Immunoproteasome in the of children with acute appendicitis, and its correlation with proteasome and UCHL1 measured by SPR imaging biosensors

Immunoproteasome in acute appendicitis measured by SPR imaging biosensor

Ewa Matuszczak1, Anna Sankiewicz2, Wojciech Debek1, Ewa Gorodkiewicz2, Robert Milewski3, Adam Hermanowicz1

1. Paediatric Surgery Department Medical University of Bialystok, Poland

2. Electrochemistry Department, University of Bialystok, Poland

3. Statistics Department Medical University of Bialystok, Poland

Corresponding author:

Ewa Matuszczak

Waszyngtona 17

15-274 Bialystok

Poland

e-mail: ewamat@tlen.pl

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List of abbreviations:

UCHL1 – ubiquitin carboxyl-terminal hydrolase L1

SPRI – surface plasmon resonance imaging

LMP – low mass protein

MECL - multicatalytic endopeptidase complex-like

ELISA - enzyme-linked immunoabsorbent test

EDTA - disodium ethylenediaminetetraacetic acid

Abstract: The aim: to determinate the immunoproteasome concentration in the blood plasma of children with appendicitis, and its correlation with circulating proteasome and UCHL1. Material and methods: 27 children with acute appendicitis, managed at the Paediatric Surgery Department, were randomly included into the study (age 2 years 9 months up to 14 years, mean age 9,5 + 1 years). There were 10 girls and 17 boys. 18 healthy, age matched subjects, admitted for planned surgeries served as controls. Results: Mean concentrations of immunoproteasome, 20S proteasome and UCHL1 in the blood plasma of children with appendicitis before surgery, 24 hours after, and 72 hours after the appendectomy - were higher than in the control group. The immunoproteasome, 20S proteasome and UCHL1 concentrations in the blood plasma of patients with acute appendicitis, were highest before the surgery. The immunoproteasome, 20S proteasome and UCHL1 concentration measured 24 hours and 72 hours after the operation, slowly decreased over time, and still did not reach the normal range ($p<0,05$). There was no statistical difference between immunoproteasome, 20S proteasome and UCHL1 concentrations in children operated on laparoscopically and children after classic appendectomy. Conclusions: The immunoproteasome concentration may reflect the metabolic response to acute state inflammation, and the process of gradual ebbing of the inflammation, thus may be helpful in the assessment of the efficacy of treatment. The method of

operation – classic open appendectomy, or laparoscopic appendectomy, does not influence the general trend in immunoproteasome concentration in children with appendicitis.

Key words: SPR imaging biosensor, immunoproteasome, proteasome, UCHL1, appendicitis, inflammation, children

Introduction

The proteasome can be found in nuclei of all eucariotic cells, and is responsible for degrading used or defective proteins, takes part in controlling of the cell cycle, and/or cell differentiation. The proteasome is already used as an target in the treatment of myeloma and mantle cell lymphoma. Moreover, according to Basler at al. the proteasome generates most ligands which are presented "on major histocompatibility complex class I molecules (MCH-1)"[1,2,3].

Proteasome 20S core particle is composed of two outer α-rings and two inner β-rings, each is made of seven distinct subunits [4]. According to Ostrowska et al. "intracellular 20S proteasome can be a free form that is latent, or forms active complexes with two regulators (PA700[19S], and PA28 [11S])" [4]. PA700 regulator binds to the outer α-rings of the 20S core, and also according to Ostrowska et al. "resulting 26S proteasome is responsible for energy-dependent degradation of polyubiquitinated proteins, including many short-lived regulatory proteins that control cell cycle progression, apoptosis, signal transduction, and gene expression" [4,5]. In humans, extracellular proteasomes have been found circulating in the plasma of patients suffering from a wide range of inflammatory, autoimmune diseases, and with neoplasms [6-11]. It is already known, that the level of proteasomes correlated with the severity of the disease in different pathologies [12]. The release of proteasome in the blood plasma may be a result of membrane disruption. Some authors postulate, that the release of active 20S proteasome is regulated [13]. In vitro studies have shown, that proteasome is released by injured

endothelial cells, smooth muscle cells of the vessels, and tubular epithelial cells [13]. Study by Ito et al. demonstrated, that "extracellular 20S proteasome not only is released from cells but also plays a functional role in physiological processes" [14].

When the cells are stimulated by inflammation or interferon-γ or TNF-α, three subunits of the constitutive proteasome β1, β2 and β5 are replaced with the immunoproteasome catalytic subunits β1i (low-molecular mass protein [LMP]2), β2i (multicatalytic endopeptidase complex-like [MECL]10, and β5i (LMP7), respectively [15]. The immunoproteasome in conjunction with PA28 or/and with PA700 activator, is responsible for processing of antigens, and expansion and/or survival of T cells, in T helper cell differentiation in patients with inflammation of the brain, in inflammatory cytokine production, and in autoimmune disease [1,4,16].

Under physiological conditions immunoproteasomes can be found in large quantities in thymus and lungs which are connected with immune system. Immunoproteasomes are practically not found in other organs [4,17]. According to animal study by Kimura et al. who investigated inflammation of the thyroid gland in mice "immunoproteasome is overexpressed", and that "its blockade restores thyroid morphology and function" [15,18]. Also in mice with inflammation of the liver caused by bacterial infection, immunoproteasomes were found to be highly expressed. In rats during ischemia or endotoxemia, immunoproteasomes were found kidneys and other organs [4,19,20]. Many authors investigated the significance of the immunoproteasomes in immune response against viruses, but current research by Kimura found that "LMP7 inhibition inhibits proinflammatory cytokine production and attenuates the development of experimental inflammatory and autoimmune diseases" [4,15,19,21-23].

Therefore we wanted to determinate the immunoproteasome concentration in the blood plasma of children with appendicitis, before and after the surgery to verify if it is a part of the metabolic response to acute state inflammation, and its correlation with circulating proteasome and UCHL1, using a surface plasmon resonance imaging (SPRI) biosensor.

To determine the immunoproteasome concentration, usually the enzyme-linked immunoabsorbent (ELISA) test and semiquantitative western blotting are used [24-29]. The drawback methods is the use of indirect labels, which may be the cause of losing of the protein functional properties. The surface plasmon resonance imaging (SPRI) biosensor can become a good alternative. According to Sankiewicz et al. “This is a label-free, surface-sensitive spectroscopic technique used to examine the interaction between biomolecules” [30-32]. SPRI is based on detection of “changes in the refractive index within a short distance from the surface of a thin metal film caused by molecules bound to the metal surface” (also Sankiewicz et al.) [32]. The researched substance is captured from the solution by the antibody or inhibitor [30-32].

So far several SPRI biosensors were used for clinical research to determinate e.g. lysosomal proteases, 20S proteasome, ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) and immunoproteasome [24,32-41].

Material and methods

27 children with acute appendicitis, who were managed at the Paediatric Surgery Department of Medical University of Bialystok, between 2015-2016, were randomly included into the study (age 2 years 9 months up to 14 years, mean age 9,5 + 1 years). There were 10 girls and 17 boys. 18 healthy, age matched subjects, admitted for planned surgeries served as controls. Exclusion criteria were: severe pre-existing infections, immunological or cardiovascular diseases that required long-term medication, and complicated cases of appendicitis with perforation of appendix and/or peritonitis. All parents of our patients, gave written informed consent for both clinical and biochemical follow-up.

Venous blood samples (1-2ml) were drawn on the admission, 24 hours after the appendectomy, and 72 hours after the appendectomy. Blood samples were collected in EDTA tubes, blood plasma prepared according to the standard protocols and stored at -80oC. After all blood samples were collected and patient data recorded, the immunoproteasome, 20S proteasome and UCHL1

concentration was assessed using Surface Plasmon Resonance Imaging by the investigators blinded to the other data.

Chip preparation

Gold chip were manufactured as described in a previous paper [32-34].

Antibody immobilization for UCHL-1 determination was done according to the method described by Gorodkiewicz et al. in their previous paper: “chips were then immersed in 20mM of cysteamine ethanolic solutions for 2h and, after rinsing with ethanol and water, dried again under a stream of nitrogen. The specific rabbit monoclonal antibody solution in a PBS buffer (10µg/ml) was activated with NHS (50 mM) and EDC (200 mM). Activation of the antibody was done by adding the mixture of NHS and EDC (1:1) in a carbonate buffer solution (pH 8.5) into the antibody solution and with vigorous stirring for 5 min at the room temperature. 3µl of this solution was placed on the active places with the amine-modified surface, and incubated at for 1h" [33].

Inhibitor - PSI immobilization for proteasome determination

PSI inhibitor (Z-Ile-Glu(OBut)-Ala-Leu-H)) at a concentration of 80nM was activated with NHS (50mM) and EDC (200mM) in a carbonate buffer (pH=8.5) environment and then placed on the thiol (cysteamine) modified surface and incubated at 37oC for 1h [33].

For receptor immobilization in the process of immunoproteasome determination we used commercial inhibitor ONX 0914, as described by Sankiewicz et al. [34]

After receptor immobilization the biosensor were rinsed with water. Next, blood plasma samples (10 x diluted) were placed directly on the prepared biosensor. The volume of the sample applied on each measuring field was 3 µl. Time of the interaction with receptor was max 10 minutes. As described by Gorodkiewicz et al. “The biosensor was washed with water and HBS-ES buffer solution pH=7.4 (0.01 M 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, 0.15 M sodium chloride, 0.005% Tween 20, 3 mM EDTA), BIOMED, Lublin, Poland) to remove unbound molecules from the surface” [35,36].

SPRI measurements were performed on self-made apparatus made, which detailed account can be found in previous paper [34].

SPRI measurements for the protein biosensor array were performed as described by Sankiewicz et al. "the measurements were performed at a fixed angle of incident light and the reflectivity was simultaneously measured cross an entire chip surface. The contrast values obtained for all pixels across a particular sample single spot were integrated. The signal was measured twice on the basis of registered images, after immobilization of the receptor (antibody or inhibitor) and then after interaction with analyte. The SPRI signal, which is proportional to coupled biomolecules, was obtained from subtraction between the signal before and after interaction with a biomolecule" [34].

Also a background correction was applied as described by Sankiewicz et al. "some of the sites on the biosensor covered with PBS buffer were used as a control. Non-specific binding was monitored by measuring the SPRI signal at a site on the chip without the receptor (ligand). Minimization of non-specific binding was achieved by preparing samples in PBS buffer (NaCl an KCl concentration ~200 mM) at pH 7.4" [34].

Blood plasma samples from healthy children admitted for planned surgeries (n=18), and from our patients after appendectomy (n=27), were 10-times diluted with PBS buffer. The drops of blood plasma were transferred onto the chip surface for 10min [34]. As described by Gorodkiewicz in previous paper "A whole section of 12 spots was covered. The surface was washed with distilled water 10-times and after drying, the SPRI measurement was performed. An average value taken from 12 pair of measurements was considered as a single result" [33]. Concentration was evaluated from the calibration curve of immunoproteasome and 20S proteasome and UCHL1 [18]. As a control surfaces, the same sample solution was run over surface derivatized with non-reacting biomolecules to delineate the level of non-specific binding.

Statistics

The Mann-Whitney U test and the Kruskal-Wallis H test with Dunn's post hoc correction to control for multiple testing were used to compare differences between groups. Statistical analyses were calculated with the STATISTICA PL release 10.0 program. A two-tailed $p<0,05$ was considered significant. Correlations were examined by linear regression (r) using the Spearman test.

Results

Mean concentrations of immunoproteasome, 20S proteasome and UCHL1 in the blood plasma of children with appendicitis before surgery, 24 hours after, and 72 hours after the appendectomy - were higher than in patients from the control group. The difference was statistically significant ($p<0,05$) (FIG.1,2,3). The immunoproteasome, 20S proteasome and UCHL1 concentrations in the blood plasma of patients with acute appendicitis, were highest before the surgery. The baseline concentrations of immunoproteasome, 20S proteasome and UCHL1 in blood plasma of children with appendicitis before surgery, were 15-folds higher for immunoproteasome, 11-folds higher for 20S proteasome, and 76-folds higher for UCHL1, than levels measured in controls, the difference was statistically significant. The immunoproteasome, 20S proteasome and UCHL1 concentration measured 24 hours and 72 hours after the operation, slowly decreased over time, and still did not reach the normal range, when compared with the concentration measured in controls ($p<0,05$). There was no statistical difference between immunoproteasome, 20S proteasome and UCHL1 concentrations in children operated on laparoscopically and children after classic appendectomy (data not shown). There was a moderate correlation between immunoproteasome and 20S proteasome concentrations before appendectomy, 24h and 72h after the operation ($r^1=0,4078$, $r^2=0,5815$, $r^3=0,3969$ respectively, $p<0,05$)). We also found a weak correlation without statistical significance between immunoproteasome and UHCL1 concentrations ($r^1=0,0967$, $p>0,05$) before appendectomy, and weak negative correlation without statistical significance between immunoproteasome and UHCL1 concentrations 24h and 72h after the surgery ($r^2=-0,2540$, $r^3=0,1097$,

p>0,05). We did not note any postoperative complications in our patients after appendectomy. All patients were discharged home on the third day after the surgery, in good general state.

Discussion

Immunoproteasome belongs to the family of proteasomes, and is responsible for regulation of the production of inflammation mediators (e.g. IL-1 and tumor necrosis factor-α), and reactive oxygen species, and can lead to the breaking of blood-brain barrier [21,22,42]. Compared to the constitutive proteasome, immunoproteasomes produce more peptides with a hydrophobic C-terminus, which are well suitable for the presentation on MHC class I molecules [43]. Mice deficient for any of the immunoproteasome subunits are protected from dextran sulphate sodium-induced colitis [1,43]. The immunoproteasome has also, additional immunological functions - immunoproteasome deficiency or inhibition affects T cell survival, expansion, and differentiation, cytokine production, and progression of autoimmune conditions [1,23,43]. Moreover, mutations in LMP7 and LMP2 in humans cause complex autoimmune and inflammatory phenotypes [39,40]. The literature about the role of the immunoproteasome in NF-κB activation is quite controversial [42,44-47]. The results of the latest study by Bitzer, clearly argue against an influence of immunoproteasomes on the canonical pathway of NF-kB activation [43]. Two different types of primary cells prepared from LMP $^{2-/-}$, L7M $^{-/-}$, and wild type mice displayed no differences in the extent and kinetic of IκBα degradation when stimulated with LPS or TNF-α. Consistent with this finding, the amount of active NF-κB in the nucleus of knockout cells as well as the transactivation activity was normal [43].

Ubiquitin C-terminal hydrolase 1 (UCHL1) is an unique enzyme, which is extremely diverse - it is able to function as both a ubiquitin ligase and a deubiquitinating enzyme. So UCH-L1 can obstruct the degradation process of the target protein. According to Wilson et el. UCHL1 “can also interact and stabilize cell proteins such as β catenin, and activate kinases such as CDK4 without the requirement for its enzymatic activity” that has both hydrolase and ligase activities [48]. According to Zhang et al. “UCHL1 is expressed predominantly in the brain and neuroendocrine systems, and accounts for 1-2%

of total brain soluble proteins" [49]. Various studies proposed UCHL1 as real-time indicator of brain damage. Furthermore, the neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, are closely related do dysregulation of UCHL1. Current researches showed that UCHL1 can be found not only in central nervous system [50-54]. According to Wilson and also other researchers "UHCL1 has also been implicated in many human diseases including kidney disease and cancers" [49-56]. The latest study by Wilson et al. show that "pharmacological inhibition of UCHL1 blocks hepatic stellate cells proliferation and when administered in vivo acts in a therapeutic way to block progression of established fibrosis despite continued liver injury" [48]. Despite many functions identified so far, there is no data available on the literature, on the correlation of expression of UCHL1 and inflammation. Therefore we wanted to determinate the UCHL1 concentration in serum of children with appendicitis and correlations between plasma levels of immunoproteasome, 20S proteasome, and UCHL1, before and after the surgery to verify if those are a part of the metabolic response to acute state inflammation.

Circulating 20S proteasomes in human serum for the first time were investigated by Wada et al. [57]. They found a positive correlation between the level of circulating proteasome and the invasiveness of hematologic malignancies [57]. Other authors, among them Sixt et al. found "correlation between the stage of malignancy and the level of c-proteasomes in patients with metastatic malignant melanoma" [57-59]. According to Sixt et al. and other authors "elevated concentrations of circulating proteasomes were detected also in patients with different solid tumors (carcinoma of breast, stomach, kidney, colon, lung, testes and liver)" [57,59,60]. Furthermore elevated concentration of c-proteasome were detected in patients after thermal injury, after trauma, with sepsis, or with liver disease [59, 61-63]. After abdominal surgery without complications, the concentration of 20S proteasome in the serum of patients was increased two-folds in comparison to values found in the serum of control group [59,63]. After thoracoscopic thymectomy, a decline in the level of c-proteasome during the surgery and a rise in its level after the procedure was noted [64]. Contrary to this observation, we observed a downward trend in immunoproteasome, 20S proteasome and

UCHL1 concentrations in blood plasma of children after appendectomy, but the baseline concentrations of immunoproteasome, 20S proteasome and UCHL1 in blood plasma of children with appendicitis before surgery, were 15-folds higher for immunoproteasome, 11-folds higher for 20S proteasome, and 76-folds higher for UCHL1, than levels measured in controls. We believe that this discrepancy is related to acute state inflammation in our patients. Other authors observed that levels of c-proteasome may be elevated up to 20-fold in patients with various autoimmune diseases e.g. autoimmune myositis, systemic lupus erythematosus, primary Sjogren syndrome, rheumatoid arthritis, vasculitis, systemic scleroderma, autoimmune hepatitis [59,64]. In those cases, there was connection between clinical state and the level of circulating proteasome. We made the same observation, connected with downward trend in the levels of immunoproteasome, 20S proteasome and UCHL1 reflecting the clinical course of appendicitis in our patients.

We also found that there were no differences between the levels of immunoproteasome, 20S proteasome and UCHL1 in children operated laparoscopically and with open technique. This opinion seems to be not in line with data published by Sixt et al., indicating that "cell damage and haemolysis are the major sources of the increased extracellular ubiquitin concentrations measured under various pathological conditions" [12, 66-69]. On the other hand Roth et al. concluded that because no significantly elevated proteasome concentrations after abdominal surgery and high levels of proteasome are found in patients suffering from trauma and sepsis, the cause of increased levels of 20S proteasome is the immunological activity rather than cellular damage [64]. Furthermore in animal studies, inhibitors of the proteasome inhibitors prevent acute ischemic acute renal insufficiency, in obstructive nephropathy slow down the development of renal fibrosis, and in vascular renal disease prevent arterial thrombosis [70-72]. According to our previous studies, the peak in the concentrations of the proteasome and UCHL1 in blood plasma of patients with thermal injury, its slow decrease over time, and its correlation with severity of the burn, suggest that 20S proteasome and UCHL1 are released from tissues damaged by burn. The observation that the dynamic of the immunoproteasome, 20S proteasome and UCHL1 are the same for open and

laparoscopic appendectomy, might suggest also that the aggression upon the abdominal wall is comparable for the 2 techniques (a single 3-4 cm long incision for the open technique vs 3 incisions of 1 cm in laparoscopic approach). Probably, the immunoproteasome concentration reflect the metabolic response to acute state inflammation more, than the systemic reaction to the surgical incisions.

In our study we did not find correlation of immunoproteasome, 20S proteasome and UCHL1 levels with gender or age, which is consistent with previous observations [9-11].

Our observation did not fully elucidate the clinical relevance of plasma levels of immunoproteasomes, 20S proteasomes and UCHL1 in appendicitis in children. Also the impact of immunoproteasome on various immunological aspects, raise an interest in understanding its cellular function in autoimmunity and inflammation. Nonetheless we believe, that evaluation of plasma levels of immunoproteasome, 20S proteasome and UCHL1, may be helpful in the assessment of the efficacy of treatment, in the course of abdominal inflammatory disease.

Conclusion

The immunoproteasome concentration may reflect the metabolic response to acute state inflammation, and the process of gradual ebbing of the inflammation, thus may be helpful in the assessment of the efficacy of treatment. The method of operation – classic open appendectomy, or laparoscopic appendectomy, does not influence the general trend in immunoproteasome concentration in children with appendicitis. The SPR imaging biosensor is an useful diagnostic tool in the assessment of immunoproteasome, proteasome and UCHL1 concentrations.

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Fig.1 Concentrations of immunoproteasome in the blood plasma of children with appendicitis g1- before surgery, g2 -24 hours after, and g3 - 72 hours after the appendectomy, ctrl – control group

Fig. 2 Concentrations of 20S proteasome in the blood plasma of children with appendicitis g1 - before surgery, g2 - 24 hours after, and g3 - 72 hours after the appendectomy, ctrl – control group

Fig.3 Concentrations of UCHL1 in the blood plasma of children with appendicitis g1 - before surgery, g2 - 24 hours after, and g3 - 72 hours after the appendectomy, ctrl – control group

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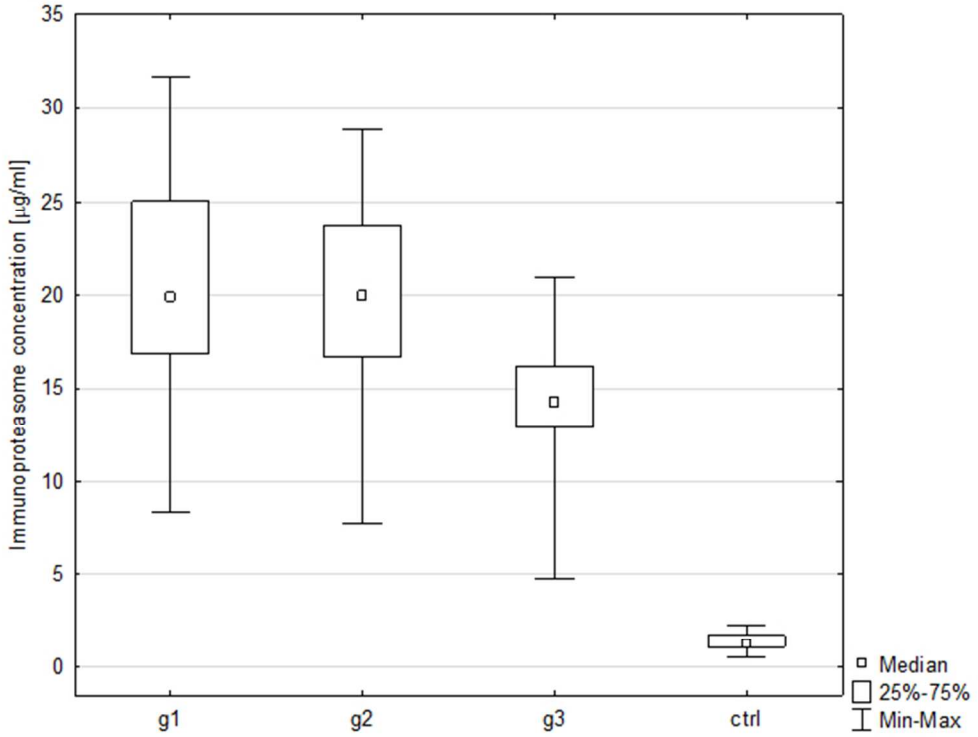

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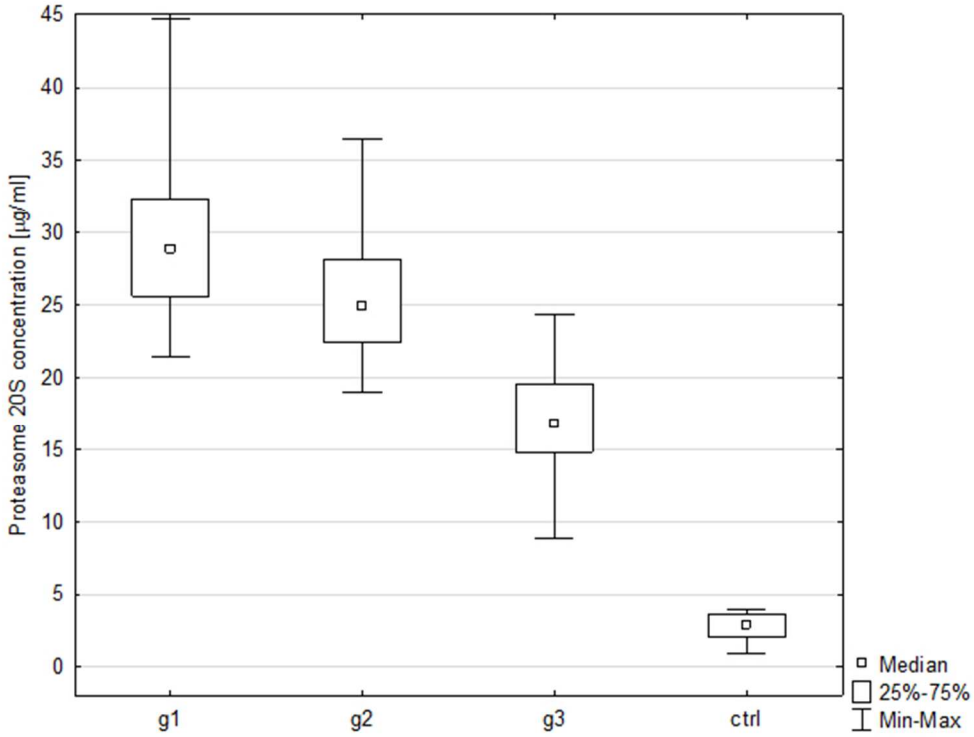

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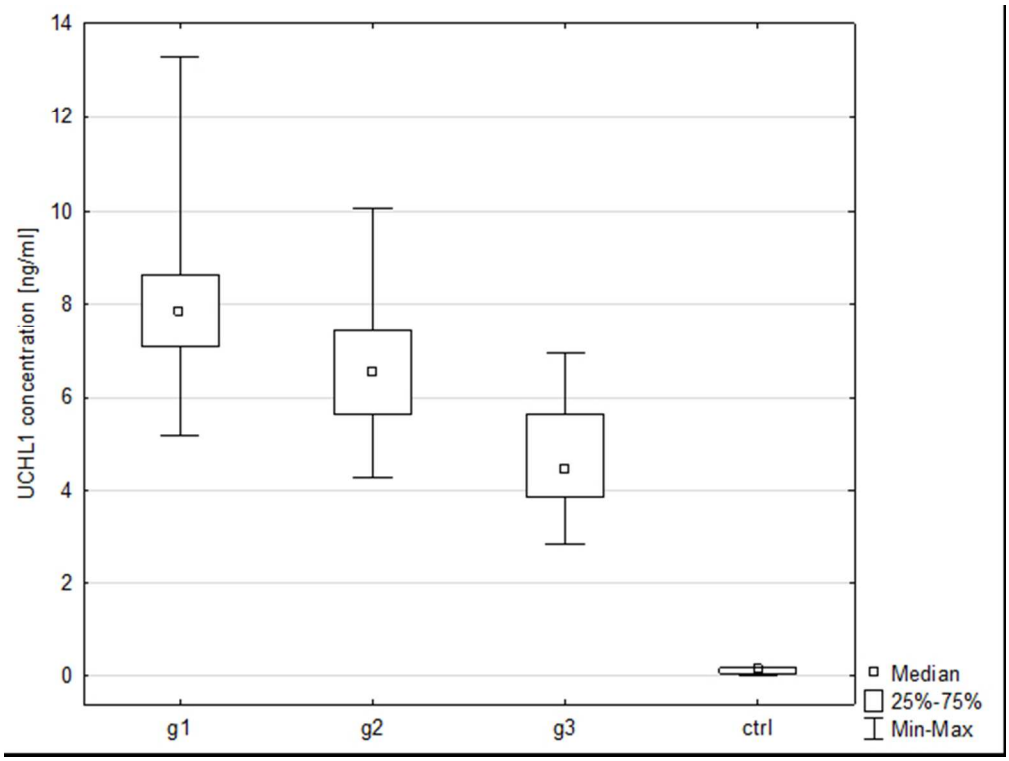

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