Final report, KH 130355

"Pathogenetikai tényezők azonosítása és jellemzése thromboticus microangiopathiákban"

In the last 12 months of the project the COVID pandemic caused significant delay in wet-lab experimentation, but during the summer period of 2020 all of the planned experiments were finally finished. However, two of the coworkers involved in the experiments and analysis (Nóa Veszeli, and Blanka Mező, is on maternity leave since 08/2020 and 10/2020), therefore some part of the analysis of final data, and summary for publication, is still ongoing. The results were partly presented in international and national meetings (but in 2020 the international complement meeting was shifted to 2021), and one part of the results were already published (see below).

Details:

The project had 4 aims, results are reported accordingly:

1, Complotype Examination in thrombotic microangiopathies

The complotye is the combination of alleles of multiple, common SNPs of complement factors and regulators, collectively claimed to play a role in the determination of the complement regulatory capacity of a given individual(1). The three SNPs determining the complotype (C3 p.R102G, CFB p.R32Q és CFH p.V62I) were genotyped in the following patient cohorts: 96 patients with aHUS, 83 with secondary HUS/TTP, 40 with STEC HUS and 80 with TTP, together with the analysis of 200 healthy controls. Minor allele frequencies in the groups (aHUS; HUS/TTP; STEC HUS; TTP) for the above SNPs were: C3 p.R102G: 0,25/0,16/0,29/0,14; CFB p.R32Q: 0.08/0.04/0.08/0.04; CFH p.V62I: 0.15/0.24/0.28/0.24. MAF in the control group was 0.16/0.08/0.23.

Of note, the rare "GG/RR/VV" haplotype seems to uniquely associate to kidney diseases, aHUS and D+HUS (Figure 1).

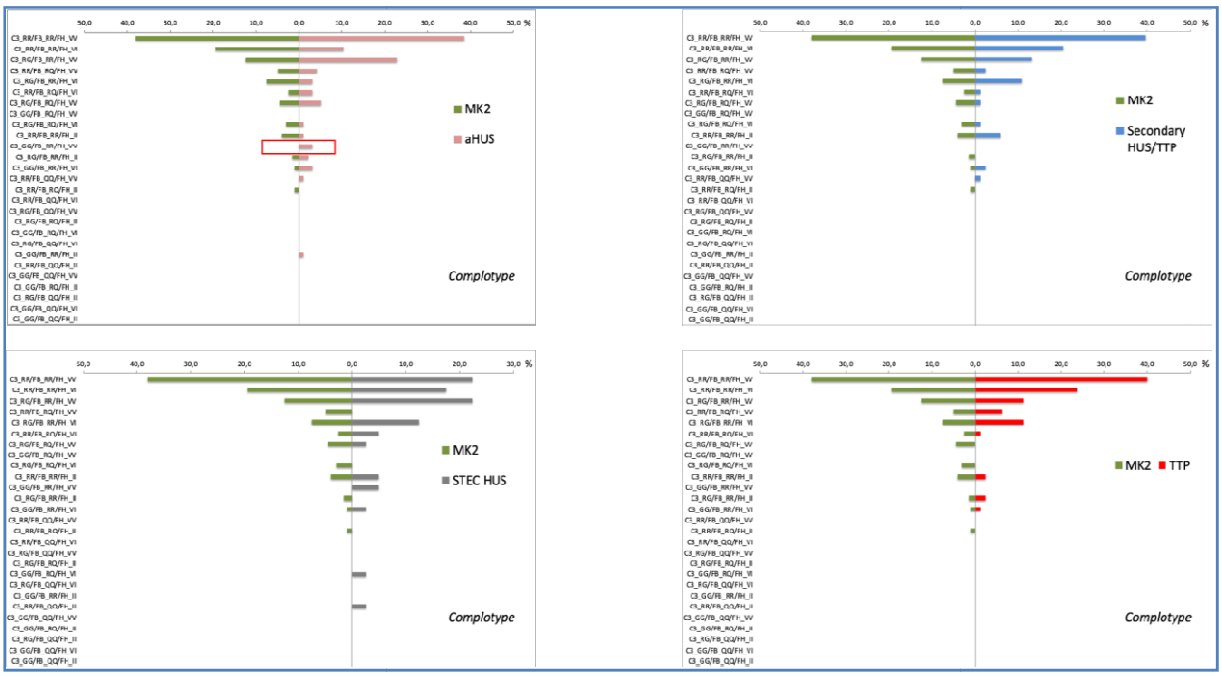


Figure 1: Haplotype distribution (frequency of carriers, X axis) among groups of healthy (MK2), atypical HUS, typical (STC) HUS, secondary TMA and TTP patients. Allelic combinations of C3 p.R102G, CFB p.R32Q és CFH p.V62I SNPs are indicated on the Y axis.

Detailed analysis of the presence and distribution of components of 'complotype' with high alternative pathway activity, clinical phenotype and outcome is ongoing, and will be finalized in 2021.

2, Characterization of potentially mRNA-damaging DGKE variants in aHUS:

The phenotypic spectrum of nephropathies (aHUS and glomerulonephritis) associated with mutations in Diacylglycerol Kinase ε (DGKE) is broad, nearly all of the affected patients/families seem to carry different mutations in *DGKE(2)*. To improve our understanding of molecular pathogenicity, we aimed to functionally characterize the novel variants observed in our diagnostic center in the past years. We set up the experimental models to determine DGKE mRNA (specific primers) and protein expression (antibodies for Western blot), and successfully finalized the preliminary experiments with human cell lines. We validated the protocol to store and analyze primary human cells for DGKE expression (obtained from the patients with DGKE variations, and control subjects), and we finalized the DGKE expression analysis (both mRNA and protein).

Messenger RNA expression was detectable in all patients and their family members (see Table 1). The degree of the expression -in term of the affected exon-was compared with one healthy individual. Interestingly and unexpectedly, all of the patients and family members had detectable DGKE protein expression, as analyzed in PBMC and platelet.

Family ID	Patient ID	Family member	Mutations/rare variations in *DGKE* gene	mRNA expression (%)	Detectable protein expression
Family 1	**HUN1993**	**Patient**	**hetero. c.35C>T (P12L) hetero. c.129G>A (Q43Q)**	**18.2**	**yes**
	HUN2199	Father	hetero.c.35C>T (P12L)	26	yes
	HUN2200	Mother	hetero. c.129G>A (Q43Q)	8	yes
	HUN2201	Sister I	hetero. c.129G>A (Q43Q)	30	yes
	HUN2202	Sister II	hetero. c.129G>A (Q43Q)	31	yes
Family 2	**HUN1338**	**Patient**	**hetero. c.1284+151A>G hetero. c.1215G>A (A405A)**	**84**	yes
	HUN1408	Mother	hetero. c.1284+151A>G	28	yes
	HUN1409	Father	hetero. c.1215G>A (A405A)	47	yes
	HUN1410	Sister	hetero. c.1284+151A>G hetero. c.1215G>A (A405A)	98	yes
	HUN1558	Aunt	hetero. c.1284+151A>G	-	yes
Family 3	**HUN1137**	**Patient**	**hetero. c.559_559 delA (p.I187Ffs*6) hetero. c.966G>A (p.W322X)**	**184**	**yes**
	HUN1167	Mother	hetero. c.966G>A (p.W322X)	40	yes
Family 4	**HUN1144**	**Patient**	**homo. c.966G>A (p.W322X)**	**13**	**yes**
	HUN1145	Brother	hetero. c.966G>A (p.W322X)	36	yes
	HUN1146	Mother	hetero. c.966G>A (p.W322X)	17	yes
-		Healthy control		100	yes

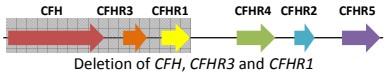
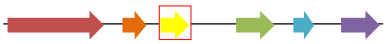
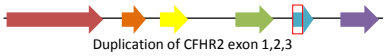
Table 1 summarizing mutations of DGKE, and DGKE mRNA/proptein expression in members of the four affected families.

Due to these rather unexpected findings detailed analysis (RNAseq) to see if aberrant transcripts are expressed, or if aberrant proteins are produced (WB with additional antibodies) are planned, and will be completed after the closure of the project (in 2021). Results will be summarized and published after these experiments are completed.

3, Examination and molecular characterization of abnormal Factor H-related (FHR) Proteins:

The regulators of complement activation gene complex (RCA) on the first chromosome contains multiple genes with high homology encoding Factor H family members. Non-allelic recombination events are frequently occurring in this region leading to the development of hybrid genes with aberrant structure and expression of dysfunctional proteins(3). MLPA analysis of the FH-related gene cluster was finalized for 177 patients with aHUS, and 183 patients with C3-glomerulopathy. Gene-rearrangement events potentially leading to the expression of aberrant, hybrid FH or FHR proteins were observed in 11 patients (5 aHUS, 6 C3G). Western blot analysis of 3 out of the 11 patients showed finally the expression of hybrid proteins, namely FHR2/FHR5, FHR3/FHR1 and FHR2/FHR1 (see Table 2). The breakpoint analysis, together with segregation analysis (family members) was finalized in 2020. The preliminary results were presented at the European Complement Meeting, and in the National Immunology Meeting. Finalization of the analysis and writing of the manuscript is in progress in 2021.

Table 2. Characterization of the five patients with large deletions, duplication and/or rearrangements in the *CFHR* genomic region. Grey background represent deletion and red frame represent duplication of indicated regions.

Patient ID	Diagnosis	Result of MLPA	Hybrid protein expression	LPVs*	Complement profile	Familial?
HUN1739	IC-MPGN	 Deletion of *CFH*, *CFHR3* and *CFHR1*	-	*CD46* A353V	AP activity, C3, FB, FH levels decreased	no
HUN1770	C3GN	 Duplication of *CFHR1*	-	-	CP and AP activity decreased	yes
HUN1504	C3GN	 Deletion of *CFHR3* exon 5-6	 $FHR3_{1\text{-}2}FHR1$	*CFI* c.13G>A *CFI* K441R	normal	yes
HUN1917	C3GN	 Deletion in *CFHR2* exon3-4 and in *CFHR5* exon 1	 $FHR2_{1\text{-}2}FHR5$	-	deficient AP activity, C3, FB levels decreased, sC5b-9 level elevated	yes
HUN1547	C3GN	 Duplication of CFHR2 exon 1,2,3	 $FHR2_{1\text{-}2}FHR1$	*CFI* K441R	AP activity decreased	no

* Likely pathogen variations in the known disease-associated genes (*CFH, CFHR5, CFI, CD46, C3, CFB, THBD*)

4, Validation of sC5b-9, as prognostic marker for TMA after allogeneic stem cell transplantation in children:

To replicate our original observations (4) we enrolled 68 completely new pediatric allogeneic HSCT patients in the validation study (October 2015-January 2019). Overall, 10/68 subjects met at least one of the different TA-TMA diagnostic criteria according to the five classification systems, typically on day 62 (median, range: 35-90). Complement activity, activation markers, and regulator levels were determined in 2019, and tested for a potential association with later development of complications. A strong association was found between early (0 to 28 day, post-HSCT) increase of sC5b-9 (10 of 10 patients with TA-TMA vs. 28 of 58 without TA-TMA; p=0.0017) and later development of TA-TMA (see Figure 3).

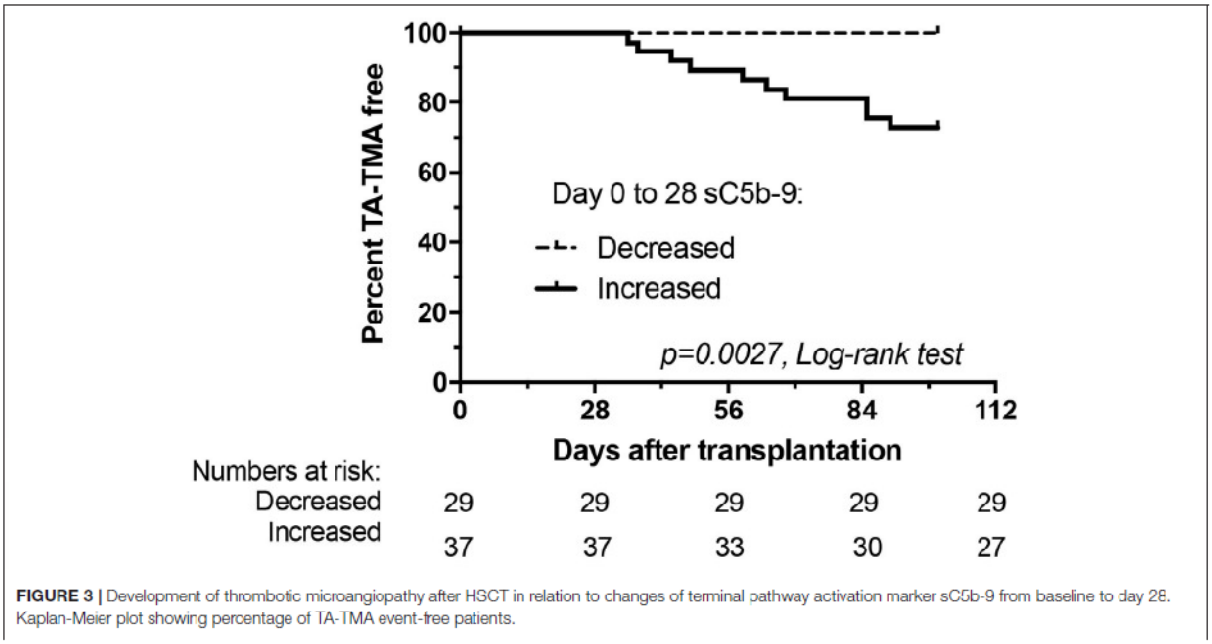


FIGURE 3 | Development of thrombotic microangiopathy after HSCT in relation to changes of terminal pathway activation marker sC5b-9 from baseline to day 28. Kaplan-Meier plot showing percentage of TA-TMA event-free patients.

An increase in sC5b-9 concentration had 100% sensitivity and 54% specificity for TA-TMA. No additional complement parameters were closely associated with the development of TA-TMA. Early raise of the sC5b-9 activation marker is predictive for later development of TA-TMA, as confirmed in this second, validation cohort. The results were published in 2020 in Frontiers in Medicine(5).

In summary, all of the planned experiments were finalized, results were published in 1 peer-reviewed article and additional congress presentations, and writing of the additional manuscripts is in progress in 2021. We thank the work of the anonymous evaluators and panel members of our proposal and report!

References cited in this report:

1. Harris CL, Heurich M, Rodriguez de Cordoba S, Morgan BP. The complotype: dictating risk for inflammation and infection. Trends in immunology. 2012;33(10):513-21. Epub 2012/07/04.
2. Azukaitis K, Simkova E, Majid MA, Galiano M, Benz K, Amann K, et al. The Phenotypic Spectrum of Nephropathies Associated with Mutations in Diacylglycerol Kinase epsilon. Journal of the American Society of Nephrology : JASN. 2017;28(10):3066-75. Epub 2017/05/21.
3. Skerka C, Chen Q, Fremeaux-Bacchi V, Roumenina LT. Complement factor H related proteins (CFHRs). Molecular immunology. 2013;56(3):170-80. Epub 2013/07/09.
4. Horvath O, Kallay K, Csuka D, Mezo B, Sinkovits G, Kassa C, et al. Early Increase in Complement Terminal Pathway Activation Marker sC5b-9 Is Predictive for the Development of Thrombotic Microangiopathy after Stem Cell Transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2018;24(5):989-96. Epub 2018/01/18.
5. Mezo B, Horvath O, Sinkovits G, Veszeli N, Krivan G, Prohaszka Z. Validation of Early Increase in Complement Activation Marker sC5b-9 as a Predictive Biomarker for the Development of Thrombotic Microangiopathy After Stem Cell Transplantation. Frontiers in medicine. 2020;7:569291. Epub 2020/10/30.