This is a detailed final report of the research project entitled „Thrombocytosis in solid tumors as an additive predictive factor of survival in diabetic and non-diabetic patients" (project ID: OTKA K-116128). The cumulative impact factor of research papers published with the help of the K-116128 grant is 17.904, and further two manuscripts are currently under review (IF: 4.988).

I. RETROSPECTIVE STUDY:

A total of 731 colorectal (CRC) and 274 breast cancer (BC) patients were included, who attended at Semmelweis University, between 2014 and 2018. Surviving patients were followed-up no later than October 31, 2019.

I/1. Preliminary results of CRC data was presented as an original article in ***ORVOSI HETILAP (2018)***. Thrombocytosis (defined as platelet counts > 400 × 10^9/L) was present in 22.1% of the study population. The frequency of thrombocytosis decreased significantly after the surgical removal of the primary tumor. Furthermore, thrombocytosis was associated with worse survival of patients, suggesting that high platelet count could indicate worse outcome. Similarly, pre-existing type 2 diabetes (T2DM) was also associated with poor survival.

I/2. A new measure of platelet change at an individual level, named personalized indicator thrombocytosis (PIT), was defined. PIT was analyzed in 100 and 150 control subjects and CRC patients, respectively. Compared to conventional thrombocytosis definition (platelet counts > 400 × 10^9/L), PIT values represented the condition of patients more precisely. In control subjects, PIT values indicated a slow decrease in platelet counts with age, while in CRC patients the opposite, a significant increase could have been observed. Higher PIT values were associated with more advanced tumor stages and poor survival. Detailed results have been reported as an original article in ***CANCERS (2020)***.

I/3. Results of BC data was published as an original article in ***ORVOSI HETILAP (2019)***. Pre-existing T2DM was associated with more severe clinicopathological stages and shorter survival times, while thrombocytosis did not affect BC. A recommendation was suggested that during routine diabetes care, women should be made aware of the importance of early routine mammography screening.

Furthermore, T2DM was suggested as a new risk factor for early BC screening and after 30 years of age mammography should be considered for T2DM patients in every two years, similarly to those with familial breast cancer.

II. PROSPECTIVE STUDY

CRC patients with and without T2DM, non-cancer T2DM patients and healthy control subjects, who attended at the Department of Internal Medicine and Hematology, Semmelweis University, and at the General Surgical Department, Szent Imre Hospital, were enrolled for this part of the project between March, 2017 and September 17, 2019. 285 basal, and a total of 398 blood samples, including baseline and all repeated measurements at 6-weeks, 6-, and 12 months after tumor removal surgery, were collected. Interleukin-6 (IL-6), CD40 ligand (CD40L), thrombopoietin (TPO), chromogranin A (CgA) and –B (CgB) level of study participant were measured.

II/1. The effect of T2DM over paraneoplastic thrombocytosis was investigated through the measurement of the biochemical markers of paraneoplastic thrombocytosis (platelet count, plasma IL-6 and TPO). Compared to healthy and T2DM control subjects, platelet counts ($p < 0.05$), plasma IL-6 ($p < 0.01$) and TPO ($p < 0.05$) levels were significantly higher in all CRC patients. In non-cancer T2DM patients, platelet count ($p < 0.05$) and IL-6 ($p < 0.01$) was significantly lower, while TPO was similar to those of CRC patients. Comparison between CRC patients with and without T2DM revealed no difference within the investigated parameters. Similar results were obtained in survival analyses: the combined disease-worsening effect of paraneoplastic thrombocytosis and T2DM could not be justified; the two conditions did not worsen the outcome of CRC together, suggesting that T2DM has no significant effect over the CRC-related thrombocytosis. Detailed results are currently under review in ***WIENER KLINISCHE WOCHENSCHRIFT***.

II/2. The role of CD40L, a glycoprotein belonging to the tumor necrosis factor-family of cytokines, is still controversy in CRC. CD40L and its relationship to thrombocytosis and disease severity was investigated in CRC. Significantly higher CD40L levels were found in CRC patients with distant metastasis ($p = 0.0055$), Stage

IV cancer ($p = 0.0233$), and thrombocytosis ($p = 0.0294$). A significant relationship was found between CD40L and the parameters of paraneoplastic thrombocytosis ($p \leq 0.0454$). Furthermore, higher pre- and postoperative CD40L level was associated with worse overall, and disease-specific survival of patients ($p \leq 0.0460$), even if the effect of thrombocytosis was eliminated. Results suggest that CD40L is just partly dependent on platelet count / thrombocytosis, and with high probability, there are further, currently unknown factors behind the CD40L elevation of CRC patients. Detailed results were sent to **WORLD JOURNAL OF GASTROENTEROLOGY *(currently under review)***.

II/3. Chromogranins are known to be involved in various cancers and in diabetes. The combined effect of thrombocytosis and CgA-positive neuroendocrine-cell differentiation (CgA^+) on CRC was never investigated previously. Serum CgA and CgB level of 42 CRC patients was measured, and CgA-specific immunohistochemical staining of resected tumor tissues was performed. CgA^+ was present in approximately third of the analyzed tissues samples, and was more frequent in right-sided tumors, and no Stage I cancer was found in its presence. Serum CgA level was higher in CgA^+, while serum CgB and plasma TPO levels were significantly higher within patients without CgA^+. IL-6 did not differ between the two cohorts. Survival predictions were significantly worse for CgA^+. Data suggested that the tumors affected by CgA^+ have a different mechanism of thrombocytosis, possibly caused by the bleeding of the tumor, while the thrombocytosis of tumors without CgA^+ can be explained by paraneoplastic thrombocytosis, with all probability. Based on these observations we proposed a new subtype of CRC, which can be characterized by CgA^+. Detailed results were published in ***CANCERS (2021)***.

III. GENETIC STUDY

III/1. Blood and colon biopsy samples were collected from four patient groups (healthy controls "controls", patients with colorectal cancer "CRC", patients with type 2 diabetes mellitus "T2DM" and patients with both type 2 diabetes and colorectal tumor "CRC + T2DM") with strict recruitment criteria ruling out comorbidities thus ensuring high levels of sample homogeneity. Altogether 22 age and sex-matched

samples were analyzed with 5 or 6 individuals per group. Following cDNA synthesis, equal amount of samples were pooled into twosomes to give 3 pooled samples per group (where a group consisted of 5 individuals, two pools and one individual sample were analyzed). Samples were analyzed by a low density OpenArray signal transduction panel, which included 574 query and 24 control transcripts. As disease-related gene expression patterns largely differed by the tissue type analyzed, data obtained from colon tissue and blood samples were processed separately.

- III/1/A. A two-tailed, unpaired t-test statistical analysis on blood sample results revealed that 49 genes were significantly differentially expressed ($p < 0.05$) in patients with CRC + T2DM as compared to CRC individuals, while 11 genes displayed altered expression in non-diabetic CRC patients compared to healthy controls. Importantly, these gene sets did not display the slightest overlap, implying that T2DM, a frequent co-morbidity afflicting lots of CRC patients, is a very important determinant of PBL gene expression signatures. In other words, the presence or absence of T2DM has to be considered in our quest for CRC biomarkers.

  The top 5 genes showing most significant expression differences in this pilot study were PCK2, MAPK9, CCND1, HMBS and TLR3 in the CRC+T2DM versus T2DM comparison, while those in the CRC versus control context were CREBBP, PPIA, NFKBIL1, MDM2 and SELPG. These gene sets deserve further consideration in larger-scale studies aimed at finding reliable CRC biomarkers.

  A systems biology approach based Reactome pathway analysis was also carried out to see which PBL pathways are most affected by CRC. These items were cytokine signaling in general and interleukin/CD28 signaling in particular in diabetic patients, while p53-dependent proapoptotic signaling and interleukin pathways turned out to be most significantly affected in normoglycemic subjects.

These results are summarized in our paper entitled „Diabetes-specific changes in peripheral blood gene expression signatures in colorectal cancer" published in ***CURRENT MOLECULAR MEDICINE***.

- III/1/B. In colon biopsy samples, possible interaction between colorectal tumor and diabetes status in establishing gene expression patterns was analyzed by two-way ANOVA. 48 transcripts showed a significant ($p < 0.05$) interaction between diabetic and colorectal carcinoma status in establishing mRNA levels. In 13 cases, the significance level was $p < 0.01$. Pathway analysis indicated that members of the Wnt, Hippo, TNF and PI3K-Akt pathways, as well as genes involved in platelet activation were over-represented in the gene set that showed significant interaction between diabetes and colorectal cancer. Furthermore, an additional 105 transcripts were differentially expressed in tumor versus non-tumor samples (regardless of diabetic status) and 75 transcript showed differential expression by diabetes status (irrespective of tumor status).

III/2. Of the genes showing significant interaction in the above study, 24 were chosen out for validation. Intra-operative tumor and surrounding tissue samples were collected from colorectal cancer patients with and without type 2 diabetes. Recruitment criteria were the same as for colorectal biopsy subjects. Finally, age and sex-matched samples of 6 persons with T2DM and 6 persons without this condition were assessed by quantitative real-time PCR. Both tumor and surrounding tissue was analyzed for each subject. Measurement data were analyzed by paired t-test. In both the diabetic and the non-diabetic group, 4-4 genes were found to show significantly different expression levels between tissue types, with only one gene overlapping between the two groups. These results thus also imply that diabetes status affects colorectal tumor-specific gene expression profiles. A manuscript summarizing both explorative and validation colorectal tissue sample data has been published in ***LIFE***.

LIST OF PUBLICATIONS

- Herold Z, Dank M, Herold M, Nagy P, Rosta K, Somogyi A: Histopathological chromogranin A-positivity is associated with right-sided colorectal cancers and worse prognosis. **CANCERS**, 13(1): 67, 2021. DOI: 10.3390/cancers13010067 **IF: 6.126 (2019)**
- Elek Zs, Rónai Zs, Keszler G, Harsányi L, Kontsek E, Herold Z, Herold M, Somogyi A, Bánlaki Zs: Correlation between expression profiles of key signaling genes in colorectal cancer samples from type 2 diabetic and non-diabetic patients. **LIFE (Basel)** 10(9): 216, 2020. DOI: 10.3390/life10090216 **IF: 2.991 (2019)**
- Molnár Zs, Bánlaki Zs, Somogyi A, Herold M, Herold Z, Guttman A, Rónai Zs, Keszler G: Diabetes-specific modulation of peripheral blood gene expression signatures in colorectal cancer. **CURRENT MOLECULAR MEDICINE** 20(10): 773-780, 2020. DOI: 10.2174/1566524020666200504084626 REAL: http://real.mtak.hu/121376/ **IF: 1.600 (2019)**
- Herold Z, Herold M, Lohinszky J, Dank M, Somogyi A: Personalized indicator thrombocytosis shows connection to staging and indicates shorter survival in colorectal cancer patients with or without type 2 diabetes. **CANCERS (Basel)** 12(3): 556, 2020. DOI: 10.3390/cancers12030556 **IF: 6.126 (2019)**
- Somogyi A, Herold M, Lohinszky J, Harsányi L, Herold Z: Survival impact of diabetes and paraneoplastic thrombocytosis in Hungarian breast cancer women. **ORVOSI HETILAP** 160(51): 2012-2020, 2019. DOI: 10.1556/650.2019.31594 **IF: 0.497**
- Herold Z, Ambrus V, Herold M, Herczeg Gy, Igaz P, Harsányi L, Somogyi A: The occurrence and impact on survival of type 2 diabetes mellitus and thrombocytosis in colorectal cancer, before and after the surgical resection of the primary tumor. **ORVOSI HETILAP**, 159(19): 756-767, 2018. DOI: 10.1556/650.2018.31038 **IF: 0.564**

Articles currently under review:

- Herold Z, Herold M, Herczeg Gy, Fodor A, Rosta K, Dank M, Lang Zs, Somogyi A: Does type 2 diabetes affect paraneoplastic thrombocytosis in colorectal cancer? **WIENER KLINISCHE WOCHENSCHRIFT, IF: 1.323 (2019)**
- Herold Z, Herold M, Herczeg Gy, Fodor A, Szasz MA, Dank M, Somogyi A: High serum CD40L level is associated with advanced stages, local and distant metastases, and worse prognosis in colorectal cancer. **WORLD JOURNAL OF GASTROENTEROLOGY, IF: 3.665 (2019)**