PROJECT TITLE: ASSESSING THE COMBINED EFFECT OF MULTIPLE POLYMORPHISMS IN ORDER TO DEFINE THE GENETIC PREDISPOSITION TO MYELOPROLIFERATIVE NEOPLASMS

PROJECT CODE: PN-III-P1-1.1-PD-2016-1414

STAGE 2018: THE DEVELOPMENT AND IMPLEMENTATION OF REAL-TIME PCR TECHIQUES IN GENOTYPING A 16 SNPs PANEL IN PATIENTS WITH BCR-ABL NEGATIVE MYELOPROLIFERATIVE NEOPLASMS AND CONTROLS.

The aim of this stage (2018) included the following activity:

GENOTYPING A 16 SNPs PANEL IN PATIENTS WITH BCR-ABL NEGATIVE MYELOPROLIFERATIVE NEOPLASMS AND CONTROLS

BCR-ABL negative myeloproliferative neoplasms are hematological malignancies that originate from myeloid progenitors or precursors and consist of: Polycythemia vera (PV), essential thrombocythemia (TE) and primary myelofibrosis (PMF). All three are chronic diseases with a median overall surviaval of 6-7 years in case of primary myelofibrosis, surpassed by polycythemia vera and essential thrombocythemia (Tefferi and Vardiman, 2008).

Common complications of these diseases are: venous and arterial thrombosis (especially splenic thrombosis - potentially life threatening) in about 20-30% of patients at diagnosis, microcirculatory events (erythromelalgia, Raynaud or blue-toe syndrome, superficial thrombophlebitis, migraine, tinnitus) and bleedings (epistaxis, upper and lower digestive tract bleedings). After a variable amount of time, 10-15% of patients with polycythemia vera or essential thrombocythemia develop secondary

myelofibrosis. Approximately 5% of patients with myeloproliferative neoplasms progress into a blastic phase (acute myeloid leukemia), with a generally bad outcome and 80% of patients report a decrease in general health. All of the above make this group of hematological malignancies an important health issue, in spite of the relatively favorable prognostic (especially in patients with polycythemia vera and essential thrombocythemia).

BCR-ABL negative myeloproliferative neoplasms are characterized by some specific somatic mutations. The JAK2 (Janus kinase 2) V617F mutation, firstly described in 2005, by 4 distinct research groups, is present in almost all PV patients and in 50-60% out of the TE and PMF patients (Kralovics et al, 2005; James et al, 2005; Baxter et al, 2005; Levine et al, 2005). Mutations in the thrombopoietin receptor gene (myeloproliferative leukemia, MPL), described in 2006, are present in 5-10% of TE and PMF patients who are JAK2 V617F negative (Pikman et al, 2006; Pardanani et al, 2006). Lastly to mention, CALR (calreticuline) mutations characterize 60-80% of JAK2 V617F negative TE and PMF patients (Klampfl et al, 2013; Nangalia et al, 2013). While only a minority of PV patients present an exon 12 – JAK2 gene mutation, the remaining TE and PMF patients (10-15%), form a heterogeneous triple negative subgroup (negative for JAK2, MPL and CALR).

In the last years, reasearchers in this field have extended their focus also on the genetic predisposition of chronic myeloproliferative neoplasms (MPN). In 2008 a large epidemiological study showed that grade I relatives of MPN patients have a 5-10 times higher risk of developing these diseases then the rest of the population (Landgren et al, 2008). In 2009, three independent research groups have reported that the JAK2 46/1 haplotype is a major constitutuional driver of MPNs, especially for JAK2 V617F positive patients (Kilpivaara et al, 2009; Jones et al, 2009; Olcaydu et al, 2009). 2010 has been the year when my research team and I, have replicated these results on a 149 romanian patient group, by genotyping rs10974944 C>G SNPs, which mark the JAK2 46/1 haplotype through an own PCR-RFLP technique (Trifa et al, 2010a,b).

Later, in 2014, the rs2736100 A>C TERT (telomerase) polymorphism, has been also described as a major driver of MPNs by a scientific research group from deCODE Iceland (Oddsson et al, 2014; Jager et al, 2014). To mention is that the same polymorphism has initially been described as having a major role in lung cancer predisposition and later also in other solid cancers, especially gliomas. It seems that

this particular polymorphism comes with an increased telomerase activity, respectively longer telomeres. In the general population, the presence of this polymorphism is associated with higher blood count of myeloid cells (erythrocytes, granulocytes, platelets). Both of these polymorphisms JAK2 46/1 haplotype and TERT rs2736100 A>C seem to have a major contribution in the development of BCR-ALB negative MPNs. The PAF (population attributable fraction) of these two polymorphisms has been evaluated at 40% and respectively at 50%.

A study from 2015 shows new polymorphisms in the context of genetic predisposition of BCR-ALB negative MPNs: JAK2 rs12339666 and MECOM rs2201862 for JAK2 V617F-negative patients, and HBS1L/MYB rs9376092 especially found in JAK2 V617F positive TE patients and CALR and MPL positive BCR-ALB negative MPNs (Tapper et al, 2015).

Later, in 2016, a GWAS study high lightens other new polymorphisms as risk factors in JAK2 V617F associated clonal hematopoiesis and MPNs (Hinds et al, 2016).

This being said, as project director, I would like to mention the results obtained so far by my team and I, on genetic predisposition in BCR-ALB negative MPNs. In 2010 we have published a study in Annals of Hematology, on 149 subjects, regarding the contribution of JAK2 46/1 haplotype in the development of JAK2 V617F positive MPNs, showing no correlations with JAK2 V617F negative MPNs (Trifa et al, 2010b). 2016 we have published in British Journal of Haematology the results of a study on 529 MPN-patients tested for JAK2 V617F and CALR (CALR hadn't been described in 2010). The study described the individual and combined contribution of the JAK2 46/1 haplotype and TERT rs2736100 A>C polymorphism in the development of MPNs (Trifa et al, 2016). We demonstrated that the JAK2 46/1 haplotype played a significant role in JAK2 V617F positive and CALR positive MPNs, with a much lesser extent in the latter. On the other hand, the TERT rs2736100 A>C polymorphism was associated in similar extent with all molecular and phonotypical types of NPMs. The two polymorphisms had a synergic effect on the development of the disease, with a peak in the case of both homozygote genotypes.

Finally in 2017, out study in American Journal of Haematology showed the individual and combined effect of five polymorphisms (JAK2 46/1, TERT rs2736100, MECOM rs2201862, HBS1L-MYB rs9376092 and THRB-RARB rs4858647) on 939

MPN patients. The MECOM rs2201862 polymorphism was associated with all MPN phenotypes and both molecular subtypes (JAK2 V617F and CALR positive), with a greater effect on the CALR positive patients. The HBS1L-MYB rs9376092 polymorphism was positively associated only with ET (on behalf of JAK2 V617F and not CALR). Lastly the THRB-RARB rs4858647 polymorphism had only a weak correlation with PMF. To sum up the study, JAK2 46/1, TERT rs2736100 and MECOM rs2201862 polymorphisms have the greatest contribution in the development of MPNs. The study had also proposed a statistic model for the prediction of these diseases by evaluating genotypes for these three polymorphisms.

Results of 2018 stage: THE DEVELOPMENT AND IMPLEMENTATION OF REAL-TIME PCR TECHIQUES IN GENOTYPING A 16 SNPs PANEL IN PATIENTS WITH BCR-ABL NEGATIVE MYLOPROLIFERITIVE NEOPLSMS AND CONTROLS.

In this stage we have elaborated the genotyping protocols by real-time PCR for the 16 SNPs panel in order to genotype the study subjects and controls.

Genotyping these polymorphisms is based on the TaqMan chemistry, using specific hydrolysis probes for each SNP. Each SNP assay contains a mix of two probes, one for each specific allele and another for the extern primers used in the amplification of the SNPs containing DNA fragments, basically forming multiplex real-time PCR type reactions.

Each of the two probes is marked with a distinct fluorochrome. The fluorochromes are masked by a quencher and can only be freed if the probe hybridizes with the targeted DNA. The wild-type allele is marked with the FAM fluorochrome, while the variant allele is marked with the VIC fluorochrome in all SNPs of the Open Array panel. The genotyping mix uses ROX as a passive reference stain.

The hybridization process leads to the hydrolysis of the probe, the fluorochrome thus being freed and excited. The emitted light signal will be quantified after each amplification cycle. The quantity of the emitted fluorescence is proportional with the number of targeted DNA sequences matching each specific probe.

The fluorescent signal is converted into a graphic signal in shape of amplification curves (one for each allele). In this sense, homozygotes have one amplification curve, specific to the wild-type or variant allele, whereas homozygotes have both amplification curves. In the end the results appear as an amplification plot, grouped in three clusters for each SNP: wild-type homozygotes, heterozygotes, variant homozygotes. The genotyping soft has an auto-call option which generates automatically the genotype of each sample, if all steps have been followed optimally.

Due to Open Array panels, patented by Thermo Fisher, genotyping more then one SNP at once is possible. These kind of panels are custom made and can contain probes for 16, 32, 64 and more probes. For this project we have ordered an Open Array panel for 16 SNPs containing the probes for the following SNPs:

- JAK2 rs10974944
- TERT rs2736100
- MECOM rs2201862
- MECOM rs3851379
- HBS/MYB1L1 rs9376092
- THRB/RARB rs4858647
- SH2B3 rs3184504
- TET2 rs1548483
- CHEK2 rs555607708
- CHEK2 rs17879961
- ATM rs1800056
- GFIB rs621940

Bioinformatic quality control of the genomic sequences for these particular SNPS has been favorable except for one. The initial panel should have contained the PINT rs58270997 SNP, which didn't pass the quality control. We will try evaluating this SNP in the next step individually, through a Taqman assay, by common real-time PCR technique. In the mean time this SNP has been replaced by CHEK2 rs17879961, which has been previously described as a moderate risc factor for many human neoplasms. Besides the mentioned 12 SNPs, the pannel contains another 4 probes for internal control (selected by the pannel producer).

The ordered panel came in shape of 10 array plates. Each one intended for 144 samples. The Open Array panels use specific reactieves and supplies:

- QuantStudio™ 12K Flex System OpenArray® Accessories Kit (12 lids and plugs, 12 immersion fluid syringes, and 2 carriers)
- Open Array Genotyping Master Mix
- OpenArray® 384-Well Sample Plates
- OpenArray® AccuFill™ System Tips

All reactions were run on a real-time PCR system dedicated to Open-Array panels: QuantStudio 12K Flex 12K.

After amplification condition optimization, we have genotyped 1000 samples of MPN patients and 400 controls.

All included study subjects had a positive molecular marker, JAK2 V617F or CALR, thus providing evidence of the malignant nature of the affection. DNA samples have been collected at the time of diagnosis, the same time as samples have been collected for molecular evidence. The controles are individuals from general population with no evidence of any hematological malignancies.

In conclusion:

- we have fulfilled 100% the aim of the activity of the 2018 stage: **GENOTYPING A 16 SNPs PANEL IN PATIENTS WITH BCR-ABL NEGATIVE MYELOPROLIFERATIVE NEOPLASMS AND CONTROLS**

Also, we have accomplished all 100% deliverables proposed in the work plan for the 2018 stage, as following:

1. Protocols for genotyping the 16 SNPs panels for patients with BCR-ABL negative myeloproliferative neoplasms and controls
2. Genotype distribution and allele frequencies for the 16 analyzed SNPs in patients with BCR-ABL negative myeloproliferative neoplasms and controls.

Following, the obtained results will be analyzed in order to build a mathematical model for the genetic predisposition of BCR-ABL negative myeloproliferative neoplasms, taking into account the genotypes and alleles for the genotyped SNPs through Open Array panel by real-time PCR. All of this will be written down and submitted in order to be published in a famous hematology paper. The above mentioned compose the 2019 stage.

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