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ASSESSMENT OF IMMUNE CHANGES IN CHRONIC HEMODIALYSIS PATIENTS – PHENOTYPIC STUDY OF LEUKOCYTE POPULATIONS INVOLVED IN THE IMMUNE RESPONSE

PHD THESIS SUMMARY

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2024

SUMMARY

List of published scientific papers:

Articles published in specialized journals

1. **Bumbea V.I.,** Bumbea H., Vladareanu A.M., Immune dysfunction in patients with end stage kidney disease; Immunosenescence – Review, Rom J Intern Med, PMID 37991332, 2023 Nov 22, DOI: 10.2478/rjim-2023-0030, ISI , factor de impact 1,6 , introducere pag 8, capitolul 1 pag 13, 16-19, capitolul 2 pag 22-28

2. **Bumbea V.,** Ardelean L., Radulescu L., Damian L., Bumbea H., Dumitru I., Lambert C., Vladareanu A-M., Proinflammatory roles of monocytes in SARS-CoV-2 infection in chronic hemodialysis patients, Front. Immunol., 3:14:1210961, 2023 Aug, **DOI:** 10.3389/fimmu.2023.1210961, ISI, factor de impact 5,7, capitolul 1 pag 20-21, capitolul 2 pag 30, capitolul 3 pag 41, capitolul 8 pag146-153 și pag 165-170

Papers presented at international scientific events

3. Proinflammatory role of monocytes in SARS-CoV-2 infection in chronic hemodialysis patients , Horia Bumbea, **Viorica Bumbea*,** Ion Dumitru, Luminita Ardelean, Luminita Radulescu, Luminita Damian, , Claude Lambert, Ana-Maria Vladareanu, prezentare orala la al 17-lea Congres National de Citometrie, 16-17 Mai 2024

4. MONOCYTE'S INVOLVEMENT IN INFLAMMATORY RESPONSE IN COVID-19 INFECTION IN CHRONIC RENAL DISEASE Horia Bumbea, **Viorica Bumbea*,** Luminita Ardelean, Luminita Radulescu, Luminita Damian, Ion Dumitru, Claude Lambert, Viola Popov, Ciprian Tomuleasa, Ana-Maria Vladareanu, Final Abstract Code: PB2259, EHA 2022 Hybrid Congress, June 9 - 12, 2022 - Vienna, Austria

CHAPTER 1. HYPOTHESIS AND OBJECTIVES OF THE STUDY

The body's resistance to foreign pathogens is achieved, among other things, through the action of the immune system, which has a complex role: defense ăr against infections, antitumor action, tissue repair. On the other hand, in the event of immune malfunction, chronic inflammation may occur with important consequences on the morbidity or even mortality of patients [1].

The body defends itself against various pathogens of the environment through physical barriers (skin, mucous membranes, etc.) through the action of cells involved in innate immunity (natural killer lymphocytes, complement or phagocytic cells) or through acquired immunity (represented by the action of B lymphocytes and T cells) [2,3,4].

Among the pathologies associated with medical practice, one of the most common is renal pathology. The kidney is connected with most of the systems in the human body, renal pathology being extremely varied, with repercussions on other apparatus and systems, the immune system and the cardiovascular system being among the most important systems affected [4].

Chronic kidney disease is defined as structural (albuminuria greater than or equal to 30 mg/day) or functional (GFR less than 60 ml/min/1.73m^2) renal impairment present for 3 or more months. This time interval differentiates between chronic kidney disease and acute kidney injury [5].

The current staging of ESRD includes, in addition to patients with low GFR, those with good renal function but with chronic changes in the urinalysis or radiological changes compatible with chronic, irreversible renal damage. Stage 1 represents patients with normal GFR but with chronic renal impairment as evidenced by microalbuminuria, proteinuria, hematuria or radiologic changes, stage 2 includes patients with mild GFR between 89-60 ml/min/1.73 m² also having chronic changes in the urinalysis or radiologic changes, stage 3 has two components: 3a with GFR between 59-45 ml/min/1.73 m² and 3b with GFR between 44-30 ml/min/1.73 m², stage 4 GFR between 29-15 ml/min/1.73 m² and stage 5 GFR below 15 ml/min/1.73 m². In stage 5 chronic kidney disease, dialysis treatment or renal transplantation is considered to sustain life in these patients [6].

The impairment of immunity in patients with renal failure is characterized both by increased susceptibility to infection, resulting in immunodeficiency, and by the presence of chronic

inflammation secondary to activation of the immune system. In patients with chronic kidney disease, there is an imbalance between pro- and anti-inflammatory factors and between pro- and anti-apoptotic factors [7].

Patients with chronic kidney disease present, on one hand, a chronic activation of the immune system, chronic inflammation, leading to atherosclerosis and increased incidence of cardiovascular disease, and on the other hand, immunodeficiency translated by a low immune response secondary to vaccination as well as increased susceptibility to infections [4].

The initial hypothesis of the research was that chronically hemodialyzed patients show changes in the cell distribution of the immune system compared to undialyzed uremic patients or healthy subjects. We also hypothesized that some of the immunity changes occurring during the course of chronic kidney disease improve, or on the contrary, worsen with the initiation of hemodialysis.

The main objective of this work was to identify phenotypically the changes in leukocytes involved in the immune response in chronic hemodialysis patients compared to healthy subjects and to patients with stage 5 chronic kidney disease before the initiation of hemodialysis. Based on similar studies found in the literature, we discussed the clinical implications of the immunophenotypic changes identified in the studied groups. The importance of this study is given by the pathology of patients with chronic kidney disease where a main trigger for cardiovascular disease and predisposition to infections is defective immunity.

The study focused on quantifying those changes in the cells involved in the immune response that have been shown in other published studies to be essential in the development of cardiovascular pathologies, immunodepression or immunosenescence. Immunophenotypic methods by flowcytometry were used to identify and evaluate the cell populations involved in the immune response and the results were compared by statistical methods.

CHAPTER 2: STATE OF THE ART

In GFR below 45 mL/min/1.73 m² the risk of cardiovascular mortality increases with decreasing GFR [8]. Cardiovascular disease mortality is 20 times higher in chronic dialysis

patients than in the general population and infections occurring in these patients result in 12-22% mortality [4,8]. In uremia both the increased incidence of cardiovascular disease and the increased risk of infection are the result of a defective immune response [4,8]. In end-stage renal disease (GFR less than 15 ml/min) and especially in dialysis patients, premature aging of the immune system, loss of thymus function, expansion of memory T lymphocytes are important factors in the development of pathology secondary to immunodepression in these patients [4].

A study published in 2016 by Fang-Fang Xiang et al performed on a group of 472 patients with chronic kidney disease at different stages of progression highlights a decrease in total lymphocyte count before the onset of stage 5 chronic kidney disease [4,9]. It also showed that patients with advanced stages of chronic kidney disease show, in addition to a decrease in total lymphocyte count, a decrease in the percentage of B lymphocytes, an increase in the percentage of NK lymphocytes, but no changes in the total number of leukocytes, monocytes, CD4+ T lymphocytes, CD8+ T lymphocytes or CD4+/CD8+ ratio [4,9].

In patients with end-stage chronic kidney disease the number of CD16+ monocytes is increased and may play a role in the systemic inflammation and increased oxidative stress observed in these patients [3,4]. There is also a decrease in the phagocytic role of monocytes in the uremic patient, which is related to the increased incidence and severity of infections [3,4].

In dialysis patients there is a positive correlation between CD16+ monocyte count and mortality, especially in patients with pre-existing cardiovascular disease [4,10]. In particular, there are predictive studies for cardiovascular disease in patients with chronic kidney disease in which a clear correlation between increased intermediate monocyte count and cardiovascular disease has been established [4,11].

Yen -Ling Chiu et al report in 2018 a study performed on 412 hemodialysis patients highlighting an accelerated aging of T lymphocytes and monocytes characterized by a decrease in CD4+ naive, CD8+ naive lymphocytes and an increase in proinflammatory CD16+ monocytes. These changes are associated with the age of the patients but also with the length of time they have been on dialysis (possibly due to prolonged exposure to the uremic environment) [4,12]. The study is based on the premise that chronically dialyzed patients show many similarities related to physiological changes and increased morbidity and mortality similar to those of the elderly (including decreased muscle mass, osteoporosis, decline in cognitive function, accelerated vascular disease and increased risk of death explained by oxidative stress,

accumulation of uremic toxins and chronic inflammation) [4,12,13] [4,12,13]. The study compared the 412 chronic dialysis patients with 57 healthy elderly patients. Chronic dialysis patients have decreased percentage of naïve CD4+ and CD8+ T lymphocytes and increased percentage of CD8+ effector memory and percentage of effector memory T lymphocytes with high differentiation (TEMRA) [4,12].

The COVID 19 pandemic has provided an opportunity to see how the immune systems of end-stage chronic kidney disease patients react to the challenge of this disease. COVID-19 is a highly contagious disease produced by the SARS-CoV-2 virus that initially emerged in China, Wuhan region in December 2019, and then rapidly spread worldwide becoming pandemic and characterized by an increased mortality rate especially in elderly or comorbid unvaccinated patients.

In 2021 Michiel G.H. Betjes publishes an article on the severity of uremia-associated COVID infection. Since the beginning of the pandemic the severity of cases has been associated with old age and the presence of comorbidities. Among them, chronic kidney disease and especially patients undergoing treatment with renal replacement therapy and those who have undergone renal transplantation play an important role in the occurrence of severe forms of COVID and mortality [14]. More than 20% of the patients who died had chronic kidney disease [15].

One explanation for the increased number of severe forms in the uremic patient would be a decrease in the number of naïve T lymphocytes and a functional decline in the entire T lymphocyte population. This results in a delayed clearance of the virus and an expansion of memory T lymphocytes through prolonged stimulation [14].Increased numbers of reactive memory T lymphocytes associated with decreased efficiency of Treg lymphocyte function lead to slower healing of viral infection [14].

CHAPTER 3: RESEARCH METHODOLOGY

3.1 Objectives of the study

The study was initiated after obtaining the approvals of the ethics committees of the University Emergency Hospital of Bucharest and the Clinical Emergency Hospital of Bucharest, with patients hospitalized in both hospitals who gave their prior consent for participation by signing the informed consent, also the paraclinical tests were performed in the laboratories of both hospitals.

In detail, the objectives of this study include:

- Immunophenotypic, numerical or percentage immunophenotypic assessment of T lymphocytes, B lymphocytes and monocytes including subpopulations representative of the immune response
- We looked at the proportion of activated cells or cells shown to be involved in chronic inflammation in each study population
- Comparison of the results obtained in chronic hemodialysis patients with the results obtained in healthy subjects and in patients with undialyzed stage 5 chronic kidney disease. Also the results of patients with chronic kidney disease stage 5 undialyzed were compared with the results of healthy subjects
- Identification of statistically significant positive or negative correlation associations between the populations studied
- Correlation of the highlighted changes with the literature and other similar studies associating these changes with the development of cardiovascular pathology, immunodeficiency or immunosenescence.
- The immune evolution of chronic hemodialysis patients during a viral infection - the COVID pandemic that occurred during the data collection period of the study gave us the opportunity to see how a viral infection influences the immune profile of the chronic dialysis patient studied

The study is a prospective case-control observational analytic prospective observational type and includes patients hospitalized in the Bucharest Emergency Clinical Hospital and the University Emergency Hospital between 2020-2021 who were compared with healthy subjects without chronic kidney disease.

3.2 Study batches

The subjects were divided into groups: a group of patients with stage 5 chronic kidney disease undergoing chronic hemodialysis in the Bucharest Emergency Clinical Hospital, a group of patients with stage 5 chronic kidney disease before being initiated on dialysis, who were admitted to the Bucharest Emergency Clinical Hospital or the Bucharest University Emergency Hospital, and a group of healthy, voluntary subjects, which represented the control group.

Thus 4 study groups were identified:

1. the group of chronic dialysis patients that included 15 chronic hemodialysis patients for at least 1 year who were already in the chronic dialysis program in the Bucharest

Emergency Hospital and who were compared with healthy subjects or with uremic patients before the initiation of dialysis.

2. uremic patients group which included 12 patients with stage 5 BCR, hospitalized in the Bucharest Emergency Clinical Hospital or in the Bucharest Emergency University Hospital for initiation in the chronic hemodialysis program. In these patients the hemograms necessary for immunological analysis were collected before the initiation of dialysis. On admission, the mean GFR was 4.35 ml/min/1.73 m^2 with limits between 2.1 and 6.8, corresponding to blood creatinine between 6.33 and 18.9 mg/dl, with a mean of 11.45 mg/dl.
3. the control group that included 27 healthy subjects for the T lymphocytes study (15 from the database of the University Emergency Hospital that had already worked on T lymphocytes phenotyping to which 12 new volunteers were added), 12 controls for the study of B lymphocytes and monocytes that were identified in the Bucharest Emergency Hospital. Healthy subjects were selected in such a way that they were similar in sex or age to dialysis patients in order to obtain homogeneous groups with the aim of reducing the incidence of confounding factors.

   These 3 initial groups were joined during the course of the study by a group of patients suffering from a medium form of Covid infection:

4. The group of 5 chronic dialysis patients drawn from group 1 who developed a medium form of Covid infection during the study, thanks to which we analyzed the immune response of these patients to a highly studied viral infection during the pandemic.

3.3 Study protocol

The flowcytometric method is used to study the changes in immunity occurring in different situations, whereby the different lymphocyte subpopulations and monocyte subtypes can be differentiated and the influence of uremia on the immune status of patients can be assessed.

The test used to analyze the T lymphocytes is a preformed assay, DuraClone IM T cell subsets T cell subsets Tube, 25 tests, RUO (REF B53328). This is a Beckman Coulter - Dura Clone IM T subset kit and contains (antibody/clone(fluorochrome): CD45RA/2H4(FITC), CD28/CD28.2(ECD), CD279(PD1)/PD1.3.5(PC5.5), CD197(CCR7)/G043H7(PE),

CD57/NC1(PacificBlue), CD27/1A4.CD27(PC7), CD4/13B8.2(APC), CD8/B9.11(A700), CD3/UCHT-1(APC-A750), CD45/J33(Krome Orange) [16].

For the identification of B lymphocyte subtypes involved in the immune response: a preformed DuraClone IM B cell subsets Tube, 25 tests, RUO (REF B53318) was also used. This Beckman Coulter - DuraClone IM B subset kit contains (antibody(clone)-fluorochrome): IgD(IA6-2)-FITC / CD21(BL13)-PE / CD19(J3-119)-ECD / CD27(1A4CD27)-PC7 / CD24(ALB9)-APC / CD38(LS198-4-3)-APC-A750 / IgM(SA-DA4)-Pacific Blue / CD45(J33)-Krome Orange [17].

The following membrane surface markers were used for the study of monocytes: CD64, CD14, CD16, CD300e, HLADR. The antibodies used for this purpose are antibody (clone) - fluorochrome: HLA-DR (L243) - V450/CD45 (HI30) - OC515/CD36 (CLB-IVC7) - FITC/CD64 (10.1) - PE/CD300e (UP-H2) - APC/CD14 (MφP9) - APCH7/CD16(Fc-Gran1)-FITC.

To perform immunophenotyping analyses we used the Gallios flowcytometer produced by Becton Dickinson, equipped with 3 lasers, and 10 colors to determine the size, type of cells examined and their fluorescent characteristics [18].

The laser system consists of a blue laser (488nm), a red laser (638nm) and a violet laser (405nm). The device simultaneously detects 10 different fluorescences which represents 10 simultaneous markers [18].

The working protocol for the study of leukocytes involved in the immune response includes:

1. Establishing the antibody protocol/panel.
2. Peripheral blood sample collection by endovenous puncture, in vacutainer with EDTA for hemoleukogram, and storage of blood samples at room temperature. After collection blood samples were transported to the laboratory at room temperature.
3. Take 100 µl of the sample (peripheral blood) and place in Falcon tubes/dry tubes for panels with lyophilized monoclonal antibodies in the tube.
4. Labeling with monoclonal antibodies 5-20 µl/tube.
5. Vortexing sample.
6. Incubate for 15 minutes in the dark at room temperature.
7. RBC lysis and cell membrane fixation +/- paraformaldehyde (if sample is kept longer) by 2 methods:

a. cu ImmunoPrep, reactivul A, B si C
b. cu FACS Lyse Solution cate 500 µl

8. Inserting the tube into the cytometer and performing the acquisition with the acquisition software of the instrument (reading in the flowcytometer after processing with ImmunoPrep or after 10-15 minutes if we used FACS Lyse solution).
9. Analyze the sample with isotype control for each fluorescence used and set the quadrant boundaries so that quadrant 3 (double negative) has at least 98% of cells. Fluorescence channel setting was performed using the Harmonemia protocol (*Harmonemia: a universal strategy for flow cytometry immunophenotyping-A European LeukemiaNet WP10 study, Leukemia volume 30, pages1769-1772 (2016).*

3.4 Statistical analysis

The database was compiled on the basis of the charts made for each individual patient, with clinical, biological and paraclinical data entered as variables. Statistical interpretation of the data was performed using Microsoft Office Excel for Windows 2007 and Jasp 0.14.1. A p-value < 0.05 was considered statistically relevant. Graphs were worked in Excel and Jasp.

The study groups were small but the patients were selected from a small population, the subjects who made up the study groups met certain inclusion and exclusion criteria so that they were comparable.

In choosing the statistical methods appropriate to these batches, it was first of all taken into account that the data obtained were numerical, so statistical methods adapted to this type of data were used. For the statistical study, the two-sample t-test was used, which is appropriate for small batches.

For the choice of the type of analysis used: parametric or nonparametric, it was taken into account that the data obtained were independent, the Shapiro-Wilk test was used for all the groups examined to check the normality of the distribution of the data and the Levene test was used to also check the homogeneity of variance. All these tests were applied in the Jasp program before selecting the type of statistical analysis.

For cases in which the data had a normal distribution and homogeneous variance the case-marker groups were analyzed using the Student test. In the case of deviation from normality or in

the case of a Levene's test indicating non-homogeneous variance the nonparametric Mann-Whitney two-sample Mann-Whitney test was preferred for comparing unpaired quantitative data.

For the paired data, the Student's paired Student's test was used if the data had a normal distribution and the Wilcoxon test if the distribution was not normal.

The correlation indices used were Spearman's′ s rho, a nonparametric correlation test was preferred because not all data sets had a normal distribution or homogeneous variance.

CHAPTER 4: PHENOTYPIC ANALYSIS OF T LYMPHOCYTES

According to the instructions for use of the panel proposed by Beckman Coulter to investigate the T lymphocyte-mediated immune response, we followed naive T lymphocytes circulating in the blood after leaving the thymus with CD45RA+CCR7+ expression, T lymphocytes that lose their CD27 or CD28 expression after exposure to foreign antigens becoming antigen-specific effector T lymphocytes, long-lived central memory T lymphocytes expressing CD45RA-CCR7+, effector memory T lymphocytes expressing CD45RA-CCR7- both providing a rapid antigen-specific immune response. T lymphocyte terminal stages of differentiation have CD45RA+CCR7- expression and are described in the literature as TEMRA cells. Some authors consider TEMRA cells as terminally differentiated effector cells that have the property of secreting interferon gamma, have high cytotoxicity, low proliferative capacity and high sensitivity to apoptosis [4 ,19]. There are authors who describe several subgroups of

TEMRA cells depending also on the expression of CD27 and CD28, the resulting subclasses having specific effects, CD27-/CD28- being the highly cytotoxic T lymphocytes with late differentiation [4,19]. TEMRA lymphocytes are identified in blood mainly CD8+ T lymphocytes, CD4+ TEMRA lymphocytes are rarely detected in blood, occur in case of infection with some viruses and are thought to have a protective role [4,20].

According to OMIP-069 CD57 is a marker of cellular senescence present in T lymphocytes with low proliferative capacity. The inhibitory PD-1 receptor is increased in activated T lymphocytes [16,21,22,23].

The results of the study show that the lymphocyte count significantly decreased (p 0.012) in the chronic dialysis patients compared to the control group and even more in the uremic group compared to the control group (p 0.002). The trend of decrease in uremic patients compared to dialysis patients is not statistically significant (p 0.2). Lymphopenia is considered in the literature to be common in patients with stage 5 chronic kidney disease [25] (Figure 4.1).

The study published in 2016 by Fang-Fang Xiang et al in a group of 472 patients with chronic kidney disease at different stages of progression shows a decrease in total lymphocyte count before the onset of end-stage (5) chronic kidney disease [4,9].

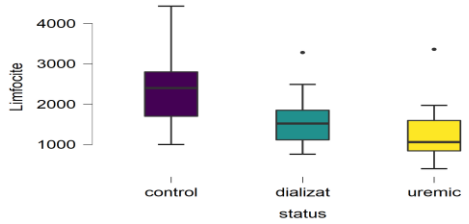

Figure 4.1: Graphical representation of lymphocyte counts between the three groups studied

T lymphocyte count has the same trend as total lymphocytes. In dialysis patients the number of T lymphocytes decreases statistically significantly (p 0.007) compared to the control group and in undialysed uremic patients the decrease is more pronounced (p less than 0.001). Although the number of T lymphocytes tends to decrease in uremic patients compared to dialysis patients this decrease is not statistically significant (p 0.1). T-lymphocyte apoptosis, increased in uremia, could explain the lymphopenia, immunodeficiency and increased risk of infection in these patients [7] (figure 4.2).

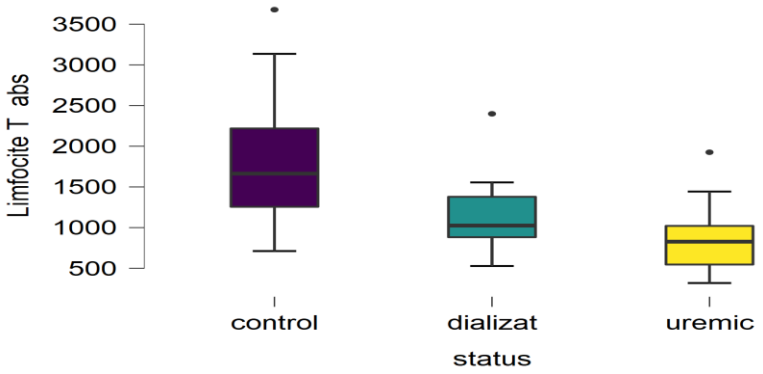

Figure 4.2: Graphical representation of T-cells counts between the three groups studied

The number of CD4+ T lymphocytes is significantly decreased in dialysis (p 0.003) and uremic (p less than 0.001) patients compared to control, with no significant differences in CD4+ T lymphocyte counts between the dialysis/uremic groups (p 0.2) (figure 4.3). CD8+ T-lymphocyte CD8+ T-lymphocyte counts do not vary significantly between dialyzed/control groups (p 0.2) or between dialyzed/uremic groups (p 0.1) but decrease statistically significantly in uremic patients compared to control (p 0.01) (figure 4.3). A similar study published in 2020 by Sampani E. et al comparing uremic patients at dialysis entry with a control group and the same patients 6 months after dialysis initiation describes the same, significant decrease in CD4+ and CD8+ T lymphocyte counts at dialysis entry (uremic patients before dialysis) compared to control group [24]. In uremic patients, as a consequence of chronic inflammation, oxidative stress, malnutrition, an irreversible involution of the thymus occurs, resulting in a decrease in the total number of CD4+ T lymphocytes and CD28 expression on the surface of the lymphocytes [25].

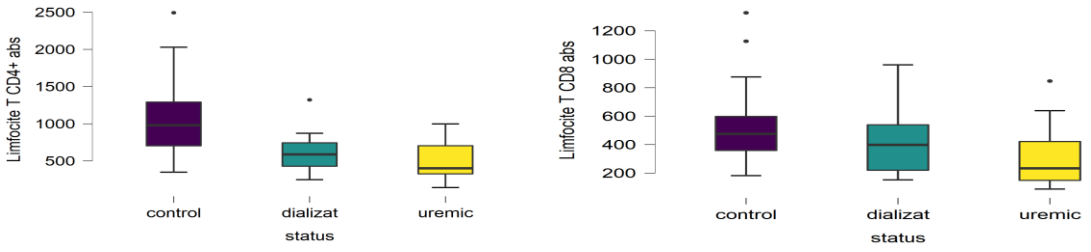

Figure 4.3: Graphical representation of CD4+ and CD8+ T-cells counts between the three groups studied

Although statistically there are no statistically significant differences in the CD4+/CD8+ T lymphocyte CD4+/CD8+ ratio between the studied groups, calculating the percentages of subunit values shows that 14.8% of the control group had subunit ratio, 26.6% of the chronically dialyzed patients had subunit ratio and 25% of the undialyzed uremic patients also had subunit ratio.

The number of central memory CD4+ T lymphocytes decreases insignificantly in dialysis patients compared to controls (p 0.2) but the decrease is significant (p 0.05) in uremic patients compared to controls. The decrease in the number of central memory lymphocytes in uremic versus chronic dialysis patients is statistically insignificant (p 0.2) (figure 4.4)

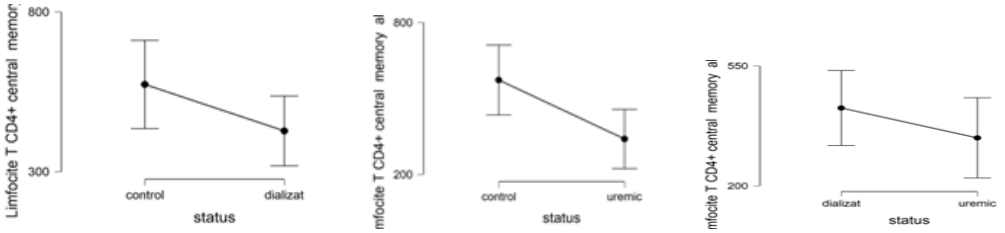

Figura 4.4 : Graphical representation of central memory CD4+ T-cells counts between the three groups studied

The number of central memory CD8+ T lymphocytes decreases in chronic dialysis patients versus control (p 0.7), in uremic patients versus control (p 0.2) and in uremic patients versus chronic dialysis patients (p 0.4) without these differences being statistically significant. (figure 4.5)

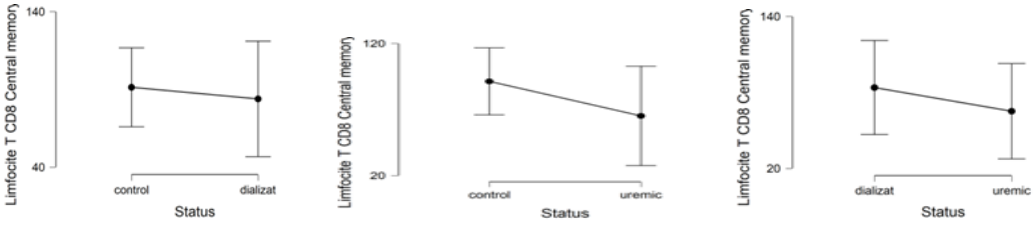

Figura 4.5: Graphical representation of central memoryCD8+ T-cells counts between the three groups studied

The number of central memory T lymphocytes correlates positively and will decrease with decreasing lymphocyte count, T lymphocyte count or CD4+ CD4+ CD8+ CD8+ which correlates with the literature.

Percentage expression, however, shows a statistically significant (p 0.003) increased percentage of CD4+ central memory T lymphocytes in chronically dialyzed patients compared to control group and in undialyzed uremic patients compared to control group (p 0.001). There were no differences between uremic and chronically dialyzed groups (p 0.89). In the case of central memory CD8+ T lymphocytes CD8+ there were no significant differences between the studied groups.

The same results appear in the study performed on 412 patients with end-stage chronic kidney disease versus 57 healthy subjects published by Chiu Y-L et al: if we look at the table centralizing the results we see that the percentage of CD4+ central memory lymphocytes significantly increases in chronic dialysis patients compared to the control group while the absolute number does not vary significantly in CD4+ and CD8+ central memory T lymphocytes compared to the control group [12].

The percentage of effector CD4+ T-lymphocytes, which correspond to the CCR7-CD45RA+ phenotype and are referred to in the literature as TEMRA cells, is small, these cells are not numerous, significantly decreased in chronic dialysis patients compared to control group (p 0.01) and significantly decreased in uremic patients compared to control group (p 0.003). There are no significant differences between uremic and chronic dialysis patients (p 0.98). As for the percentage of CD8+ TEMRA T lymphocytes, it is significantly increased in chronic dialysis patients compared to control (p 0.009), although uremic has fewer effector CD8+ T lymphocytes compared to chronic dialysis patients the percentages are not statistically significantly different (p 0.09) and between the control group and the uremic patients there do not seem to be significant differences (p 0.7). Due to the premature aging of the immune system, in the chronically dialyzed patient, more highly differentiated memory T lymphocytes appear which have a proinflammatory action destabilizing the atherosclerotic plaque and maintaining the chronic inflammatory status in the uremic patient [7]. In the study published by Chiu Y-L, there is a significant increase in both the number and percentage of terminally differentiated CD8+

lymphocytes in patients with stage 5 chronic kidney disease. In contrast, the number and percentage of terminally differentiated CD4+ T lymphocytes do not appear to be significantly influenced by chronic kidney disease [12] (Figure 4.6).

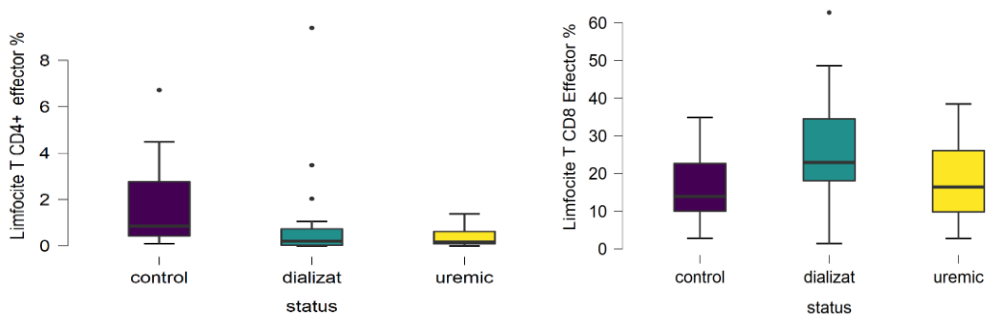

Figura 4.6 : Graphical representation of TEMRA (effector) CD4+ and CD8+ T cells percentage between the three groups studied

The percentage of naïve CD4+ lymphocytes is significantly decreased in chronic dialysis patients compared to the control group (p less than 0.001), also this percentage is significantly decreased in uremic patients compared to the control group (p less than 0.001). Between uremic and chronic dialysis groups there are no significant differences (p 0.9). There is also a statistically significant decrease in naïve CD8+ T-lymphocytes in the chronically dialyzed patients compared to the control group (p 0.01) and a statistically significant decrease in the uremic patients compared to the control group (p 0.002) (figure 4.7)

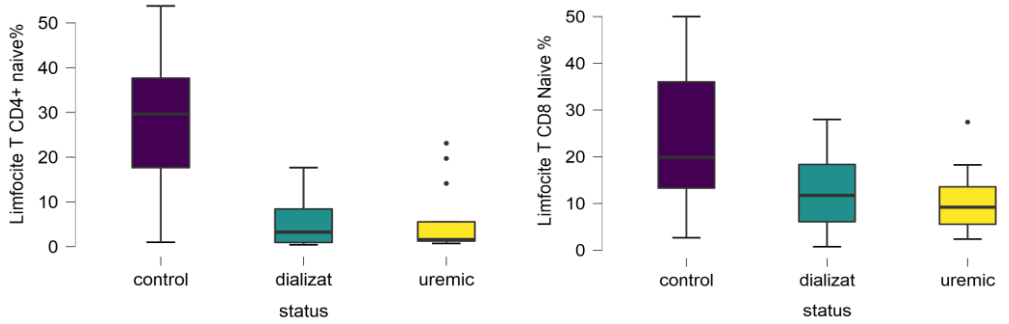

Figura 4.7 : Graphical representation of naive CD4+ and CD8+ T cells percentage between the three groups studied

The study published in 2018 by Chiu Y-L highlights a significant percentage and numerical decrease in both naive CD4+ and naive CD8+ T lymphocytes, the latter, together with the increased percentage of CD8+ TEMRA T lymphocytes in patients with end-stage chronic kidney disease, being considered important elements in the diagnosis of immunosenescence involved in the atherosclerosis process [12].

CD4+CD57+ T lymphocytes show a significant percentage increase in chronic dialysis patients (p 0.01) and uremic patients (p 0.02) compared to the control group, while there is no difference between uremic and chronic dialysis patients (p: 1). In case of CD8+CD57+ T lymphocytes the percentage increase in chronic dialysis patients is insignificant (p 0.6) compared to the control group, uremic patients have insignificantly more percentage of CD8+CD57+ T lymphocytes compared to the chronic dialysis patients (p 0.1) and undialyzed uremic patients have significantly more percentage of CD8+CD57+ T lymphocytes compared to the control group (p 0.03) (figure 4.8).

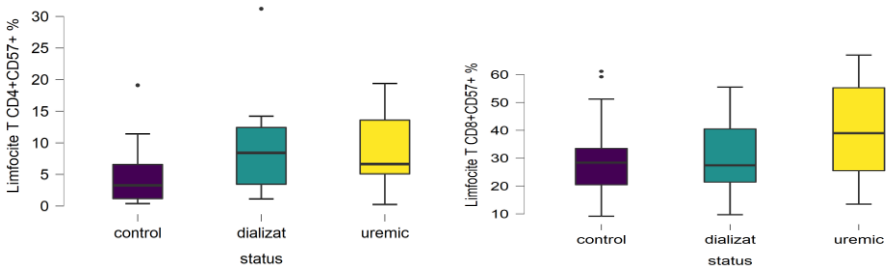

Figura4.8 : Graphical representation of CD57+ CD4+ and CD8+ T cells percentage between the three groups studied

In the case of CD4+CD27-CD28- T lymphocytes, a statistically significant percentage increase is observed in the chronically dialyzed patients compared to the control group (p 0.02), also uremic patients have significantly more CD4+CD27-CD28- T lymphocytes compared to the control group (p 0.01). The chronically dialyzed and undialyzed uremic groups were not statistically different (p 0.6). CD8+CD27-CD27-CD28- T lymphocytes increased significantly in the chronically dialyzed versus control group (p 0.02), which was repeated in the uremic versus control group (p 0.004). The increase in the uremic versus chronically dialyzed group was statistically insignificant (p 0.3)

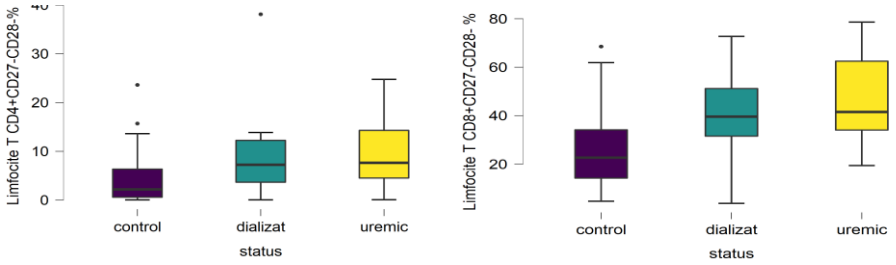

Figura 4.9 : Graphical representation of CD27-CD28- CD4+ and CD8+ T cells percentage between the three groups studied

In our study, in patients undergoing hemodialysis, age correlates only with the percentage of naive lymphocytes, whereas the increased percentages of senescent, proinflammatory, terminally differentiated lymphocytes correlate with each other but are independent of age, which argues for the immunosenescence of dialysis patients regardless of their age.

CHAPTER 5: PHENOTYPIC ANALYSIS OF B LYMPHOCYTES

The number of B lymphocytes does not differ statistically between dialysis and control (p:0.981), between dialysis and uremic (p: 0.683) or between control and uremic (p:0.669)

In terms of transitional B lymphocytes there is no statistically significant correlation between control and chronically dialyzed patients (p:0.82). The uremic patients have a statistically significantly lower percentage of transitional lymphocytes compared to the dialysis patients (p:0.04) and compared to the control group the decrease is also statistically significant with a p less than 0.001. So uremic patients have significantly fewer transitional B lymphocytes which seems to correct with the initiation of chronic dialysis.

As regards naive B lymphocytes, if we statistically analyze the dialyzed group compared to the control group, we can observe a significantly increased percentage (p:0.03) of naive B lymphocytes in the group of dialysis patients. Also patients on in chronic hemodialysis program have a significantly higher percentage (p:0.05) of naive B lymphocytes (p:0.05) than uremic patients. As for the group of patients with end-stage chronic kidney disease undialyzed there are no significant differences from the control group (p:0.893). These results highlight the increase in the percentage of naïve B lymphocytes only in dialyzed patients which is not found in uremic patients. (Figure 5.1). The studies performed so far describe a higher percentage of immature B lymphocytes in chronic dialysis patients compared to uremic patients which is confirmed in our study for both transitional and naive lymphocytes [26]

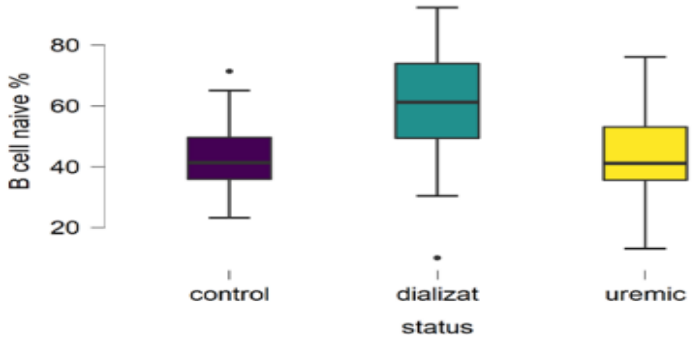

Figura 5.1: Graphical representation of naive B cells percentage between the three groups studied

The percentage of unswitched B-lymphocytes is significantly increased in uremic patients compared to those on hemodialysis (p: 0.005) and to the control group (p:0.035), while there are no statistically significant differences between the dialysis and control groups (p 0.3) although the dialyzed patient tends to have fewer unswitched B lymphocytes compared to the control group (Figure 5.2).In other words unswitched B lymphocytes are elevated in uremic patients before the start of dialysis and show a significant decrease once they enter hemodialysis.

In the case of the percentage of B lymphocytes switched statistically no significant differences were found between the studied groups: p:0,217 for control/dialyzed, p:0,139 for dialyzed/uremic and p:0,887 for control/uremic

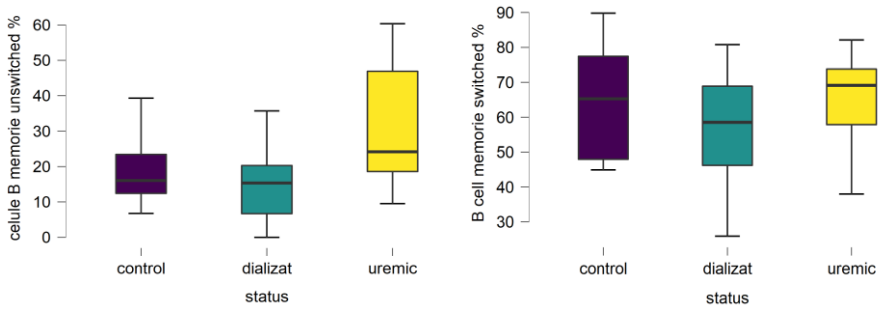

Figura 5.2: Graphical representation of unswitched and switched B cells percentage between the three groups studied

If we statistically analyze the differences between the dialysate/control, uremic/dialysate and uremic/control groups for CD21lowCD38low B lymphocytes, it can be observed that between the dialysate and control groups there is no statistically significant difference (p:0.103) instead undialyzed uremic patients have a significantly higher (p:0.010) percentage of CD21lowCD38low B lymphocytes CD21lowCD38low than dialysis patients and non-significantly higher (p:0.143) than control possibly being an indicator of chronic inflammation in

these patients. In other words, with the start of hemodialysis as a treatment method the percentage of CD21lowCD38low B lymphocytes shows a significant decrease in dialysis patients (Figure 5.3).

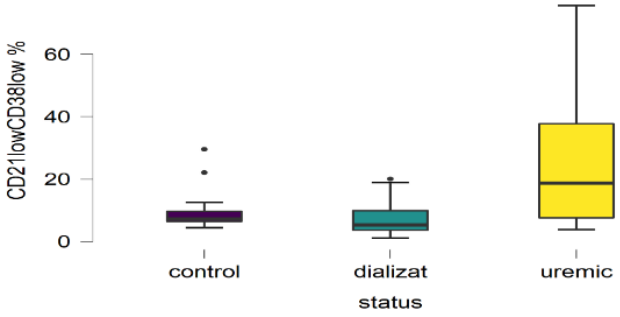

Figura 5.3: Graphical representation of CD21lowCD38low B cells percentage between the three groups studied

The percentage of plasmablasts is significantly lower in uremic patients than in dialysis patients but not in the control group, which also has a lower but statistically insignificant percentage of plasmablasts, which is an indicator of inadequate immune response in patients with stage 5 chronic kidney disease and improves with the start of dialysis (Figure 5.4).

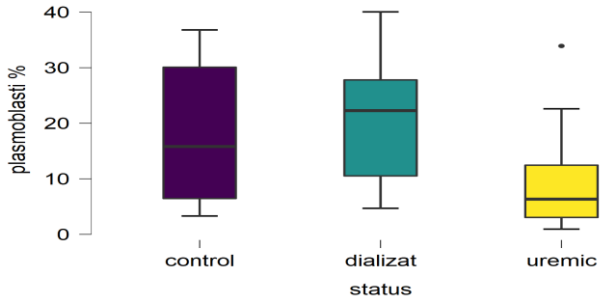

Figura 5.4: Graphical representation of B plasmablasts percentage between the three groups studied

In our study the number of lymphocytes decreases in both uremic and chronically dialyzed patients compared to control, the percentage of naive B lymphocytes is increased in dialyzed patients compared to uremic or control, the percentage of unswitched memory B lymphocytes increases in uremic patients compared to dialyzed and control but unswitched memory B lymphocytes remain unchanged, the percentage of plasmablasts is low in uremic versus dialysis patients and it is possible that there are differences in response to vaccination in uremic versus chronic dialysis patients, and transitional B lymphocytes are low in uremic versus dialysis or control group. These results are comparable to those obtained by Kyoung Woon Kim

et al in 2012 when they publish a study comparing B lymphocyte subpopulations between 27 chronic hemodialysis patients, 17 chronic kidney disease patients on pre-dialysis and 27 healthy volunteers based on the observation that only 50-75% of end-stage chronic kidney disease patients develop protective antibodies after hepatitis B vaccination so there are changes in B lymphocyte immunity[4,26]. The results of the study showed that the percentage of CD19+ B lymphocytes did not differ in the three groups, the percentage of immature B lymphocytes was higher in the hemodialysis group than in the predialysis group and the percentage of memory B lymphocytes was higher in the predialysis group than in the hemodialysis group [4,26]. According to the authors of this study the decrease in the percentage of immature B lymphocytes and the increase in the percentage of memory B lymphocytes in predialysis patients would be the consequence of the imbalance of balance between effector and regulator action [4,26]. Unexpectedly in the present study there were no statistically significant differences between the healthy-control group and predialysis patients [4,26]. In dialysis patients there was a decrease in memory B lymphocytes compared to the control group [4,26] which was not observed in our study.

Uremia is responsible for the reduction in the number of naive and memory B lymphocytes leading to a deficient humoral immune response in patients with end-stage chronic kidney disease in the setting of infection or vaccination [3, 4] . As observed in our study undialyzed uremic patients have fewer naïve B lymphocytes compared to dialyzed patients but have more CD21lowCD38low (proinflammatory) B lymphocytes and more unswitched memory B lymphocytes compared to dialyzed patients. Study published in 2010 by Madeleine V. Pahl et al performed on 21 stable patients undergoing hemodialysis versus 21 control patients showed decreased numbers of all B lymphocyte subpopulations except transitional B lymphocytes, without demonstrating increased B lymphocyte apoptosis in the chronically hemodialyzed versus non hemodialyzed patient[4,27].This decrease is attributed to down-regulation of the BAFF receptor on transitional B lymphocytes. The result is a resistance to the action of BAFF (B-cell-activating factor of the tumor necrosis factor family) which plays an important role in B lymphocyte differentiation and survival [4,27]. In our study we did not show these decreases, but it should be taken into account that the dialyzers used nowadays are more and more efficient, the number of hemodiafiltration sessions has increased, the efficiency of hemodialysis sessions is

continuously increasing, which probably reflects in the improvement of immunity disorders in chronically dialyzed patients.

CHAPTER 6: PHENOTYPIC ANALYSIS OF MONOCYTES

Results of the study show that the percentage of classical monocytes decreases significantly in both dialysis and uremic groups compared to control with a more pronounced non-significant decrease in the dialysis group and the percentage of proinflammatory CD16+ monocytes (non-classical and intermediate) are increased in both dialysis and uremic patients which supports the proinflammatory status of the end-stage kidney disease patient.

Highlighting the preferential increase of proinflammatory monocytes is important for the clinician because this increase is directly related to the pathology of the patient with chronic kidney disease.

Classical monocytes have a role in tissue repair and immune response (phagocytosis), intermediate monocytes are responsible for T cell stimulation and proliferation, produce the largest amount of ROS and have a role in angiogenesis and non-classical monocytes produce CD4+ T cell stimulation and proliferation, produce proinflammatory cytokines and migrate through the vascular endothelium [28].

Increased intermediate monocyte burden is associated with progression of chronic kidney disease [28]. The number of intermediate monocytes in the dialyzed patient is an important predictor of cardiovascular events [28]. Also an increased number of nonclassical monocytes are found in patients with end-stage chronic kidney disease [28].

The identification of monocytic subpopulations and their proinflammatory action could help as biomarkers in various diseases including cardiovascular diseases in uremic patients for risk quantification, also they can be markers of evolution under different treatments applied including uremic patients (e.g. anti-inflammatory) [28]. Also finding a therapeutic modality to decrease proinflammatory monocytes in uremic patients may influence the progression of cardiovascular disease in these patients.

A study by V. Liakopoulos et al and published in 2018 on 15 hemodialyzed patients and 16 healthy individuals-control subjects highlights a significant increase in monocyte counts and a different distribution of monocyte subgroups corresponding to proinflammatory status: classical monocytes decrease significantly, while intermediate and nonclassical monocytes increase statistically significantly as in our study [29].

Another study published in 2017 by Yachung Jeng et al on a group of 136 chronic hemodialysis patients shows that increased CD16+ monocyte CD16+ count increases the risk of cardiac mortality and mortality from other causes especially in dialysis patients with a history of coronary artery disease [10]. Assessment of microinflammation could help clinicians to recognize at-risk patients, reduce inflammation, in the future may regulate immune system function thereby reducing mortality [10].

A study published in 1998 by W.A. Nockher et al. showed an increase in CD16+ monocytes in chronically hemodialyzed patients, which show an additional increase in case of infection. In addition they express a higher level of HLA-DR on the surface [30].

The increase of CD16+ monocytes in hemodialyzed patients is followed by endothelial injury and thus plays a role in the increased morbidity and mortality rate of these patients (CD14+CD16+ monocytes produce proinflammatory cytokines and have an increased ability to migrate into the vascular wall, express a large number of adhesion molecules) [31].

Patients with chronic kidney disease and especially hemodialyzed patients show oxidative stress, increased ROS production and defective phagocytosis capacity possibly explained by increased intermediate monocytes and decreased classical monocytes [30]. Recent research shows that monocytes express components of the angiotensin system [30]. Study published in 2012 by A Merino et al highlights that Losartan-type angiotensin receptor blocker treatment decreases monocyte activation in dialysis with a decrease in CD14+CD16+(nonclassical monocytes) monocyte numbers during treatment [32].

Microinflammation is common in patients with chronic kidney disease and plays an important role in the development of cardiovascular disease. Patients with chronic kidney disease frequently associate inflammation with malnutrition and atherosclerosis, the presence of which is referred to as MIA syndrome, these being partly due to the expansion of proinflammatory CD14+CD16+ monocytes. CD14+CD16+ monocytes also correlate with the grade of chronic

kidney disease and with the vascular stiffness index, with vascular stiffness being reported in end-stage renal disease, particularly in chronically dialysed patients [33].

Study published in 2018 by S.D. Naicker et al highlights the increase of intermediate monocytes with the progression of chronic kidney disease and especially HLADR high intermediate monocytes show significant increase [34]. Heine et al showed that the number of intermediate monocytes still increases in the stage of pre-dialysis patients and the number of non-classical monocytes increases only in dialysis patients [34]. These results were also evidenced in the present study with the difference that in our study percentage increases of both intermediate and nonclassical monocytes were reported in dialyzed and uremic patient.

In our study the classical monocytes decreased significantly in percentage in the dialyzed (p less than 0.001) and uremic (p 0.003) patients compared to the control group with a trend to decrease in the dialyzed patients compared to the uremic (p 0.08) (Figure 6.1), these decreases being also reported in the literature [29].

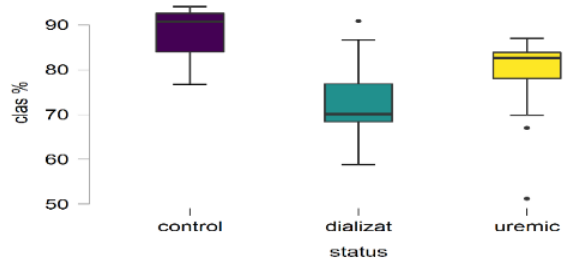

Figura 6.1 Graphical representation of classical monocytes percentage between the three groups studied

Nonclastic monocytes are significantly increased in percentage of dialysis (p less than 0.001) and uremic (p 0.04) patients compared to control, nonclastic monocytes are also significantly increased in percentage of dialysis group compared to uremic (p 0.04) (Figure 6.2). These results are also consistent with the literature [28,29].

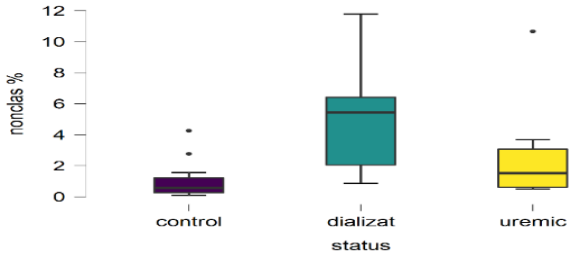

Figura 6.2 Graphical representation of nonclassical monocytes percentage between the three groups studied

In the case of intermediate monocytes there are no significant differences between the dialyzed and uremic groups (p 0.9) but there is a significant percentage increase in both the dialyzed (p less than 0.001) and uremic (p 0.004) groups compared to the control group. (Figure 6.3), and these results are also consistent with data obtained from previous studies[28,29].

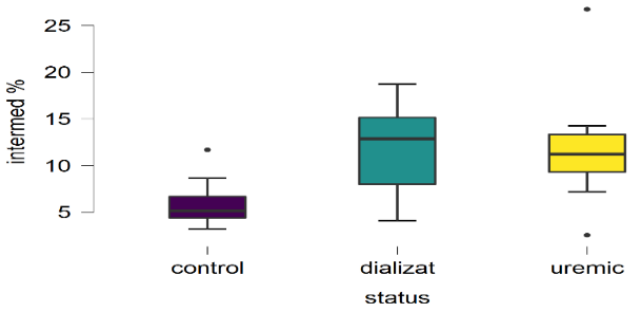

Figura 6.3 Graphical representation of intermediate monocytes percentage between the three groups studied

In our study, the percentage of CD300e+ monocytes is significantly higher in the dialyzed patient than in the uremic patient (p 0.03) (Figure 7.9).

Figura 6.4: Graphical representation of CD300e+ monocytes percentage between the three groups studied

The percentage of CD300e+ classical monocytes is also significantly increased in the dialyzed patient (p 0.003) compared to the uremic patient (Figure 6.5).

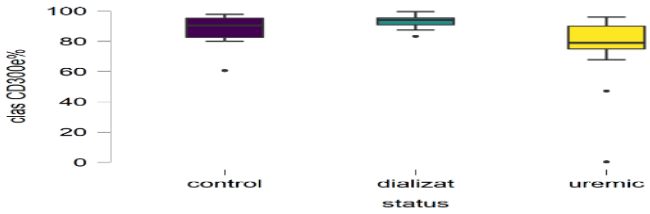

Figura 6.5: Graphical representation of CD300e+ classical monocytes percentage between the three groups studied

The percentages of CD300e+ non-classic CD300e+ monocytes are significantly higher in dialysis patients compared to uremic patients (p 0.004) and lower in uremic patients compared to control patients (p 0.02) (Figure 6.6).

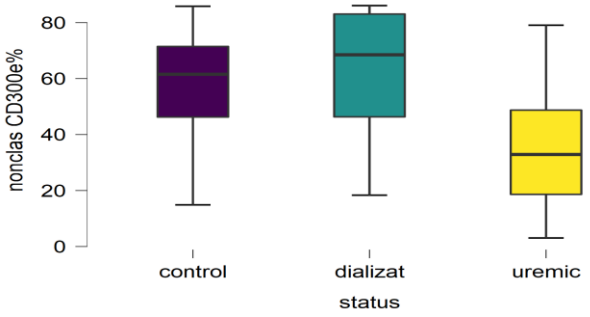

Figura 6.6: Graphical representation of CD300e+ nonclassical monocytes percentage between the three groups studied

The percentages of CD300e+ intermediate monocytes are not statistically different statistically comparing the three groups (uremic, dialysis and control) (Figure 6.7).

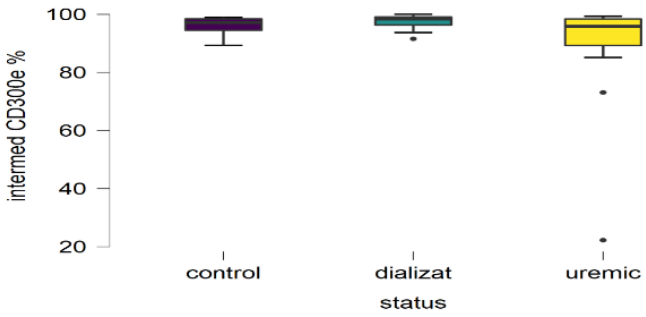

Figura 6.7: Graphical representation of CD300e+ intermediate monocytes percentage between the three groups studied

CD300e is considered an immune-activating receptor, able to modulate the inflammatory response, its activation being followed by the release of proinflammatory cytokines [35]. CD300e activation is followed by monocyte activation, increases monocyte survival, increases the production of proinflammatory cytokines and increases the expression of costimulatory molecules which finally leads to T lymphocyte activation [36]. In our study we noticed an increase in the percentage of CD300e+ monocytes especially in chronically dialysis patients, also we noticed an important increase in the percentage of nonclassical C D300e+ monocytes in

chronically dialysis patients. In other words, with chronic dialysis treatment nonclassical monocytes are immune activated and the production of proinflammatory cytokines increases.

The age of the patients correlated only in the control group negatively with the percentage of classical monocytes (p:0.002), positively with the percentage of non-classical monocytes (p:0.020) and positively with the absolute value of non-classical monocytes (p:0.044). There were no significant age-related correlations for any parameter in the uremic or dialysis patient groups.

We could say that the changes in monocyte populations are age-independent in patients with chronic kidney disease, possibly related to uremia or dialysis-induced changes and are one of the causes of immunosenescence in these patients.

CHAPTER 7: PHENOTYPIC ANALYSIS OF LEUKOCYTE POPULATIONS DURING COVID INFECTION

In our study, during Covid infection the number of leukocytes (p 0.02) and lymphocytes (p 0.02) decrease statistically significantly compared to the period before infection. There are no significant differences between leukocyte counts during infection/post Covid (p 0.2) or before infection compared to after infection (p 0.1) Similarly lymphocyte counts do not differ significantly between Covid/post Covid (p 0.3) or ante Covid/post Covid (p 0.5) determinations (figure 7.1)

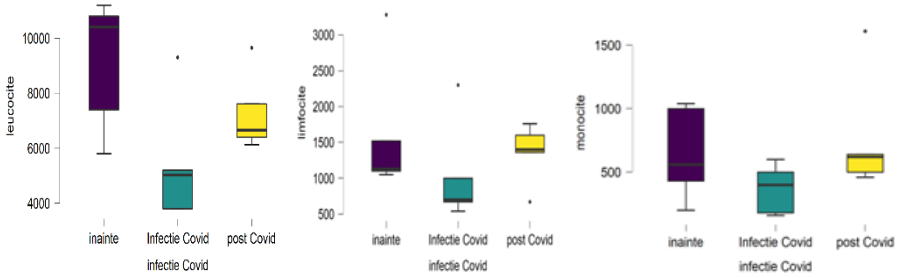

Figura 7.1: Graphical representation of leukocytes, lymphocytes and monocytes count before, during and after Covid infection

7.1 T lymphocytes in Covid infection

The number of T lymphocytes decreases statistically significantly (p 0.04) in Covid-infected patients compared to the pre-Covid period with no statistical significance between the during- and post-Covid determinations (p 0.3). There is also no statistical significance between pre Covid infection and post Covid determinations (p 0.3). From a percentage point of view we have comparable values in pre Covid/Covid (p 0.3), Covid/post Covid (p 0.8) and post Covid there is a significant percentage decrease in T lymphocytes compared to pre Covid (p 0.01) (figure 7.2).

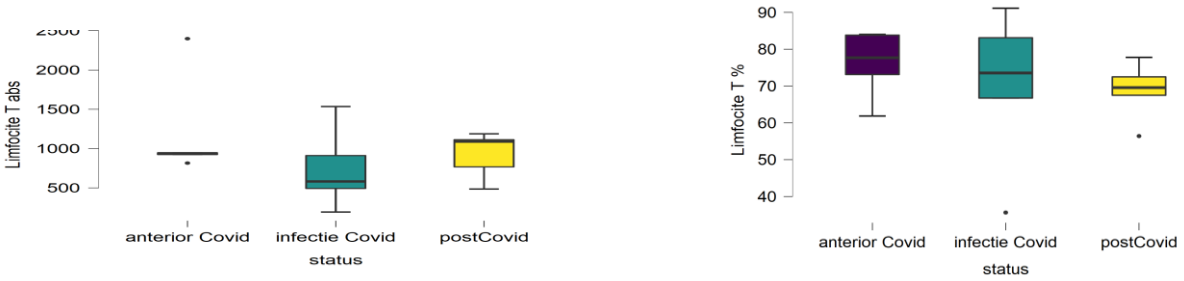

Figura 7.2: Graphical representation of T cells count and percentage before, during and after Covid infection

In the case of CD4+ T lymphocytes there are no statistically significant changes between the values studied, p ranging between 0.11 and 0.88. In other words Covid infection does not seem to influence CD4+ T lymphocytes. The number of CD8+ T lymphocytes also does not seem to be influenced by the Covid infection: they decrease insignificantly (p 0.07) during the infection compared to before, we obtain a p 0.3 between the values during infection compared to post Covid and p 0.1 if we compare the pre Covid values with the post Covid values. The percentage values show us a non-significant increase during Covid infection compared to pre Covid (p 0.1) but we observe significant decreases in CD8+ T lymphocytes post Covid compared to infection (p 0.002) and also significant percentage decrease post Covid compared to pre Covid values (p 0.05) (figure 7.3).

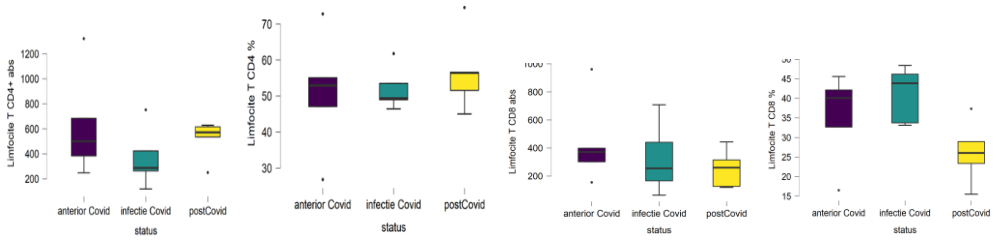

Figura 7.3: Graphical representation of CD4+ and CD8+ T cells count and percentage before, during and after Covid infection

The CD4+/CD8+ T lymphocyte ratio was not influenced by Covid infection, we obtained p values between 0.2 and 0.9.

CD4+ effector memory T lymphocytes do not vary significantly during Covid infection with p between 0.1 and 0.2. In the case of CD8+ effector memory T lymphocytes, there is an increase during Covid infection without statistical significance (p 0.1) and the percentage decreases significantly (p 0.02) post Covid compared to the values obtained during infection.

The results suggest that Covid infection does not influence T lymphocytes effector memory, also they are not affected post Covid.

In the case of effector T lymphocytes, TEMRA, CD4+ TEMRA T lymphocytes, although the percentages are small, increase insignificantly during Covid infection (p 0.4), followed by a statistically insignificant increase post Covid compared to the values during infection (0.4), and then in the post Covid period we identify a significant increase of these lymphocytes compared to the period before the disease (p 0.01). CD8+ effector T-lymphocytes vary to the same extent, increasing slightly, insignificantly during the illness (p 0.4), but increasing post Covid compared to values during the illness (p 0.2) to identify statistically significantly increased percentage values post Covid compared to the period before the illness (p 0.01) (figure 7.4).

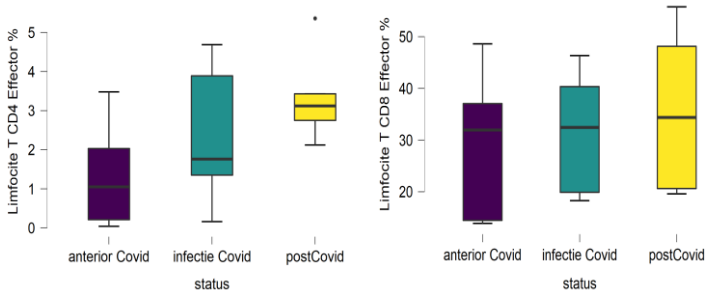

Figura 7.4: Graphical representation of effector CD4+ and CD8+ T cells percentage before, during and after Covid infection

From the results presented we have leukopenia, lymphopenia, decreased T lymphocyte count during Covid infection all being reversible post Covid. Post Covid there is however a significant decrease in the percentage of T lymphocytes compared to before the disease. The percentage of CD8+ T-lymphocytes post Covid also decreases significantly compared to before or during the disease. The most interesting results are however in the CD8+ lymphocyte compartment. TEMRA CD4+ and CD8+ T-lymphocytes are significantly increased post Covid compared to the pre-illness period. During Covid infection the percentage of naive CD8+ T lymphocytes also decreases significantly and the percentage of CD57+ CD8+ T lymphocytes increases significantly, probably due to increased inflammation, but these changes are reversible post Covid. The most important result of this study I think is the increase in the percentage of highly differentiated lymphocytes at a distance from Covid infection, we can suggest that this infection independently contributes to the immune system semescence and probably accelerates the atherosclerosis process.

7.2 B lymphocytes in Covid infection

B-lymphocytes, essential elements of the humoral immune response, of the body's defense against viruses, are an important element in the body's immune protection mechanism against SARS-CoV-2. After infection with SARS-CoV-2, naive B lymphocytes are activated and transform into short-lived plasma cells secreting low affinity antibodies. Some of the B lymphocytes enter the germinal center where they undergo the switch phenomenon to transform into long-lived, high-affinity antibody-producing plasma cells and memory B lymphocytes [37]. In particular, it has been observed that in SARS-CoV-2 infection IgG production occurs almost simultaneously with IgM and IgA production within the first 2 weeks after the onset of symptoms. Another peculiarity is that the amount of protective antibodies is higher in patients with severe disease than in patients with moderate disease or those with reinfections also with moderate disease [38].

The results of our study show that the number and percentage of B lymphocytes is decreased during SARS Cov 2 infection, this decrease being statistically significant: p:0.020 for the number of B lymphocytes and p:0.006 for the percentage of B lymphocytes (Figure 7.5).

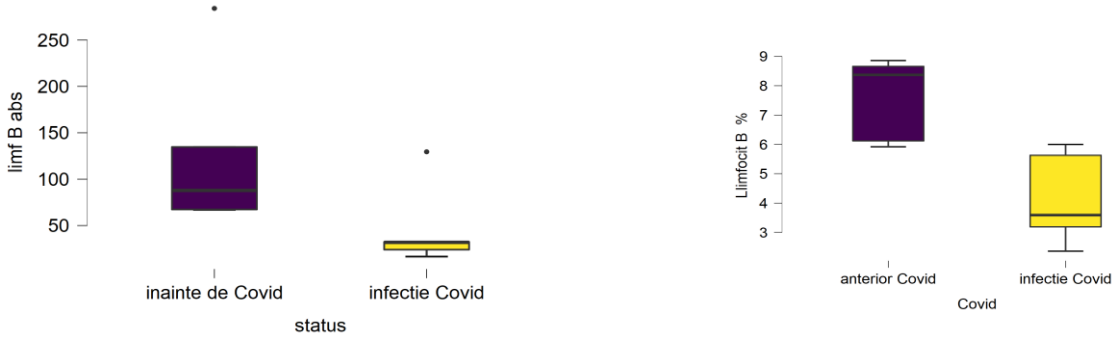

Figura 7.5: Graphical representation of B cells count and percentage before and during Covid infection

Unswitched B lymphocytes decreased slightly in percent during Covid infection but without statistical significance (p:0.471).

The percentage of switched memory B lymphocytes decreases percent during infection, the decrease being statistically significant (p:0.021) (Figure 7.6).

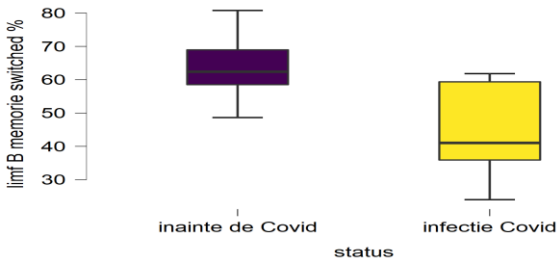

Figura 7.6: Graphical representation of switched memory B cells percentage before and during Covid infection

The percentage of Plasmoblasts shows an increase during COVID infection which is statistically significant (p:0.042) (Figure 7.7).

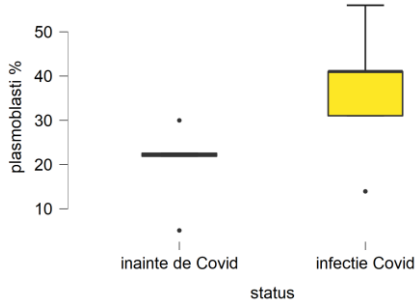

Figure 7.7: Percentage of Plasmoblasts before and during Covid infection

In conclusion during Covid infection dialysis patients show a significant decrease in lymphocyte and B-lymphocyte counts. The percentage of marginal B lymphocytes, naive B lymphocytes and transitional B lymphocytes do not change statistically significantly but memory switched B lymphocytes decrease during infection while the percentage of plasmablasts increases statistically significantly during Covid infection suggesting an increased immune activity with emphasis on the transformation of B lymphocytes into antibody-producing plasma cells with a probable focus on short-lived plasma cells if the percentage decrease of switched B lymphocytes is to be taken into account. This could be explained by the fact that sampling was performed immediately after the onset of disease symptoms and positive Covid tests i.e. within the first days of infection.

A study published in 2022 but excluding patients with chronic kidney disease or significant comorbidities showed a correlation between the mortality risk of Covid 19-infected patients and low B-lymphocyte counts, low naïve B-lymphocyte counts and low memory switched B-lymphocyte counts. As in the present study the patients were analyzed in the acute period of the disease [39]. The currently studied patients diagnosed with renal failure and Covid infection have a similar profile of B lymphocyte damage.

7.3 Monocytes in Covid infection

A study published by Arianna Gatti et al in July 2020 showed a decrease in the number of nonclassical and intermediate monocytes, thus in the number ofCD16+ monocytes in severe forms of Covid's disease and their paradoxical increase in moderate forms of the disease [40,41].

In our study if we analyze the monocyte subsets in patients with moderate forms of Covid infection and end-stage chronic kidney disease, a different distribution of the inflammatory response is observed with a decrease in the percentage of non-classical monocytes similar to severe forms of the disease but without worsening of the patient's condition. Also, nonclassical monocytes express a higher level of HLA-DR in our patients contrary to the reports for patients with intermediate forms of Covid infection without end-stage chronic kidney disease [40,42]. The percentage of intermediate monocytes tends to increase as in intermediate forms of Covid infection without chronic kidney disease. HLA-DR expression in intermediate monocytes also increases, contrary to the decrease observed and reported in other studies in patients with intermediate forms of Covid infection without kidney disease [40,42]. These changes might suggest increased antiviral activity in nonclassical monocytes and increased proinflammatory and phagocytic activity in intermediate monocytes in patients with end-stage chronic kidney disease and intermediate forms of Covid infection. Analyzing the results obtained approximately 10 months after Covid infection it appears that the proinflammatory status of monocytes is maintained months after infection [40].

Classical monocytes seem to be less affected during Covid infection and we cannot say in this study that they show increased activity during infection or after healing [40].

As for nonclassical monocytes, they show a significant decrease during Covid infection and also show a significantly increased expression of HLADR+ expression and a trend to increased expression of CD300e expression contrary to other studies occasionally reporting a

decrease in HLADR+ expression in patients with intermediate forms of disease without end-stage chronic kidney disease [40,42]. In conclusion, although low in percentage, they show increased activation.

Approximately 10 months after healing of the infection, the percentage of non-classical monocytes continued to decline and to lose the expression of activation markers due to Covid infection [40] (Figure 7.8).

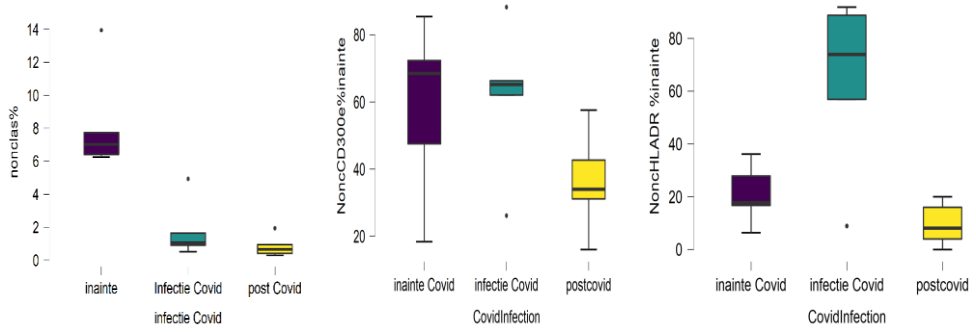

Figura 7.8: Graphical representation of nonclassical monocytes percentage, CD300e+ and HLADR+ expression before, during and after Covid infection

Contrary to nonclassical monocytes, the percentage of intermediate monocytes has a tendency to increase during the course of the disease comparable to other studies performed on patients with undialyzed intermediate forms of the disease but with a tendency to increase HLADR+ expression contrary to other studies showing a decrease in HLADR+ expression in patients with undialyzed intermediate forms of the disease and the increase in HLA-DR expression persists after the infection is cured despite the fact that the percentage of intermediate monocytes decreases after infection. [40,42].

Thus, as in the case of nonclassical monocytes, the percentage of intermediate monocytes decreases after Covid infection to lower values than before infection but they acquire an important proinflammatory activity with a significant increase in HLA-DR expression compared to before infection. We consider this as the most important result of the study of monocytes during Covid infection in patients with end-stage chronic kidney disease: the persistence of the proinflammatory character of intermediate monocytes because they are involved in increased morbidity and mortality from cardiovascular causes in the chronic dialysis patient.

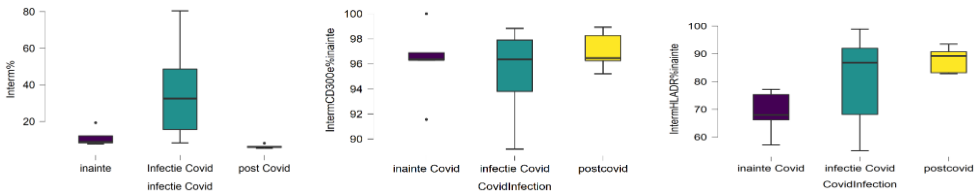

Figura 7.9: : Graphical representation of intermediate monocytes percentage, CD300e+ and HLADR+ expression before, during and after Covid infection

We could say that Covid infection acts as a trigger for the proinflammatory activation of intermediate monocytes [40] (Figure 7.9).

HLADR+ and CD300e+ expressions in the whole monocyte population during Covid infection show an increasing trend for both CD300e and HLADR expression similar to other studies performed in patients with intermediate forms of disease but undialyzed. We could consider that end-stage chronic kidney disease treated by hemodialysis does not change the inflammatory response against SARS-Cov2 in the middle forms of disease [40].

On the other hand, determining the expression of CD300e and HLA-DR in the whole monocytic population shows a significant increase in their expression after the cure of Covid infection, which would be a further indicator of proinflammatory activation of monocytes secondary to Covid infection. It is as if the infection alters the long-term proinflammatory status of monocytes with all the consequences that derive from this [40]. Interesting is the observation of the increase, at a distance after the cure of the COVID infection, of the percentage expression of CD300e+ (p:0.025) monocytes (p:0.025) compared to the values before infection and p=0.036 compared to the values during infection) and HLADR+ (p=0.009) monocytes (p=0.009) compared to the values before infection). The percentage of HLADR+ monocytes did not differ significantly during COVID infection compared to post-infection values (p=0.625). These results would indicate that after COVID infection the monocytes of chronically hemodialyzed patients remain with a significant proinflammatory status which certainly further contributes to the progression of immunosenescence [40] (Figure 7.10).

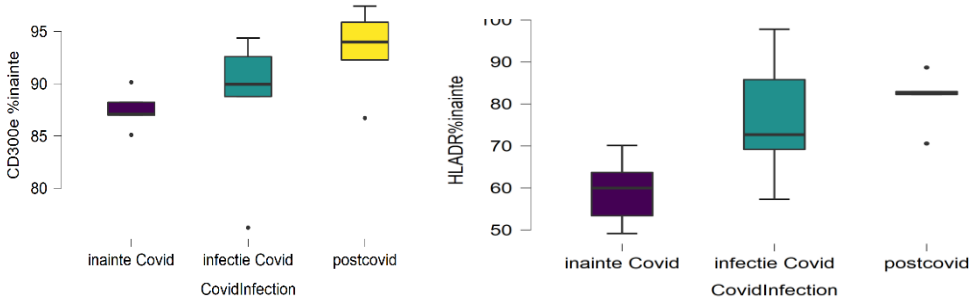

Figura 7.10: Graphical representation of CD300e+ and HLADR+ monocytes percentage before, during and after Covid infection

In the coming years these patients should be monitored to see if this proinflammatory status persists, what are the consequences, what comorbidities occur and in particular cardiovascular events [40].

CHAPTER 8: CONCLUSIONS AND PERSONAL CONTRIBUTIONS

The aim of the research work was to determine to what extent immunity is impaired in patients with stage 5 chronic kidney disease prior to dialysis entry and to what extent it is altered in stable patients already on chronic dialysis. After enrolling patients in the study and performing the analyses we were confronted with the Covid pandemic which provided the opportunity to see how the immune system of chronic dialysis patients reacts to this challenge, before they could be vaccinated thus without immunization against the disease. Also, as these patients were chronically dialyzed and thus could be continuously monitored, they could be retested at a distance from the time of illness to see what the immune system might be affected by this infection.

T-lymphocyte, B-lymphocyte and monocyte subtypes were considered and the analyses were carried out in accordance with the recommendations of the immunophenotyping groups for the diagnosis, screening and classification of immunodeficiencies published in the EuroFlow educational book.

Many of the results obtained were comparable with those in the literature but we also obtained a number of different results. The immunophenotyping technique by which the patient samples were processed is a rapid diagnostic method, reliable but somewhat limited by high cost.

The results obtained during the study show a higher leukocyte count in the undialyzed uremic patient, which returns to values comparable to those of the control group after dialysis initiation. In contrast, both dialysis and uremic patients show significant lymphopenia compared to the control group, a decrease which is more pronounced in the uremic patients. In other words, hemodialysis treatment partially improves the lymphopenic status of uremic patients without resolving the lymphopenia secondary to the uremic environment.

T-lymphocytes are low in undialyzed uremic patients in particular, slightly increased in chronically dialyzed patients but remain statistically significantly low compared to the control group. In contrast, B lymphocyte counts do not differ statistically significantly between the three groups studied. In conclusion, we can say that the lymphopenia occurring in uremic patients is mainly due to a decrease in the number of T lymphocytes, an immunodeficiency in cellular immunity.

The analysis of T lymphocyte subtypes shows that CD4+ T lymphocytes are significantly decreased in uremic patients (undialysed and chronically dialysed) compared to control, and CD8+ T lymphocytes are significantly decreased only in uremic patients compared to control, and after the initiation of dialysis the values are comparable to control. The explanation for the unaffected CD8+ T-lymphocyte CD8+ T-lymphocyte count is the increase in the population of highly differentiated lymphocytes, in other words the count is maintained by the increase in that lymphocyte population with high cytotoxicity. Percentage-wise there are no significant variations between the three studied batches neither for CD4+ nor for CD8+ T lymphocytes, the batches have the same disposition of lymphocyte populations for CD4+ and CD8+.

We have comparable values of the CD4+/CD8+ T lymphocyte ratio but here we should mention a higher subunit percentage in chronic dialysis and non-dialysis uremic patients showing a tendency to inversion of the ratio in uremic patients that does not correct itself, but on the contrary, seems to increase with the chronic hemodialysis treatment.

In the statistical analysis of the different T lymphocyte subpopulations, percentage analysis was preferred because it better reflects their distribution, decreasing or increasing trends with significantly different CD4+ or CD8+ T lymphocyte counts between the populations studied.

Unexpectedly, the percentage of CD4+ central memory T lymphocytes increases significantly in uremic and chronically dialyzed patients compared to the control group, the percentage of CD8+ central memory T lymphocytes does not change in the three groups studied and if we analyze the number of CD4+ central memory T lymphocytes they decrease significantly only in uremic patients compared to the control group (result in correlation with the literature) and the number of CD8+ central memory T lymphocytes remains unchanged in our study among the three groups studied. The study results do not show a significant impairment of central memory T lymphocytes in chronically dialyzed patients in contradiction with the literature.

Highly differentiated CD8+ T-lymphocytes are significantly higher in chronically dialyzed patients than in controls. In conclusion, as uremic patients undergo chronic hemodialysis treatment, the proportion of cytotoxic CD8+ T lymphocytes increases, immunosenescence progresses. In the case of CD4+ T lymphocytes, which are poorly studied,

appear to have a protective role and are associated with viral infections, they are significantly decreased in both undialyzed uremic and chronically dialyzed uremic patients compared to controls.

The results were clear in terms of CD4+ and CD8+ naïve lymphocytes, which were significantly, lower in both undialyzed and chronically dialyzed uremic patients.

In other words, in the studied groups immunosenescence occurs as chronic kidney disease progresses and worsens with the initiation of renal dialysis. In the present study the most important changes in T lymphocyte distribution showing aged lymphocyte populations in uremic patients are the decline of naïve T lymphocytes and the expansion of highly differentiated or senescent T lymphocytes.

Senescent CD57+ T lymphocytes increase significantly only in the CD4+ T lymphocyte compartment in both chronic dialysis and uremic patients and in the CD8+ compartment the increase is significant only in undialyzed uremic patients, the percentage seems to improve in dialysis-treated patients.

CD4+ PD1+ T-lymphocytes are decreased in percentage of uremic patients in the two groups probably resulting in a dysfunction in the regulation of T-lymphocyte activity in immune tolerance.

The results highlight the increased percentage of CD28-CD27- T lymphocytes in both CD4+ and CD8+ groups studied, which could be correlated with the pathology dominated by chronic inflammation and atherosclerosis of the beaver patients, a situation that does not improve with chronic dialysis.

Correlation analyses yielded interesting results. Age correlated in the control group, as expected, with the percentage of CD4+ central memory, naïve, CD57+, CD27-CD28- T lymphocytes and in the CD8+ group with central memory, naïve, CD57+, CD27-CD28- T lymphocytes. In the group of dialyzed patients age correlated only with CD4+ and CD8+ naive lymphocytes and in the undialyzed uremic patients age did not correlate at all. The conclusion is that the changes that occur in the immunosenescent context in uremic patients are largely independent of the age of these patients, a situation that is not found in the control group. On the other hand, in the control group, undialyzed uremic patients as well as chronically dialyzed patients, we observe a correlation between the number of central memory lymphocytes with the number of lymphocytes, T lymphocytes and TCD4+ and CD8+ lymphocytes, the conclusion

being that the lymphopenia described in the literature for central memory lymphocytes is related to the lymphopenia of the uremic patient. For the decrease in the percentage of naive T lymphocytes apart from the mentioned correlation related to age we have no other correlations, it seems to occur independently, it is possible that it is related to the uremic environment and immunosenescence. Expected positive correlations also appear between the percentages of highly differentiated lymphocytes.

Covid infection is dominated by leukopenia, lymphopenia and T-lymphocyte count decrease, which subsequently remits. The percentage of CD4+ TEMRA CD4+ T-lymphocytes increases significantly post Covid compared to the pre-Covid period probably as a reaction to the viral infection but most importantly the increase in the percentage of CD8+ TEMRA CD8+ T-lymphocytes post Covid suggesting that this viral infection worsens the immunosenescence of chronically dialyzed patients. Covid infection affects naive CD8+ lymphocytes which decrease significantly in percentage during infection but this decrease is reversible postCovid. The changes occurring in CD57+ or CD27-CD28- T lymphocytes during Covid infection were reversible after cure.

As for the B lymphocyte, it is less affected, there are no differences between the studied groups.

Analysis of B lymphocyte subtypes showed surprising results. The percentage of naive B-lymphocytes is unexpectedly increased in the chronically dialyzed group compared to the control or uremic undialyzed group.

Undialyzed uremic patients have a significantly higher percentage of CD21lowCD3838low proinflammatory B lymphocytes than chronically dialysis patients, concluding that dialysis as a treatment method is effective in this proinflammatory subtype.

Also the percentage of unswitched B lymphocytes is significantly increased in uremic patients compared to control and dialysis patients.

Plasmablasts decrease significantly in uremic patients compared to dialysis patients, after starting chronic dialysis this percentage tends to be comparable to the control group which would suggest a dysfunction in humoral immunity especially in the undialyzed uremic patient.

Uremic patients also have significantly fewer transitional B-lymphocytes than controls or dialysis patients, and this decrease seems to correct with the start of dialysis.

In conclusion, the unedialyzed uremic patient presents important distribution changes at the level of B lymphocyte subpopulations with an increase in the percentage of proinflammatory lymphocytes, an increase in the percentage of unswitched lymphocytes, a decrease in the percentage of transition lymphocytes and plasmablasts but these changes occur in the B lymphocyte tend to improve after the start of hemodialysis treatment.

During Covid infection the number of B lymphocytes decreases, the percentage of switched memory B lymphocytes decreases significantly during infection, the percentage of plasmablasts increases significantly during infection and this increase suggests increased immune activity.

The number of monocytes is comparable in the three groups but chronically dialyzed patients have a significantly higher percentage of CD300e monocytes than undialyzed uremic patients, the percentage of HLADR+ monocytes being comparable in all three groups studied. CD300e is considered an immune activating receptor, capable of modulating the inflammatory response, its activation being followed by the release of proinflammatory cytokines. Activation of CD300e is followed by monocyte activation, increases monocyte survival, increases production of proinflammatory cytokines and increases expression of costimulatory molecules which ultimately leads to T lymphocyte activation.

If we analyze the monocyte subgroups it is observed that the percentage of classical monocytes decreases in uremic patients in both groups compared to control, non-classical monocytes increase significantly in dialysis and uremic patients compared to control with a significant increase in the dialysis group compared to undialysed uremic patients, also the percentage of intermediate monocytes is increased in dialysis and uremic groups compared to control. We conclude that the percentage of proinflammatory monocytes is increased in uremic patients with the mention that chronic dialysis patients have a higher proinflammatory profile than undialyzed uremic patients. The identification of monocyte subpopulations, which does not require complicated techniques, could be used to identify uremic patients predisposed to an increased risk of chronic inflammation and thus cardiovascular events.

The percentage of CD300e+ classical monocytes is significantly increased in the dialyzed versus the uremic patient and, as with monocytes overall, there are no significant differences in the percentages of HLADR+ classical monocytes between the groups studied.

The percentage of CD300e non-classical monocytes is significantly higher in dialysis patients compared to uremics but also in the control group compared to uremics. These values can in fact be interpreted as a decrease in the percentage of CD300e non-classical monocytes occurring in undialyzed uremic patients. Also the percentages of HLADR+ non-classical monocytes do not vary between the studied groups.

For intermediate monocytes, there were no significant differences for CD300e or HLADR+ monocytes between the three groups studied.

In conclusion, undialyzed uremic patients have a low percentage of CD300e classical and nonclassical monocytes but an increased percentage of monocytes of patients on chronic hemodialysis express more CD300e in monocytes in general, nonclassical and classical monocytes in particular. In other words, with chronic dialysis treatment monocytes and in particular non-classical monocytes are immune activated and the production of proinflammatory cytokines increases.

Another conclusion of the study is that the changes in monocyte populations are age-independent in patients with chronic kidney disease, possibly related to uremia- or dialysis-induced changes and are one of the causes of immunosenescence in these patients.

An observation of monocytic changes during Covid infection is the significant decrease in the percentage of nonclassical monocytes during infection, this decrease being reported in the literature in severe cases of the disease in patients without chronic kidney disease, which may explain the fragility of chronic dialysis patients in the face of this disease with the possibility of having transformed at any time into a lethal form.

These nonclassical monocytes show a significantly increased expression of HLADR+ expression and a trend toward increased expression of CD300e contrary to other studies occasionally reporting a decrease in HLADR+ expression in patients with intermediate forms of disease without end-stage chronic kidney disease. In conclusion, although low in percentage, they show increased activation, both CD300e and HLADR being markers of monocyte activation.

In conclusion, if we analyze the monocyte subsets in patients with medium forms of Covid infection and end-stage chronic kidney disease, a different distribution of the inflammatory response is observed with a decrease in the percentage of non-classical monocytes similar to severe forms of the disease but without worsening of the patient's condition. The

percentage of intermediate monocytes and HLADR expression in intermediate monocytes tended to increase as in intermediate forms of Covid infection without chronic kidney disease. These changes might suggest increased antiviral activity in nonclassical monocytes and increased proinflammatory and phagocytic activity in intermediate monocytes in patients with end-stage chronic kidney disease and intermediate forms of Covid infection. If we analyze the results obtained approximately 10 months after Covid infection it appears that the proinflammatory status of monocytes is maintained months after infection.

Approximately 10 months after healing of the infection, the percentage of non-classical monocytes continued to decrease and to lose the expression of activation markers due to Covid infection.

As with nonclassical monocytes, the percentage of intermediate monocytes decreases after Covid infection to lower values than before infection but they acquire an important proinflammatory activity with a significant increase in HLA-DR expression compared to before infection. We consider this as the most important result of the study of monocytes during Covid infection in patients with end-stage chronic kidney disease: the persistence of the proinflammatory character of intermediate monocytes because they are involved in increased morbidity and mortality from cardiovascular causes in the chronic dialysis patient. We could say that Covid infection acts as a trigger for the proinflammatory activation of intermediate monocytes.

If we determine the expression of CD300e and HLA-DR in the whole monocyte population, we notice a significant increase in their expression after the cure of Covid infection, which would be a further indicator of proinflammatory activation of monocytes secondary to Covid infection. It is as if the infection alters the long-term proinflammatory status of monocytes with all the consequences that derive from this. Of interest is the observation of an increase in the percentage expression of CD300e+ ($p=0.025$ compared to pre-infection values and $p=0.036$ compared to pre-infection values) and HLADR+ ($p=0.009$ compared to pre-infection values) monocytes, at a distance after the cure of COVID infection. The percentage of HLADR+ monocytes did not differ significantly during COVID infection compared to post-infection values ($p=0.625$). These results would indicate that after COVID infection the monocytes of chronically hemodialyzed patients remain with a significant proinflammatory status that certainly further contributes to the progression of immunosenescence.

In the coming years these patients should be monitored to see if this proinflammatory status persists, what are the consequences, what comorbidities occur and in particular cardiovascular events.

The **personal contribution of** this paper is to highlight the worsening of immunosenescence especially in chronic dialysis patients independent of the age of these patients (Chapters 6.4 and 8.5). Also the study performed on chronic dialysis patients with moderate forms of Covid infection showed a worsening of immunosenescence and proinflammatory status of chronic dialysis patients at a distance (about one year) after recovery, results that were partially published in Frontiers of Immunology, as no other study of this kind was found in the literature at the time of publication (chapters 6.4, 7.4 and 8.5).

Studies concerning the immune changes in uremic patients are extremely important because on their basis, knowing that these patients show a marked and irreversible involution of the thymus followed by immunosenescence, therapeutic methods could be found to stop or make reversible this involution phenomenon. Further research should focus on factors that could ameliorate the demonstrated changes in immune system cell distribution, whether they concern improving the quality of dialysis and reducing the uremic environment, general methods such as physical activity or nutrition, or pharmacological methods, and here we are discussing hormonal treatments or administration of interleukins (IL-7sor IL-22).

At the same time, the work opens a way to better understand the pathophysiologic mechanisms of COVID infection, guiding the study of persistent inflammatory mechanisms and the role of monocytes in a hitherto unexplored view.