 e no perfil tensional (dipping vs nom dipping) – um estudo observacional.

Requerente: João Pedro Loureiro de Almeida Grilo, Raquel Chorão e Catarina Reis Santos.

Título: Diálise peritoneal: relação entre os níveis sanguíneos e no efluente peritoneal de Lth17 e no perfil tensional (dipping vs nom dipping) – um estudo observacional.

Investigador: João Pedro Loureiro de Almeida Grilo, Raquel Chorão e Catarina Reis Santos

População do Estudo: É convidado(a) a participar voluntariamente num estudo concebido e coordenado pelo Serviço de Nefrologia do HAL, com o objetivo de caracterizar o perfil tensional dos doentes no programa de diálise peritoneal do Serviço de Nefrologia do HAL e estabelecer a sua correlação com o perfil imunológico dos doentes.

Serviço onde decorre o estudo: Serviço de Nefrologia do HAL

Data do pedido: datado no HAL a 07/07/2022

A Comissão de Ética da ULSCB, EPE, concorda com a aplicação do referido estudo desde que seja mantida a confidencialidade dos sujeitos do mesmo e todos os princípios éticos inerentes ao processo de investigação sejam respeitados.

ULS de Castelo Branco, E.P.E., 8 de julho de 2022

A Comissão de Ética