SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Etiological factors, prevention and treatment alternatives of oral mucositis during autologous peripheral stem cell transplantation

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The Examination takes place at the Rm 205 Faculty of Dentistry, University of Debrecen, 26 November, 2021, 11.00 a.m

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1 Background and aims of the degree of doctor of philosophy (PhD)

The incidence of malignant hematological diseases increases from year to year and, sadly, occurs increasingly in younger age. Autologous and allogeneic transplantation has been applied in their treatment more and more widely and successfully (1). Mucosal barrier injury (MBI), oral (OM) and enteral mucositis (EM), is one of the most common and severe toxic symptoms and complications of high dose intensive cytostatic treatment (myeloablative conditioning regimen) and total body irradiation (TBI) by appropriate indication (2). OM can not only cause severe decline of quality of life (severe pain, xerostomy, taste alteration, ulcer formation requiring parenteral nutrition), but, due to mucosal barrier injury, dissemination of oral microbes can lead to fatal sepsis during prolonged neutropenia. Prolonged hospitalization, increase in nosocomial infections risk are expected, burdening the health care system (3). In addition to therapeutic benefits, increasing risks of secondary malignancies, primarily oral cancers, are also likely as long term side effects of the applied pre-and conditioning therapies (4).

Oral mucositis is a disease of multifactorial ethiopathogenic origin with several patient and treatment related risk factors (5). Its treatment is supportive and palliative in spite of extensive research and laborious work (6). Everyday routine includes maintenance of basic oral care, analgesia, provision of proper nourishment as well as prevention and treatment of infections (7). Furthermore, it has no validated biomarker (8).

In view of the above, our aim was to assess risk factors, identify biomarkers, examine the effect of autologous peripheral stem cell transplantation (APSCT) on local immunity and assign new research directions.

1.1 Hematopoietic stem cell transplantation (HSCT)

The number of hematopoietic stem cell transplantations increase every year (1). In 2019, 48512 HSCT were conducted in 700 centers of 51 countries. The main indications for autologous HSCT were lymphoid malignancies (90%) with plasma cell disorders, comprising 55% of all autologous HSCT patients.

In HSCT the patient's own hematogenesis and immune system are destroyed the use of appropriate conditioning and immunosuppressive therapy (intensive cytostatic treatment and/or radiotherapy), then healthy mononuclear cells, including CD34+ multipotent hematopoietic stem cells, are administered, which are able to reorganize the myelo-and lymphopoietic systems (9,10). Autologous HSCT has three main steps: stem cell harvesting, conditioning and giving back stem cells (9,10), as part of the peritransplantation period that optimally lasts from diagnosis to care. In terms of oral dentistry, prevention of the acute exacerbation of chronic dental inflammations during cytopenia through appropriate removal, reducing the risks of the development and severity of OM and timely recognition of secondary oral cancers and oral graft-versus-host disease (GVHD) during posttransplantational care are especially important (11-13).

1.2 Oral mucositis, is a mucosal barrier injury during HSCT

Varying degrees of oral mucositis occur in 60-100 % of patients during HSCT (14). Development of mucosal barrier injury is a result of a complex and dynamic biological process involving in several molecular and cellular events and affecting all layers of the mucosa ('panmucosal') (15). Its development has 5 phases: 1. initiation 2. primary damage 3. signalling and amplification 4. ulceration, and 5. healing. More than 14 inflammatory and cell apoptotic pathways have been identified in the pathobiology of OM (16). Knowledge of these is

especially important in the mapping of new prevention and therapeutic alternatives.
Exact classification of OM enables optimal follow-up of patients' general status and the assessment of the success of the transplantation. At the same time it is one of the basic pillars of efficient research (17). Several scales are known nowadays, of which the classifications of the World Health Organization (WHO) and Oral Assesment Guide (OAG) are most common in clinical practice (18).
Oral mucositis is a disease of multifactorial ethiopathogenic origin (19) with several patient (low neutrophil granulocyte count, female sex, poor oral hygiene, etc.) and treatment (TBI, high dose cytostatic therapy) related risk factors (20).
Of several prevention and treatment alternatives human recombinant keratinocyte growth factor (hrKGF) is the only prevention alternative approved by the Food and Drug Administration (FDA). However, its routine administration.is limited by side effects and high costs (21).
Prediction of OM is especially important regarding the efficient and individual plan of oncotherapy. In view of this, therapy modifiation due to toxicity, dose reduction or potential hospitalisation time can be reduced, significantly improving therapeutic needs. There is only a small number of relevant clinical data regarding OM development during HSCT as a result of conditioning regimen. Potential, non-validated biomarkers of OM caused by radiotherapy have been classified into eight groups by *Normando et al 2017*: 1. growth factors 2. cytokins 3. markers of acute phase reactants 4. genetic factors 5. general proteins 6. plasma antioxidants 7. apoptotic proteins 8. cells (22).

1.3 Basic pillars of oral immunity

Basic pillars of oral immunity are the oral epithelium, leukocytes, saliva and the periodontium (23). The oral

epithelium and its associated lamina propria provide a physical barrier that protects the underlying tissues. Action of the immune system's soldiers (macrophages, dentritic cells, natural killer cells, and polymorphonuclear cells) make the protective mucosal barrier stronger by producing inflammatory mediators, cytokins and chemokins. Several defensive contents and functions of saliva and gingival crevicular fluid are essential to the complex and optimal function of oral immunity. Oral mucosal immunity neutralizes the agents which damage the oral cavity, limits the colonization of pathogenic microorganisms and provides the maintenance of commensal homeostasis. Dysregulation of oral mucosal immunity may result in the development of oral immunopathogenic reactions, common infections, acute-and chronic inflammations, and possibly plays an auxiliary role in the development of oral cancer in case of permanent persistation (23).

1.4 Effects of sex hormones on the physiology of oral cavity

Sex hormones play a pivotal role in the maintenance of homeostasis of the oral cavity and in its regulation. As a consequence of the sex hormone receptors' tissue specific localization hormones affect the whole oral milieu directly and indirectly. Effects of sex hormones on the oral epithelium, periodontium, microbiome, consumption of saliva and the function of the immune system can be demonstrated (24,25). Estrogen is primarily an immunostimulant. It regulates the growth, differentiation and proliferation of lymphocytes, polymorphonuclear's (PMN's) chemotaxis, antigene presentation, production of citokines and antibodies, and cell survival. It enhances bloodstream and capillary permeability (24), stimulates the proliferation and keratinization of epithelial cells. Estrogene (E2) regulates production of the extracellular matrix and enhances proliferation of gingival fibroblasts. It

affects wound healing, and plays a role in the localization of the dissemination of the dentoalveolar infection by modulating the production of IL-1 (26). On the other hand progesterone (P4) and androgens are immunosupressants (27). P4 stimulates the production of inflammatory mediators, including prostaglandine E2. It enhances vascular permeability in the gingival structures (24). Besides its elevated levels, a decrease in keratinised cells can be observed (24). Keratinization and karyopicnotic index decrease significantly as a consequence of daily regular administration of synthetic progestins. P4 inhibits proliferation of gingival fibroblasts (24). It suppresses the mucosal immune response, inhibiting IgA-associated immune response (28). Antibacterial activity of neutrophil granulocytes is decreased as a result of the administration of high dose progesterone (29). Recent findings suggest that P4 decreases the permeability of enteral mucosa, systemic microbial translocation, and inflammation during pregnancy as a result of the inhibition of NF-κB and upregulation of occludin expression (30).

1.5 Role of secretory immunoglobulin A (sIgA) and serum IgA in mucosal protection. Importance of immunoglobulins' glycosylation

Alterations in N-linked carbohydrate structures of glycoproteins can serve as indicators for several key biochemical mechanisms (31,32) and offers new paths for biomarker research (32,33). Besides IgG, IgA is one of the most abundant glycoproteins in serum and saliva (34). However, while the N-glycosylation of IgG is well published, not so much is revealed about IgA and sIgA, especially in saliva (35,36). Glycosylation is essential for the functions of immunoglobulins, such as secretory immunoglobulin IgA (sIgA) dimerization, polymeric Ig receptor-mediated

transcytosis, and adhesion of pathogens to the mucosal surface, and is responsible for antibody binding to the mucus layer (36). The biochemical and immunochemical properties of serum and secretory IgA are different (37).

Serum IgA as an anti-inflammatory antibody, plays the role of "silent housekeeper" in regulating infective-inflammatory processes (38). It has been shown to prevent activation of complement system and to inhibit phagocytosis, chemotaxis and antibody-dependent cellular cytotoxicity. These results suggested that the predominant role of serum IgA was the removal of antigenic substances without the generation of an inflammatory response (37).

The role of the secretory immunoglobulin A (sIgA) is more complex. Salivary sIgA is crucial in immune exclusion via direct interaction with microbial antigens, and eliminates viruses by non-virulent immune complex formation. It also neutralizes bacterial lipopolysaccharide (LPS), and maintains commensal homeostasis, thereby preventing disseminating pathogens (39).

Immunoglobulin A has two known isotypes of IgA1 and IgA2, with several further subtypes of IgA2m(1), IgA2m(2) and IgA2n. The main structural difference between the subtypes lies in the hinge region and the number and distribution of N- and O- glycosylation sites, leading to different functional properties (37,40). In serum, the IgA1 isoform is predominant, which is primarily produced in the bone marrow and, to a lesser extent, in marginal zone B and B1 cells, entering the blood without reaching the mucosal surface (37,38). In external secretions, like in saliva, IgA2 is predominant, mostly as a dimer (41).

As a result of chemotherapy there is decreased sIgA secretion. During APSCT, serum IgA, also decreases (42). While serum IgA usually returns to the normal level within six or seven months, salivary sIgA level needs up to five years to recover (43).

1.6 Osteopontin (OPN)

OPN is a multifunctional, chemokine-like, sialic-acid rich phosphoglycoprotein, plays a pivotal role in tumour development, progression, inflammation and mucosal protection impacts on cell survival, proliferation and invasion (44). It is classified as a member of the Small Integrin-Binding Ligand *N*-linked Glycoprotein (SIBLING) family (45). It OPN is expressed by many cell types such as immune, neural, epithelial and endothelial cells, fibroblasts and secreted in body fluids including blood, cerebrospinal fluid and saliva. OPN gene expression is modulated by several factors such as cytokines (e.g., IL-1ß, IL-6), hormones (e.g., oestrogen, progesterone (P4)) and growth factors (44). Overexpression of OPN in several cancers such as breast cancer, malignant haematological diseases (acute leukemia, lymphoma, multiple myeloma) or oral squamous cell carcinoma (OSCC) (46,47) predicts poor overall survival, suggesting its role as a prognostic biomarker (44). OPN is an effective regulator of the hematopoietic stem cell homeostasis and neutrophil migration (48). It plays a crucial role in several non-neoplastic processes, including GVHD after allogeneic hematopoietic stem cell transplantation (49). Its role in mucosal defence, especially against viral pathogens (50) and in tissue destruction with subsequent repair process, is also essential (51). Osteopontin may be required for their interaction and co-operative effects in promoting the transition from innate to adaptive responses and the initiation of repair (51).

1.7 Definition and importance of oral and peripheral blood engraftment

Peripheral blood neutrophil engraftment is defined as peripheral absolute neutrophil count of ≥0.5 G/L, whereas thrombocyte engraftment as ≥20 G/L thombocytes, on three consecutive days after cytopenia during transplantation; and

oral engraftment is defined as oral mucosal neutrophil count (OMNC) of $\geq 0.25 \times 10^4$/ml on three consecutive days after cytopenia during transplantation (52,53). The type of transplantation (allogeneic vs autologous), its source (bone marrow vs peripheral blood) and the underlying malignant hematological disease which indicates HSCT, affect the time of development of both oral (OE) and blood engraftment (BE) (52). OE is a more efficient indicator of OM improvement than BE. Although OM shows rapid improvement after the development of BE, neutrophil penetration and occurrence in the oral cavity, namely OE development contributes to OM's resolution earlier and indicates the initiation phase of bone marrow regeneration earlier (52). OMNC is an earlier indicator of the processes taking place in the organism, susceptibility of infection and complications that accompany neutropenia (such as neutropenic fever, OM, etc) than absolute neutrophil count (ANC)(54).

2 Materials and methods

2.1 Retrospective study population and design

We conducted a restrospective analysis of 192 patients over a period of 4 years who had required and undergone APSCT due to malignant haematological disorder in the Haematopoietic Transplantation Centre of the Clinical Centre of the University of Debrecen, Hungary. The study was approved by the Regional Istitutional Research Ethics Committee, Clinical Center, University of Debrecen (Ethical licence: DE RKEB/IKEB 4948-2018). The study was conducted in accordance with the Declaration of Helsinki. Diagnoses were obtained from the institutional electronic clinical database

eMedSolution (T-Systems Inc. Budapest, Hungary) in accordance with the ethical approval.

Two large patient groups were created, lymphoma (Hodgkin (HL) and non-Hodgkin's lymphoma (NHL)) and multiple myeloma (MM). Regarding stage at disease onset all patients were early and advanced, respectively, while regarding stage prior to the transplantation we established two groups: complete remission (CR) and very good partial remission (VGPR) were merged as a single group, whereas partial remission (PR) represented a separate group. Response categories were determined in accordance with the International Myeloma Working group (IMWG) uniform response criteria (55). In Hodgkin lymphoma we categorised the conditioning treatments applied during the transplantation into four groups (1. BEAM (bischloronitrosourea, etopozid, cytosin arabinozid, melphalan) 2. R-BEAM (Rituximab-BEAM), 3. R-BEAM-Adcetris, and 4. other). In NHL, in group 1 R-BEAM and in group 2 Z-BEAM (Zevalin-BEAM) conditioning regimen was used, whereas group 3 represented cases with any other conditioning regimen. In MM conditioning was administration of 140 mg/m^2 melphalan in 12 patients and 200 mg/m^2 in the rest of the patients. After transplantation all patients received granulocyte colony stimulating factor (G-CSF) with the antimicrobial prohylaxis. OM was classified according to the WHO guidelines (Grade 0–4) and the most severe appearence defined the stage in the individual patient (2). For the statistical analysis there were two separate groups, (1) non-ulcerative (OM0-1) and (2) ulcerative (OM2-4) mucositis (56). The time required for neutrophil engraftment was calculated as number of days with <0.5 Giga (10^9) per Litre (G/L) absolute neutrophil count (ANC), and for thrombocyte engraftment as number of days with <20 G/L thrombocyte (THR) count.

We analysed the relationship between oral mucositis developed during transplantation and the following continuous variables: age at time of transplantation, time elapsed between diagnosis

and transplantation (DG-TX time/month), amount of stem cells administered (10^6/body mass kg), stem cell viability (%), number of viable cells (10^6/body mass kg) and mononuclear cells (MNC) (10^8/body weight kg), engraftment time (ANC < 0.5 G/L, THR <20 G/L-days) and lactate dehydrogenase (LDH) (U/L). Of the categorical variables we analysed the relationship between oral mucositis and sex; stage of the disease at diagnosis (early *versus* advanced) and prior to transplantation (PR, VGPR, CR); the type of conditioning applied; outcome (dead or alive); infectious complications in the early post-transplantation stage (positive haemoculture); and correlation with disease subtype, where applicable.
VGPR and CR, the stages immediately preceding transplantation, were conflated according to standard practice.
Based on their presumed hormonal status, the 85 female patients of the study were separated into premenopausal (≤50 years) (n=19) and postmenopausal (≥51 years) (n=66) groups, respectively, according to published criteria (57). All premenopausal APSCT women were on hormone replacement therapy (HRT) (5–10 mg norethisterone-acetate on cycle days 3–27, prior to and after the day of the transplantation, as long as cytopenia persisted in order to supress cycles.

2.2 Prospective study population and design

Our prospective study was carried out at the Hematopoietic Transplantation Centre collaborating with the Dental Outpatient Care, University of Debrecen, Hungary including 10 patients who had required and undergone APSCT and 23 respective healthy controls.
All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Regional Institutional Research Ethics

Committee, Clinical Centre, University of Debrecen (Ethical license: DE RKEB/IKEB 4948-2018) and Regional and Institutional Committee of Science and Research Ethics (ethical licence: 5570-1/2018/EKU). Inclusion criterion was the presence of malignant haematological diseases requiring APSCT in the patient population, while participants with severe chronic disease (diabetes, autoimmune diseases, acute or chronic inflammatory diseases, etc.) and previous malignancy were excluded from the study in both groups. Three age groups were created based on the literature (58): young adults (25–34 years), middle-aged (35–59 years) and elderly (60+ years). All participants (both patients and healthy controls) answered a questionnaire containing data about age, sex, peritransplantation time interval, smoking habits, alcohol consumption, dental rounds and hormonal status. Based on their hormonal status reflected by their answers in our questionnaire women were divided into two groups, pre-and postmenopausal. OM was classified according to the WHO guidelines (Grade 0–4), OAG and Oral Mucositis Assessment Scale (OMAS) scores each day before samplings (18). All patients received combined antimicrobial prophylaxis.

Serum, unstimulated whole saliva (UWS) and exfoliative cytologic buccal samplings were performed at the same time (between 7 and 8 am) on specific days of the peritransplantation period as follows: day of hospital admission (day −3/−7), day of transplantation (day 0) and day +7 and day +14 post-transplantation (59). Saliva and peripheral blood samplings to determine white blood cell counts were performed each morning. We took photos each day according to the predilectation sites.

Saliva collection was performed according to the standard methods (60). Both controls and patients were in a sitting position during the sampling with eyes open and a slightly tilted head. Following oral cavity rinse with 25 mL of physiological saline solution (B. Braun Melsungen AG, Melsungen,

Germany) for 30 s, saliva was collected for 5 min in an externally pre-disinfected 15 mL lockable Falcon tube (Sigma-Aldrich, St. Louis, MO, USA). Participants adapted to the test condition for 5 min prior to sample collection. Taking into account the diurnal variation of saliva constituents, samplings were done at a specified time window: between 7 a.m. and 8 a.m., one hour after eating, drinking, or tooth-brushing in order to avoid contamination. Within one hour of collection, Halt Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA) was added proportionally to the saliva samples. After homogenization, saliva samples were aliquoted into 1.5 mL Eppendorf tubes and stored at −70 °C until further processing. Blood samples were centrifuged after collection at 1200 g for 30 min and the serum fractions were also stored at 70 °C until further processing within one hour of collection.

2.2.1 Detection of salivary and blood sample E2 and P4 levels

Estradiol and progesterone levels in serum and saliva were determined in respective healthy controls (7 pre- and 7 postmenopausal women) and in 7 postmenopausal female patients undergoing APSCT, at four stages of transplantation (day − 7/−3, 0, +7, +14).
Saliva and serum samples stored at −70 °C were thawed at room temperature and centrifuged at 4 °C for 10 min at 3,000 rpm. Serum (500 µl) and diluted saliva supernatant (150 µl in 450 µl Hanks' Balanced Salt solution (Sigma-Aldrich, St. Louis, MO, USA) were filtered through 70 µm EASYstrainer cell sieve (Greiner Bio-One, Frickenhausen, Germany). Hormone levels were determined using electrochemiluminescence immunoassay (ECLIA) (Roche, Basel, Switzerland) (Laboratory Medicine, Clinical Center, University of Debrecen).

2.2.2 Detection and *N*-glycomic analysis of serum and salivary IgA

Eight patients with malignant hematological disease who required APSCT and 10 age-and sex-matched patients as controls were included in the study. Serum IgA levels were detected using Sysmex XN-2000 Hematology Analyzer (Sysmex Hungary, Budapest, Hungary). Salivary IgA levels were measured by IDK sIgA ELISA kit (Immundiagnostik, Bensheim, Germany) according to the manufacturer's instructions. We determined the salivary IgA secretion rate (μg/min), because it is a more stable value than IgA concentration (41).

We had to devise a special method for glycomic analysis to help us get IgA from serum and saliva without any significant loss and injury. First we planned a special IgA binding protein. We had to make an optimal gene construction, optimise the protein production protocol, and devise an efficient protein purifying method which helped us produce sufficiently pure Z(IgA1) in sufficient quantity (61). We thank Hajnalka Jankovics and Ferenc Vonderviszt (The Faculty of Engineering, Pannon University, Veszprém) for the implementation of the workflow. The steps of the glycomic analysis were then the following: IgA partitioning, *N*-glycan release and fluorophore labeling, exoglycosidase based carbohydrate sequencing, capillary electrophoresis.

2.2.3 Detection of salivary and serum OPN levels

10 patients who had required and undergone APSCT and 23 healthy controls were selected in the study. Serum and saliva samples stored at 70 °C were thawed at room temperature and centrifuged at 4 °C for 30 min at 1200 rpm and at 4 °C for 10 min at 3000 rpm. Two and fourfold dilutions of serum and

saliva samples, respectively, were used. OPN levels were measured by Human Osteopontin ELISA Kit RAB0436-KT (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Osteopontin concentrations were normalized to total protein oncentration determined by BCA protein assay kit ThermoScientific, Waltham, MA, USA).

2.2.4 Detection of oral mucosal and peripheral blood absolute neutrophil count. Assessment of oral and peripheral blood engraftment

Saliva samples gained through rinsing and swishing were centrifuged at 200 x g for 15 minutes. The pellet was suspended after decanting supernatant with 1ml diluted (3.6%) formaldehide solution. This dilution was filtered through 70 μm EASYstrainer cell sieve (Greiner Bio-One, Frickenhausen, Germany) for 10 min. The samples were stored at 4 °C for futher processing (measuring by Sysmex XN-2000 Hematology Analyzer) (Sysmex Hungary, Budapest, Hungary) (Laboratory Medicine, Clinical Center, University of Debrecen).

2.3 Statistical analysis

Statistical analysis was performed using IBM SPSS22 software (IBM, Armonk, NY, USA). Kolmogorov–Smirnov test was used to investigate the normal distribution of data. In case of normal distribution, we compared the two groups using independent sample *t*-test in the continuous variables, whereas in non-normal distribution we applied Mann–Whitney and Wilcoxon tests. For distribution of categorical variables *Chi*-square test and, in case of a low number of cases, Fischer exact test were used. Overall survival (OS) was calculated from the

time of diagnosis to the last follow-up visit or death. Survival data were analyzed using the Kaplan–Meier method with log-rank test. Odds Ratios (OR) were obtained using binary logistic regression models.

In glycomic analysis Shapiro–Wilk test was performed to investigate the normal distribution of data. If it passed the normality test ($p>0.05$), analysis of variance (ANOVA) followed by Tukey *post hoc* test was used to compare peak intensities between experimental groups, otherwise the Kruskall–Wallis test followed by Dunn's multiple comparison was used.

$p<0.05$ was considered significant.

3 Results

3.1 Retrospective analysis

3.1.1 Retrospective analysis of primary outcome of patients who underwent APSCT

Multivariable analysis revealed that in the all patient group and in the lymphoma group neutrophil engraftment (OR 1.492, 95% CI 1.228–1.813, $p<0.001$; OR 1.476, 95% CI 1.061–2.052, $p=0.021$) and female sex (OR 2.301, 95% CI 1.124–4.714, $p=0.023$; OR 4.190, 95% CI 1.081–16.240; $p=0.038$) could be considered independent predictive factors (Classification ratio: 67.7%, Nagelkerke coefficient: 0.172); (Classification ratio: 78.8%, Nagelkerke coefficient: 0.236) in the development of OM2-4. In the myeloma group neutrophil engraftment (OR 1.39, 95% CI 1.09–1.773, $p=0.008$) (Classification ratio: 62.6%, Nagelkerke coefficient: 0.105) appeared as an independent prognostic factor.

Time to neutrophil engraftment (7.774±2.10 days) was significantly (p<0.001) longer in the lymphoma group than in the MM (5.80±2.07 days). At the same time, neutrophil engraftment was the strongest predictive factor in the NHL group (OR 1.598, 95% CI 1.101–2.321, p=0.014); (thrombocyte engraftment: OR 1.239, 95% CI 1.004–1.529, p=0.046; female sex: OR 5.320, 95% CI 1.077–26.276, p=0.040).

3.1.2 Secondary outcomes of retrospective analysis of 85 women who underwent APSCT

As next, since female sex has been shown to have a major role in the development of OM2-4, we conducted further analysis.
The 85 female patients were classified into two groups based on their hormonal status (57). Patients ≤50 years were grouped as premenopausal and those ≥51 years as postmenopausal, as we mentioned earlier. Based on this classificiation 19 women were premenopausal and 66 postmenopausal. Of the 19, 15 (78.95%), and of the 66, 49 (74.24%) patients developed ulcerative mucositis (OM2-4). There was no significant difference between the two groups (p=0.771). We did our calculations for the total patient group and the individual patient group, respectively, and got similar results. In the lymphoma subgroup 16 (88.89%) out of 18 premenopausal women, while 14 (93.33%) out of 15 postmenopausal women developed severe OM. The difference was not significant here, either (p= 1). In the myeloma group, 33 out of 49 postmenopausal female patients developed ulcerative OM while the corresponding ratio in the premenopausal group was 1 out of 3 (33.33%). No significant difference was found here, either (p=0.114).
However, ulcerative mucositis was significantly (p=0.009) more frequent in the lymphoma group than in the MM group

(30 of the 33 patients with lymphoma-90.9%, while 34 out of the 53 patients with MM-65.38%, p=0.009).

3.1.3 Overall survival with and without ulcerative mucositis

Correlation was assessed between average post-transplantational survival time and ulcerative mucositis. OS was 5.17 months shorter in the combined patient group (HL, NHL, MM) if ulcerative mucositis developed (OM2-4: 35.34 months (31.83–38.85); OM0-1: 40.51 months (26.42–44.61) (p =0.101).

3.2 Results of prospective studies

3.2.1 Determination of serum and salivary E2 and P4 levels in controls and during APSCT

A physiological decrease was observed in serum E2 level in the postmenopausal control group compared to the premenopausal (p=0.004), while there was no significant difference in salivary E2 level (p=0.069). Both in serum and saliva, P4 levels were significantly decreased in the postmenopausal controls compared to the premenopausal group (p=0.017, p=0.004). Serum P4 was more elevated in the transplantated patients compared to the postmenopausal controls at all four stages of transplantation, at day + 7 significantly (p=0.026). Salivary P4 was higher, although not significantly at days +7 and +14 compared to the two other stages of APSCT and to controls ($\geq$ 51), respectively. Although decrease in salivary P4 level was significant in the postmenopausal controls compared to the premenopausals, a tendency for increase was observed in postmenopausal APSCT patients at day +7 and day +14

compared not only to the postmenopausal controls, but also to the premenopausals. Serum E2 decreased significantly (p= 0.004) in the patient group compared to the premenopausal controls, while there was no significant difference between E2 serum and salivary hormone levels of postmenopausal controls and patients undergoing APSCT in the postmenopause. In summary, we didn't find any significant changes of E2 in relation to underlying disease and/or due to the APSCT in the patient group.

3.2.2 Assessment of serum and salivary IgA

3.2.2.1 Determination of serum and salivary IgA levels during APSCT and their correlation with the grade of OM

There was a continuous significant decrease in serum IgA levels during APSCT (day 0, day +7, day +14) as compared to the control group (p=0.024; p=0.005; p=0.004) and to the day of admission (p=0.027; p=0.028; p=0.028). The IgA secretion rate was lower in the remission stage than in controls at the first sampling (day −3/−7 prior to transplantation). At the further stages of APSCT (day 0, day +7, day +14), significant differences were observed between the controls and patients (p=0.015; p=0.001; p<0.001). There was no correlation between serum IgA (g/L) or salivary IgA secretion rate (µg/min) and the degree of oral mucositis (p= 0.685; p=0.1729).

3.2.2.2 Identified *N*-glycan structures. Comparison of serum and salivary *N*-glycome profile of controls and patients undergoing APSCT

We identified 44 *N*-glycan structures during the *N*-glycomic analysis of serum (n=31) and salivary (n=38) IgA. From the 38 *N*-glycan structures identified in the saliva sample, 13 were salivary specific and 25 were the same as found in the serum sample.
From the 25 overlapping structures in serum and saliva, eight were sialylated; fifteen were neutral and two were high mannose type structures. The salivary-specific *N*-glycan structures showed the following distribution: one structure was sialylated; out of the four neutral glycans three were afucosylated and one was antennary and core fucosylated, five oligosaccharides were high mannose type and three unknown.
Fourteen *N*-glycan structures showed significant differences ($p<0.05$) in serum between controls and any stages of APSCT.
The core fucosylated, sialylated bisecting biantennary glycan (FA2BG2S2) was the single significantly different structure between any two specified time points of the peritransplantation period (day −3,−7 and +14; $p=0.0279$), A1[3] only between control and day +7.
There were six significantly changed salivary IgA *N*-glycan structures in the control as well as in the patient group at the four stages of transplantation. None of the structures changed significantly between any two specified time points of the peritransplantation period.

3.2.2.3 Sialoform to neutral carbohydrate ratio (SF/NF) in serum and saliva

We calculated the ratios of sialylated and neutral structures in all three possible scenarios (present in serum; in saliva; and in both (i.e., 'overlapping structures') in the control and patient group at four stages of transplantion. This ratio was significantly higher in serum in all examined stages of APSCT

as compared to the control group (p=0.002; p=0.001; p=0.002; p=0.043). A significant change of the SF/NF ratio was observed between two specified time points of the transplantation (day −3/−7 and day 0; p=0.05). This ratio was also significantly higher in saliva samples at the day of admission and day 0 compared to the controls (p= 0.021; p=0.009). The SF/NF ratio of the overlapping structures in serum was significantly higher in all examined stages of the APSCT compared to the controls (p<0.001; p<0.001; p<0.001; p=0.006) and significantly lower between day 0 and day +14 (p=0.036).

3.2.3 Assessment of osteopontin

3.2.3.1 Changes of serum OPN levels in the control groups and in patients during APSCT

There was no significant difference in serum OPN levels regarding either age or pre- and postmenopausal hormonal status in the control group. Considerable overexpression could be observed during APSCT at all four stages of transplantation (day −3/−7, day 0, day +7, day +14) compared to the control group (p=0.013, p=0.02, p=0.011, p=0.028).

3.2.3.2 Changes of salivary OPN levels in the control groups and in patients during APSCT

Salivary OPN level was significantly lower in the elderly control group compared both to the middle-aged and the young adults group (p=0.001, p=0.01), while there was no difference between the middle-aged, and the young adults group (p=0.305). Premenopausals showed significantly higher

salivary OPN level than postmenopausal controls (p=0.001). There was no significant difference in salivary total protein concentration neither in relation to age nor to hormonal status. OPN/total protein concentration ratio (i.e., normalized OPN concentrations) in the elderly control group was lower compared both to the middle-aged and the young adults groups (p=0.003, p=0.012). There was no significant difference in the middle-aged group compared to the young adults group (p= 0.945) and it was lower in the postmenopausals compared to the premenopausals (p<0.001), in concert with the changes of absolute (non-normalized) OPN levels. These indicate that decrease in OPN is not due to a decrease in protein content in general. During APSCT there was a significant increase at day +7 and day +14 in salivary OPN levels compared to the control group (p=0.011, p=0.034) and at day +14 compared to the day of admission (day −3/−7) and transplantation (day 0) (p= 0.039, p=0.011).

3.2.3.3 Results of correlation analyses

There was a significant negative correlation between both salivary and serum OPN levels and grade of OM during APSCT (r =−0.791, p=0.019; r =−0.973, p=0.001). Salivary P4 level (62) showed a significant positive correlation with salivary OPN level in the postmenopausal control group (r=0.944, p=0.001). Neither pre-transplantational serum LDH level (activity), nor pre-treatment time showed a significant correlation with serum OPN levels at the day of admission (day −3/−7). Nor did we find any significant correlation between OPN levels and the returned amount of stem cells, stem cell viability, viable cell count and amount of mononuclear cells at the day of transplantation (day 0). CRP level showed a significant positive correlation with serum OPN level only at day +14 of the four stages of transplantation (r=0.700, p=0.036).

3.2.4 Changes of unstimulated whole saliva (USW) flow rate in the controls and in patients during APSCT

We determined and examined the changes of unstimulated whole saliva in all three parts of the prospective study.

During the hormonal analysis there was no significant difference (p=0.628) in UWS flow rate between the pre- and postmenopausal control groups. During APSCT, significant decrease was observed at day 0, day +7 and day +14 in UWS flow rate between the pre- and postmenopausal control groups (p=0.004, p=0.004, p=0.004); (p=0.048, p=0.030, p=0.018), and between the day of admission of APSCT (day − 3/− 7) (p= 0.043, p=0.043, p=0.043), respectively. There was a significant positive correlation (p=0.008, r=0.928) between serum E2 level and UWS flow rate in the premenopausal group.

In the study of IgA contrary to expectations, the amount of UWS did not decrease in the patients in pre-APSCT remission compared to the control group. During APSCT, there was a significant decrease at day 0, day +7 and day +14 in UWS flow rate as compared to the control group (p=0.008; p=0.004; p= 0.001) and the day of admission (p=0.012; p=0.012; p=0.012), respectively. There was negative correlation (r= −0.3622; p=0.0416) between decreased salivary flow rate (mL/min) and increasing severity of OM.

During the analysis of OPN levels in serum and saliva, we also examined the changes of UWS flow rate. There was no significant difference in UWS flow rates neither between the three age groups nor between the pre-and postmenopausals. No significant difference was observed in UWS flow rate at the day of admission (day −3/−7) compared to the control group whereas the amount of UWS decreased significantly at day 0, day +7 and day +14 compared both to the control group and the day of admission (p=0.008, p=0.004, p=0.001, p=0.012, p= 0.012, p=0.012) (63).

3.2.5 Analysis of relationship between oral and peripheral blood engraftment

We determined the time to engraftment. Time to oral engraftment was 14.14±5.815 days, and to blood engraftment was 12.13±2.532 days. We examined salivary and peripheral blood leukocytes also and we established that changes in salivary leukocytes was significant only at day +7 ($p=0.005$) of APSCT, while it was significant at all four stages of transplantation in the peripheral blood.

4 Discussion, summary

Injury of the protective mucosal barrier is one the most severe, sometimes fatal complications of hematopoietic stem cell transplantation. Its management is mostly supportive and palliative care; there are no valid biomarkers for the condition.
Retrospective analysis has revealed that female sex is an independent prognostic factor in the development of oral mucositis in lymphoma. We examined the changes of the two main female sex hormones (estrogen, progesterone) in serum and saliva during transplantation and assessed its correlation with the development of OM. We concluded that elevated progesterone levels may play a role in the weakening of the mucosal barriers not only in pre-, but also in postmenopause. Our results indicate that monitoring serum progesterone levels in women undergoing APSCT may be a suitable tool in the assessment of mucosal immunity, function and risk of severe OM.
The next section of the study we examined the *N*-glycosylation alteration of serum and salivary immunoglobulin A. To do this, first a special IgA binding protein had to be designed and produced. Then we confirmed that the developed Z(IgA1) affibody and the high resolution capillary electrophoresis with laser-induced fluorescent detection (CE-LIF) based

glycoanalytical methods provided an efficient and sensitive workflow to detect and monitor IgA glycosylation alterations in serum and saliva. We determined that *N*-glycosylation alteration of serum and salivary immunoglobulin A is a possible biomarker in oral mucositis.

The role of osteopontin in mucosal immunity and preservation of epithelial barrier integrity is essential. In our study we confirmed the importance of osteopontin in mucosal defense during APSCT, too. We concluded that salivary osteopontin could serve as a potential biomarker for oral mucositis and could be a suitable and efficient tool to screen and monitor different endocrine abnormalities. Serum osteopontin has been identified as an efficient marker of malignant hematological diseases durnig APSCT, too.

We set an easy and well applicable method for the detection of salivary leukocytes in the daily clinical rutin. Using this method, we determined that individual pool of salivary leukocytes are more resistant againts cytotoxic agents than peripheral blood leukocytes.

In our pilots, during in-depth examination of saliva, one of the main pillars of oral immunity, we identified new aetiological factors that play an important role in the development of oral mucositis and potential biomarkers which could also serve as therapeutic alternatives. Glycoanalytics has been a widely used, easily and efficiently applicable method in oral diagnostics and pathology. We have managed to appoint new research pathways in the recognition of oral immunity and the pathogenesis of oral inflammatory processes with the help of above.

5 New findings and considerations of the dissertation

1. P4 levels increase in postmenopause during APSCT both in serum (at day +7 significantly) and in saliva (at day +7 and +14) compared to the pre-and postmenopausal control groups. Monitoring serum progesterone levels in women undergoing APSCT may be a suitable tool in the assesment of mucosal immunity, function and risk of severe OM.
2. IgA, which is necessary for glycomic analysis, may be obtained from serum and saliva without significant loss and injury with the help of the developed IgA specific binding Z(IgA) affibody.
3. The high resolution CE-LIF based glyocoanalytical methods provided an efficient and sensitive workflow to detect and monitor IgA glycosylation alterations.
4. N-glycosylation alteration of serum and salivary immunoglobulin A could be a possible biomarker in oral mucositis during APSCT.
5. In our study there was a significant negative correlation between both salivary and serum OPN levels and OM grade during APSCT, highlighting the pivotal role of OPN in mucosal protection.
6. Salivary osteopontin could be a potential biomarker in oral mucositis.
7. Assessment of salivary osteopontin could serve as a suitable tool for screening endocrine abnormalities.
8. Serum OPN is a reliable biomarker for the presence of haematological malignancies during APSCT as well.
9. Individual pool of salivary leukocytes proves to be more resistant against cytotoxic agents than that in the peripheral blood.

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7 Keywords

oral mucositis, saliva, progesterone, immunoglobulin A, secretory IgA, osteopontin, engraftment, glycoanalitics, Z(IgA1), *N*-glycan profile, biomarker, hematopoietic stem cell transplantation

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Appendix

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List of other publications

5. Jakab, Á., Antal, K., Emri, T., Boczonádi, I., Imre, A., **Gebri, E. Z.**, Majoros, L., Pfliegler, V. P., Szarka, M., Balla, G., Balla, J., Pócsi, I.: Effects of hemin, CO2, and pH on the branching of Candida albicans filamentous forms.
Acta Microbiol. Immunol. Hung. 63 (4), 387-403, 2016.
DOI: http://dx.doi.org/10.1556/030.63.2016.023
IF: 0.921

6. **Gebri, E. Z.**, Kiss, A., Hegedűs, C., Baksa, B.: Symptoms of acute leukemias in the oral cavity.
Remedy OA. 1, 1-7, 2016.

Total IF of journals (all publications): 16,695
Total IF of journals (publications related to the dissertation): 15,774

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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