GENETIC SUSCEPTIBILITY TO MYELOPROLIFERATIVE NEOPLASMS AND THERAPEUTIC EFFICACY

ANA PAULA DA SILVA AZEVEDO

Tese para obtenção do Grau de Doutor em Medicina

Na Especialidade de Genética, Oncologia e Toxicologia Humana

Na NOVA Medical School | Faculdade de Ciências Médicas

Dezembro, 2017

GENETIC SUSCEPTIBILITY TO MYELOPROLIFERATIVE NEOPLASMS AND THERAPEUTIC EFFICACY

Ana Paula da Silva Azevedo

Orientador: Professor Doutor José Rueff, Professor Catedrático

Tese para obtenção do Grau de Doutor em Medicina

Na Especialidade de Genética, Oncologia e Toxicologia Humana

Dezembro, 2017

Aos meus pais,

Aos meus filhos e ao meu marido,

Todo o meu amor.

"O Homem é do tamanho do seu sonho"

Fernando Pessoa

TABLE OF CONTENTS

FIGURE INDEX

TABLE INDEX

AGRADECIMENTOS

O trabalho desenvolvido nos últimos anos e que possibilitou a elaboração desta dissertação, teve a colaboração de um conjunto de pessoas e instituições que, de alguma forma, me acompanharam e a quem gostaria de expressar o meu sincero reconhecimento e agradecimento.

Ao Professor Doutor José Rueff, Diretor do Centre for Toxicogenomics and Human Health Genetics, Oncology and Human Toxicology, da Universidade Nova de Lisboa agradeço a orientação e a oportunidade que me concedeu para a realização do meu projecto no seu Laboratório, todo o apoio, empenho e dedicação incansáveis e o contributo científico na elaboração da minha tese. Agradeço a amizade e o entusiasmo com que aborda a Genética e a Medicina, que me contagiou enquanto sua aluna na Faculdade e que me conduziu até aqui.

À Professora Susana Nunes da Silva agradeço toda a dedicação, apoio, empenho e disponibilidade sem limites e o contributo científico na elaboração da minha tese. Agradeço a amizade demonstrada desde o início e que se consolidou ao longo destes anos, a compreensão e as palavras de alento e de incentivo que chegaram sempre no momento certo e que marcaram a diferença em diversas ocasiões.

Ao Professor Doutor Jorge Gaspar, que infelizmente já não se encontra entre nós, agradeço a amizade e o voto de confiança que depositou em mim ao lançar-me este desafio, incentivando-me continuamente a prosseguir, sobretudo nos momentos mais difíceis.

À Dra. Alice Reichert agradeço a amizade, o contributo para a minha formação na área das neoplasias mieloproliferativas e todo o apoio prestado na seleção e acompanhamento dos doentes incluídos no estudo.

Ao Dr. Fernando Lima, Diretor do Serviço de Hematologia Clínica do Centro Hospitalar de Lisboa Ocidental e a todos os colegas deste Serviço e do Serviço de Imunohemoterapia, entre os quais destaco a Dra. Paula Santos, a Dra. Filipa Mousinho e a Dra. Amélia Pita, agradeço a amizade e a colaboração no seguimento dos doentes envolvidos neste projecto, permitindo a sua realização.

À Dra. Esmeraldina Correia Júnior e ao Dr. João Faro Viana, respectivamente anterior e actual Diretores do Serviço de Patologia Clínica do Centro Hospitalar de Lisboa Ocidental, agradeço a amizade e incentivo, permitindo que o trabalho fosse executado.

À Dra. Sância Ramos e à Dra. Vanessa Henriques agradeço a simpatia e disponibilidade na cedência das imagens de exame histológico de medula óssea, incluídas nesta tese.

Ao Professor Sebastião Rodrigues e às colegas da Genomed, Dra. Sónia Matos e Professora Sónia Santos agradeço a disponibilidade e apoio prestado sempre que solicitado.

Às Técnicas Luísa Limão, Sílvia Amaro e Inês Santos agradeço a simpatia e colaboração.

A todos os doentes que participaram neste trabalho e que constituiram a principal motivação para a sua realização, agradeço a simpatia, interesse e disponibilidade.

À Professora Doutora Julieta Esperança Pina agradeço a amizade, o exemplo de força e preseverança e o apoio no “abraço” a este desafio e à atividade académica.

Ao Dr. Valdemar Teixeira e à Dra. Cristina Gamelas agradeço a amizade, o exemplo de dedicação e entusiasmo pela Hematologia e os ensinamentos que me transmitiram e que foram fulcrais no período inicial da minha formação, fazendo despertar o meu interesse por esta área da Medicina.

À Dra. Ana Batalha Reis, ao Dr. Cândido Silva, à Dra. Susana Matos e à Dra. Flora Meireles agradeço o apoio, a amizade e a equipa de trabalho que juntos formamos, pautada pelo rigor, exigência e diferenciação, contribuindo para o meu “crescimento” como especialista.

À Dra. Maria Ana Pessanha, à Dra. Maria Jorge Arroz, à Professora Doutora Teresa Marques e ao Professor Doutor Miguel Seabra agradeço o apoio e amizade demonstrados.

Aos vários colegas, entre os quais destaco o Professor António Almeida, agradeço pela qualidade e inspiração das inúmeras palestras às quais tive o privilégio de assistir e que muito contribuiram para o meu interesse e conhecimento sobre o tema.

A todos os meus colegas do Serviço de Patologia Clínica do Centro Hospitalar de Lisboa Ocidental, do Centre for Toxicogenomics and Human Health Genetics, Oncology and Human Toxicology da Universidade Nova de Lisboa, e amigos agradeço a simpatia, amizade e apoio ao longo dos anos.

Aos meus filhos e ao meu marido, agradeço todo o carinho, apoio e compreensão pela minha pouca disponibilidade em muitos momentos destes últimos anos.

Aos meus pais, agradeço por nunca se terem poupado a esforços para me proporcionarem a melhor educação e formação, por sempre me apoiarem e incentivarem incondicionalmente. Aos dois devo tudo aquilo que hoje sou…

Muito obrigada a todos!

LIST OF ACRONYMS

AML	Acute Myeloid Leukemia
ARMS-PCR	Amplification Refractory Mutation System Polymerase Chain Reaction
BER	Base Excision Repair
BM	Bone Marrow
CALR	Calreticulin
CASP	Caspase
CI	Confidence Interval
CML	Chronic Myeloid Leukemia
DNA	Desoxirribonucleic Acid
ELN	European Leukemia Net
EMH	Extramedullary Hematopoiesis
EPO	Erythropoietin
EPO-R	Erythropoietin-Receptor
ET	Essential Thrombocythemia
FISH	Fluorescent *In Situ* Hybridization
G-CSF	Granulocyte Colony Stimulating Factor
GWAS	Genome-Wide Association Studies
HRM-PCR	High Resolution Melting Polymerase Chain Reaction
HSC	Hematopoietic Stem Cell
HU	Hydroxyurea
IFN	Interferon
IPSS	International Prognostic Scoring System
IWG-MRT	International Working Group-Myeloproliferative Neoplasms Research and Treatment
JAK	Janus Kinase family
LD	Linkage Disequilibrium
LOH	Loss of heterozygosity
MAF	Minor Allele Frequency
MDS	Myelodysplastic Syndromes
MPL	Myeloproliferative Leukemia virus oncogene
MPN	Myeloproliferative Neoplasms
OR	Odds Ratio
PB	Peripheral Blood
PCR	Polymerase Chain Reaction
Ph	Philadelphia chromosome

PLT	Platelets
PMF	Primary Myelofibrosis
PN-MPNs	Philadelphia-negative Myeloproliferative Neoplasms
PV	Polycythemia Vera
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RBC	Red Blood Cells
ROS	Reactive Oxygen Species
SNP	Single Nucleotide Polymorphism
STAT	Signal Transducers and Activators of Transcription
TPO	Thrombopoietin
TPO-R	Thrombopoietin Receptor
UPD	Uniparental Disomy
UTR	Untranslated Region
WBC	White Blood Cells
WHO	*Word Health Organization*

SYMBOLS OF AMINO ACIDS

A or Ala	Alanin	**M or Met**	Metionin
C or Cys	Cystein	**N or Asn**	Asparagin
D or Asp	Aspartat	**P or Pro**	Prolin
E or Glu	Glutamic acid	**Q or Gln**	Glutamin
F or Phe	Phenilalanin	**R or Arg**	Arginin
G or Gly	Glycin	**S or Ser**	Serin
H or Hys	Hystidin	**T or Tre**	Treonin
I or Ile	Isoleucin	**V or Val**	Valin
K or Lys	Lysin	**W or Trp**	Triptophan
L or Leu	Leucin	**Y or Tyr**	Tyrosin

RESUMO

As neoplasias mieloproliferativas são classicamente divididas em neoplasias mieloproliferativas *BCR-ABL1* (cromossoma Philadelphia) positivas, como é o caso da leucemia mieloide crónica, e *BCR-ABL1* negativas, incluindo a policitémia vera, a trombocitémia essencial e a mielofibrose primária. Estas entidades nosológicas têm origem na transformação maligna das *stem-cells* hematopoiéticas, levando a amplificação anormal e à proliferação das células das linhagens mielóides.

A diversidade fenotípica das neoplasias mieloproliferativas Philadelphia negativas resulta da combinação de mutações somáticas já identificadas e caracterizadas, variabilidade genética hereditária, regulação pós-genética e condicionantes individuais. De acordo com a literatura, vários polimorfismos genéticos foram identificados, podendo influenciar os mecanismos de apoptose e de capacidade de reparação do DNA, comprometendo a transcrição genética e/ou a atividade celular, conferindo predisposição genética para a doença, incluindo o caso das neoplasias mieloproliferativas, e condicionando a resposta terapêutica, a evolução clínica e o prognóstico.

O presente trabalho teve como objetivo caraterizar a população portuguesa de acordo com o tipo de neoplasia mieloproliferativa Philadelphia negativa, a sua prevalência e a presença da mutação *JAK2*, dando especial ênfase a alguns doentes em particular; investigar o papel dos polimorfismos nos genes envolvidos nas vias de apoptose e de reparação por excisão de bases na susceptibilidade para estas doenças e, juntamente com outros fatores de risco, na sobrevivência, evolução clínica e prognóstico.

Foram realizados estudos caso-controlo numa amostragem de 133 doentes Portugueses caucasianos e 281 indivíduos saudáveis correspondentes e foram selecionados nove e oito polimorfismos de genes envolvidos nas vias de apoptose e de reparação por excisão de bases, respectivamente, que foram genotipados utilizando a técnica de *Polymerase Chain Reaction* em tempo real. A análise estatística foi realizada com recurso ao *software* SPSS versão 22.0.

Os resultados deste estudo revelaram uma distribuição dos doentes por patologia de 60,2% com trombocitémia essencial, 29,3% com policitémia vera e 10,5% com mielofibrose primária, um discreto predomínio nas mulheres e 75,0% dos doentes positivos para a presença da mutação *JAK2* V617F, evidenciando uma incidência mais elevada na trombocitémia essencial e mais reduzida na policitémia vera, relativamente ao esperado.

Entre os doentes, alguns casos em particular foram destacados e apresentados nesta tese: o caso de um doente com policitémia vera *JAK2* exão 12 positivo, apresentando uma nova mutação - c.1605G> T (p.Met535Ile) - associada a outra mutação já anteriormente descrita, evidenciando um fenótipo clínico atípico; dois doentes com trombocitémia essencial, com suspeita da coexistência rara da mutação *JAK2* V617F e da translocação *BCR-ABL1*; e um doente com leucemia mieloide crónica,

apresentando uma nova mutação no domínio kinase *BCR-ABL1* - c.839T> G (p.Val280Gly) - que poderá estar associada a resistência ao imatinib.

Quanto aos polimorfismos investigados, os nossos resultados revelaram potenciais associações entre alguns dos polimorfismos e a susceptibilidade individual para estas doenças, sugerindo o potencial envolvimento de polimorfismos do gene *CASP9* (Phe136Phe) e dos genes *XRCC1*_399 (Gln399Arg) e *MUTYH* (Gln335His), após estratificação por patologia para os doentes com trombocitémia essencial e quando se tratam de mulheres. Nos doentes *JAK2* positivos verificou-se uma associação com os polimorfismos do gene *CASP9* (Phe136Phe) e do gene *XRCC1_399* (Gln399Arg). Nos genes de reparação por excisão de bases, a combinação de alelos também demonstrou associação com doença para um haplogrupo específico.

Em relação à evolução clínica dos doentes sob tratamento, na maioria dos casos com hidroxiureia, de acordo com nossos resultados: 17 doentes apresentaram progressão para mielofibrose secundária/leucemia mieloide aguda, influenciando a sobrevivência e estando associada à exposição a agentes citoredutores. Nestes doentes, houve também evidência global de associação com polimorfismos do gene *CASP8* (3'UTR) e do gene *XRCC1_194* (Arg194Trp), e após estratificação para trombocitémia essencial com polimorfismos do gene *CASP9* (Arg173His) e dos genes *APEX1* (Asp148Glu) e *XRCC1_194* (Arg194Trp); 11 doentes desenvolveram uma neoplasia não mieloide primária *de novo*, evidenciando uma associação global com polimorfismos do gene *CASP8* (Asp270His) e do gene *XRCC1_399* (Gln399Arg); 22 doentes apresentaram eventos trombóticos e uma associação global com polimorfismos do gene *XRCC1_399* (Gln399Arg).

Os resultados apresentados na presente dissertação ajudaram a caracterizar uma população de doentes com neoplasias mieloproliferativas Philadelphia negativas, podendo refletir a realidade portuguesa, e revelaram o papel potencial de determinados polimorfismos e outros fatores na susceptibilidade para a doença e na evolução clínica dos doentes sob terapêutica, com impacto prognóstico.

Embora sejam necessários estudos mais alargados para confirmar estes resultados, estes novos dados podem contribuir para um melhor conhecimento da fisiopatologia e, no futuro, para uma escolha mais racional e eficiente das estratégias terapêuticas a adotar nestas patologias.

ABSTRACT

Myeloproliferative neoplasms are classically divided into *BCR-ABL1* (Philadelphia chromosome) positive chronic myeloid leukemia and *BCR-ABL1* negative myeloproliferative neoplasms, including polycythemia vera, essential thrombocythemia and primary myelofibrosis. These disorders have origin from the malignant transformation of a hematopoietic stem-cell, leading to abnormal amplification and proliferation of myeloid lineages.

The phenotypic diversity of Philadelphia chromosome negative myeloproliferative neoplasms results from the combination of somatic mutations already identified and characterized, inherited genetic variability, postgenetic regulation, and individual conditioners. According to the literature, several genetic polymorphisms have been identified, which may influence the apoptosis mechanisms and the DNA repair capacity, compromising genetic transcription and/or cell function, conferring genetic predisposition to disease, including myeloproliferative disorders, and conditioning the therapeutic response, the clinical outcome and prognosis.

The present work had the purpose of characterize the Portuguese population according to the type of Philadelphia chromosome negative myeloproliferative neoplasm, its prevalence and the presence of *JAK2* mutation, with special emphasis on some particular patients; investigate the role of polymorphisms in genes involved in apoptosis and base excision repair pathways in these disorders susceptibility and, along with other risk factors, in survival, clinical outcome and prognosis.

Case-control studies were carried out in a Caucasian Portuguese sample of 133 patients and 281 matched control subjects, nine and eight polymorphisms of genes involved in apoptosis and base excision repair pathways, respectively, were selected and genotyped using real time polymerase chain reaction technique and statistical analysis was performed with SPSS version 22.0.

The results from this study revealed a pathology distribution of 60.2% of patients with essential thrombocythemia, 29.3% with polycythemia vera and 10.5% with primary myelofibrosis, with a discrete predominance in females, and a total of 75.0% of patients positive for the presence of the *JAK2* V617F mutation, with an increased incidence in essential thrombocythemia and a decreased incidence in polycythemia vera patients than expected.

Among the patients, some particular cases were highlighted and presented in this thesis, namely the case of a polycythemia vera patient *JAK2* exon 12 positive, presenting a novel mutation – c.1605G>T (p.Met535Ile) - associated with another mutation previously described, evidencing an atypical clinical phenotype; two patients with essential thrombocythemia, with suspicion of the rare coexistence of *JAK2* V617F mutation and *BCR-ABL1* translocation; and a patient with chronic myeloid leukemia, presenting a new *BCR-ABL1* mutation kinase domain mutation – c.839T>G (p.Val280Gly) – which might be associated with resistance to imatinib.

Concerning the polymorphisms investigated, our results revealed potential associations between some polymorphisms and individual susceptibility to these disorders, suggesting the potential

involvement of *CASP9* (Phe136Phe) gene and *XRCC1*_399 (Gln399Arg) and *MUTYH* (Gln335His) genes polymorphisms after stratification by pathology diagnosis for essential thrombocythemia patients and when they are women. When patients are *JAK2* positive an association with *CASP9* (Phe136Phe) gene and *XRCC1_399* (Gln399Arg) gene polymorphisms was found. In base excision repair genes, combination of alleles also demonstrated an association with the disease for one specific haplogroup.

Regarding the clinical outcome in patients under treatment, the majority of them with hydroxyurea, according to our results: 17 patients showed progression to secondary myelofibrosis/acute myeloid leukemia, influencing survival and being associated with exposure to cytoreductive agents. In these patients, there was also global evidence of association with *CASP8* (3'UTR) gene and *XRCC1_194* (Arg194Trp) gene polymorphisms, and after stratification for essential thrombocythemia with *CASP9* (Arg173His) gene, and *APEX1* (Asp148Glu) and *XRCC1_194* (Arg194Trp) genes polymorphisms; 11 patients developed a new primary nonmyeloid neoplasm, evidencing a global association with *CASP8* (Asp270His) gene and *XRCC1_399* (Gln399Arg) gene polymorphisms; 22 patients presented thrombotic events and a global association with *XRCC1_399* (Gln399Arg) gene polymorphisms.

The results shown in the present dissertation helped to characterize a population of Philadelphia negative myeloproliferative neoplasms patients that could reflect Portuguese reality, and have revealed the potential role of polymorphisms and other factors in disease susceptibility and clinical outcome of patients under treatment, with prognostic impact.

Although larger studies are required to confirm these results, these new data may contribute to a best knowledge of the pathophysiology and, in the future, to a more rational and efficient choice of therapeutic strategies to be adopted in these disorders.

LIST OF PUBLICATIONS

This thesis was based on the following publications:

Articles in International Scientific Periodicals with Referees (Indexed)

1) **Azevedo AP**, Silva SN, Reichert A, Lima F, Júnior E, Rueff J. Effects of polymorphic DNA genes involved in BER and caspase pathways on the clinical outcome of myeloproliferative neoplasms under treatment with hydroxyurea.
   Submitted for publication in Molecular Medicine Reports

2) Mousinho F*, **Azevedo AP***, Mendes T, Santos PS, Cerqueira R, Ramos S, Viana JF, Lima F. Concomitant presence of *JAK2* V617F mutation and BCR-ABL translocation in two patients: a new entity or a variant of myeloproliferative neoplasms.
   * Both authors contributed equally; Submitted for publication in Molecular Medicine Reports

3) **Azevedo AP**, Silva SN, Reichert A, Lima F, Júnior E, Rueff J. The role of caspase genes in genetic susceptibility to Philadelphia-negative myeloproliferative neoplasms in a Portuguese population. (2017) Accepted for publication in Pathology and Oncology Research

4) Pita A*, **Azevedo AP***, Reichert A, Silva C, Henriques V, Mendes D, Reis A, Cerqueira R, Torres F, Viana JF. Atypical haematological presentation in a case of polycythemia vera with a new variant mutation detected in exon 12 – c.1605G>T (P.Met535Ile). (2017) BMJ – Journal of Clinical Pathology Oct 11 (Epub). DOI: 10.1136/jclinpath.2017.204556
   * Both authors contributed equally

5) **Azevedo AP**, Silva SN, Lima JP, Reichert A, Lima F, Júnior E, Rueff J. Prevalence of the Janus kinase 2 V617F mutation in Philadelphia-negative myeloproliferative neoplasms in a Portuguese population. (2017) Biomedical Reports 7:370-376. DOI: 10.3892/br.2017.977

6) **Azevedo AP**, Silva SN, Lima JP, Reichert A, Lima F, Júnior E, Rueff J. DNA repair genes polymorphisms and genetic susceptibility to Philadelphia-negative myeloproliferative neoplasms in a Portuguese population: the role of base excision repair genes polymorphisms. (2017) Oncology Letters 13:4641-4650. DOI: 10.3892/0l.2017.6065

7) **Azevedo AP**, Reichert A, Afonso C, Alberca MD, Tavares P, Lima F. BCR-ABL V280G mutation, potencial role in imatinib resistance: first case report. (2017) Clinical Medicine Insights: Oncology 11:1-5. DOI: 10.1177/1179554917702870

Chapters in Scientific Books of national circulation

1) Reichert A**, Azevedo AP,** Lima F. Mutação V280G e resistência ao Imatinib – primeiro caso descrito. (2015) Casos Clínicos em Leucemia Mielóide Crónica – a experiência portuguesa, Edição Novartis Farma – Produtos Farmacêuticos, S.A.

Abstracts in International Scientific Periodicals with Referees (Indexed)

1) **Azevedo AP**, Silva S, Reichert A, Lima F, Júnior E, Rueff J. Caspases and base excision repair genes polymorphisms and therapeutic response to hydroxyurea in Philadelphia-negative myeloproliferative neoplasms: a study in a Portuguese population. (2017) Accepted for publication in Annals of Medicine

2) Pita A, Silva C, Mendes D, Reichert A, **Azevedo AP**, Reis A, Cerqueira R, Torres F, Viana JF, Henriques V. Apresentação hematológica atípica num caso de policitémia vera com mutação variante detetada no exão 12 – c.1605G>T (P.Met535Ile). (2017) Blood Transfusion 15 (Suplement 2):s415

In the context of this thesis, the following presentations at conferences and national and international scientific meetings were also submitted:

1) **Azevedo AP**, Silva S, Reichert A, Lima F, Júnior E, Rueff J. Effects of polymorphic DNA genes involved in BER and apoptosis on the clinical outcome of Philadelphia-negative myeloproliferative neoplasms under treatment. Accepted for presentation at Scientific Toxomics Meeting (date to be defined)

2) **Azevedo AP**, Silva S, Reichert A, Lima F, Júnior E, Rueff J. Effects of polymorphic DNA genes involved in BER and apoptosis on the clinical outcome of Philadelphia-negative myeloproliferative neoplasms under treatment. III Genetics Workshop (Lisboa, 2017)

3) **Azevedo AP**, Silva S, Reichert A, Lima F, Júnior E, Rueff J. Caspases and base excision repair genes polymorphisms and therapeutic response to hydroxyurea in Philadelphia-negative myeloproliferative neoplasms: a study in a Portuguese population. 2nd International Congress of CiiEM (Costa da Caparica, 2017)

4) Pita A, Silva C, Mendes D, Reichert A, **Azevedo AP**, Reis A, Cerqueira R, Torres F, Viana JF, Henriques V. Apresentação hematológica atípica num caso de policitémia vera com mutação variante detetada no exão 12 – c.1605G>T (P.Met535Ile). 2º Congresso Hispano-Português de Medicina Transfusional e Terapia Celular, 2º Encontro Hispano-Luso Afro-Latino-Americano de Transfusão de Sangue, X Congresso Nacional da Associação Portuguesa de Imuno-hemoterapia (APIH) e 28º Congresso da Sociedade Espanhola de Transfusão Sanguinea e Terapia Celular (SETS) (Porto, 2017)

5) Mousinho F, Santos P, Cerqueira R, **Azevedo AP**, Ramos S, Lima F. Atypical Essential Thrombocythemia with leukocytosis, positive JAK2 V617F mutation and BCR/ABL translocation and a normal karyotype. 7th International Conference on Myeloproliferative Neoplasms, European School of Haematology (Estoril, 2016)

6) **Azevedo AP**, Silva S, Lima J, Reichert A, Lima F, Júnior E, Rueff J. Apoptosis deregulation expression in Philadelphia-negative Myeloproliferative Neoplasms in a Portuguese population. 7th International Conference on Myeloproliferative Neoplasms, European School of Haematology (Estoril, 2016)

7) **Azevedo AP**, Silva S, Reichert A, Lima F, Júnior E, Rueff J, Gaspar J. Identification of genetic determinants associated with susceptibility and therapeutic efficacy in Philadelphia-negative Myeloproliferative Neoplasms. I Genetics Workshop (Lisboa, 2015)

8) **Azevedo AP**, Silva S, Reichert A, Lima F, Júnior E, Rueff J, Gaspar J. Identification of genetic determinants associated with susceptibility and therapeutic efficacy in Philadelphia-negative Myeloproliferative Neoplasms. Scientific Toxomics Meeting (Lisboa, 2015)

9) **Azevedo AP**, Silva S, Lima J, Reichert A, Lima F, Junior E, Rueff J, Gaspar J. The role of apoptosis polymorphisms in individual susceptibility to Philadelphia-negative myeloproliferative neoplasms. 18th Annual Reunion of Sociedade Portuguesa de Genética Humana (Lisboa, 2014)

10) **Azevedo AP**, Silva S, Lima J, Reichert A, Lima F, Junior E, Rueff J, Gaspar J. The role of base excision repair polymorphisms in individual susceptibility to Philadelphia-negative

myeloproliferative neoplasms. 6th International Conference on Myeloproliferative Neoplasms, European School of Haematology (Estoril, 2014)

11) Reis A, Rodrigues R, Narciso S, Silva C, **Azevedo AP**; Afonso C, Reichert A, Gomes P, Santos P, Silveira M, Lima F, Diamantino F, Junior E. La morbilidad asociada con la aspiración de médula ósea y biopsia ósea – resultados de un año en el Centro Hospitalar de Lisboa Ocidental – CHLO. LV Congresso Nacional SEHH; XXIX Congreso Nacional SETH, SEHH / SETH (Sevilha, 2013)

12) Reichert A, **Azevedo AP**, Afonso A, Alberca M, Torres F, Tavares P, Lima F. V280G mutation and imatinib resistance – first case described. 15th International Conference on Chronic Myeloid Leukemia: biology and therapy, European School of Haematology (Estoril, 2013)

13) **Azevedo AP**, RodriguesR, Freire I, Gago T, Júnior E. Promoting an Active Role of Clinical Laboratory in the Management of Patients with a Coagulation Disorder. 1º Congresso Nacional do Laboratório Clínico (Lisboa, 2010)

This thesis also used methods/technics of the following articles:

1) Santos L, Branco S, Silva S, **Azevedo AP**, Gil O, Manita I, Ferreira T, Limbert E, Rueff J, Gaspar J. Polymorphisms in base excision repair genes and thyroid cancer risk. (2012) Oncology Reports 28(5):1859-68

2) **Azevedo AP**, Silva P, Marcelo C, Gamelas C, Teixeira V, Vieira A, Francisco M, Cruz M, Costa T. Valores de referência para hemograma na população da zona metropolitana de Lisboa. (2010) Acta Médica Portuguesa 23:597-604

3) Gomes B, Silva SN, **Azevedo AP**, Manita I, Gil OM, Ferreira TC, Limbert E, Rueff J, Gaspar JF. The role of common variants of non-homologous end-joining repair genes XRCC4, LIG4 and Ku80 in thyroid cancer risk. (2010) Oncology Reports 24:1079-1085

4) **Azevedo AP**, Gamelas C, Teixeira V, Contreiras M, Monteiro R, Vale M, Durães F. Neutropénia Crónica Benigna da Infância – um Caso Clínico. (2010) Acta Médica Portuguesa 23:521-526

5) **Azevedo AP**, Marcelo C, Meireles F, Gamelas C, Teixeira V. Hemoglobinopatias e Talassémias: oito anos de casuística no Hospital de São Francisco Xavier. (2009) Arquivos do Egas Moniz 5(1):14-21

6) Silva SN, **Azevedo AP**, Teixeira V, Pina JE, Rueff J, Gaspar JF. The role of GSTA2 polymorphisms and haplotypes in breast cancer susceptibility: a case-control study in the Portuguese population. (2009) Oncology Reports 22:593-598

7) Conde J, Silva SN, **Azevedo AP**, Teixeira V, Pina JE, Rueff J, Gaspar JF. Association of common variants in mismatch repair genes and breast cancer susceptibility: a multigene study. (2009) BMC Cancer 9:344

8) Bastos HN, Antão MR, Silva SN, **Azevedo AP**, Manita I, Teixeira V, Pina JE, Gil OM, Ferreira TC, Limbert E, Rueff J, Gaspar JF. Association of polymorphisms in genes of the homologous recombination DNA repair pathway and thyroid cancer risk. (2009) Thyroid 19:1067-1075

9) Silva SN, Moita R, **Azevedo AP**, Gouveia R, Manita I, Pina JE, Rueff J, Gaspar JF. Menopausal age and XRCC1 gene polymorphisms: role in breast cancer risk. (2007) Cancer Detection Prevention Journal 31:303-309

CHAPTER 1:
GENERAL INTRODUCTION

CHAPTER 1: GENERAL INTRODUCTION

1.1 – MYELOPROLIFERATIVE NEOPLASMS: PHILADELPHIA CHROMOSOME NEGATIVE DISORDERS

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic malignancies resulting from the transformation of hematopoietic stem cells, leading to abnormal amplification of physiological signal-transduction pathways and proliferation of one or more myeloid lineages. With regard to MPNs, the *Word Health Organization (WHO) Classification of Tumors of Hematopoietic and Lymphoid Tissues* includes chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) (Vardiman, Harris, & Brunning, 2002), chronic neutrophilic leukemia, chronic eosinophilic leukemia not otherwise specified and MPNs unclassifiable (Swerdlow et al., 2008). In addition to primary (*de novo*), myelofibrosis can be secondary to PV (post-PV) or ET (post-ET) (Tefferi & Barbui, 2017). In the last revision of WHO classification, in 2016, mastocytosis is no longer listed under the heading of MPNs (Arber et al., 2016).

This chapter intends to focus on the insights and give a brief description of Philadelphia negative myeloproliferative neoplasms (PN-MPNs; PV, ET and PMF), the group of disorders which was the subject of study and whose results will be presented throughout this dissertation. It will be addressed to their molecular pathophysiology, clinical manifestations and diagnosis criteria, prognosis and predictive factors, risk stratification and therapy management.

HISTORICAL PERSPECTIVE

In 1951, William Dameshek (1900-1969) was the first to conceptualize these group of disorders, highlighting the clinical and morphologic similarities between CML, PV, ET and PMF (Dameshek, 1951). He recognized that these disorders are caused by hyperproliferation of multiple hematopoietic lineages in the bone marrow that proliferate ‘‘as a unit’’, and he proposed the term “myeloproliferative disorders” to indicate that these entities may represent a continuum of related syndromes. Moreover, he also proposed that the proliferative activity could be perhaps due ‘‘to a hitherto undiscovered stimulus’’. However, the finding that bone marrow and peripheral blood cells from MPNs patients can produce erythroid colonies in vitro in the absence of added growth factors indicated the cell autonomous nature of these diseases (Skoda, Duek, & Grisouard, 2015).

But the “story” about MPNs had begun a few years before. Previously in 1845, John Hughes Bennett (1812–1875), an English pathologist working in Edinburgh, had described CML, and in 1879,

a German surgeon, Gustav Heuck (1854–1940), underlined the morphological distinguishing features between PMF and CML, namely the presence of bone marrow fibrosis, osteosclerosis, and extramedullary hematopoiesis in the former. Some years later in 1892, Louis Henri Vaquez (1860–1936), a French physician, was the first to describe PV, about a patient with marked erythrocytosis and hepatosplenomegaly, and in 1903 William Osler (1849–1919) took another step forward, distinguishing PV from both relative polycythemia and secondary polycythemia. The first description of ET is credited to Emil Epstein (1875–1951) and Alfred Goedel, two Austrian pathologists, who in 1934 published a case report of an "hemorrhagic thrombocythemia" in the absence of marked erythrocytosis.

In 1960, Peter Nowell (b. 1928) and David Hungerford (1927–1993), two American scientists working in Philadelphia, described the association between the Philadelphia (Ph) chromosome and CML (Figure 1.1) (Nowell & Hungerford, 1960), in contrast to PN-MPNs (PV, ET and PMF).

Finally, the establishment of all four classic MPNs as clonal stem cell diseases was achieved by Philip Fialkow (1934–1996), an American physician scientist, through his studies developed between 1967 and 1981, on X chromosome inactivation patterns in women with PV, ET, PMF or CML carrying a polymorphic variant of the X-linked glucose-6-phosphate dehydrogenase (G-6-PD) gene (Adamson, Fialkow, Murphy, Prchal, & Steinmann, 1976; Fialkow, 1977; Fialkow, Faguet, Jacobson, Vaidya, & Murphy, 1981; Tefferi, 2016).

In 1972, Janet Rowley (1925–2013), an American geneticist, described the Ph chromosome as a reciprocal translocation between chromosomes 9 and 22; t(9;22)(q34;q11) and in 1985-6, the *BCR-ABL1* transcript and its P210 fusion protein product were identified, contributing to distinguish CML from other MPNs. In 1996, in the era of the Human Genome Project development, these discoveries led Nicholas Lydon (b.1957), a British scientist, and Brian Druker (b. 1955), an American physician scientist, to the elaboration and therapeutic use of imatinib (a tyrosine kinase inhibitor) in CML (Figure 1.1) (Tefferi, 2016).

The discovery of somatic mutations in Janus kinase 2 gene (*JAK2*), namely exon 14 V617F gain-of-function mutation, identified in 2005 by several independent groups of investigators (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005), in most patients with PV, but also with ET or PMF, was one of the major genetic insights into the pathogenesis of the PN-MPNs (Figure 1.1). The fact that approximately 50% of ET and PMF patients are JAK2 V617F negative, led the investigators to continue to study whether other genes in the JAK-STAT signaling pathway could be mutated in these patients. Thus, in 2006, Pikman and colleagues (Pikman et al., 2006) identified the mutations of thrombopoietin receptor (TPO-R) in myeloproliferative leukemia virus oncogene (MPL). Moreover, only one year later, in 2007, Scott and colleagues identified a set of *JAK2* exon 12 mutations in *JAK2* V617F negative patients with PV (Scott, Tong, et al., 2007). Nevertheless, one of the latest discoveries was made by Kralovics in 2013, with the identification of calreticulin (CALR) mutation in 73% of MPNs patients who do not bear the *JAK2* or *MPL* mutation (Figure 1.1) (Klampfl et al., 2013).

The discovery of all these mutations, that directly or indirectly activate JAK-STAT pathway, contributed to the establishment of the WHO diagnostic criteria and risk stratification in PN-MPNs.

Mutations other than in these driver genes have also been described in PN-MPNs and have shown to provide additional prognostic information (Tefferi, 2016), as it will be discussed below in this dissertation.

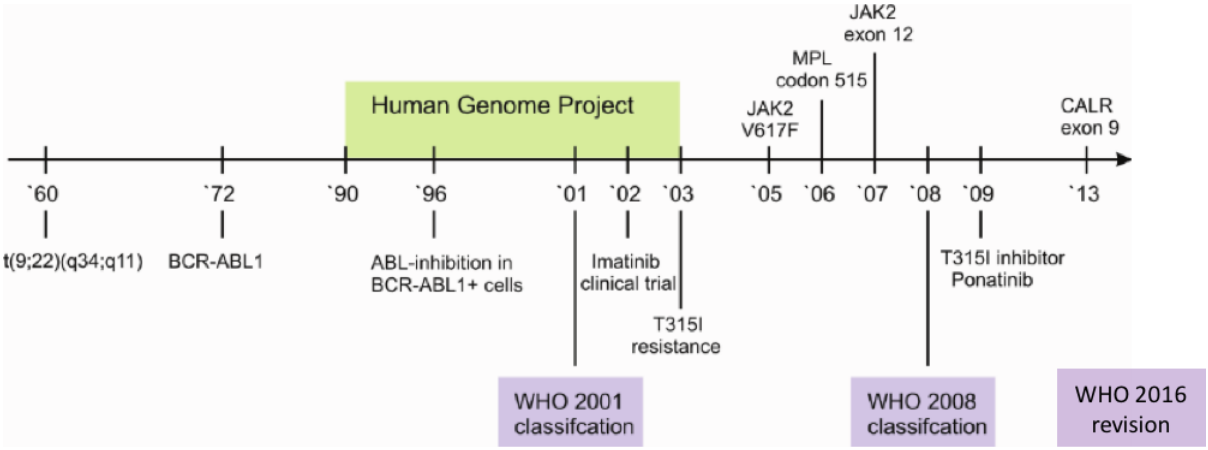


Figure 1.1 – Timeline of the elucidation of genomic alterations in myeloproliferative neoplasms.
Note the significant impact of the Human Genome Project on the elucidation on Ph negative specific genomic alterations. In 2008, the WHO has revised its classification of myeloid malignancies and coined the term "myeloproliferative neoplasms" (Adapted from (Hasan et al., 2013; ResearchGate.net).

HEMATOPOIESIS AND PN-MPNS EPIDEMIOLOGY

Hematopoiesis (Figure 1.2) is the process by which multipotent bone marrow-based stem cells (HSC) differentiate and mature into fully formed blood cells (namely lymphoid, erythroid, megakaryocytes and other myeloid cells), in response to external stimulus, such as erythropoietin (EPO), thrombopoietin (TPO), granulocyte-macrophage-colony stimulating factor (GM-CSF), other stimulating growth factors and several interleukins. Growth factors initiate signal transduction pathways (ex: JAK-STAT pathway), which lead to activation of transcription factors, and elicit different outcomes depending on the combination of factors and the cell's stage of differentiation.

In a healthy adult person, approximately 10^{11}–10^{12} new blood cells are produced daily in order to maintain steady state levels in the peripheral circulation. Besides bone marrow, in some cases and if necessary, the liver, thymus, and spleen may resume their hematopoietic function, if necessary, which is called extramedullary hematopoiesis, causing these organs to increase in size substantially.

The PN-MPNs are characterized by the clonal proliferation of one or more myeloid cell lineages (erythrocytic, granulocytic or megakaryocytic) (Figure 1.2), without altering the hematopoietic stem-cell hierarchy, predominantly in the bone marrow and involving JAK-STAT pathway (discussed below), in some cases associated with the presence of extramedullary hematopoiesis. There is evidence of a normal and effective maturation, resulting in peripheral blood increased erythrocytes, granulocytes and platelets counts (Passamonti, Mora, & Maffioli, 2016).

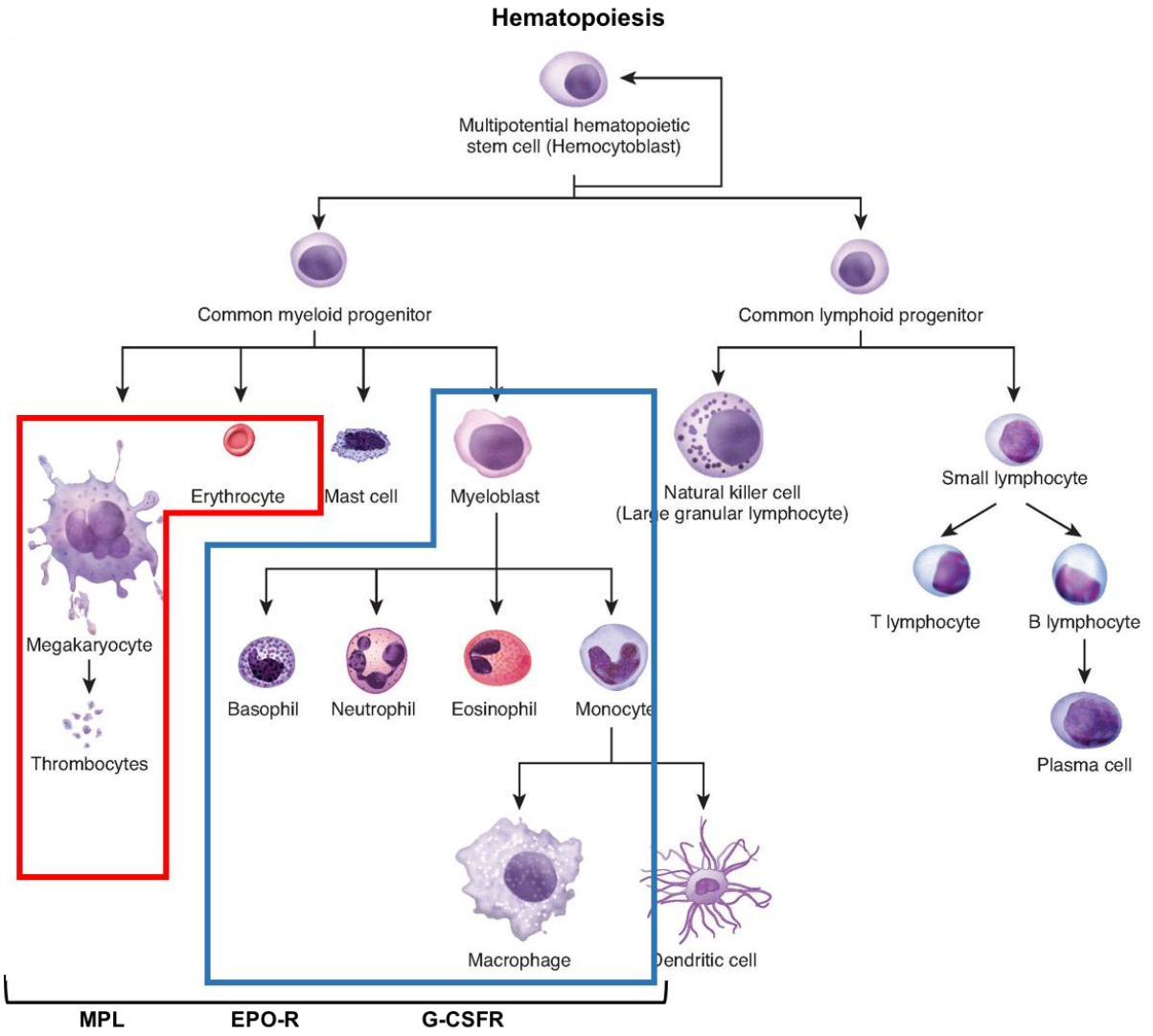


Figure 1.2 – Overview of hematopoiesis and affected myeloid cell lineages in PN-MPNs.
The hematopoietic lineages begin at a pluripotent hematopoietic stem cell (HSC). Myeloid differentiation occurs through the common myeloid progenitor, differentiating into megakaryocyte, red blood cell, mast cell, or granulocyte-monocyte progenitor, which in turn differentiate into a basophil, neutrophil, eosinophil, or monocyte followed by macrophage. Differentiation in the lymphocytic pathway (not involved in PN-MPNs) proceeds through the common lymphoid progenitor, ultimately ending as a B cell and plasma cell, T cell, or natural killer cell.
MPL, EPO-R and G-CSFR - Receptors of bone marrow progenitor cells (Adapted from http://oerpub.github.io).

Among the different PN-MPNs entities there is a frequent overlap of clinical, laboratory and morphological data. Leukocytosis with neutrophilia, thrombocytosis, excessive megakaryocytic proliferation, myelofibrosis, splenomegaly and hepatomegaly can occur in any of these diseases. Despite insidious clinical onset, all PN-MPNs are at risk of clonal evolution, with disease progression that may end in medullary failure (myelofibrosis or ineffective hematopoiesis) or transformation into acute leukemia. These aspects will be further explored below in this section.

PN-MPNs are considered as rare disorders, because their combined incidence is lower than 6 per 100 000 individuals per year (Rumi & Cazzola, 2017).

Among the existent registries in European Union, the PN-MPNs have an annual incidence rate per 100 000 individuals per year ranging from 0.4-2.8 (while the literature estimated 0.68-2.6) for PV, from 0.38-1.7 for ET (in the literature 0.6-2.5) and from 0.1-1.0 for PMF, respectively (Moulard et al., 2014; Titmarsh et al., 2014).

There are few European studies reported on the MPNs' prevalence (Moulard et al., 2014). However, according to American data published in 2014, the prevalence per 100 000 individuals of PV (44-57) and ET (38-57) was much higher than that of MF (4-6) or subgroups with MF features (post-PV MF = 0.3-0.7; post-ET MF = 0.5-1.1) (Mehta, Wang, Iqbal, & Mesa, 2014).

These group of disorders occur in middle- or advanced-age adults, with a medium age of diagnosis of 65-67 years for PV, 65-70 years for ET and 67-70 years for PMF (Srour et al., 2016), although it can be diagnosed in younger individuals, particularly if there is a familial predisposition (Bellanné-Chantelot et al., 2006).

There are reports in the literature indicating that ET is more common in women (particularly at younger ages) and PV more common in men, while in PMF both sexes are nearly equally affected (Bai et al., 2008; Passamonti et al., 2008; Swerdlow et al., 2008).

As demonstrated by European and international studies (Hultcrantz et al., 2012; Tefferi, Guglielmelli, et al., 2014), the distinction of MPNs in three nosological entities has a relevant prognostic significance. From the various published reports, generally PN-MPNs patients have a reduced life expectancy compared with general population, with PMF having the lowest overall survival (5.7 years), followed by PV with 15 years survival in 65% of cases, and ET with an overall survival of more than 18-20 years (Rumi & Cazzola, 2017; Tefferi, 2008a). As will be discussed below, it was also reported that mortality in PN-MPNs patients is generally attributed to disease progression to other hematologic malignancies (the most common being acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS)), bacterial infections and cardio- and cerebrovascular diseases, especially in younger patients (Hultcrantz et al., 2015). Fortunately, mortality due to these complications has been decreasing in the last few years (Rumi & Cazzola, 2017).

In Portugal epidemiological data is very scarce, however several efforts have been made in the last years in order to organize a national registry on these disorders and develop consensus meetings where several related aspects can be debated, such as diagnostic classification, high-risk mutations and treatment strategies. One of the achieved advances respects to the development and validation of a scale of evaluation of symptomatic load (MPN10) designed specifically for these patients, in Portuguese language, based on international ones already existing. The sequential use in time of these scales is an useful instrument of symptom evaluation in clinical practice, may give us a perspective of disease evolution and treatment response, in order to a standardize criteria for multicentric studies.

In Chapters 3 and 9 of this dissertation, will be presented the results obtained from the study of a group of Portuguese patients, which were characterized according to the type of PN-MPN, general features, prevalence of *JAK2* mutation, clinical outcome and therapy management.

1.1.1 – MOLECULAR PATHOPHYSIOLOGY

Until 2005 little was known about the etiology of PN-MPNs, but the discovery of *JAK2* V617F mutation, which turned out to be the most important and most frequently recurring somatic mutation in PN-MPNs, was crucial and revolutionized the understanding of these disorders.

After *JAK2* V617F discovery in the majority of PN-MPNs patients, there may have been an assumption of genetic uniformity. However, major genetic insights into the pathogenesis of the PN-MPNs were achieved with the identification of other driver mutations, such as *JAK2* exon 12, *MPL* and *CALR* mutations, which contributed to a better clarification of the pathophysiology of these disorders, their diagnostic tools and therapeutic management (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Scott, Tong, et al., 2007; Tefferi, 2016). It soon became clear that this group of diseases was far more genetically heterogeneous and complex than CML. In the majority of PN-MPNs cases *CALR, MPL* and *JAK2* mutations are mutually exclusive, however rare exceptions can occur (Kang et al., 2016; Spivak, 2017).

The present section is a review of the mutations more commonly found in PN-MPNs (Figure 1.3). A particularly interesting note is that the majority of those mutations fall into one of two categories - activation of the JAK-STAT pathway (*JAK2* V617F, *JAK2* exon 12, *MPL*, *LNK* and probably *CALR*) or aberrant epigenetic modification (*TET2*, *ASXL1*, and *EZH2*) (Tefferi & Pardanani, 2011). A combination of mutations in these genes and environmental factors are likely the determining factors of the development of each one of these disorders.

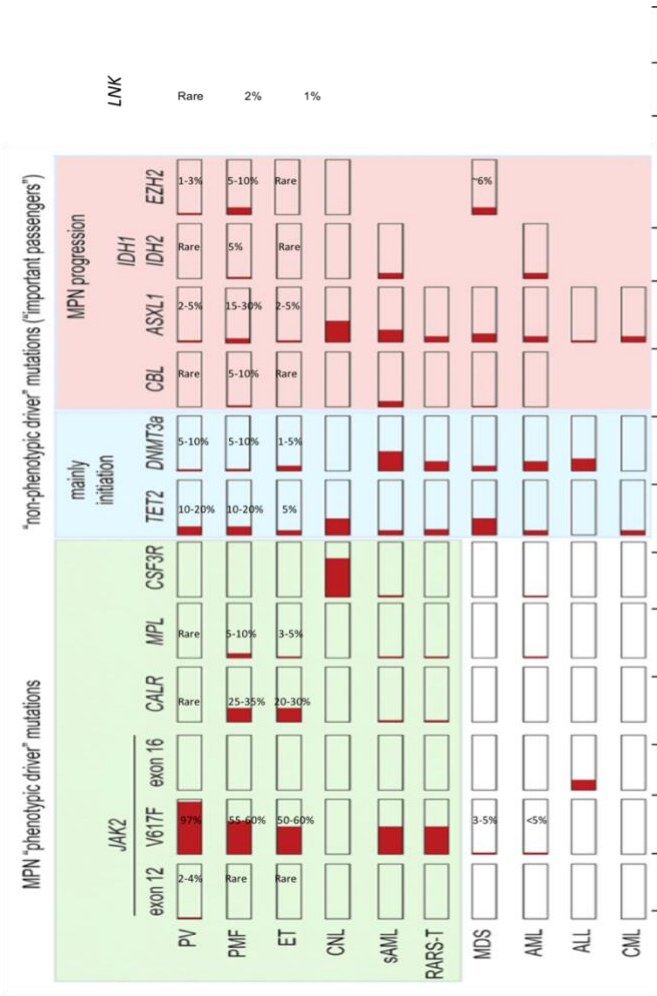
PN-MPNs DRIVER MYELOID GENES

The presence of somatic mutations in the three driver genes, namely *JAK2*, *MPL* and *CALR*, represent a major diagnostic criteria, in combination with clinical-hematological and morphological abnormal features (Rumi & Cazzola, 2017).

Variation frequency of *JAK2* V617F and exon 12, *MPL* W515L/K and *CALR* mutations is represented in Figures 1.3 and 1.4.

JAK2 V617F MUTATION

*JAK*2 gene, located at chromosome 9 and first identified in 1993, is a member of the Janus kinase family and the most common MPN driver gene. As will be discussed below in this Chapter, *JAK*2 serves as the cognate tyrosine kinase for the EPO and TPO receptors and can also be used by the G-CSF receptor, all of which lack an intrinsic kinase domain (Spivak, 2017; Tefferi, 2016).



	Chrom. location	Mutation location and type	Significance
10%	12q24.12	Exon 2 (missense deletion)	Germ-line or somatic mutation cooperate with V617F or *CALR;* Wild-type *LNK* is a negative regulator of JAK2 signaling
--	7q35-q36.1	All coding regions (missense, indel)	Wild-type is part of a histone methyltransferase (polycomb repressive complex - PRC2); in MPN might have a tumor suppressor activity, which contrasts with the gain-of-function activity for lymphoma-associated *EZH2* mutations; initiation, progression; possible myelofibrotic transformation in ET and PV; PMF bad prognosis
20%	2q33.3/ 15q26.1	Exon 4 (missense, hotspot)	Converts ketoglutarate into hydroxiglutarate and inhibits *TET2;* initiation and late event associated with leukemic transformation
18%	20q11.1	Exon13 and 12 (nonsense, indel)	Wild type is involved in normal hematopoiesis; can act as activator and suppressor of transcription; age related clonal hematopoiesis; initiation and rapid progression; In PMF leukemic progression and bad prognosis
--	11q23.3	Exon 8-9 (missense)	E3 ubiquitin ligase that marks mutant kinases for degradation; cytokine receptor internalization; late event associated with leukemic transformation, requires loss of this function
15%	2p23	Exons 7-23 (missense, hotspot)	DNA methyltransferase responsible for the maintenance of DNA in the methylated state; age-related clonality; impact initiation, clinical phenotype and progression; prevent eradication MPN clone
17%	4q24	All coding regions (missense, nonsense deletion)	Catalyzes conversion of 5-methylcytosine to hydroxi-methylated cytosine, which favors demethylated DNA; age-related clonality; impact initiation, clinical phenotype and progression; prevent eradication MPN clone
--	1p34.3	Exon 14 and 17	Plays a crucial role in the proliferation, differentiation and survival of cells along the neutrophilic lineage; strongly associated with chronic neutrophilic leukemia
5%	1p34	Exon10 (MPL515L/K/A/R MPL5505N; other missense)	Cytokyne-independent growth and TPO hypersensitivity; contributes to primarily megakaryocytic myeloproliferation
--	19p13.2	Exon 9 (indel)	Good prognosis; wild-type CALR is a multi-functional Ca^{2+} binding protein chaperone mostly located in the endoplasmic reticulum; activator of MPL; increased platelet production
--	9p24	Exon 16	Exclusive for Down Syndrome-acute lymphoblastic leukemia patients
50%	9p24	Exon 14	Tyrosine kinase associated with cytokine receptor; abnormal myeloproliferation and progenitor cell growth factor hypersensitivity
--	9p24	Exon 12	Tyrosine kinase associated with cytokine receptor; mainly erythroid myeloproliferation

Figure 1.3 – Frequency, distribution and pathogenetic relevance of acquired gene mutations in hematologic malignancies.
Frequency represented by red portions of the horizontal bars. ALL-acute lymphocytic leukemia; AML-acute myeloid leukemia; BP-blastic phase; CML-chronic myeloid leukemia; CNL-chronic neutrophilic leukemia; ET-essential thrombocythemia; MDS-myelodysplastic syndromes; MPN-myeloproliferative neoplasms; PMF-primary myelofibrosis; PV-polycythemia vera; RARS-T-refractory anemia with ring sideroblasts and thrombocytosis (WHO 2016: MDS-RS - myelodysplastic syndrome with ring sideroblasts); sAML-secondary acute myeloid leukemia. (Adapted from (Barbui et al., 2012; Koopmans, Schouten, & van Marion, 2015; Rumi & Cazzola, 2017; Skoda et al., 2015; Tefferi, 2008b; Vainchenker & Kralovics, 2017).

JAK2 V617F was the first driver mutation to be described in 2005 (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005), and is the most commonly involved in PN-MPNs pathogenesis, with the highest frequency (up to 95%) in PV, and 50–60% in ET and PMF patients (Figures 1.3 and 1.4) (Cazzola & Kralovics, 2014; Hinds et al., 2016; Levine, 2009; Meyer & Levine, 2014; Oh & Gotlib, 2010; Skoda et al., 2015; Tefferi, 2016; Tefferi & Pardanani, 2015).

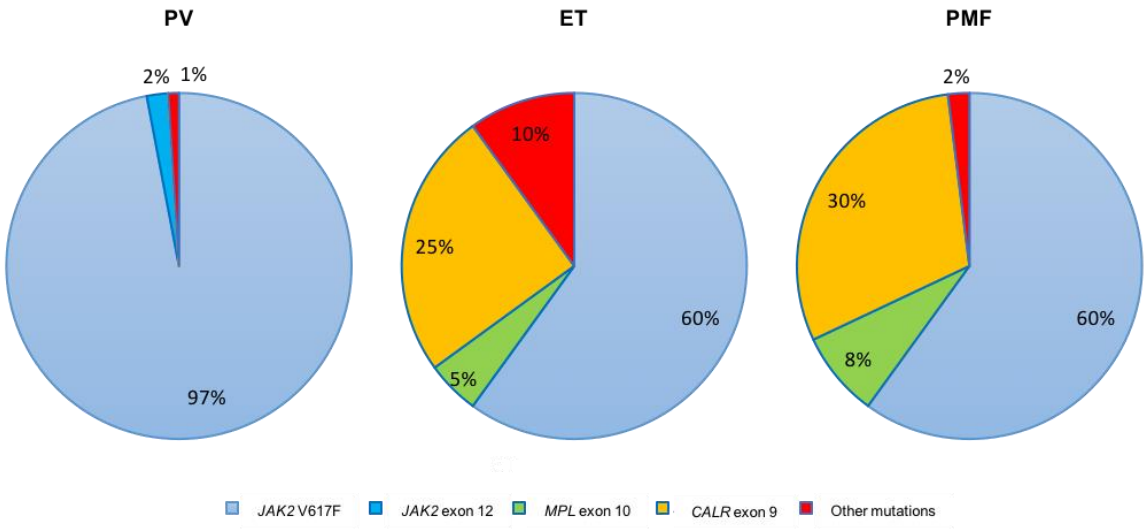


Figure 1.4 – Variation frequency of driver and other mutations in PN-MPNs.
(Langabeer et al., 2015; Nangalia & Green, 2014; Rumi & Cazzola, 2017).

V617F consists of a gain-of-function missense mutation with a G to T (guanine to thymidine) substitution at nucleotide 1849, in exon 14 of the *JAK2* gene, resulting in the substitution of valine with phenylalanine at codon 617 in the inhibitory JH2 domain (Figure 1.5). In humans, *JAK2* V617F occurs at the stem cell level and is present in hematopoietic stem cells progenitors from affected individuals, but not usually in the germline, suggesting that this mutation is acquired as a somatic disease allele in the hematopoietic compartment (Tefferi, 2008b). It is believed to be myeloid lineage specific because it is present in erythroid and granulocyte-macrophage progenitors. However, some reports have suggested *JAK2* V617F clonal involvement of B (Ishii, Bruno, Hoffman, & Xu, 2006), T (Ishii et al., 2006), and NK lymphocytes (Bellanné-Chantelot et al., 2006), also confirming the stem cell nature of *JAK2* V617F MPN's (Tefferi, 2008b).

Lower frequencies of V617F mutation occur in PN-CML, chronic myelomonocytic leukemia, megakaryocytic AML and juvenile myelomonocytic leukaemia. Other mutations in the JAK2 pseudokinase domain (including point mutations involving R683) have been detected in about 20% of Down Syndrome-associated and other acute lymphoblastic leukaemia and AML. A number of JAK2 fusion proteins lead to activation of JAK kinase activity and have also been associated with myeloid and lymphoid leukemia or atypical CML. They include the TEL-JAK2, PCM1-JAK2 and BCR-JAK2 fusion proteins (Babon, Lucet, Murphy, Nicola, & Varghese, 2014; Meyer & Levine, 2014).

Although the prevalence of *JAK2* V617F mutation differs among PN-MPNs, one of the most challenging aspects of the study of these disorders still is the explanation of phenotypic heterogeneity and mechanism of progression of the PN-MPNs (Hinds et al., 2016).

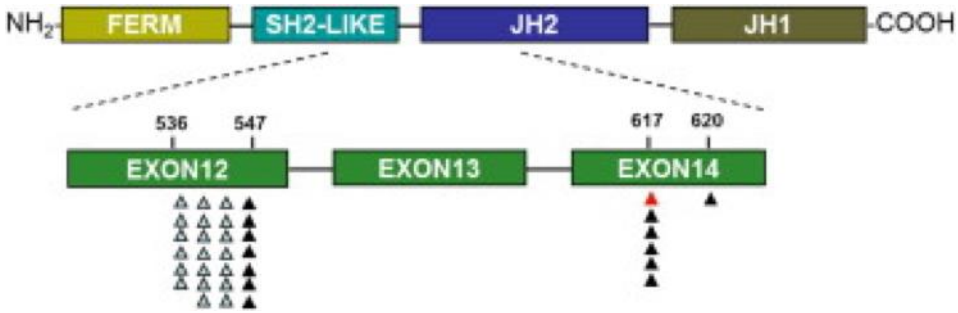


Figure 1.5 – Schematic representation of *JAK2* gene and mutation hot spots.
Representation of mutations: red triangle for V617F, black triangles for other SNP mutations, and blue triangles for insertions/deletions. FERM, four-point-one, ezrin, radixin, moesin domain; JH1, kinase domain; JH2, pseudokinase domain; SH2-like, SH2-like domain (Adapted from (Gong et al., 2013).

It is known that *JAK2* V617F mutation is present in myeloblasts, granulocytes, erythroblasts and in all EPO-independent erythroid colonies, affecting the inhibitory function of the pseudokinase JH2 domain of *JAK2* gene. The JH2 domain is believed to be auto-inhibitory and valine 617 plays an important role in JAK2 kinase auto-inhibition (Tefferi, 2008b). When V617F mutation occurs, the result is an increased activity in myeloid progenitor cells, which leads to proliferation and excessive production of mature cells (Figure 1.6) (Anand et al., 2011; Chen & Mullally, 2014; Ebid, Ghareeb, Salaheldin, & Kamel, 2015; Green & Llambi, 2015; Steensma et al., 2006).

The receptors of bone marrow progenitor cells are hypersensitive to EPO (stimulates erythroblasts), TPO (stimulates proliferation and differentiation of megakaryocytes), stem cell factor (SCF, induces proliferation and selfrenewal of multipotent hematopoietic progenitors), granulocyte stimulating factor (GSF, stimulates proliferation and differentiation of granulocytes) and interleukins. The hypersensitivity to these cytokines results in monoclonal stimulation of the erythropoiesis, megakaryopoiesis and granulopoiesis.

JAK2 V617F mutation activates signaling through the three main homodimeric receptors EPO-R, MPL, and G-CSFR, which are involved in erythrocytosis, thrombocytosis, and neutrophilia, respectively (Vainchenker & Kralovics, 2017). In addition, expression of *JAK2* V617F results in constitutive activation of downstream signaling pathways including the JAK-STAT, PI3K/AKT and MAPK/ERK pathways (discussed below in this section) (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). However, studies have shown that expression of *JAK2* V617F results in transformation of Ba/F3 cells, characterized by IL-3 independent growth, unlike wild-type JAK2 (James et al., 2005). Due to *JAK2* V617F mutation and other mutations, hematopoietic progenitor cells can proliferate without the presence or induction by cytokines, resulting in factor independent growth of the erythroid cell line and activation of signal transduction (Tefferi, 2008b), mostly in PV homozygous cases. Nevertheless, it is essential the presence of receptors (Ebid et al., 2015; Michiels, 2005).

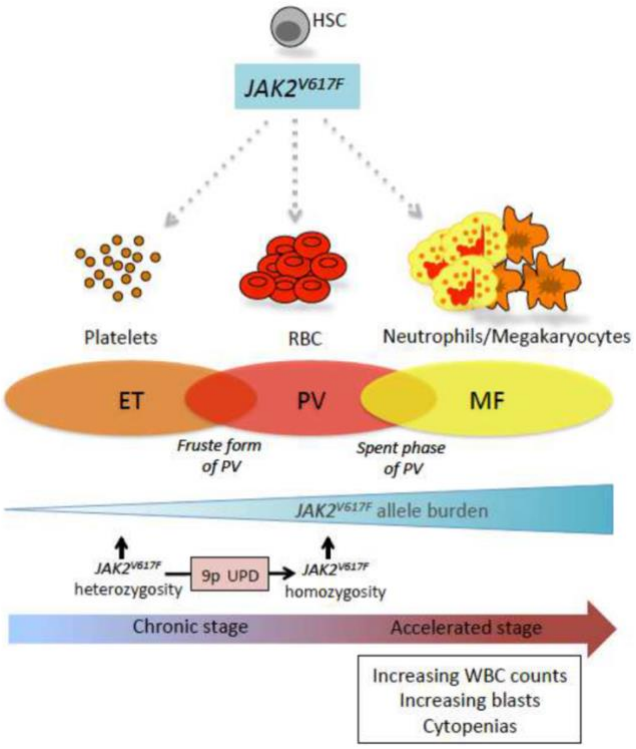


Figure 1.6 – Schematic representation of *JAK2* V617F positive PN-MPNs.
The mutation *JAK2* V617F arises in HSC and results in clonal proliferation of terminally mature myeloid cells. This mutation is found in three clinically distinct MPNS (ET, PV and PMF), that are characterized by the hyper proliferation of platelets, red blood cells/neutrophils and neutrophils/megakaryocytes, respectively. However, a small proportion of ET may overlap PV phenotype depicting the fruste form of PV and PV can evolve into myelofibrosis by passing through a spent phase featuring some symptoms of MF. Of note, PV is also characterized by hyperproliferation of granulocytes and platelets in contrast to erythrocytosis that is characterized by the sole hyperproliferation of erythroid cells. *JAK2* V617F heterozygosity is associated with ET and homozygosity due to chromosome 9 uniparental disomy (9p UPD) is associated with PV. *JAK2* V617F allele burden increases across the spectrum of PN-MPNs, lowest in ET and highest in MF. These disorders form a continuum where ET and PV depicts the chronic phase of disease and accelerated phase may encompass the transformation of ET and PV into myelofibrosis along with other manifestations such as increasing white blood cells, blasts and neutropenia and thrombocytopenia (Adapted from (Hasan et al., 2013).

JAK2 V617F is a somatically acquired mutation and a subset of patients with PV are homozygous for the *JAK2* V617F allele as a result of mitotic recombination and duplication of the mutant allele, leading to uniparental disomy (UPD) (Figure 1.6). Uniparental disomy of chromosomal locus 9p24, including *JAK2*, had previously been noted in PV, before identification of the *JAK2* V617F allele (Tefferi, 2008b). *JAK2* V617F mutation occurs in a homozygous state in 25% to 30% of patients with PV and 2% to 4% with ET (Tefferi, 2008b; Vannucchi et al., 2007). These observations suggest that mitotic recombination, which leads to *JAK2* V617F homozygosity, is more likely to occur in PV patients with mutation in exon 14 of the *JAK2* gene than in those with exon 12 mutations (Pietra et al., 2008), and is an early genetic event in the development of PV, but not ET (Tefferi, 2008b). Although *JAK2* V617F homozygous subclones can be detected both in PV and ET patients, expansion of a dominant homozygous subclone is almost exclusive in PV patients (Rumi & Cazzola, 2017). In the heterozygous

state, *JAK2* V617F–bearing receptors are still responsive to growth factors. Only with JAK2 V617F homozygosity, do these receptors become autonomous with respect to growth factor (Spivak, 2017).

Several published data have shown the contribution and influence of *JAK2* V617F mutation allelic burden, in the definition of phenotype and prognostic impact in PN-MPNs (Larsen, Pallisgaard, Møller, & Hasselbalch, 2007; C. Nielsen, Bojesen, Nordestgaard, Kofoed, & Birgens, 2014). *JAK2* V617F allelic burden corresponds to the ratio between mutant and wild type *JAK2* in hematopoietic cells and is on the basis of a stronger activation of intracellular signaling pathways (Vannucchi, Pieri, & Guglielmelli, 2011). Between MPNs patients there is a variability in the number of cells carrying the *JAK2* V617F mutation and there is a variability in the alleles that carry the mutation.

It is recognized that the allele burden tends to be higher in PV (due to the higher number of homozygous cases) and PMF, with defined hematological and clinical markers indicative of a more aggressive phenotype (Vannucchi et al., 2011), whereas a lower allele burden is generally observed in ET patients (Figure 1.6) (Chen & Mullally, 2014; Duletić et al., 2012; Ha, Kim, Jung, Jung, & Chung, 2012; Hinds et al., 2016; Larsen et al., 2007). Indeed, some ET patients with increasing allele burden transform over time to PV or PMF. Importantly, ET patients with the mutation have a "PV-like" phenotype compared to ET patients without this mutation. However, patients carrying *JAK2* V617F mutation do not have a higher risk of developing post-PV and post-ET myelofibrosis, compared to patients without the mutation (Koopmans et al., 2015).

Another possible explanation concerns the concept of a 'pre-JAK2' phase in which additional somatic mutations or inherited predisposing alleles present before the mutation are responsible for the clonal hematopoiesis, determine the phenotype, influence the risk of progression to AML, and might even be responsible for generating the mutation or act synergistically (Koopmans et al., 2015; Skoda et al., 2015). In fact, although *JAK2* V617F mutation is central to the pathogenesis of PV, ET and PMF, the presence of the same allele in three clinically distinct MPN's suggests that there might be additional inherited or acquired genetic predisposition. Indeed, a familial tendency has been reported in 72 families, which is consistent with an inherited genetic predisposition to MPN (Pardanani, Lasho, McClure, Lacy, & Tefferi, 2006).

On the other hand, mutations in epigenetic regulators, transcription factors, and signaling components modify the course of the disease and can contribute to disease initiation and/or progression (Skoda et al., 2015). Some studies performed in mice, conducted to the consideration of the 'host genetic factors', which act as modifiers of the phenotype in combination with the mutation, for instance, singe nucleotide polymorphisms (SNPs) (focused below in this section). Even gender could be an independent modifier, with women having a lower allele burden than men (Koopmans et al., 2015).

Also, the coexistence of independent *JAK2*-mutant and *JAK2* wild-type clonal expansions in the same patient can be an explanation. It is observed that *JAK2* positive AML patients are preceded by transformation to myelofibrosis during their disease course, in contrast to *JAK2* wild-type AML, which is preceded by chronic-phase ET and PV patients (Koopmans et al., 2015).

JAK2 V617F mutation, along with other driver mutations connected with clonal expansion of

hematopoietic cells, might also represent a feature of the aging hematopoietic system in individuals without a malignant disease (Jaiswal et al., 2014; C. Nielsen, Birgens, Nordestgaard, & Bojesen, 2013). There is increasing evidence that *JAK2* V617F is relatively frequent in the aging healthy population and is presently estimated to be 0.5% (Vainchenker & Kralovics, 2017). This share of the population usually presents higher erythrocyte, platelet and leucocyte counts. Moreover, it has been observed that individuals without malignant disease but who are positive to the *JAK2* V617F somatic mutation were 44/28 risk fold more likely to develop an hematological cancer, since aging is generally associated with a deregulation of hematopoietic stem cells, which lose their function and become myeloid-biased and less quiescent as a consequence of intrinsic and environmental changes, with *JAK2* V617F hematopoietic stem cells having higher competitive properties in this context (C. Nielsen et al., 2013; Vainchenker & Kralovics, 2017).

Although there is no gold standard and the choice of methodology is dependent on the application, quantitative real-time PCR is a useful method for detecting V617F mutation in *JAK2* gene (Bench, Baxter, & Green, 2013).

The description of the developed work within the scope of this dissertation, begins precisely in Chapter 3, were the study carried out for *JAK2* V617F evaluation is discussed. Moreover, in Chapter 5 it is reported the case of two ET patients included in our study population, bearing *JAK2* V617F mutation, with the suspicion of a very rare association with *BCR-ABL* translocation.

JAK2 EXON 12 MUTATION

A small proportion of patients with PV are *JAK2* V617F negative when tested by sensitive allele-specific assays (Tefferi, 2008b), which motivated a search for additional *JAK2* mutations. Thus, in 2007, a set of *JAK2* exon 12 mutations were described by Scott and colleagues, in *JAK2* V617F negative patients with PV (Scott, Tong, et al., 2007).

Unlike V617F where only a single codon is affected, exon 12 frameshift mutations comprise more than forty different small deletions/duplications and substitutions of one or more amino acids between phenylalanines F533 and F547 (ex: lysine for leucine at codon 539 - K539L), which are located in a linker between the JH2 pseudokinase and the SH2 domains (Figure 1.5) (Vorechovsky, Jones, & Cross, 2013).

From literature review, almost the totality of the patients diagnosed with PV negative for *JAK2* V617F mutation are exon 12 positive (95% *vs* 2-4%, respectively; Figures 1.3 and 1.4) (Arber et al., 2016; Butcher et al., 2008; C. H. Park et al., 2016; Passamonti, Rumi, et al., 2010; Rumi et al., 2014; Scott, 2011; Scott, Beer, Bench, Erber, & Green, 2007; Scott, Tong, et al., 2007). Some studies have reported that the Chinese PV patients have a relatively lower *JAK2* V617F mutation frequency (82%), while the mutations in *JAK2* exon 12 are much more pervasive (13%), when compared to Westerns, and other East Asians (C. H. Park et al., 2016; Z. Wu et al., 2014). Unlike *JAK2* V617F, which can be

detected in any of the PN-MPNs, *JAK2* exon 12 mutations are almost exclusive of *JAK2* V617F negative PV patients (Tefferi, 2008b).

PV patients who present *JAK2* exon 12 mutations, unlike those who are V617F positive, are not commonly homozygous (Godfrey et al., 2016; Scott, 2011; Scott, Tong, et al., 2007; Spivak, 2017). However, just like *JAK2* V617F mutation, also exon 12 mutant alleles induce cytokine-independent/hypersensitive proliferation in EPO receptor (EPO-R) expressing cell lines and constitutive activation of JAK-STAT signaling (Tefferi, 2008b).

There are also reports of the coexistance of *JAK2* V617F and *JAK2* exon 12 mutations as two separate clones (Passamonti et al., 2011; Spivak, 2017).

The *JAK2* exon 12 mutations contribute primarily to erythroid myeloproliferation, associated with increasing levels of phosphorylated JAK2, STAT5 and Erk1/2, compared to patients with wild type *JAK2*, and even higher activated JAK2 and Erk1/2 levels compared to patients with the *JAK2* V617F mutation (Godfrey et al., 2016; Koopmans et al., 2015; Scott, Tong, et al., 2007).

PV patients with the *JAK2* exon 12 mutations are usually younger than those with the *JAK2* V617F mutation, and have a phenotype usually more benign than that of JAK2V617F, usually without panmyelosis (Arber et al., 2016), with normal leukocyte and platelet counts (Koopmans et al., 2015; Spivak, 2017). However, there are exceptions as will be demonstrated in the clinical case presented in Chapter 4.

PN-MPNs *JAK2* exon 12 positive patients appear to be associated with a distinct syndrome, specific to patients with higher hemoglobin concentrations, without concomitant leukocytosis or thrombocytosis (or minimal thrombocytosis), and isolated bone marrow erythroid hyperplasia (Godfrey et al., 2016), independently of the mutational variant (Godfrey et al., 2016; Passamonti et al., 2011). The fact that exon 12 mutations are more frequently associated with erythrocytosis is compatible with their absence in ET, but possible presence in PMF or AML secondary to PV (Scott, 2011).

However, despite the phenotypical difference, the clinical course and outcome seems similar between *JAK2* V617F and *JAK2* exon 12 positive patients, with similar incidences of thrombosis, myelofibrosis, leukemia and death (Passamonti et al., 2011).

Although there is no gold standard and the choice of methodology is dependent on the application, quantitative real-time PCR and high resolution melt-curve analysis are useful methods for detecting this type of mutation in *JAK2* gene (Bench et al., 2013).

In Chapter 4 of this dissertation it is presented the interesting case of the only known *JAK2* exon 12 positive patient included in the studied population, presenting a novel mutation – c. 1605G>T (p.Met535Ile) and an atypical hematological phenotype, characterized by the presence of thrombocytosis.

MYELOPROLIFERATIVE LEUKEMIA ONCOGENE (MPL)

Approximately 50-60% of ET and PMF patients are *JAK2* V617F negative. This led Pikman and colleagues (Pikman et al., 2006), one year after the discovery of the *JAK2* V617F mutation, to study whether other genes in the JAK-STAT signaling pathway might be mutated in *JAK2* V617F negative ET and PMF patients. They described mutations of the myeloproliferative leukemia oncogene (*MPL*), located at chromosome 1p34, which encodes for TPO-R, and is a key factor for growth and survival of megakaryocytes. The two *MPL* W515L exon 10 missense mutations consist in a G (glycin) to T (treonin) transition at nucleotide 1544, resulting in a switch from tryptophan to leucine or lysine or, less frequently, to arginine or alanine at amino acid 515 (*MPL* W515L/K or W515R/A) in the *MPL* juxtamembrane domain (Figure 1.7) (Tefferi, 2008b). Mutations can also occur in the MPL extracellular distal cytokine domain.

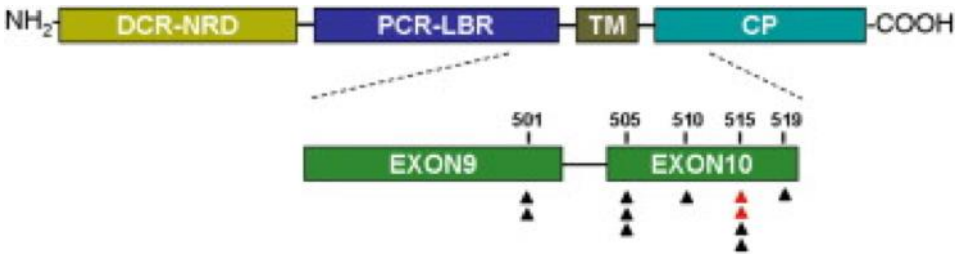


Figure 1.7 – Schematic representation of *MPL* gene and mutation hot spots.
Representation of mutations: red triangles for W515L and W515K, and black triangles for other SNP variant mutations. CP, cytoplasmic domain; DCR-NRD, distal cytokine receptor domain-negative regulatory domain; PCR-LBR, proximal cytokine receptor domain-ligand binding region; TM, transmembrane domain (Adapted from (Gong et al., 2013).

MPL mutational frequency in MPNs is substantially lower (<10%) than the other driver mutations (Figures 1.3 and 1.4) (Tefferi, 2008b). With rare exceptions, *MPL* mutations are absent in PV and other myeloid malignancies (Pardanani et al., 2006), being found in approximately 15% of *JAK2* V617F negative MPNs, with a frequency of 3–4% in ET, and 6–7% in PMF patients (Figures 1.3 and 1.4) (Langabeer et al., 2015; Tefferi, 2016).

These acquired gain of function mutations are stem cell-derived events, which constitutively force a change in the TPO-R activating JAK2, STAT3, STAT5, Erk and Akt pathway in the absence of TPO binding. Like *JAK2* and *CALR* mutations, however, *MPL* mutations require a hematopoietic growth factor, in this case TPO, for complete kinase activation in the heterozygous state (Spivak, 2017).

Overall, compromised MPL function due to incomplete glycosylation and impaired MPL cell-surface expression may have a more important role in the pathophysiology of myeloproliferative neoplasms than any *MPL* mutation. *JAK2* V617F impairs MPL maturation, increasing the proportion of

immature receptors in the plasma membrane, reduces MPL recycling, and increases its degradation. Impaired MPL cell-surface expression, which is also a feature of *CALR* and *MPL* mutations, results in elevated plasma TPO levels, as a result of reduced clearance of TPO from the plasma by megakaryocytes and platelets, and may also be involved in the emigration of involved hematopoietic stem cells from their marrow niches (Spivak, 2017).

Somatic *MPL* mutations and germline single-nucleotide variants are not mutually exclusive of *JAK2* V617F, though they are not in the same clone (Spivak, 2017).

Patients with *MPL* exon 10 mutations have a distinct clinical phenotype tending to have lower hemoglobin levels, when compared to *JAK2* V617F positive MPNs patients (Koopmans et al., 2015; Langabeer et al., 2015). ET patients with an *MPL* mutation are of older age, and are also reported to have higher platelet counts, isolated megakaryocytic proliferation and higher serum erythropoietin levels compared to *JAK2* V617F positive ET patients. *MPL* mutated PMF patients are also of older age with lower hemoglobin levels.

Compared to *JAK2* V617F mutations, there seems to be no prognostic difference with respect to thrombosis, hemorrhage, myelofibrotic (except in ET patients) and leukemic transformation or survival. Patients carrying the W515K mutation have higher allelic burdens than those with the W515L allele (that may be in the range of 3–15%), suggesting a functional difference between these variants (Langabeer et al., 2015). The mutant allele burden is often greater than 50% and homozygosity has been described, suggesting biallelic mutation or loss of heterozygosity (LOH).

CALRETICULIN (CALR) MUTATION

The second most common and most recent driver gene to be described in MPNs, by Kralovics in December 2013, was calreticulin (*CALR)* (Klampfl et al., 2013; Tefferi, 2016).

CALR is a multi-functional highly conserved Ca binding protein chaperone, with pleiotropic roles related to its distribution in the endosplasmic reticulum (ER), cytosol, and on the cell surface. It is also involved in glycoprotein folding and in cellular functions such as proliferation, phagocytosis, and apoptosis (Spivak, 2017).

To date, more than 50 different CALR mutations have been identified, which often consist of exon 9 frameshift mutations (chromosome 19) with somatic insertions or deletions, resulting in one base pair reading frameshift, an altered C-terminal that is missing the KDEL (lysine, aspartic acid, glutamic acid and leucine) ER retention motif and is positively rather than negatively charged, and Ca^{2+} impaired function. These mutations substantially alter CALR cellular distribution because mutant CALR is able to bind MPL through the receptor's extracellular domain and chaperone it to the plasma membrane. The mutant, positively charged CALR C terminal domain is obligatory for both MPL binding and cellular transformation, but how MPL JAK2 signaling is activated by mutant *CALR* is unknown. As is the case with *JAK2* and *MPL* mutations, the proportion of immature MPL in the cell is increased. Like

receptors containing *JAK2* V617F, MPL bound by mutant *CALR* still requires growth factor stimulation for complete *JAK2* activation in the heterozygous state (Spivak, 2017; Vainchenker & Kralovics, 2017). The *CALR* mutants mainly activate MPL and at a low level the G-CSFR but not the EPOR, explaining the thrombocytosis associated with these mutants (Vainchenker & Kralovics, 2017).

With rare exceptions, *CALR* (and *MPL*) mutations are absent in PV. Somatic *CALR* mutations have been identified in 20–25% of ET and PMF patients (Figures 1.3 and 1.4) (Klampfl et al., 2013; Nangalia et al., 2013; Skoda et al., 2015; Tefferi, 2016).

About 80-85% of *CALR* mutated patients harbor one of two mutation variants: type 1, a 52-bp deletion (p.L367fs*46) or type 2, a 5-bp TTGTC insertion (p.K385fs*47). Type 1 mutations are more common in PMF, whereas type 1 and type 2 occur with similar frequency in ET (Spivak, 2017; Tefferi, 2016). *CALR* variants that are neither type 1 nor type 2 are operationally classified into “type 1-like” and “type 2-like” variants, based on their structural similarities to each type, which is in turn based on alpha-helix content of the mutant C-terminus (Tefferi, 2016).

CALR mutations are occasionally homozygous as a result of chromosome 19 UPD, usually with type 2 mutations. They are not mutually exclusive of *JAK2* V617F, and can be detected in PV in rare cases (Spivak, 2017).

Whereas *JAK2* and *MPL* mutations are believed to directly activate JAK-STAT, *CALR* mutations might do the same indirectly in megakaryocytic and granulocytic precursor cells (Klampfl et al., 2013), with aberrant and preferential expansion for the megakaryocyte lineage. How these changes result in JAK-STAT or other pathway activation is currently being investigated. Moreover, some mouse models suggest a primary effect on platelet production (Tefferi, 2016).

In some but not all studies, the type 1 *CALR* mutation appeared to be associated with a survival advantage as compared with *JAK2* V617F and *MPL* mutations, but the three mutations did not differ with respect to leukemic transformation (Spivak, 2017).

In ET, type 2 *CALR* mutation is associated with significantly higher platelet count and a higher tendency to thrombotic events, compared to patients with type 1 *CALR* mutations (Kang et al., 2016; Spivak, 2017). In PMF, it is associated with higher risk category, circulating blast percentage, leukocyte count, and inferior survival (Tefferi, 2016).

Compared with ET patients bearing *JAK2* or *MPL* mutations, *CALR* mutated ET patients are more commonly male, and exhibit lower white blood cell and hemoglobin levels, higher platelet counts and better clinical outcome, with a lower risk of thrombosis and no progression to PV. However, there is no meaningful difference in the rate of transformation to pos-ET MF, nor in overall survival (Rumi et al., 2014; Vannucchi et al., 2014).

In PMF, carrying a *CALR* mutation is associated with better survival compared to patients with *JAK2* or *MPL* mutations. The prognostic benefit of *CALR* mutations may be limited to type 1 or type 1–like *CALR* variants (Tefferi & Vannucchi, 2017); patients with type 2 or type 2–like *CALR* variants exhibit comparably worse survival, which is comparable with that of PMF patients with *JAK2* V617F mutation (Rotunno et al., 2014). In PMF, cumulative incidence of thrombosis at 10 years was 11,0% in *CALR*

mutated *vs* 21,0% in *JAK2* mutated patients (Klampfl et al., 2013; Rotunno et al., 2014; Rumi et al., 2014).

While *CALR* is a clonal marker that can provide diagnostic clarity in cases of ambiguous thrombocytosis, it does not have a bearing on general therapeutic decisions in established cases of ET or PMF (discussed below). For example, *CALR* mutation does not affect the International Prognostic Score for predicting the risk of thrombosis in ET (Tefferi, Lasho, et al., 2014). Moreover, PMF patients with mutant *CALR* exhibit responsiveness to *JAK* inhibitor therapy, even with unmutated *JAK2* (Tefferi, Guglielmelli, et al., 2014). Nevertheless, the prognostic impact of mutated *CALR* needs to be weighed in the context of dynamic genetic-based scoring systems that layer additional prognostic information, such as karyotype and other molecular abnormalities (especially poor-risk mutations in *ASXL1*, *EZH2*, *SRSF2*, *IDH 1/2*), on top of the clinical and laboratory variables (DIPSS-PLUS prognostic score). Such refined prognostic schemes are particularly relevant to decision making about hematopoietic stem cell transplantation, mostly in PMF patients.

In the following subsection it will be discussed how the cytokine receptor/JAK2 pathway is activated and their downstream effectors.

JAK-STAT SIGNALLING PATHWAY

Not the least, the cellular and molecular mechanisms involved in the pathophysiology of PN-MPNs have not yet been fully clarified (Beer et al., 2010; Björkholm, Hultcrantz, & Derolf, 2014; Bolufer et al., 2006; Campregher, Santos, Perini, & Hamerschlak, 2012; Delhommeau, Jeziorowska, Marzac, & Casadevall, 2010; Hinds et al., 2016; Kilpivaara & Levine, 2008; Rice et al., 2011; Rueff & Rodrigues, 2016). However, in PN-MPNs patients, hyperactive JAK/STAT signaling pathway appears to be a constant, even in the presence of *CALR* mutations and the so-called ‘‘triple-negative’’ MPNs (nonmutated *JAK2*, *CALR*, and *MPL*), where the driver gene mutation is still unknown (Reuther, 2016; Skoda et al., 2015).

Due to their essential roles as intracellular signaling effectors of cytokine receptor activation, the Janus Kinase (JAK) family of tyrosine kinases have aroused much interest since their discovery more than 20 years ago (Babon et al., 2014).

This family comprises four members, JAK1, JAK2, JAK3 and TYK2, originally named ‘just another kinase’. They owe their name due to the homology of kinase (JH1) and pseudokinase (JH2) symmetrical domains with Janus, the Roman god of two faces (Figure 1.8) (Becerra-Díaz, Valderrama-Carvajal, & Terrazas, 2011; McLornan, Percy, & McMullin, 2006). The first JAK identified was TYK2 by Krolewski in 1990, using libraries of complementary DNA from human T lymphocytes, while JAK1, JAK2 and JAK3 where identified through conserved motif clonation of the catalytic domain (Becerra-Díaz *et al.*, 2011). They comprise seven homologous JH domains organized into four regions; kinase (JH1), pseudo-kinase (JH2), FERM (four-point-one, ezrin, radixin, moesin; includes the N-terminal JH7, JH6,

JH5 and part of JH4) and SH2-like (JH3 and part of JH4). The carboxy-terminal portion of these molecules includes the distinctive kinase domain (JH1) which is catalytically active and the catalytically inactive pseudo kinase domain (JH2) which is felt to regulate the activity of JH1. The other amino-terminal JH domains, JH3-JH7, mediate association with receptors. In humans, JAK1 is located on chromosome 1p31.3, JAK2 on 9p24, JAK3 on 19p13.1 and TYK2 on 19p13.2 (Tefferi, 2016).

JAK proteins interact with different intracellular domains of cytokine receptors and are present in a variety of cell subtypes. Expression is ubiquitous for JAK1, JAK2 and TYK2 but restricted to hematopoietic cells for JAK3 (Tefferi, 2016).

Furthermore, a seven-member family of transcription factors named Signal Transducers and Activators of Transcription (STAT) (Figure 1.8) are also involved in many cytokine signaling pathways. In 1994, Darnell and colleagues identified the first two members of the family, STAT1 and 2, by purification of factors linked to interferon (IFN) stimulated genes, and the other family members were described subsequently (Becerra-Díaz *et al.*, 2011). These proteins act as transcriptional factors when they form homo- and heterodimers among them, by phosphorylation at tyrosine residues in their SH2 domain, induced by upstream JAK proteins, activating different genes and regulating downstream the JAK/STAT signaling pathway (Becerra-Díaz *et al.*, 2011).

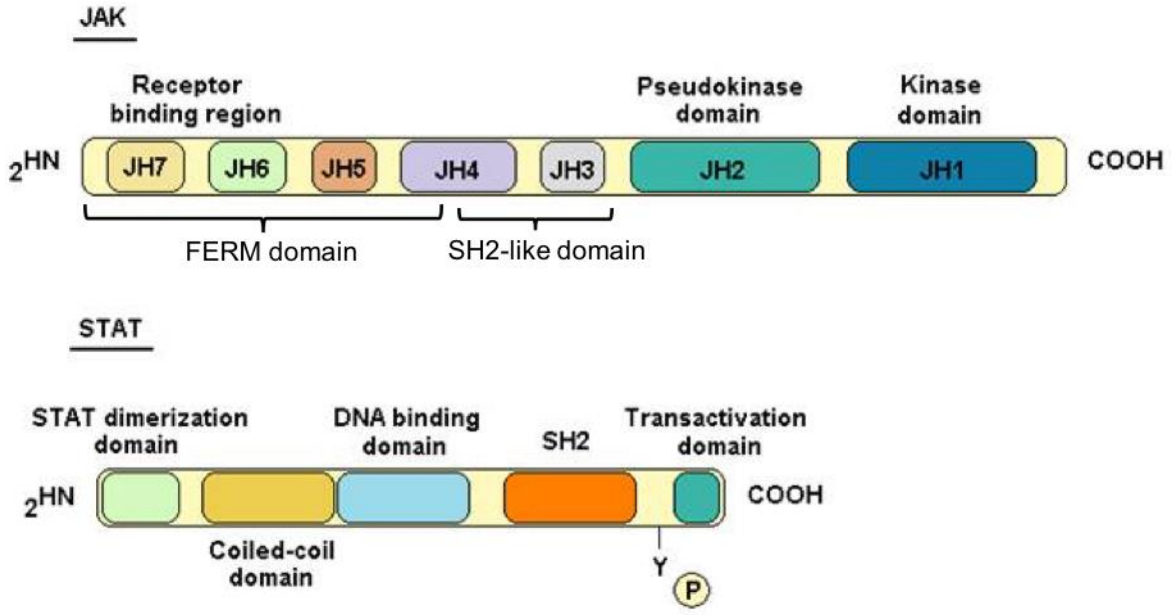


Figure 1.8 – JAK and STAT protein structures.
JAK proteins are composed of seven regions (JH1-JH7), in which JH1 domain has kinase activity and JH2 domain is homologous to JH1 but associated with regulatory functions, without the residues required for kinase activity (pseudokinase domain). STAT proteins are transcription factors constituted by five conserved domains (Adapted from Becerra-Díaz *et al.*, 2011).

The Janus kinase/signal transducers and activators for transcription (JAK/STAT) pathway regulate a large plethora of biological processes including cell proliferation, differentiation, cell migration and apoptosis (Becerra-Díaz *et al.*, 2011).

All of these proteins are constitutively present in the cytoplasm without previous stimuli, but can be quickly activated from the cellular membrane to the nucleus, by the binding of cytokines, growth factors or hormones on cell surface receptors (Table 1.1) (Becerra-Díaz *et al.*, 2011).

Table 1.1 – Cytokine and factors stimuli for JAK and STAT families activation.
(Adapted from (Becerra-Díaz *et al.*, 2011).

	Cytokine or factor
	JAK Family
JAK1	IL-2, IL-7, IL-9, IL-15, IL-4, IL-13, IL-6, IL-11, IFN-α, IFN-β, IFN-γ, IL-10, CT-1
JAK2	IL-3, IL-12, IL-13, IL-6, IL-11, IFN-γ, CT-1, Growth hormone, Prolactin, Eritropoyetin
JAK3	IL-2, IL-7, IL-9, IL-15, IL-4
TYK2	IL-6, IL-11, IL-12, IL-13, CT-1, IFN-α, IFN-β, IL-10
	STAT Family
STAT1	IL-2, IL-6, IL-10, IFN-α, IFN-β, IFN-γ, IL-27
STAT2	IFN-α, IFN-β
STAT3	LIF, IL-10, IL-6, IL-27, Growth hormone
STAT4	IL-12
STAT5a/b	Prolactin, Growth hormone, Thrombopoietin
STAT6	IL-4, IL-13

Typically, JAK kinases function through their interaction with cytokine receptors that lack intrinsic kinase activity.

As illustrated in Figure 1.9, with the example of JAK2, cytokines initiate signaling when occurs ligand binding (ex: EPO, TPO) to the appropriate cytokine receptor (type 1 or type 2 cytokine receptors; ex: EPO-R, MPL), which results in juxtaposition of JAKs and bind to their specific cell surface receptors, inducing several important conformational changes mainly oligomerization or multimerization of their receptors. Follows JAKs recruitment to the cytoplasmatic domain of the cytokine receptor and phosphorylation of a tyrosine residue in the receptor, creating a docking site for the recruitment and activation of cytoplasmic signal transducers and activators of transcription (STATs; STAT3 and STAT5 in the case of JAK2), through their SH2 domain. While STAT proteins are attached to the cytokine receptor, JAK2 proteins undergo autophosphorylation at a tyrosine residue, detaching the STAT protein from the cytokine receptor so that the STATs form homo- and heterodimers through their SH2 domain, that will translocate to the nucleus. There they bind to the promoter region of genes via specific DNA binding domains to promote gene transcription.

The net result of STAT3 and STAT5 activation is apoptosis inhibition and a proliferative activity (Koopmans et al., 2015), playing an important role in growth factor induced myeloid differentiation. STAT3 regulates cell growth through regulation of cyclins promoting cell-cycle progression, as cyclin D1, and induces Bcl-2, resulting in an anti-apoptotic signal. May promote cellular differentiation by upregulating the expression and enhancing the transcriptional activity of CCAAT/enhancer binding protein alpha (C/EBPα), a key transcription factor that drives myeloid differentiation (Numata et al., 2005). STAT3 was also shown to play an important role in megakaryopoiesis, mainly through the expansion of megakaryocytic progenitor cells.

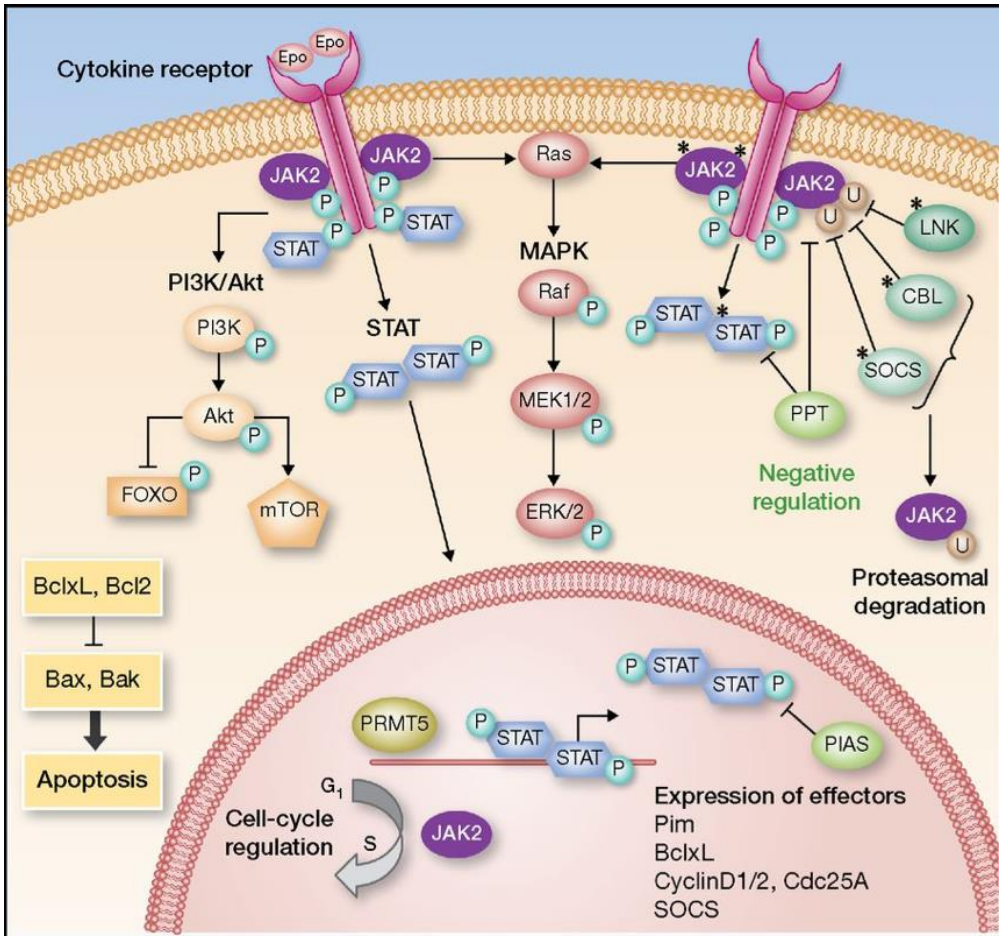


Figure 1.9 – Overview of molecular JAK signaling and regulation (ex: JAK2) and acquired genetic alterations involved in activation of components of JAK/STAT pathway.
In the absence of ligand, JAK2 molecules bind to cytokine receptor (EPO-R) as an in active dimer. Ligand (EPO) binding to cytokine receptor induces the conformational change in the receptor resulting in auto and trans phosphorylation on JAK2 and cytokine receptor. Once activated, cytokine receptor then recruits and phosphorylates several signal transmitter molecules that lead to signaling through signal transducers and activators (STAT), phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. JAK2 signaling is regulated by dephosphorylation (SHP-1), by competing for STAT binding (PIAS or SOCS), cytokine receptor ubiquitination (c-CBL) and JAK2 inactivation (LNK) (Adapted from (Babon et al., 2014; Becerra-Díaz et al., 2011; Levine, Pardanani, Tefferi, & Gilliland , 2007; Meyer & Levine, 2014).

Successful differentiation of neutrophils, induced by G-CSF, is perturbed by expression of a dominant negative form of STAT5. It has been suggested that STAT5 may be responsible for promoting the survival of myeloid progenitors via transcriptional upregulation of the anti-apoptotic protein BclxL and Pim kinase, inhibiting apoptosis of megakaryocytes, and mediates cell growth through induction of cyclin D1, thereby allowing myeloid differentiation to proceed (Kieslinger et al., 2000).

EPO is secreted by interstitial kidney cells in response to reduction in blood oxygen concentration, transported to the bone marrow where it binds its receptor, EPO-R, and transmits an intercellular signal through a receptor conformational change, which stimulates an increased production of red blood cells (Jacobson, Goldwasser, Fried, & Plzak, 1957; Maxwell, Lappin, Johnston, Bridges, & McGeown, 1990; Remy, Wilson, & Michnick, 1999). The *JAK2* FERM domain constitutively binds the EPO-R. EPO-induced EPO-R conformational change facilitates cross-phosphorylation and activation of the JAK2 proteins (Witthuhn et al., 1993).

The amino-terminal extracellular TPO-R domain has a similar structure to EPO-R, which is critical in ligand binding, resulting in a significant overlap between EPO and TPO stimulated pathways. As in EPO signaling, TPO stimulation causes the JAK2-dependent phosphorylation of STAT3 and STAT5, activation of the MAP kinase pathway, activation of the PI3K / Akt survival pathway indirectly, and can induce transcription of the pro-survival factor BclxL through STAT5- and PI3K-dependent pathways, promoting megakaryocyte differentiation. Overall, discovery of STAT, MAP kinase, and PI3K pathway stimulation downstream of the TPO-R gave a framework to understand the considerable overlap in phenotypic response to TPO and EPO (Kralovics et al., 2003; Li et al., 1994).

JAK2 also serves as an endoplasmic reticulum chaperone for the EPO and TPO receptors, transporting them to the cell surface, and increases the total number of TPO receptors by stabilizing the mature form of the receptor, enhancing receptor recycling, and preventing receptor degradation (Spivak, 2017). On the other hand, nuclear JAK2 is involved in epigenetic modifications ((Babon et al., 2014; Becerra-Díaz et al., 2011; Levine et al., 2007; Meyer & Levine, 2014).

The JAK/STAT pathway is tightly regulated and inhibited at multiple levels by several protein families - tyrosine phosphatases, suppressors of cytokine signaling (SOCS) and protein inhibitors of activated STATs (Figure 1.9) (Tefferi, 2016):

1) Suppressors of cytokine signaling proteins (SOCS), most notably SOCS1 and SOCS3, and CBL interact with activated JAKs and phosphorylated receptors or mark JAK for proteasomal degradation. CIS, SOCS1, SOCS2 and SOCS3 are members of the SOCS protein family. The synthesis of SOCS is induced by activated STATs resulting in a negative feedback loop, through interaction with activated JAKs and consequent inhibition of STAT recruitment to the binding sites (Espert, Dusanter, Fourt, & Chelbi Alix, 2005; Rawlings, Rosler, & Harrison, 2004);

2) Hematopoietic cells express SHP1. SHP1 belongs to the family of phosphotyrosine phosphatases (PTP); PTP dephosphorylates activated JAKs, STATs and cytokine receptors (Valentino & Pierre, 2006);

3) Protein inhibitors of activated STATs (PIAS), interact with activated STATs, inhibit their

dimerization and prevent their binding to target DNA (Meyer & Levine, 2014);

4) LNK sequesters JAK2 by direct binding (Meyer & Levine, 2014).

Mutations in all four JAKs have been associated with human diseases. Inherited mutated JAK alleles lead to inactivated JAK3 and TYK2 in human immune deficiency syndrome, while somatic mutations in JAK1, JAK2 and JAK3 result in constitutively active kinases in myeloproliferative diseases and leukemia/lymphomas (Babon *et al.*, 2014; Meyer & Levine, 2014).

A qualitative difference in the signaling state of STAT proteins has been described in MPN. ET progenitors have high phosphorylation levels of STAT1 and STAT5, whereas PV progenitors have only phosphorylated STAT5. The reasons behind this and other phenotypic differences are unclear, but are potentially the result of a complex interplay between acquired and/or inherited variation, and possibly environmental exposure, all unique to each MPN patient (Jones & Cross, 2013).

The most frequent mutation, *JAK2* V617F, activates the three main myeloid cytokine receptors (EPO-R, G-CSFR and MPL) whereas *CALR* or *MPL* mutants are restricted to MPL activation, explaining why *JAK2* V617F is associated with PV, ET and PMF, whereas *CALR* and *MPL* mutants are found in ET and PMF (Vainchenker & Kralovics, 2017).

The *JAK2* V617F mutation also activates the MAPK and phosphatidylinositol-3-kinase (PI3K)-Akt pathway (Figure 1.9), by interacting with p85, a regulatory subunit of PI3K, promoting proliferation and survival. Activated PI3K activates Akt, which in turn activates mammalian target of rapamycine (mTor) on Ser2448 which directly phosphorylates ribosomal p70S6Kinase (p70S6k). p70S6K and mTor are involved in angiogenesis by activation of vascular endothelial growth factor (VEGF) (Koopmans et al., 2015; Meyer & Levine, 2014). It is known that this pathway is commonly activated in leukemia and lymphoma and is involved in inhibiting apoptosis in normal human erythroblasts. The PI3K/Akt pathway also induces the phosphorylation of BAD, a pro-apoptotic member of the Bcl2 family, via phosphorylated Akt (pAkt) and p70S6k, inhibiting BAD function and resulting in inhibition of apoptosis. BclxL is also activated by this pathway, resulting in inhibition of megakaryocyte apoptosis (Koopmans et al., 2015).

On the other hand, it was also demonstrated in PV patients an increased activation of Ras-Erk signalling pathway (Figure 1.9). Ras is activated and activates Raf-1, which mediates the activation of MEK, which in turn activates extracellular signal-regulated kinase (Erk), one of members of the MAPK families. The result of Erk phosphorylation is the inhibition of apoptosis, by blocking the function of BAD and activation of Bcl2. Therefore, the activation of the Erk pathway is suggested to be one of the mechanisms responsible for the hypercellularity seen in the bone marrow of MPNs patients, also possibly secondary to the *JAK2* V617F mutation (Koopmans et al., 2015). Due to the inactivation of the pro-apoptotic factor BAD and activation of BclxL, Akt together with *JAK2* V617F mutation suppresses apoptosis and promotes cell survival, up-regulating megakaryocytes and erythropoiesis (Koopmans et al., 2015).

In contrast to its effect on the EPO receptor, *JAK2* V617F appears to increase the quantity of immature MPL while increasing MPL degradation through ubiquitination and reducing its cell-surface expression (Spivak, 2017).

Recently, at the beginning of 2017, Yao *et al.* demonstrated that activation of *JAK2* mutants can differentially couple to selective cytokine receptors and change the signaling repertoire, revealing the molecular basis for phenotypic differences elicited by *JAK2* V617F or mutations in exon 12. On the basis of these findings, receptor-JAK2 interactions could represent new targets of lineage-specific therapeutic approaches against MPN, which may be applicable to other cancers with aberrant JAK-STAT signaling (Yao et al., 2017).

Recent data indicate that the *JAK2V617F* allele might escape negative feedback by SOCS3 (Meyer & Levine, 2014).

NON-MPN-DRIVER MYELOID GENES

Pathogenic mutations have been identified in more than 90% of patients with PN-MPNs, 50 to 60% of them having only a driver mutation (*JAK2* V617F, *CALR*, *MPL*, or in rare cases, *LNK*) (Figure 1.4) (Spivak, 2017). However, about 10–15% of patients who meet the WHO criteria mostly for PMF or ET, do not express any one of the three mutations and are referred to as being “triple-negative” (nonmutated *JAK2*, *CALR*, and *MPL*), carrying a poor prognosis and a high rate of disease progression and leukemic transformation (Delhommeau et al., 2010; Nangalia et al., 2013; Rumi & Cazzola, 2017; Rumi et al., 2014; Tefferi & Pardanani, 2015).

It is known that the different driver mutations involved in the pathogenesis of MPNs can lead to different clinical effects and that a single mutation may be associated with distinct phenotypes and clinical outcomes (Rumi & Cazzola, 2017). Moreover, none of the driver *JAK2* V617F, *MPL* W515L/K, or *CALR* mutations is specific of a particular MPN subtype, and cannot fully explain the phenotypic heterogeneity of PN-MPNs, nor the susceptibility of progression to MF, AML or MDS (Hinds et al., 2016).

More than a decade on from the original description of *JAK2* V617F, attributing the PN-MPNs phenotype only to constitutive activation of JAK2, is to over simplify the complex molecular interactions that regulate the JAK/STAT pathway. Although the pathogenic mechanism of PN-MPNs is not completely clarified, it is well known that, in addition to the “phenotypic driver mutations” referred above, there are other somatic mutations directly associated with hyperproliferation of hematopoietic cells, that could be involved in these disorders (Figures 1.3 and 1.4). This can occur due to different variants within the same gene or between variants in different genes. The latter must be considered, as the number of passenger mutations in a tumor may modulate the effect of driver mutations, thus acting as putative modifier genes (Rueff & Rodrigues, 2016).

Along with the recent description of the CALR mutation, differences in the epigenetic landscape have been hypothesized to be a key component, and additional mutations in genes involved in epigenetic regulation, splicing, signal transduction and tumor-suppression have been investigated (McPherson, McMullin, & Mills, 2017; Spivak, 2017; Vainchenker & Kralovics, 2017).

Epimutations are included in different categories and constitute epigenetic regulators (DNA methylation: *TET2*, *IDH1*, *IDH2*, *DNMT3A*; histone methylation: *EZH2*, *ASXL1*), transcription factors (tumor suppressor: *TP53*; *IKZF1*, *NF-E2*, *CUX1, ETV6, RUNX1*), RNA splicing (*SRSF2*, *U2AF1*, *SF3B1*) or signaling molecules (*CBL, NF1, LNK*) (Figure 1.10) (Spivak, 2017; Tefferi, 2016; Vainchenker & Kralovics, 2017). Mutations that can silence tumor suppressor genes must be taken into account (Rueff & Rodrigues, 2016), which highlights the concept that, probably more important than the genes, are their levels of expression.

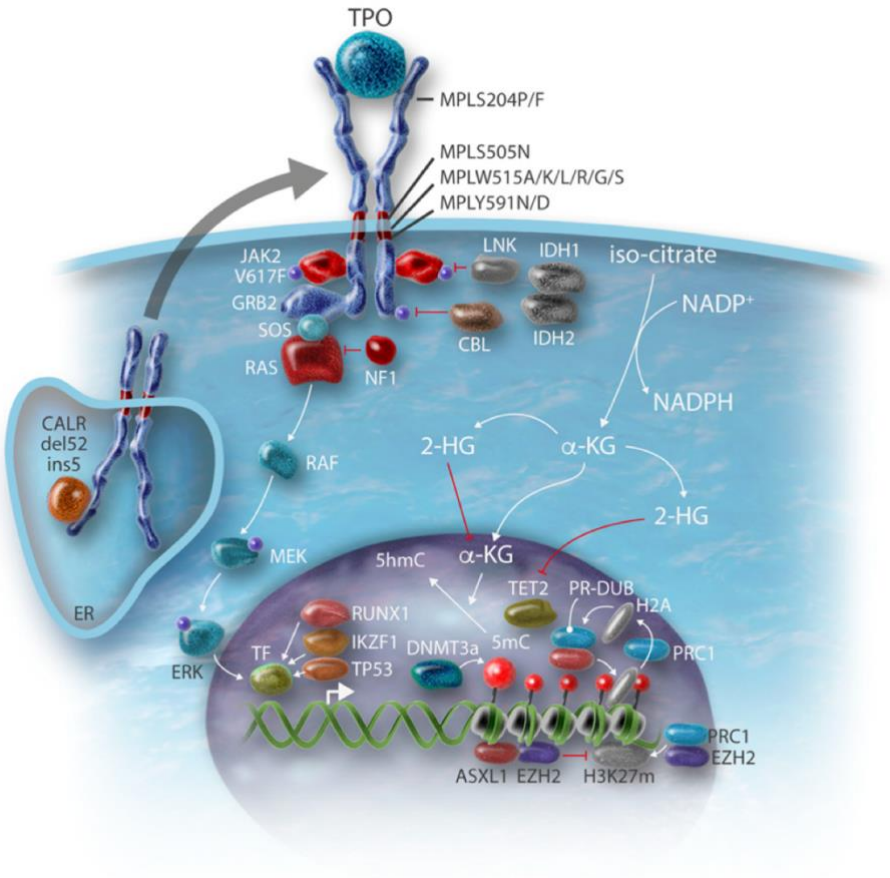


Figure 1.10 – Epigenetic regulators.
Some of these genes are involved in leukemic transformation (ex: *NRAS, TP53, RUNX1*) (Adapted from (Vainchenker & Kralovics, 2017).

The pathogenic role of these other mutations is incompletely understood and might include cooperation with the driver mutations in defining phenotype and facilitating disease progression (Tefferi, 2016; Vainchenker & Kralovics, 2017).

Usually, they are detected in patients with a very different phenotype and disease evolution profile, with variable frequencies (Figure 1.3), appearing that their presence alone is not sufficient to

cause, nor explain the clinical presentation and complications observed in those PN-MPNs patients (Hermouet, Bigot-Corbel, & Gardie, 2015).

Epimutations occurrence may chronologically precede or follow the acquisition of driver mutations. Identification of this great number of mutations in PN-MPNs patients suggests that they are primarily background mutations, several of them involved in the same pathways and in the same "stage" of the disease (Table 1.2). On the other hand, many of them are sequentially acquired in cells that already bear a mutation in one of the phenotypic driver genes, most frequently *JAK2* V617F, and are thought to be involved in disease progression (Skoda et al., 2015; Spivak, 2017; Vainchenker & Kralovics, 2017).

Table 1.2 – Frequencies of gene mutations co-occurrence in the chronic phase of PN-MPNs, according to phenotype and driver mutation.
(Adapted from (Spivak, 2017; Vainchenker & Kralovics, 2017).

Gene mutation	Polycythemia vera			Essential thrombocythemia				Primary myelofibrosis			
	JAK2 V617F	*JAK2* exon 12	*CALR*	*JAK2* V617F	*CALR*	*MPL*	Triple negative	*JAK2* V617F	*CALR*	*MPL*	Triple negative
DNA methylation											
TET2	++/+++	NA	NA	++	NA	NA	NA	+++	+	+	?
DNMT3A	++	NA	NA	++	NA	+	NA	++	-	NA	-
IDH1/2	+	NA	NA	-/+	NA	NA	NA	+	+/++	-	++
Histone methylation											
ASXL1	+/++	NA	NA	+	NA	+	NA	+++/ ++++	+++/ ++++	++++	++++
EZH2	+	NA	NA	-/+	+	NA	NA	+/++	++	+++	++
SUZ12	+	NA	NA	-	NA	NA	NA	+	NA	NA	-
Spliceosome											
U2AF1	-	NA	NA	-	NA	NA	NA	+++/ ++++	-/+	++++	++
SRSF2	-	NA	NA	-	NA	NA	NA	+++	+	+++	++++
SF3B1	++/+++	NA	NA	-	NA	NA	NA	+/++	-/+	++	+++
ZRSR2	-	NA	NA	-	NA	NA	NA	+	NA	NA	-
Tumor suppressors											
TP53	++	NA	NA	++	NA	NA	NA	++	NA	NA	-
Transcription factors											
NF-E2	+	NA	NA	?	NA	NA	NA	?	?	?	NA
Activated signaling											
NRAS/KRAS	+	NA	NA	-	NA	NA	NA	++	NA	NA	-
CBL	+	NA	NA	+	NA	NA	NA	++	NA	NA	?
NF1	+	NA	NA	-	NA	NA	NA	+	NA	NA	-
SH2B3(LNK)	?	NA	NA	?	NA	NA	?	?	NA	NA	NA

-: 0%; +: <5%; ++: 5-9%; +++: 10-19%; ++++: ≥20%; NA: not available; ?: Unknown

Some mutations are involved in early phase of disease initiation MPNs (*TET2, DNMT3A, IDH1/2, ASXL1, EZH2*), in chronic phase (*TET2, DNMT3A, IDH1/2, ASXL1, EZH2, U2AF1, SRSF2, SF3B1*) and some in disease progression (*TET2*, *IDH1/2, EZH2, ASXL1, SRSF2, LNK*; progression to leukemia – *CBL, NRAS, NF1, FLT3, TP53, RUNX1*) (Table 1.2 and Figure 1.3) (McPherson et al., 2017; Spivak, 2017; Vainchenker & Kralovics, 2017). Other mutations may be associated with phenotypic change (ex: *SF3B1* and *U2AF1* related to anemia) (Vainchenker & Kralovics, 2017).

In addition, some of these mutations are not mutually exclusive, making the hierarchy complex and unpredictable. This makes it more difficult to predict MPN patient outcomes at a single time-point (McPherson et al., 2017).

Ten-Eleven-Translocation2 (***TET2***) mutations have been implicated in several myeloid disorders (Figures 1.3 and 1.10), consisting in a group of frameshift, nonsense, and missense mutations that may induce DNA hypermethylation, resulting in gene silencing and impaired myelopoiesis (Tefferi & Pardanani, 2011). The role of *TET2* mutations in disease progression varies according to disease, appearing not to be a prognostic indicator in PN-MPNs in general (Jankowska et al., 2009; Tefferi et al., 2009).

Represented in Figure 1.10, additional mutations in other epigenetic modifiers are Sex Comb-Like1 (***ASXL 1***), Isocitrate Dehydrogenase 1/2 (***IDH1/IDH2***) and Enhancer of Zeste Homolog 2 (***EZH2***) and DNA cytosine methyltransferase 3a (***DNMT3A***) mutations, which influence the regulation of transcription involving DNA and histone modifications. These mutations are predominantly found in PMF patients and blast phase MPNs patients (Figure 1.3), suggesting that they occur late in disease progression or collaborate with mutations in other genes, and are associated with poor survival in PN-MPNs patients.

By a higher frequency of some mutations in myelofibrosis patients (PMF and post-ET/PV MF) they might predict disease progression to myelofibrosis in PV and ET patients (ex: *ASXL1*). Other mutations are found in higher frequency in AML patients preceded by a MPN (*LNK*, *DNMT3A*) (Koopmans et al., 2015). *IDH1* and *IDH2* mutations are associated with higher risk of *de novo* leukemia (Guglielmelli et al., 2014).

In PMF, several mutations were also associated with shortened leukemia-free survival, namely *ASXL1*, *SRSF2*, *EZH2*, *IDH1/2*, and *U2AF1*. *CALR* mutations favorably affected survival, independently of both number of mutations and IPSS/DIPSS-plus. The 'number' of detrimental mutations provides added value in the combined molecular and clinical prognostication of PMF (Guglielmelli et al., 2014).

In addition, *TP53*, *IDH2*, *SRSF2*, and *SH2B3* mutations have been reported to be over-represented in blast phase MPNs, suggesting their relevance in disease progression. Most noteworthy in this regard is *TP53* loss, which is believed to make *JAK2*-mutated patients vulnerable to leukemic transformation (Tefferi, 2016).

They are also frequently found in other hematologic malignancies, such as MDS and *de novo* acute leukemia (Figure 1.3) (Skoda et al., 2015). An example are the **LNK mutations**, also found in other myeloid malignancies and therefore not specific for PN-MPNs (low frequency, but 10% in leukemic transformation), that result in neutralization of the inhibition of *JAK*2 V617F and *MPL* activity induced by wildtype *LNK* via the TPO-R (Koopmans et al., 2015).

According to WHO classification criteria, in the absence of any of the three driver mutations, the patients should be tested for the presence of the most frequent accompanying mutations, in order to establish clonality (Arber et al., 2016; Rumi & Cazzola, 2017).

The majority of MPN patients do not have **cytogenetic abnormalities** and cytogenetic

assessment is not part of routine work-up. Cytogenetic abnormalities are found in 43%, 15%, and 7% of patients with PMF, PV and ET, respectively (Gangat et al., 2008; Gangat et al., 2009; Hussein et al., 2009; Tefferi & Vannucchi, 2017). The most recurrent is gain of part or all of chromosome 9 in PV, and this is associated with a copy number gain of *JAK2* V617F mutation. Gain of chromosome 8, partial trisomy for 1q, and interstitial deletions of 13q and 20q have also been recorded in all MPNs subtypes. Many of these are seen in other hematological malignancies, most frequently in MDS, but abnormalities +9 and 13q are relatively specific to MPNs (Tefferi, 2012).

Acquired UPD 1p, 4q 7q, 9p, and 11q is usually associated with homozygosity for mutations in *MPL, TET2, EZH2, JAK2* and *CBL*, respectively (Jones & Cross, 2013).

Prognostic significance has been described for cytogenetic abnormalities in PMF, but not in PV or ET patients (Bittencourt et al., 2012; Guglielmelli et al., 2014; Hussein et al., 2009).

Genetic variation across the genome and **single nucleotide polymorphisms (SNPs)**, including *JAK2, CALR, MPL* and other genes, have also been investigated, through genomewide association studies (GWAS) for their association with disease susceptibility, phenotype definition and disease progression, additionally to sex and age (Batar, Guven, Baris, Celkan, & Yildiz, 2009; Bolufer et al., 2006; Bănescu et al., 2014; da Silva Silveira et al., 2009; Hernández-Boluda et al., 2012; Jiang, Zhang, Yang, & Wang, 2009; Jones & Cross, 2013; Joshi, Korgaonkar, Shanmukhaiah, & Vundinti, 2015; Pakakasama et al., 2007; Pardanani, Fridley, Lasho, Gilliland, & Tefferi, 2008; Seedhouse et al., 2002). A predisposing haplotype (46/1) in the *JAK2* locus has been described (Jones et al., 2009; Olcaydu et al., 2009), Another example is a single-nucleotide variant in *TERT*, which is associated with all three PN-MPNs, most significantly with their familial forms, having an additive effect on susceptibility to these disorders and in predisposition to the co-occurrence of solid tumors in these patients (Spivak, 2017).

On the other hand, these genetic predispositions may explain the co-occurrence of stem-cell clones harboring *JAK2*, *MPL*, or *CALR* mutations in the same person (Spivak, 2017).

In addition to the above mentioned genetic changes, familial aggregation of MPNs has been described with a 5-7 fold increased risk in first-degree relatives (Landgren et al., 2008). In a study developed by Landgren *et al*, no anticipation was seen; however, Rumi *et al* have observed a lower median age in second-generation compared to first-generation MPNs patients in their family studies (Rumi et al., 2007; Rumi et al., 2008). More recently, a number of germline *JAK2* or *MPL* mutations associated with familial syndromes characterized by a thrombocytosis phenotype have been identified (Skoda et al., 2015; Spivak, 2017).

In this dissertation (Chapters 7, 8 and 9) will be presented the results obtained from the application of case-control studies to a Portuguese population of PN-MPNs, for the investigation of caspases and base excision repair pathway polymorphisms association with genetic susceptibility for disease and their impact on disease progression in patients under treatment.

Besides these molecular aberrations, other mechanisms are believed to be involved in the pathogenic mechanism initiating PN-MPNs disease. Bone marrow mutant and non-mutant cells,

including stem cells, megakaryocytes and myeloid cells, as well as nonhematopoietic cells such as mesenchymal stromal cells, can produce aberrant **cytokines**, activating signaling pathways. Moreover, STAT3 and NF-κβ signaling have been found to regulate cytokine transcription in other diseases. This is the reason why unmutated JAK patients are also capable of clinically respond on JAK inhibitory therapy, since JAK inhibitors and also STAT3 deletion can modulate cytokine expression (Koopmans et al., 2015; Vainchenker & Kralovics, 2017).

On the other hand, the inflammatory cytokines might also be involved in extramedullary hematopoiesis by favoring colonization of *JAK2* V617F hematopoietic stem cells and progenitors in the spleen (Vainchenker & Kralovics, 2017).

1.1.2 – DIAGNOSIS, PROGNOSIS AND PREDICTIVE FACTORS

PN-MPNs DIAGNOSIS

Although defined as distinct disorders, PN-MPNs share some phenotypic characteristics among themselves, as well as with other myeloid neoplasms and even benign hematopoietic disorders. In addition to phenotypic mimicry, each type of PN-MPNs is capable of evolve into another type and transform to an aggressive form of bone marrow failure or acute leukemia, which makes diagnosis, risk assessment, and therapeutic decisions very difficult (Spivak, 2017).

Current diagnosis is based on WHO-criteria and, since the earlier classifications, involves a composite assessment of clinical manifestations, laboratory abnormalities, peripheral blood smear characteristics, bone marrow morphology and molecular and cytogenetic features (Table 1.3) (Arber et al., 2016; Swerdlow et al., 2008).

In the most recent revision (Arber et al., 2016), WHO classification was modified to incorporate the recent advances in the molecular characterization of these diseases, in which these clonal markers are considered as one of several major criteria in the diagnosis of PV, ET and PMF. Proof of clonality and diagnostic and prognosis determination were provided by the discovery of novel molecular findings, such as *CALR* mutation, in addition to *JAK*2 and *MPL* mutations (Arber et al., 2016). Driver mutations were effectively integrated into the WHO diagnostic criteria (Table 1.3) used to distinguish MPNs from secondary/reactive erythrocytosis or thrombocytosis. Additional differential diagnosis includes chronic myeloid leukemia, chronic neutrophilic leukemia, chronic myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia (Arber et al., 2016).

Globally, the establishment of the molecular mutational profiles of MPNs patients is of major importance in the classification of MPNs subtypes and determination of prognosis, influencing therapeutic decisions (Skoda et al., 2015). Figure 1.11 represents a practical algorithm for the diagnosis

of PN-MPNs, specially based on the screening for the three driver mutations, *JAK2*, *CALR*, and *MPL* (Langabeer et al., 2015).

Current WHO guidelines do not recommend further testing once *JAK2* V617F mutation is detected in PN-MPNs. However, Nussenzveig *et al*. have recently published data that indicate that quantification of *JAK2* V617F allele burden may be clinically relevant in PN-MPNs and that in those with low allelic burden additional testing for *JAK2* exon-12 and *MPL* exon-10 mutation should be pursued (Nussenzveig et al., 2016).

Table 1.3 – WHO classification criteria for PN-MPNs diagnosis.

	WHO 2008 (Swerdlow et al., 2008)	**WHO 2016 (Arber et al., 2016)**
Polycythemia vera	**Both major criteria and one minor criterion or the presence of the first major criterion together with two minor criteria**	**All three major criteria, or the first two major criteria and the minor criterion***
	Major criteria 1. Hemoglobin > 18.5 g/dL in men, 16.5 g/dL in women or other evidence of increased red cell volume 2. Presence of *JAK2* V617F or other functionally similar mutation such as *JAK2* exon 12 mutation	**Major criteria** 1. Hemoglobin > 16.5 g/dL in men, 16.0 g/dL in women or, hematocrit > 49% in men, > 48% in women or, increased red cell mass (RCM, > 25% above mean normal predicted value) 2. BM biopsy showing hypercellularity for age with trilineage growth (panmyelosis) including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size) 3. Presence of *JAK2* V617F or *JAK2* exon 12 mutation
	Minor criteria 1. BM biopsy showing hypercellularity for age with trilineage growth (panmyelosis) with prominent erythroid, granulocytic and megakaryocytic proliferation 2. Serum erythropoietin level below the reference range for normal 3. Endogenous erythroid colony formation in vitro	**Minor criteria** 1. Subnormal serum erythropoietin level
Essential thrombocythemia	**All four criteria**	**All four major criteria or the first three major criteria and the minor criterion**
	1. Sustained platelet count ≥ 450x10^9/L 2. BM biopsy specimen showing proliferation mainly of the megakaryocytic lineage with increased number of enlarged, mature megakaryocytes. No significant increase or left-shift of neuthrophil granulopoiesis or erythropoiesis 3. Not meeting WHO criteria of Polycythemia vera, primary myelofibrosis, *BCR-ABL1* positive chronic myelogenous leukemia or myelodysplastic syndrome or other myeloid neoplasm 4. Demonstration of *JAK2* V617F or other clonal marker, or in the absence of *JAK2* V617F, no evidence for reactive thrombosis	**Major criteria** 1. Platelet count ≥ 450x10^9/L 2. BM biopsy showing proliferation mainly in the megakaryocyte lineage with increased numbers of enlarged mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in neuthrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers 3. Not meeting WHO criteria for *BCR-ABL*1$^+$ CML, PV, PMF, myelodysplastic syndromes, or other myeloid neoplasms 4. Presence of *JAK2*, *CALR*, or *MPL* mutation
		Minor criteria 1. Presence of a clonal marker or absence of evidence for reactive thrombocytosis

	All three major criteria and two minor criteria	All three major criteria, and at least one minor criterion, confirmed in two consecutive determinations**
Primary myelofibrosis	**Major criteria** 1. Presence of megakaryocytic proliferation and atypia, usually accompanied by either reticulin and/or collagen fibrosis, or in the absence of significant reticulin fibrosis, the megakaryocyte changes must be accompanied by an increased bone marrow cellularity, characterized by granulocytic proliferation and often decreased erythropoiesis (i.e. prefibrotic cellular-phase disease) 2. Not meeting WHO criteria for polycythemia vera, *BCR-ABL*1$^+$ CML, myelodysplastic syndrome, or other myeloid neoplasms 3. Demonstration of *JAK2* V617F or other clonal marker (e.g. *MPL* W515K/L), or in the absence of a clonal marker, no evidence that the bone marrow fibrosis or the changes are secondary to infection, autoimmune disorder or other chronic inflammatory condition, hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy, or toxic (chronic) myelopathies	**Major criteria** ***Prefibrotic PMF*** 1. Presence of megakaryocytic proliferation and atypia, without reticulin fibrosis > grades 1, accompanied by increased age-adjusted bone marrow cellularity, granulocytic proliferation, and often decreased erythropoiesis 2. Not meeting WHO criteria for ET, PV, *BCR-ABL*1$^+$ CML, myelodysplastic syndromes, or other myeloid neoplasms 3. Presence of *JAK2*, *CALR*, or *MPL* mutation or in the absence of these mutations, presence of another clonal marker, or absence of minor reactive BM reticulin fibrosis (minor reticulin fibrosis secondary to infection, autoimmune disorder, or other chronic inflammatory conditions, hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy, or toxic myelopathies) ***Overt PMF*** 1. Presence of megakaryocytic proliferation and atypia, accompanied by either reticulin and/or collagen fibrosis grades 2 or 3 2. Not meeting WHO criteria for ET, PV, *BCR-ABL*1$^+$ CML, myelodysplastic syndromes, or other myeloid neoplasms 4. Presence of *JAK2*, *CALR*, or *MPL* mutation or in the absence of these mutations, presence of another clonal marker, or absence of minor reactive BM reticulin fibrosis (minor reticulin fibrosis secondary to infection, autoimmune disorder, or other chronic inflammatory conditions, hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy, or toxic myelopathies)
	Minor criteria 1. Leukoerythroblastosis 2. Increase in serum lactate dehydrogenase level 3. Anemia **4.** Splenomegaly	**Minor criteria** ***Prefibrotic PMF*** 1. Anemia not attributed to a comorbid condition 2. Leucocytosis (WBC count ≥ 11x10^9/L) 3. Palpable splenomegaly 4. LDH increased to above upper normal limit of institutional reference range ***Overt PMF*** 1. Anemia not attributed to a comorbid condition 2. Leucocytosis (WBC count ≥ 11x10^9/L) 3. Palpable splenomegaly 4. LDH increased to above upper normal limit of institutional reference range 5. Leukoerythroblastosis

* PV: Criterion number 2 (BM biopsy) may not be required in cases with sustained absolute erythrocytosis: hemoglobin levels >18.5 g/dL in men (hematocrit, 55.5%) or >16.5 g/dL in women (hematocrit, 49.5%) if major criterion 3 and the minor criterion are present. However, initial myelofibrosis (present in up to 20% of patients) can only be detected by performing a BM biopsy; this finding may predict a more rapid progression to overt myelofibrosis (post-PV myelofibrosis).
** PMF: In the absence of any of the 3 major clonal mutations, the search for the most frequent accompanying mutations (ex: ASXL1, EZH2, TET2, IDH1/IDH2, SRSF2, SF3B1) are of help in determining the clonal nature of the disease.
See Table 1.5 for myelofibrosis grading definition; BM – Bone marrow.

Establishing PN-MPNs diagnosis is of immediate clinical importance with selection, implementation and the continual evaluation of the appropriate laboratory methodology to achieve this goal, whilst many of these mutations appear to have also prognostic implications. The advantages and

limitations of these approaches in identifying and quantitating the common PN-MPNs-associated mutations are considered with particular regard to their clinical utility.

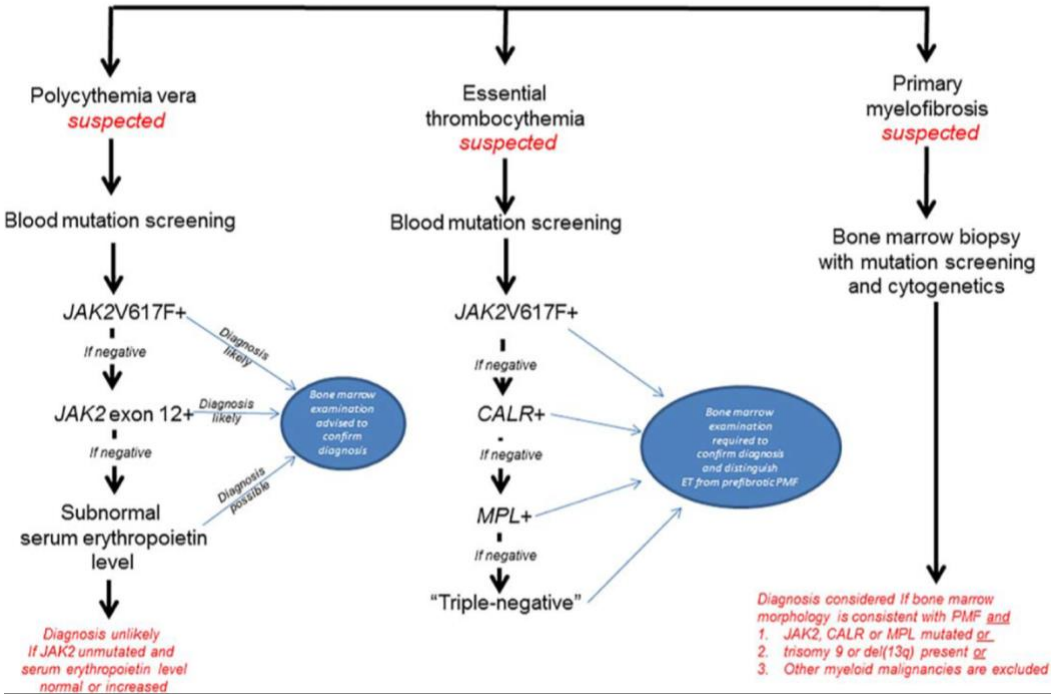


Figure 1.11 – Practical workflow for the diagnosis of PN-MPNs.
(Adapted from (Tefferi & Barbui, 2017).

The evolution of molecular diagnostic applications and platforms has occurred in parallel with the discovery of PN-MPNs-associated mutations. Quantitative , real-time, allele specific PCR (AS-qPCR) assays have been widely adopted in the study of *JAK2* V617F mutation and other associated mutations, including quantification of mutant allele burden (Langabeer et al., 2015). Probably, emerging technologies such as next-generation sequencing (NGS) and digital PCR will in the future play an increasing role and improve the molecular diagnosis of PN-MPNs (Langabeer et al., 2015). Most recently, whole exome sequencing revealed the presence of somatic or germline *MPL* or *JAK2* variants in 3 of 8 triple-negative patients with MPNs and subsequent *MPL/JAK2* entire codon sequencing among 62 ET and 49 PMF triple-negative cases revealed novel somatic or germline *MPL* variants in 5 of 62 cases and *JAK2* variants in 5 of 57 cases (Tefferi, 2016).

In routine practice, clonality is usually determined by the presence of an acquired mutation or cytogenetic abnormality, however additional clinical, laboratory and morphological information play an important role in the diagnosis and distinction of each specific subtype (Cross, 2011; Duletić et al., 2012; Tefferi & Pardanani, 2015).

An accurate boné marrow cytological/histological exam is fundamental for a well defined diagnosis and prognosis prediction in PN-MPNs, highlighting the importance of establishing

standardized morphologic criteria in this group of disorders, enhancing interobserver reproducibility and consensus of morphologic diagnosis, which was reported to range from 76% to 88% among several studies performed (Arber et al., 2016).

The inclusion of bone marrow morphology as a major criterion for the 2016 WHO classification (Table 1.3) has allowed lowering of the threshold hemoglobin level for the diagnosis of PV from 18.5 g/dL in men and 16.5 g/dL in women to a hemoglobin level of 16.5 g/dL in men and 16 g/dL for women (or a hematocrit level of 49% in men and 48% in women), reducing the risk of underdiagnose of PV patients when using the previous WHO 2008 classification hemoglobin levels (Arber et al., 2016). Hematocrit and qualitative *JAK2* V617F mutation allowed a reliable diagnosis of PV, but the incorporation of EPO and/or *JAK2* V617F mutant load did not improve the diagnostic accuracy (Ancochea et al., 2014). On the other hand, histologic evidence of the absence of reticulin fibrosis at disease onset, along with other features, allowed to differentiate between the patients with ET and those with masked PV or prefibrotic/early PMF, having prognostic implications (Tefferi & Pardanani, 2015). The minor clinical criteria in prefibrotic PMF were also well defined, having a major impact on accurate diagnosis and prognosis (Table 1.3) (Arber et al., 2016).

Characterization and classification of PN-MPNs is based on which myeloid cell lineage (erythrocytic, granulocytic or megakaryocytic) is predominantly expanded in the peripheral blood, depending on the type of driver and additional mutations acquired , resulting in very different phenotypic readouts (Figure 1.12) (Tefferi, 2016). As mencioned above, regarding the influence that the order of mutation acquisition might have in clinical phenotype definition, the opinions are divergent (Spivak, 2017; Vainchenker & Kralovics, 2017).

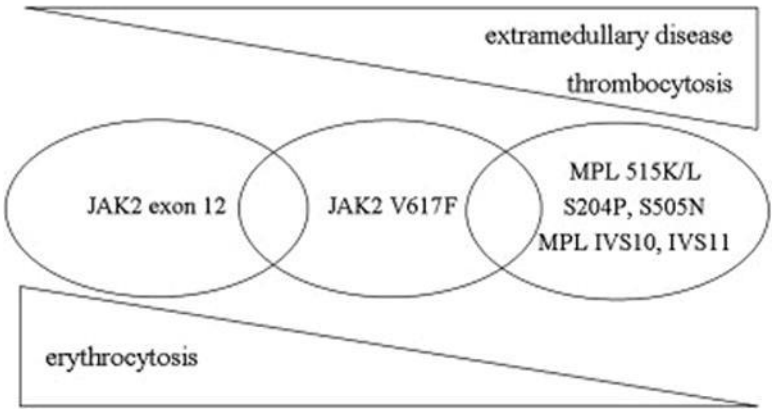


Figure 1.12 – Spectrum of PN-MPNs phenotypes associated with *JAK2* and *MPL* lesions.
(Adapted from (Williams, Kim, Rogers, Spivak, & Moliterno, 2007).

Table 1.4 is a summary of most prominent clinical, morphological and molecular features of each one of classical PN-MPNs.

Table 1.4 – Classical PN-MPNs clinical, morphological and molecular features.
(Adapted from (Rumi & Cazzola, 2017).

	Clinical and morphological features	Molecular features
Polycythemia vera	Erythrocytosis frequently combined with thrombocytosis and/or leukocytosis (that is, polycythemia) and typically associated with suppressed endogenous erythropoietin production. ***Bone marrow:*** hypercellularity for age with trilineage growth (panmyelosis)	• *JAK2* V617F in about 96% of patients • *JAK2* exon 12 mutations in about 4% of patient (isolated erythrocytosis in most of these patients) • Patients with wild-type *JAK2* extremely rare, if any
Essential thrombocythemia	Thrombocytosis ***Bone marrow:*** normocellular bone marrow with proliferation of enlarged megakaryocytes	• *JAK2* V617F in 60-65% of patients • *CALR* exon 9 indels in 20-25% of patients • *MPL* exon 10 mutations* in about 4-5% of patients • Noncanonical *MPL* mutations* in 1% of patients • About 10% of patients do not carry any of the above somatic mutations (the so-called triple-negative cases)
Primary myelofibrosis	**Prefibrotic PMF** • Various abnormalities of peripheral blood • ***Bone marrow:*** granulocytic and megakaryocytic proliferation, with lack of reticulin fibrosis **Overt PMF** • Various abnormalities of peripheral blood • ***Bone marrow:*** megakaryocytic proliferation with atypia, accompanied by either reticulin and/or collagen fibrosis grades 2/3. • Abnormal stem cell trafficking with myeloid metaplasia (extramedullary hematopoiesis in the liver and/ or the spleen)	• *JAK2* V617F in 60-65% of patients • *CALR* exon 9 indels in 25-30% of patients • *MPL* exon 10 mutations* in about 4-5% of patients • Noncanonical *MPL* mutations* in 1% of patients • About 5-10% of patients do not carry any of the above somatic mutations (the so-called triple-negative cases)

*Canonical *MPL* exon 10 mutations include W515L/K/A/R, S505N/C, and V501A (transmembrane domain of MPL); noncanonical *MPL* mutations (outside exon 10) include T119I, S204F/P, E230G, Y252H (extracellular domain) and Y591D/N (intracellular domain) AML, acute myeloid leukemia.

Although these diseases are defined as distinct entities, they have similar biological features, as already mentioned. They share a common stem cell-derived clonal origin, arising from a primitive bone marrow progenitor cell, with myeloproliferation leading to an excess of abnormal cells in peripheral blood, and together with extramedullary hematopoiesis often causing splenomegaly and hepatomegaly (Figure 1.13) (Passamonti et al., 2016).

The major presenting symptoms of PN-MPNs are related to hypertension or other cardiovascular abnormalities, however approximately half of all PN-MPNs patients are reported to be asymptomatic at diagnosis (Jones & Cross, 2013).

The most common PN-MPN, **PV**, is the ultimate phenotypic consequence of *JAK2* gene mutations and, in rare cases, *CALR* or *LNK* mutations. It is clinically characterized by excessive production of erythrocytes, independent of the mechanisms that normally regulate erythropoiesis (with subnormal EPO levels), granulocytes and megakaryocytes as well, increased red cell mass (increased hemoglobin or hematocrit) and extramedullary hematopoiesis, leading to splenomegaly, and thrombohemorrhagic complications (Figure 1.13 and Table 1.4) (Arber et al., 2016; Jones & Cross, 2013; Rumi & Cazzola, 2017; Swerdlow et al., 2008; Tefferi & Vardiman, 2008). PV symptoms include

headache, dizziness, paraesthesias, visual disturbance, pruritus (due to activated basophils), gouty arthritis, and when patients become overtly polycythemic they can have plethora and enlarged spleen and liver (Swerdlow et al., 2008). In about 20% of cases it is reported a venous or arterial thrombotic event, such as deep vein thrombosis, myocardial ischemia or stroke, which could be the first symptom. As it will be discussed later, in some patients it occurs transformation to bone marrow failure, myelofibrosis, and acute leukemia (Spivak, 2017).

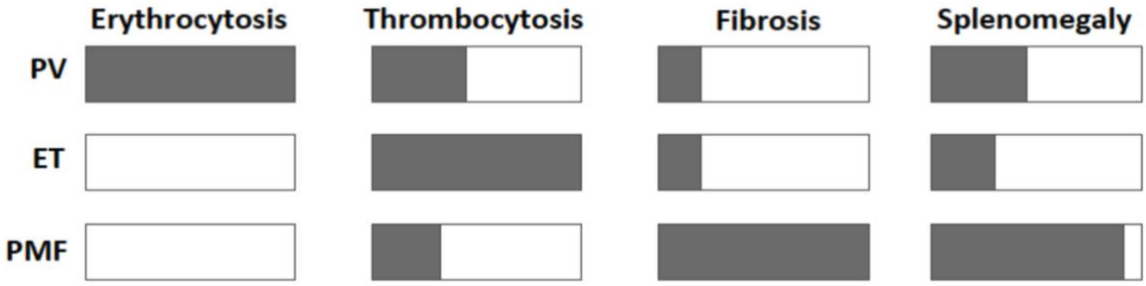


Figure 1.13 – Clinical presentation in classic PN-MPNs.
Clinical features are shared over the spectrum of PN-MPNs. Grey bars represent the proportion of specific found overlapping in ET, PV and PMF. Leukocytosis often present in PV and PMF patients and cytopenia of some PMF patients is not represented (Adapted from (Kiladjian et al., 2012).

PV can be divided in a prodromal pre-polycythemic phase (with a borderline or mild erythrocytosis associated with thrombocytosis, mimetizing ET, and low EPO levels), followed by a polycythemic phase (with significant erythrocytosis, palpable splenomegaly in nearly 70% and hepatomegaly in 40% of cases) and a post-polycythemic myelofibrosis phase (post-PV MF; with cytopenias, including anemia associated with ineffective hematopoiesis, medullary fibrosis, extramedullary hematopoiesis and hypersplenism). Blood count show increase of hemoglobin, normal erythrocytes and hematocrit levels, but microcytosis and hypochromia due to iron deficiency may occur with lower hemoglobin and hematocrit values. Leukocytosis with neuthrophilia is frequent (up to $20x10^9$/L), with absolute neutrophilia in about 2/3 of patients and frequent basophilia (<3%). Occasional immature granulocytes may be present in the overt polycythemic phase, but blasts are not usually observed. Thrombocytosis is also frequent, occurring in about 50% of patients at the time of diagnosis, with more than $1000x10^9$/L in 10% of cases (Swerdlow et al., 2008).

Both in the pre-polycythemic and polycythemic phases of PV, the major features observed in the peripheral blood and bone marrow result from the effective proliferation of erythroid, granulocytic and megakaryocytic lineages (panmyelosis) (Table 1.4). Bone marrow usually reveal accentuated hypercellularity, especially in the subcortical space (an area which is normally hypocellular), with hyperplasia of the three hematopoietic strains (particularly the erythroid and megakaryocytic), although less prominent in the pre-polycythemic than in the overt polycythemic phase. Erythropoiesis and

granulopoiesis are morphologically normal, but megakaryocytic morphology is abnormal, with megakaryocytes evidencing a significant pleomorphism, varying from small and hypolobate to large and hyperlobulated, with increased mean size and lobulation (Figure 1.14). Megakaryocytes tend to form loose clusters or to lie close to bone trabeculae, and reticulin fibre network is normal in about 80% of patients. Generally, iron deposits are absent.

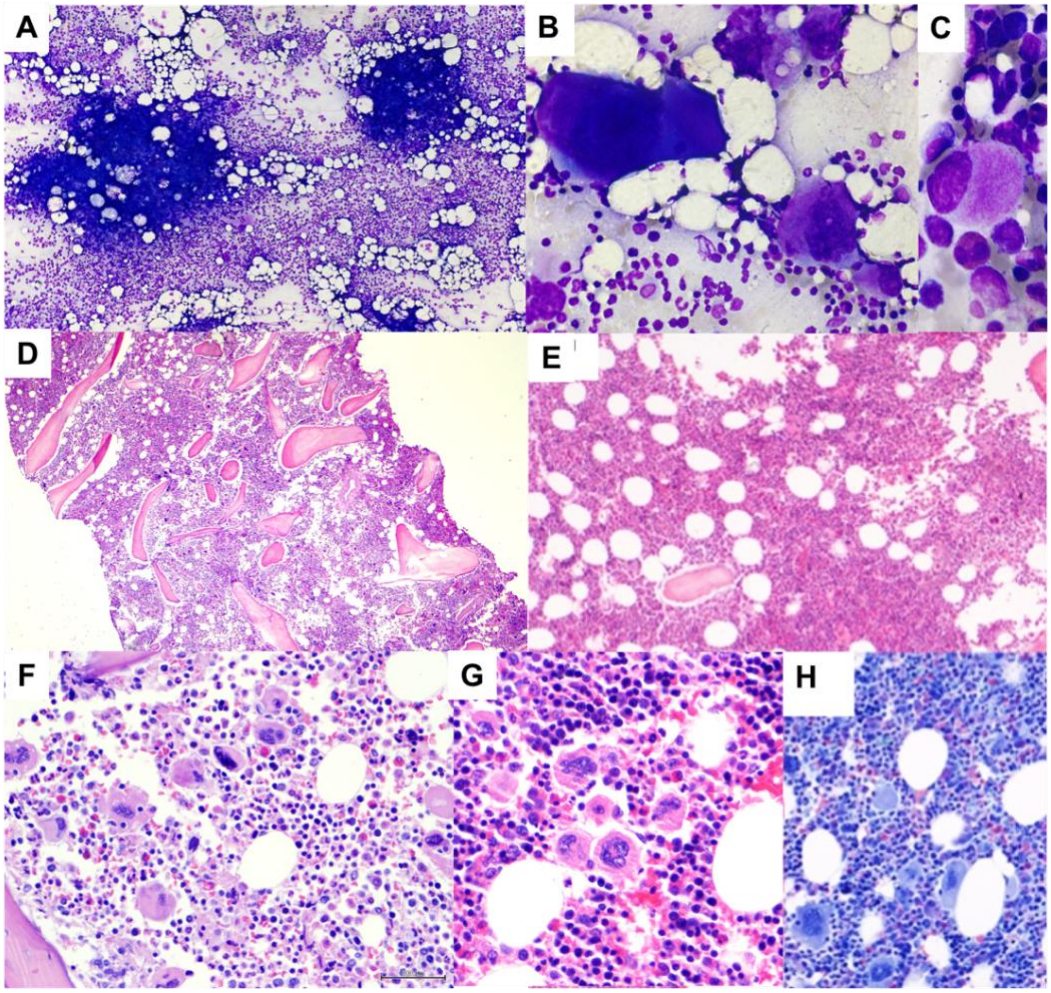


Figure 1.14 – Bone marrow morphologic and histologic aspects from a PV patient.
Bone marrow aspirate **A)** to **C)** and biopsy **D)** to **H)** showing trilinear hyperplasia and pleomorphic mature megakaryocytes.
A) x10, **B)** x40 and **C)** x100, May-Grünwald-Giemsa stain; **D)** x40, **E)** x100, **F)** x200 and **G)** x200, Hematoxylin eosin stain; **H)** x200, Giemsa stain.
Bone marrow aspirate slides were obtained and scored by the author; biopsy images were courtesy of Pathology Department of CHLO.

PV differential diagnosis includes other PN-MPNs (ET, pre-fibrotic PMF), relative hemoconcentration, secondary polycythemia to tissue hypoxia (ex: heart or lung disease, hemoglobinopathies with increased affinity for oxygen, carbon monoxide intoxication, acute smoking

habits) and polycythemia due to non-physiological EPO production (ex: anabolics, tumors, benign nephropathies).

ET is caused by *JAK2* V617F, *CALR*, or *MPL* mutations and infrequently by germline single-nucleotide variants. It involves primarily the megakaryocytic lineage and is characterized by an excessive production of platelets (thrombocytosis), with increased numbers of large, mature megakaryocytes in the bone marrow and thrombohemorrhagic complications (Table 1.4) (Arber et al., 2016; Jones & Cross, 2013; Rumi & Cazzola, 2017; Swerdlow et al., 2008; Tefferi & Vardiman, 2008). Bone marrow and peripheral blood are the main sites of involvement, whereas the spleen is a sequestration site for platelets, but does not evidence significant extramedullary hematopoiesis (Swerdlow et al., 2008).

ET is generally an indolent disease, characterized by long symptom free intervals. More than half of the patients are asymptomatic at diagnosis and the remainder present clinical signs related to thrombotic and hemorrhagic events. Occlusion of microvasculature may lead to transient ischemic attacks, digital ischemia with paresthesias and gangrene, but major arteries and veins may also be affected, causing splenic or hepatic vein thrombosis (Swerdlow et al., 2008). Bleeding occurs most frequently at mucosal surfaces from gastrointestinal and upper respiratory tracts (Swerdlow et al., 2008). At diagnosis, mild splenomegaly is present in about 50% and hepatomegaly in 15-20% of ET patients (Swerdlow et al., 2008). Some cases evidence disease transformation to bone marrow failure, myelofibrosis, and acute leukemia. ET is a diagnosis of exclusion because the TPO-R, MPL, is the only hematopoietic growth factor receptor expressed by hematopoietic stem cells, and isolated thrombocytosis may be the first manifestation of PV or PMF (Spivak, 2017).

Peripheral blood smear shows marked thrombocytosis, platelet anisocytosis, giant platelets, sometimes hypogranulous, fragments of megakaryocytes or small nuclei of megakaryocytes (Figure 1.15). Leukocytes and differential counts are normal or borderline leukocytosis, and mild myelemia may occur (<5% of cases), with absent or minimal basophilia. Hypochromia and microcytosis due to iron deficiency may be present if there is recurrent hemorrhage, and about 50% of cases present mild splenomegaly at the time of diagnosis (Swerdlow et al., 2008).

Bone marrow is generally, normocellular or, moderately, hypercellular, megakaryocytes are present in increased numbers, and granulopoiesis and erythropoiesis are no more than slightly increased (Table 1.4). Usually there is abnormal megakaryocytic morphology, revealing megakaryocytes with increased mean size and lobulation, mainly large megakaryocytes to giants, displaying abundant, mature cytoplasm, and deeply lobulated/hyperlobated (stag-horn like), but less pleomorphic than in PV (and in the hypercellular phase of primary myelofibrosis), with no increase of small megakaryocytes number (Figures 1.15 and 1.16). Iron stores are present in 40-70% of patients at diagnosis (Swerdlow et al., 2008).

The megakaryocytes are usually distributed throughout the bone marrow, but may occur in loose clusters. The reticulin network is normal or only minimally increased (Swerdlow et al., 2008).

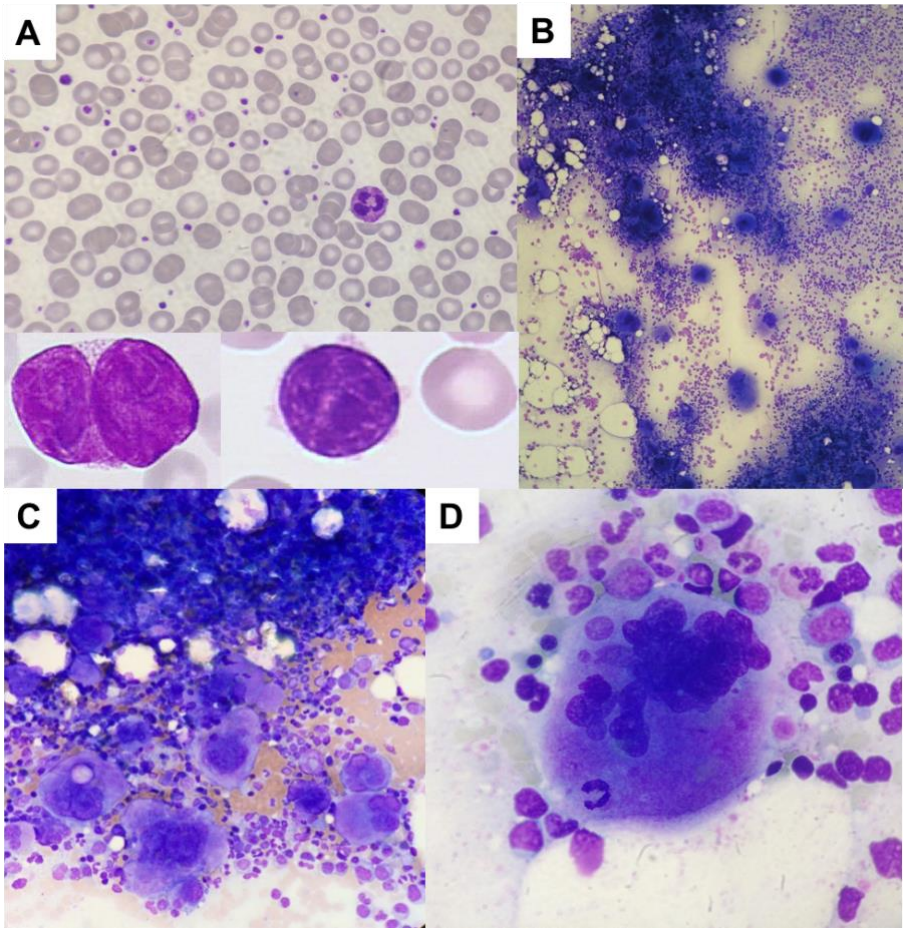


Figure 1.15 – Peripheral blood smear and bone marrow morphologic aspects from an ET patient.
A) Peripheral blood smear showing marked thrombocytosis with platelets anisocytosis and small nuclei of megakaryocytes; **B)** to **D)** Bone marrow aspirate showing megakaryocytic hyperplasia and enlarged/giant megakaryocytes with hyperlobulated nuclei, and no abnormalities of the myeloid and erythroid series.
A) x40 and image magnification, Wright stain; **B)** x10, **C)** x40 and **D)** x100, May-Grünwald-Giemsa stain.
Slides were obtained and scored by the author.

Bone marrow morphological findings are essential to distinguish ET from other conditions associated with thrombocytosis. If granulocytic and erythroid proliferation is present should prompt concern for prodromal stage of PV. When granulocytic proliferation is associated with bizarre or atypical megakaryocytes should raise consideration of pre-fibrotic stage of PMF. On the other hand, significant dyserythropoiesis or dysgranulopoiesis raises the suspicion of MDS (isolated del(5q), MDS with ring syderoblasts). Megakaryocitic morphology is essential in distinction between ET and MDS, chromosomal abnormalities and CML (Swerdlow et al., 2008). Other differential diagnosis includes reactive thrombocytosis (infectious, inflammatory, malignant neoplasms, surgery and trauma) and acute leukemia (associated with cytogenetic abnormalities involving the 3q21 and 3q26 regions).

On the other hand, bone marrow fibrosis is the hallmark of **PMF**, the least common and most aggressive MPN, resulting in a variable count of myeloid series cells, with a proliferation of predominantly megakaryocytes and granulocytes, associated with extramedullary hematopoiesis and

hepatosplenomegaly (Table 1.4) (Arber et al., 2016; Jones & Cross, 2013; Rumi & Cazzola, 2017; Swerdlow et al., 2008; Tefferi & Vardiman, 2008).

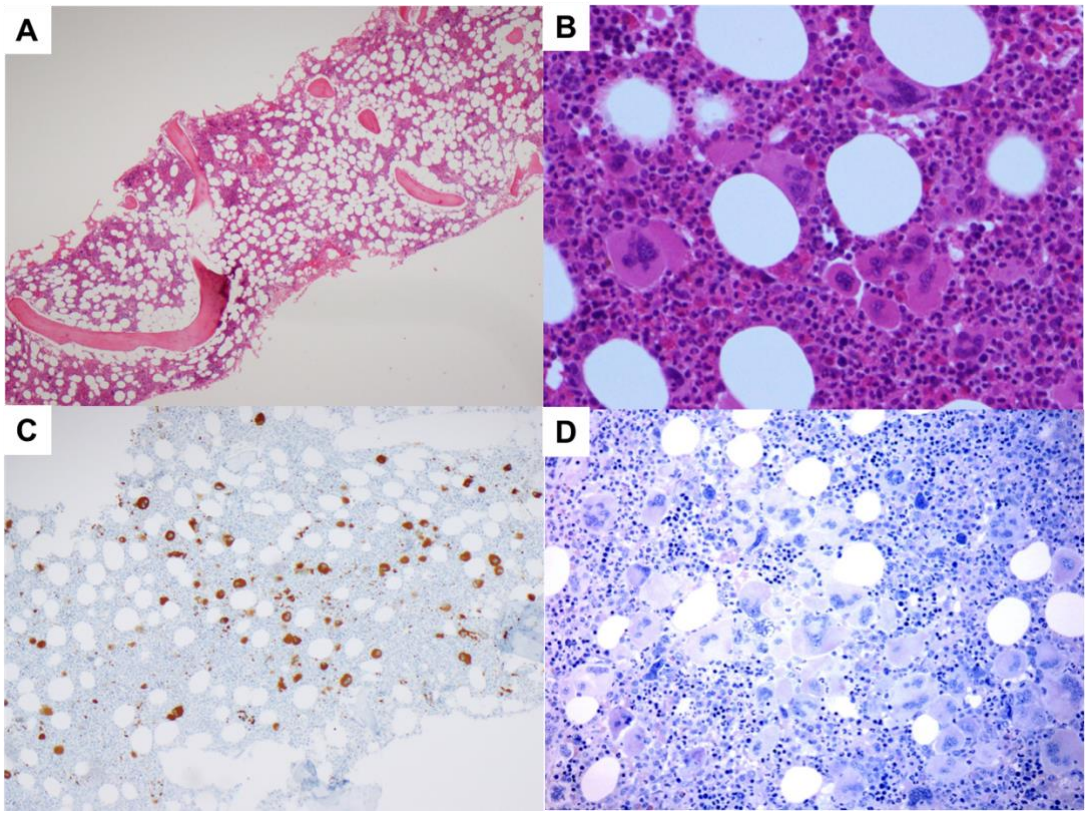


Figure 1.16 – Bone marrow histological aspects from an ET patient.
Bone marrow biopsy shows a normocellular in **A)** and a hypercellular marrow in **B)** and **D)** with megakaryocytic and granulocytic hyperplasia; **C)** immunohistochemical CD61 marker for megakaryocytes.
A) x40 and **B)** x200, Hematoxylin eosin stain; **C)** x100, Immunohistochemical CD61 marker; **D)** x200, Giemsa stain.
Images were courtesy of Pathology Department of CHLO.

There is a stepwise evolution from an early pre-fibrotic phase, in which there is an hypercellular bone marrow with absent or minimal reticulin fibrosis, followed by disease progression with accumulation of fibrous tissue, to a fibrotic phase with marked reticulin or collagen fibrosis in the bone marrow and often osteosclerosis, and transformation to acute leukemia (Swerdlow et al., 2008).

PMF morphological and clinical data vary considerably at the time of diagnosis, depending on whether the patient is in the pre-fibrotic or fibrotic stage. Up to 30-40% of patients are asymptomatic at the time of diagnosis and the disease is suspected due to splenomegaly, anemia, leukocytosis and / or thrombocytosis, or less often due to unexplained leukoerythroblastosis or an increased lactate dehydrogenase. Constitutional symptoms, due to inflammatory cytokine production, such as fatigue, dyspnea, weight loss, night sweats, low-grade fever, bleeding episodes, gouty arthritis and

hyperuricemia may be present. Splenomegaly is present nearly in 90% of patients and may be severe, whereas about 50% have hepatomegaly (Swerdlow et al., 2008).

Peripheral blood and bone marrow are always involved (Table 1.4). Blood count usually shows evidence of normocytic normochromic anemia <10 g/dL in 60% of patients, initial leukocytosis with neuthrophilia (up to 50 to $100x10^9$/L) with moderate myelemia (10 to 15%), some blasts (1 to 5%), anisopoikilocytosis with "teardrop" cells or dacrocytes and nucleated red blood cells (5 to 10/100 leukocytes) (Figure 1.17). This disease may be initially more proliferative with leukocytosis and then progressively cytopenic (ineffective granulopoiesis and splenomegaly). Platelets are present in varying numbers, from mild to moderate thrombocytopenia (in about 1/3 of patients at diagnosis) to thrombocytosis (in about 40% of patients, sometimes higher than $1000x10^9$/L, which may be the only change observed in the pre-fibrotic phase, simulating ET). Pancytopenia may occur in 10% of patients (Swerdlow et al., 2008).

Bone marrow can evidence different stages of the disease (Figure 1.17 and Tables 1.4 and 1.5). In the prefibrotic phase (fibrosis grade 0 and 1) there is normal or slightly increased reticulin, absence of collagen, and vascular proliferation, associated with hypercelularity due to increased granulopoiesis, presence of markedly atypical megakaryocytes, including with abnormal patterns of chromatin clumping, cloud-like or balloon-like nuclei, hyperchromatic and increased nude nuclei, organized in dense aggregates, some adjacent to bone trabeculae or vascular sinuses, and reduced erythropoiesis. In fibrotic phase (fibrosis grade 2 and 3) the presence of markedly increased reticulin, collagen, sometimes with osteosclerosis, results in a cellularity focally increased, normal or hypocellular, with megakaryocytes often increased, in which large dense aggregates of atypical megakaryocytes may persist, often within dilated vascular sinuses, with increased proportion of small megakaryocytes and prominence of hyperchromatic nuclei.

PMF differential diagnosis includes other MPNs (CML, ET, PV), other hematological diseases (hairy cell leukemia, some lymphomas, MDS with myelofibrosis, systemic mastocytosis), autoimmune myelofibrosis, solid tumors with spinal metastases and disseminated tuberculosis.

Irrespective of clinical diagnosis, several authors have pointed that homozygous *JAK2* V617F mutation is more symptomatic and is generally associated with older age, higher hemoglobin level and hematocrit value, leukocytosis, lower platelet count, larger spleen volume, increased risk of thrombosis (in ET) and cardiovascular events, and more frequent fibrotic transformation in both PV and ET (Tefferi, 2016; Tefferi & Vardiman, 2008; Vannucchi et al., 2007), also evidenced in Medline- and Embase-guided meta-analyses (Dahabreh et al., 2009; Lussana et al., 2009).

Compared to PV patients with *JAK2 V617F* mutation, patients with exon 12 mutations (and also in mouse models), do not usually present panmyelosis (Arber et al., 2016; Rumi & Cazzola, 2017). *JAK2* exon 12 positive patients appear to be associated with a distinct syndrome, mainly characterized by isolated marked erythrocytosis, with higher hemoglobin concentrations, lower white cells and platelets counts (minimal thrombocytosis), and isolated bone marrow erythroid hyperplasia (Godfrey et

al., 2016), independently of the mutational variant (Godfrey et al., 2016; Passamonti et al., 2011; Scott, 2011).

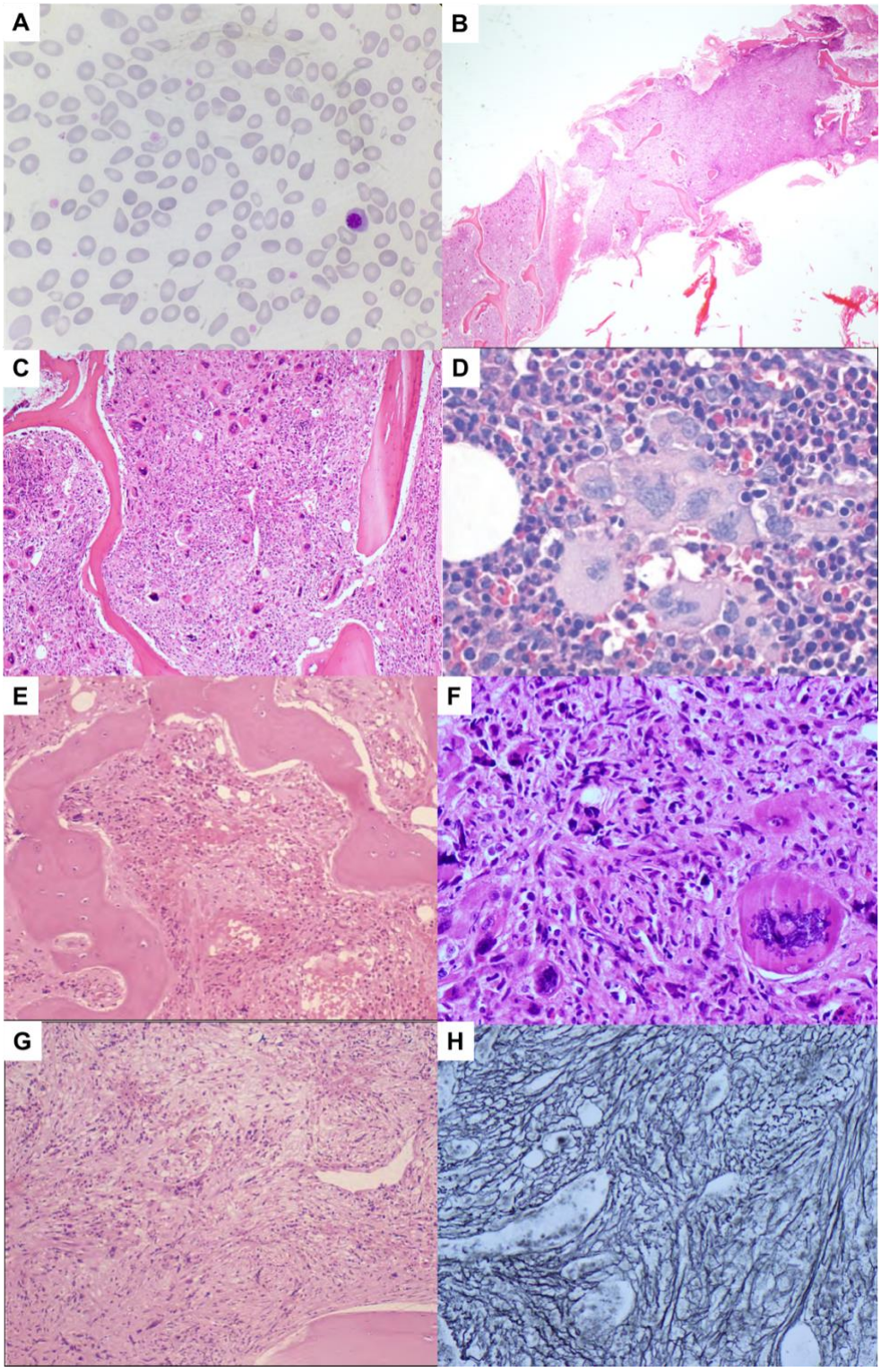


Figure 1.17 – Peripheral blood smear morphological and bone marrow histological aspects from a PMF patient.

A) Peripheral blood smear showing many "teardrop" cells or dacrocytes and a red nucleated cell; Bone marrow biopsy showing a pack marrow in **B)** and **C)**, with a group of megakaryocytes with morphological atypia in **D)** and **F)**; **E)** Irregular thickening of bone trabeculae and marked fibrosis of collagen; **G)** Fibrotic phase with marked collagen fibrosis with reduction of all hematopoietic cells; **H)** Fibrotic phase with grade 3 reticulin fibrosis.
A) x100, Wright stain; **B)** x40, **C)** x100, **D)** x400, **E)** x100, **F)** x400 and **G)** x100, Hematoxylin eosin stain; **H)** x400, Reticulin stain.
Periphearl blood smear slide was obtained and scored by the author; bone marrow biopsy images were courtesy of Pathology Department of CHLO.

Table 1.5 – Myelofibrosis grading.
(Arber et al., 2016).

Semiquantitative grading of BM fibrosis*	
MF – 0	Scattered linear reticulin with no intersections (crossovers), corresponding to normal BM
MF – 1	Loose network of reticulin with many intersections, especially in perivascular areas
MF – 2**	Diffuse and dense increase in reticulin with extensive intersections, ocasionally with focal bundles of thick fibers mostly consistent with collagen, and/or focal osteosclerosis
MF – 3**	Diffuse and dense increase in reticulin with extensive intersections and coarse bundles of thick fibers consistent with collagen, usually associated with osteosclerosis

* With minor modifications concerning collagen and osteosclerosis; Fiber density should be assessed only in hematopoietic areas
** Additional trichrome stain is recommended
BM – Bone marrow

According to literature, even though *JAK2* exon 12 mutations are mostly associated with erythrocytosis, up to 25% percent of patients present with elevated counts of other lineages (Scott, 2011). The basis for this different phenotype is not completely known (J. Li et al., 2014). Scott *et al* suggested that genetic factors might be involved, such as the nature of the mutation or other additional MPN-associated mutations (Scott, 2011). An enhanced STAT1 activation might cause superior megakaryopoiesis and, therefore, possibly be a contributing factor to the understanding of the small share of PV patients with exon 12 mutation who behave differently. On the other hand, the absence of thrombocytosis in the majority of patients may be related to STAT1-independent mechanisms that might cause, for instance, increase platelet destruction (Godfrey et al., 2016). The fact that exon 12 mutations are more frequently associated with erythrocytosis is compatible with their absence in ET, but possible presence in PMF or AML secondary to PV (Scott, 2011).

Mutant *CALR* in ET is associated with younger age, male sex, higher platelet count, lower hemoglobin level and lower leukocyte count and, in PMF, with younger age, higher platelet count and lower frequencies of anemia, leukocytosis, and spliceosome mutations (Tefferi, 2016). Type 2 *CALR* mutation in ET patients is associated with significantly higher platelet count and, in PMF, with higher circulating blast percentage and leukocyte count (Tefferi, 2016).

Myeloproliferative disease burden in each of the *JAK2*, *CALR* and *MPL* mutant PN-MPNs is best reflected by the degree of anemia, splenomegaly, mutation allele burden, bone marrow cellularity and myelofibrosis degree (Jan Jacques et al., 2016).

PROGNOSIS AND PREDICTIVE FACTORS

Although there has been a clear improvement in survival in the last years, globally, PN-MPNs patients have a reduced life expectancy, compared with the general population, due to several causes, such as other hematologic malignancies, bacterial infections, cardiovascular and cerebrovascular diseases (Rumi & Cazzola, 2017).

In a study recently published by the Italian collaborative group of Mayo Clinic, involving 826 patients, the median survival for ET was 20 years, for PV 14 years and 6 years for PMF, with an average increase of ten years in each disorder for patients younger than 60 years old. The study also revealed an inferior life expectancy in ET patients, when compared to age and sex-matched individuals (Rumi & Cazzola, 2017; Tefferi, 2016; Tefferi & Vannucchi, 2017).

Clinical course and disease progression depend on the contribution of several risk factors, influencing survival (Table 1.6) (Arber et al., 2016; Jones & Cross, 2013; Mesa et al., 2016; Rumi & Cazzola, 2017; Tefferi, 2016).

Age is the most important determinant of survival with the corresponding median of 33, 24, and 15 years in patients younger than 60 years.

Genetic information is the second most important prognostic tool and includes karyotype, driver mutational status, and presence of specific other mutations, which used in combination may enhance genetic risk stratification. Globally, karyotype has been shown to have prognostic relevance in PV (abnormal vs normal) and PMF (unfavorable vs favorable abnormalities). Driver mutations are prognostically most relevant in PMF (type 1/type 1-like *CALR* has been associated with superior survival) (Tefferi & Vannucchi, 2017).

Table 1.6 – Classical PN-MPNs conventional and molecular risk factors influencing clinical outcome and affecting prognosis.
(Adapted from (Rumi & Cazzola, 2017; Tefferi, 2016).

	Relationships between genotype, phenotype, and clinical outcome	**Main risk factors**
Polycythemia vera	• PV patients are at increased risk of thrombosis • PV may progress to myelofibrosis and less commonly to a blast phase similar to AML, sometimes preceded by a myelodysplastic phase	***Thrombosis:*** • previous thrombosis • age ≥ 60 years • *JAK2* V617F mutation • blood hyperviscosity ***Myelofibrotic transformation:*** • *JAK2* V617F mutant allele burden >50% • co-operating mutations in myeloid genes ***Leukemic transformation:*** • age ≥ 60 years • leukocytosis • co-operating mutations in myeloid genes ***Survival:*** • previous thrombosis • leukocytosis • co-operating mutations in myeloid genes

Essential thrombocythemia	• ET involves increased risk of thrombosis and bleeding, and may progress to more aggressive myeloid neoplasms • *JAK2* V617F-mutant ET involves a high risk of thrombosis, and may progress to PV or myelofibrosis • *CALR*-mutant ET involves lower risk of thrombosis and higher risk of progression to myelofibrosis • Triple-negative ET is an indolent disease with low incidence of vascular events	***Thrombosis:*** • previous thrombosis • age ≥ 60 years • *JAK2* V617F mutation ***Bleeding:*** • previous major bleeding • high PLT count (≥1500 x 10^9/L) ***Polycythemic transformation:*** • *JAK2* V617F mutation ***Myelofibrotic transformation:*** • age ≥ 60 years • anemia • *CALR* mutation • co-operating mutations in myeloid genes ***Leukemic transformation:*** • previous thrombosis • extreme thrombocytosis (PLT count >1000x10^9/L) • co-operating mutations in myeloid genes ***Survival:*** • previous thrombosis • leukocytosis • co-operating mutations in myeloid genes
Primary myelofibrosis	• PMF is associated with the greatest symptom burden and the worst prognosis within MPNs, with a variable risk of progression to AML • *CALR*-mutant PMF is associated with longer survival compared with other genotypes • *JAK2* V617F- and *MPL*-mutant PMF have worse prognosis than *CALR*-mutant PMF • Triple-negative PMF is an aggressive myeloid neoplasm characterized by prominent myelodysplastic features and high risk of leukemic evolution	***Survival & leukemic transformation:*** • age >65 years • presence of constitutional symptoms • anemia (Hb <10 g/dL) • leukocytosis (WBC count >25 x 10^9/L) • thrombocytopenia (PLT <100 x 10^9/L) • circulating blasts (≥1%) • degree of bone marrow fibrosis • unfavorable karyotype • driver mutation (triple negative vs *JAK2/MPL* vs *CALR* mutation) • co-operating mutations in myeloid genes

AML – Acute myeloid leukemia; Hb – Hemoglobin; WBC – White blood cells; PLT – Platelets count

PN-MPNs have high morbidity and mortality with thrombohemorrhagic complications (the most frequent), and risk of progression (Figure 1.18). Signs of progression of these diseases include genetic evolution, increased splenomegaly, increased or decreased cell counts, myelofibrosis/myelodysplasia, 10 to 19% of blasts in the peripheral blood or bone marrow (accelerated phase) and finally presence of more than 20% blasts in peripheral blood or bone marrow (blast phase).

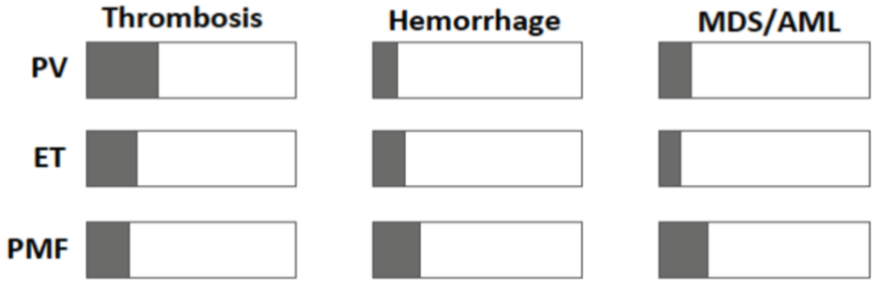


Figure 1.18 – Clinical course in classic PN-MPNs.
Complications are shared over the spectrum of PN-MPNs. Grey bars represent the proportion of specific complications found overlapping in ET, PV and PMF (Adapted from (Kiladjian et al., 2012).

ET can phenotypically develop into PV (Passamonti et al., 2008) and both PV and ET can progress to secondary MF. PV, ET, and PMF can at any time transform to AML, in occasions preceded by a phase of myelofibrosis or MDS (Figure 1.19) (Swerdlow et al., 2008).

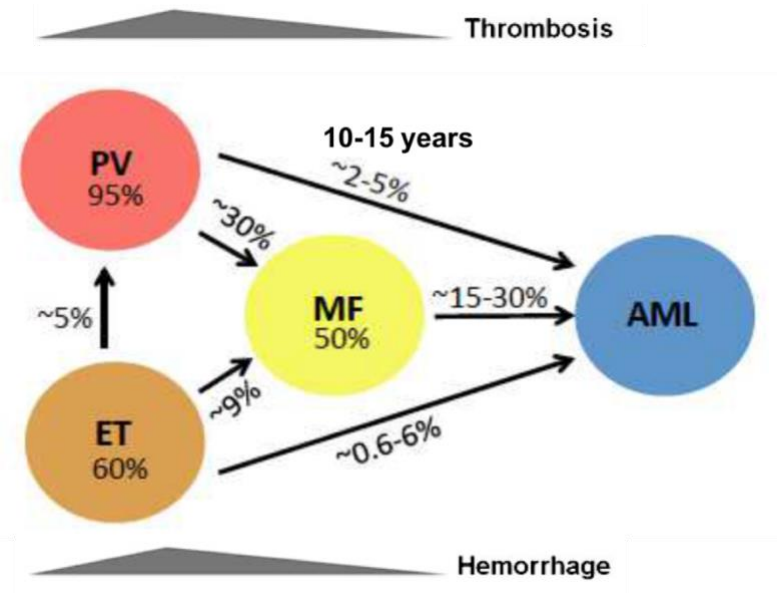


Figure 1.19 – Disease course and complications associated with PN-MPNs.
Around 95% of PV and 50-60% of ET and PMF patients harbor *JAK2* V617F mutation. In the natural course of disease progression ET may evolve into PV and PV into secondary myelofibrosis and all three of them can directly transform into AML. Value on each flash represents the approximate percentage of each type of progression (Adapted from (Swerdlow et al., 2008).

Patients with PN-MPNs have a high risk of both **arterial and venous thromboembolic** complications. The largest epidemiologic study in PV, the European Collaboration on Low-dose Aspirin (ECLAP), revealed that about 41% of all deaths in PN-MPNs (1.5 deaths per 100 persons per year) were due to cardiovascular complications.

In PV, 11-39% of patients present with a major thrombosis and around 20% develop thrombosis during follow-up (De Stefano et al., 2008; Papadakis, Hoffman, & Brenner, 2010). The cumulative rate of nonfatal thrombosis in PV was 3.8 events per 100 persons per year, without a significant difference between arterial and venous events (Barbui, Finazzi, & Falanga, 2013).

In patients with ET, the frequency of thromboembolic events in different studies ranges from 10% to 30% at diagnosis and between 8% and 31% during follow-up (Carobbio et al., 2007) and the rate of fatal and nonfatal thrombotic events ranged from 2% to 4% patient-years, with a predominance of arterial events (Barbui et al., 2013), whose risk is higher in patients with *JAK2* or *MPL* mutations (Tefferi & Vannucchi, 2017)

PMF seems less susceptible for thrombotic events as the overall cumulative rate of cardiovascular death and nonfatal thrombotic complications was 2.23 events per 100 persons per year (Andıç et al., 2016; Barbui et al., 2010; Barbui et al., 2013).

The pathogenesis of thrombosis in PN-MPNs patients is complex, involving clinical factors such as age, previous history of thrombotic events, obesity, hypertension, and hyperlipemia, as well increased blood cell counts (ie, leukocytosis, erythrocytosis, and thrombocytosis) (Barbui et al., 2013). The most important risk factor for future arterial and venous thrombosis in MPNs is previous history of arterial or venous thrombosis, respectively (Tefferi, 2016). High hematocrit, leukocytosis and *JAK2* mutation are shown to be additional risk factors (Table 1.6). The influence of the *JAK2* V617F mutational status and allele burden on the thrombotic risk has been evaluated and established in several studies among PN-MPNs, however regarding the presence of *MPL* mutation, the published results are discrepant (Barbui et al., 2013). Older (age >60 years) patients are no longer considered “high risk,” unless they have a history of thrombosis or are *JAK2*-mutated (Table 1.6) (Barbui et al., 2013; Tefferi, 2016).

It was reported that PV patients with a white blood cell count >15 × 10^9/L, had a significant 70% increase of myocardial infarction, when compared with those with a WBC count <15 × 10^9/L. It was also demonstrated that an increased baseline leukocyte count was an independent risk factor for both thrombosis and inferior survival in ET patients and an elevated WBC count developing during follow-up was also correlated with major thrombosis (Barbui et al., 2013; Carobbio et al., 2007; De Stefano et al., 2010; Papadakis et al., 2010). However, in the ECLAP study, neither the currently proposed therapeutic target of 400 × 10^9/L, nor any of the other platelet-count thresholds evaluated predicted a higher risk of thrombosis (Barbui et al., 2013).

Conventional risk factors for atherosclerosis, including hypertension, hyperlipidemia, diabetes, and smoking, have been assessed in multivariable analysis in PN-MPNS with variable results (Barbui et al., 2013).

Besides clinical factors, the pathogenesis of thrombosis in PN-MPNs involves a number of complex cellular and molecular mechanisms, as illustrated in Figure 1.20. Clonal proliferation of hematopoietic stem cells induces both quantitative and qualitative changes in blood cells, resulting in cellular activation and expression by the blood cells themselves (ie, erythrocytes, platelets, and leukocytes) of procoagulant and proteolytic properties, secretion of inflammatory cytokines, and expression of adhesion molecules. At the same time, in response to inflammatory cytokines, hyperviscosity, and leukocyte-derived proteases (ie, elastase, cathepsin-G, and myeloperoxidase), there are prothrombotic changes occurring in the normal vascular endothelium, with the upregulation of endothelial adhesion receptors, which favors the attachment of platelets, erythrocytes, and leukocytes to the vascular wall, with subsequent localization of clotting reactions and fibrin deposition (Barbui et al., 2013). Once activated, neutrophils can release proteolytic enzymes and reactive oxygen species capable of activate or damage platelets and endothelial cells and impair some coagulation proteins. Activated platelets express P-selectin and tissue factor and release membrane fragments,

which have procoagulant properties. The increased expression of CD11b on the neutrophil surface allows the adhesion of neutrophils to endothelial cells and platelets and the assembly of coagulation proteases on the neutrophil surface. In addition, biochemical changes in the cell membrane and content of erythrocytes may impair blood flow also through the formation of aggregates, which induce platelet and leukocyte interaction with the vessel wall (Barbui et al., 2013).

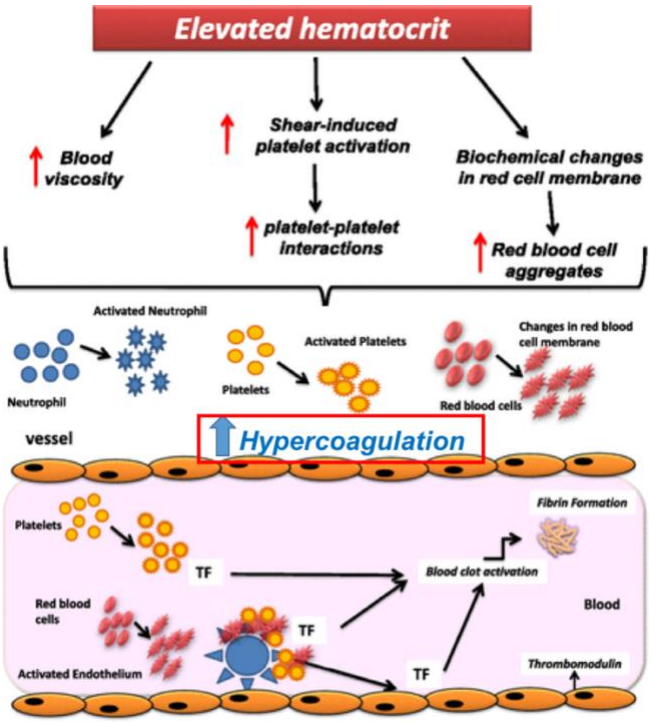


Figure 1.20 – Mechanisms involved in the pathogenesis of thrombosis in PN-MPNs.
(Adapted from (Barbui et al., 2013).

Moreover, PN-MPNs patients present a hypercoagulable background state, not only for the increased levels of circulating procoagulant microparticles, but also due to the presence of an acquired activated protein C resistance, result of Protein S cleavage (Barbui et al., 2013).

On the other hand, the risk of major **bleeding** is elevated in MPN patients, although bleeding complications are not as common as thromboembolic complications (Finazzi et al., 2012). Unprovoked hemorrhage is uncommon (Spivak, 2017).

The cumulative incidence of bleeding in PV and ET is around 5-6% and in PMF is about 12% (Finazzi et al., 2012; Papadakis et al., 2010).

Risk factors for bleeding are history of previous bleeding event, thrombocytopenia, and the use of aspirin and vitamin K antagonists (Table 1.6) (Finazzi et al., 2012). A higher risk of bleeding has also been noted in patients treated with a combination of aspirin and anagrelide (C. N. Harrison et al., 2005).

Extreme thrombocytosis (platelets count over 1000 or 1500x10^9/L) was found to be related with a lower risk of thrombosis but increased risk of hemorrhagic complications, because of associated acquired von Willebrand syndrome, as a result of platelet proteolysis of high-molecular weight von Willebrand multimers (Andıç et al., 2016; Campbell et al., 2012; Michiels et al., 2006; Spivak, 2017; Tefferi, 2016).

The most common pattern of disease progression is overt reticulin and collagen **fibrosis** of the bone marrow (see Table 1.5 above).

The range of reported frequencies for fibrotic transformation for PV is 4.9-6% at 10 years and 6-14% at 15 years and for ET is 0.8-4.9% at 10 years and 4-11% at 15 years, according to recent updates (Figure 1.19) (Cerquozzi & Tefferi, 2015; Swerdlow et al., 2008).

Risk factors for fibrotic transformation in PV include *JAK2* V617F allele burden of >50%; in ET include advanced age and anemia, with the presence of *JAK2* V617F being associated with a lower risk of fibrotic transformation and *CALR* with a higher risk (Table 1.6) (Tefferi, 2016). Moreover, *MPL*-mutated patients might be at risk for accelerated fibrotic progression (Tefferi & Vannucchi, 2017).

Recent published works have reported that genomic abnormalities are present in megakaryocytes in MPNs and that these appear to be associated with progression to bone marrow fibrosis (Guo et al., 2017; Vainchenker & Kralovics, 2017).

In addition to WHO classification, the International Working Group for MPN Research and Treatment (IWG-MRT) criteria are used for diagnoses of post-PV and post-ET myelofibrosis (Table 1.7) (Tefferi & Pardanani, 2015).

During post-PV and post-ET myelofibrosis installation, erythropoiesis progressively decreases, peripheral blood smear evidences a leucoerythroblastic reaction and poikilocytosis with “teardrop” shaped erythrocytes, and the spleen further enlarges, due to extramedullary erythropoiesis (Table 1.7). Bone marrow hypocellular specimens are common, with a decrease of erythropoiesis and granulopoiesis, and clusters of megakaryocytes bearing very dysmorphic nuclei are prominent (Swerdlow et al., 2008).

PMF may show progressive aggravation of myelofibrosis (Table 1.5) and evolve with infectious and hemorrhagic complications resulting from pancytopenia, thrombosis, hepatosplenomegaly (aggravation of pancytopenia, portal hypertension, heart failure, digestive compression), post-transfusion hemochromatosis and progression to AML in 15% of cases. Development of myelofibrosis in PMF is related to disease progression and is not significantly influenced by treatment options other than allogeneic stem-cell transplantation (Swerdlow et al., 2008).

Although the clinical characteristics of post-PV and post-ET myelofibrosis patients are not substantially different from those with PMF, their outcomes differ and prognostic scoring systems for post-PV and post-ET myelofibrosis should be improved (Masarova et al., 2017).

Table 1.7 – International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) recommended criteria for post-PV and post-ET myelofibrosis.

	IWG-MRT (Tefferi & Barbui, 2017)	
Post-PV myelofibrosis	**Required criteria:**	**Additional criteria (two are required):**
	1. Documentation of a previous diagnosis of PV as defined by the WHO criteria 2. BM fibrosis grade 2–3 (on 0–3 scale) or grade 3–4 (on 0–4 scale) (see footnote for details)	1. Anemia or sustained loss of requirement for phlebotomy in the absence of cytoreductive therapy 2. A leukoerythroblastic peripheral blood picture 3. Increasing splenomegaly defined as either an increase in palpable splenomegaly of ≥ 5 cm (distance of the tip of the spleen from the left costal margin) or the appearance of a newly palpable splenomegaly 4. Development of ≥1 of three constitutional symptoms: >10% weight loss in 6 months, night sweats, unexplained fever (>37.5ºC)
Post-ET myelofibrosis	**Required criteria:**	**Additional criteria (two are required):**
	1. Documentation of a previous diagnosis of ET as defined by the WHO criteria 2. BM fibrosis grade 2–3 (on 0–3 scale) or grade 3–4 (on 0–4 scale) (see footnote for details)	1. Anemia and a ≥ 2 g/dL decrease from baseline hemoglobin level 2. A leukoerythroblastic peripheral blood picture 3. Increasing splenomegaly defined as either an increase in palpable splenomegaly of ≥ 5 cm (distance of the tip of the spleen from the left costal margin) or the appearance of a newly palpable splenomegaly 4. Increased lactate dehydrogenase 5. Development of ≥ 1 of three constitutional symptoms: >10% weight loss in 6 months, night sweats, unexplained fever (>37.5ºC)

See Table 1.5 for myelofibrosis grading definition; BM – Bone marrow

In PN-MPNs, although the cells mature relatively normally, there is potential for further evolution of the neoplastic clone, which may result, besides bone marrow failure, in transformation to accelerated phase and/or myelodysplasia (with more than 10% blasts in peripheral blood or bone marrow or significant myelodysplasia) or transformation to AML if blasts are higher than 20%. The risk of transformation to **AML or MDS** in ET and PV is associated with a bad prognosis, and has been reported to occur in 0.7-3% and 2.3-14.4% of patients, respectively, 10 years following the initial diagnosis, and increases with time after diagnosis (2.1-5.3% and 5.5-18.7%, respectively, at 15 years) (Figure 1.19) (Cerquozzi & Tefferi, 2015; Rampal et al., 2014; Swerdlow et al., 2008). The reported frequency of AML in PMF is highest and ranges from 5 to 30% (Figure 1.19) (Swerdlow et al., 2008).

According to IWG-MRT, risk factors for leukemic transformation in PV include older age, leukocytosis and abnormal karyotype, in ET include thrombosis and extreme thrombocytosis (platelets >1000x10^9/L) and in PMF they include some of the above outlined clinical and genetic risk factors such as age, constitutional symptoms, hemoglobin levels, leukocytes/platelets count, circulating blasts, red blood cells transfusion need and karyotype (Table 1.6) (Tefferi, 2016). In addition, several mutations have been associated with leukemic transformation, namely *ASXL1, SRSF2, EZH2, IDH1/2*, *IKZF1*, *TP53*, *NF1*, *RUNX1, NRAS*, *SRSF2*, and *DNMT3A* (Abdel-Wahab et al., 2010; Vainchenker & Kralovics, 2017). Prognostic significance has been described for cytogenetic abnormalities in PMF, but not in PV and ET patients (Bittencourt et al., 2012; Guglielmelli et al., 2014; Hussein et al., 2009).

On the other hand, *JAK2* V617F has not been correlated to an increased risk of transformation to AML, nevertheless, patients *JAK2* V617F positive at MPN diagnosis can transform to *JAK2* V617F negative AML (Theocharides et al., 2007). AML secondary to MPNs is often characterized by a more complex karyotype compared to *de novo* AML, indicating a worse prognosis (Milosevic et al., 2012).

The risk of transformation seems to increase following certain types of cytotoxic therapy (Swerdlow et al., 2008), increasing the incidence to 20% (Spivak, 2017). In a small subset of patients, it was evidenced that the presence of inherited DNA mutations involved in repair pathways are associated with the development of therapy related AML/MDS (McNerney et al., 2017). Moreover, abnormal function of the bone marrow niche, previously or following treatment, may induce lesions in the local microenvironment, favoring the expansion of preleukemic clones (McNerney et al., 2017). However, there are no conclusive randomized studies on treatment and AML transformation in patients with PN-MPNs, due the late-appearing and rare events in a long-term disease course, and reluctance to randomly assign patients to receive potentially leukemogenic therapies.

JAK2 V617F mutational status may have prognostic significance in PV, ET and PMF (Tefferi, 2008b). In PV, despite the phenotypic differences, the clinical course seems similar between *JAK2* V617F and *JAK2* exon 12 positive patients, with similar incidences of thrombosis, myelofibrosis, leukemia and death (Passamonti et al., 2011). *JAK2/CALR* mutational status did not affect survival in ET (Tefferi, 2016). In PMF and ET, triple-negative patients appear to have a less favorable prognosis than patients with a driver mutation (*JAK2*, *CALR*, or *MPL*), whereas patients with *CALR* mutations tend to have a better prognosis than patients with *JAK2* or *MPL* mutations. Type 2 *CALR* mutation in PMF patients is associated with higher risk category, and inferior survival (Tefferi, 2016). Most noteworthy is the survival advantage shown by PMF patients with type 1 or type 1-like *CALR* variants, compared to all other genotypes (Table 1.6) (Tefferi, 2016). *ASXL1* and *SRSF2* mutations have been associated with inferior overall, leukemia-free, or fibrosis-free survival in both PV and PMF, and a recent targeted sequencing study has identified additional other adverse mutations in both these disorders, as well as in ET (Tefferi & Vannucchi, 2017).

Another important concern refers to the increased risk of development of **new nonhematological and nonmyeloid neoplasms** in MPNs, with an incidence ratio of 1.2-1.4 and 3.4, respectively, compared to the general population (Hernández-Boluda et al., 2012). There is evidence that this risk is higher when *JAK2* V617F mutation is present and other patient related factors may be also involved. However, the association with cytoreductive therapy is controversial. It has been hypothesized that ET patients have the intrinsic potential to evolve into malignant diseases, because of the presence of an impaired “tumor immune surveillance,” persistent inflammatory state, accumulation of reactive oxygen species, and favorable role of platelets to promote cellular growth, angiogenesis, and metastasis (Santoro et al., 2017).

In Chapter 9 of this dissertation will be presented part of the developed work, which was dedicated to the evaluation of clinical outcome of the patients under treatment with hydroxyurea included in the studied population, in terms of the presence of thrombotic events, occurrence of new

nonmyeloid neoplasms and progression to myelofibrosis or AML transformation, and their relation with caspases and BER polymorphisms.

1.1.3 – RISK STRATIFICATION, THERAPY MANAGEMENT AND RESPONSE EVALUATION

PN-MPNs RISK STRATIFICATION

Over the last 50 years, Louis Wasserman from the Unites States and Tiziano Barbui from Italy developed a crucial work and skillfully organized a number of important clinical trials, whose results constituted the basis for the creation of international prognostic systems and current treatment algorithms in PN-MPNs (Tefferi, 2016).

The therapeutic strategy to be adopted in each disorder is decided according to the stratification of risk for each patient, using the prognostic models and risk criteria described in Table 1.8. For each one of PN-MPNs different prognostic models were developed, which were subsequently upgraded in the recent years, based on the risk factors previously mentioned in Table 1.6, known to influence disease progression.

As shown in Table 1.8, in the case of PV and ET patients risk stratification is based on IPSS and IPSET scores, respectively, depending on the age, *JAK2* mutation status, leukocytes/platelets count and previous history of thrombotic events (Rumi & Cazzola, 2017; Tefferi, 2016). For PMF, risk stratification depends on DIPSS-plus score (including previously mentioned risk factors such as age, constitutional symptoms, hemoglobin levels, leukocytes/platelets count and circulating blasts, in addition to red blood cells transfusion need and karyotype), molecular status for *CALR* and *ASXL1* or other high risk mutations (*IDH1/2, EZH2, SRSF2*), and increased serum IL-8, IL-2R or serum free light chain levels (Rumi & Cazzola, 2017; Tefferi, 2016).

Table 1.8 – Prognostic models and risk stratification for PN-MPNs patients.
(Adapted from (Rumi & Cazzola, 2017).

	Prognostic model	**Risk groups and clinical relevance**
Polycythemia vera	**Conventional thrombosis score (European LeukemiaNet recommendations) (Barbui et al., 2011)**	
	At least 1 of the following risk factors: • Age ≥60 y • Previous thrombosis	***Low risk*:** age <60 y AND no history of thrombosis, that is, no risk factors **High risk:** age ≥60 y AND/OR history of thrombosis, that is, at least 1 risk factor Low-risk patients are given low-dose aspirin and undergo regular phlebotomy to keep hematocrit <45%; high-risk patients are given also a cytoreductive treatment

Essential thrombocythemia	**IPSS for overall survival in PV (Tefferi, Rumi, et al., 2013)**	
	Risk factors (weight): • Age ≥67 y (5 point) • Age 57-66 y (2 points) • WBC count ≥15 x 10^9/L (1 point) • Previous venous thrombosis (1 point)	***Low risk*:** 0 (median survival, 28 y) ***Intermediate risk*:** 1-2 points (median survival, 19 y) ***High risk*:** ≥3 points (median survival, 11 y)
	Conventional score for prediction of vascular complications (European LeukemiaNet recommendations) (Barbui et al., 2011)	
	At least 1 of the following risk factors: • Age ≥60 y • Previous thrombosis or major bleeding • PLT count ≥1500 x 10^9/L	***Low risk*:** age <60 y AND no history of thrombosis or major bleeding AND PLT count <1500 x 10^9/L, that is, none of the 3 major risk factors ***High risk*:** age ≥60 y AND/OR history of thrombosis or major bleeding AND/OR PLT count ≥1500 x 10^9/L, that is, at least 1 of the 3 major risk factors While low-risk patients are just followed (observation alone) or given low-dose aspirin, high-risk patients are given a cytoreductive treatment plus low-dose aspirin
	IPSET-thrombosis (International Prognostic Score for ET: estimates the risk of thrombosis) (Barbui et al., 2012)	
	Risk factors (weight): • Age ≥60 y (1 point) • Previous thrombosis (2 points) • Cardiovascular risk factors* (1 point) • *JAK2* V617F mutation (2 points) *Cardiovascular risk factors include hypertension, diabetes, and active tobacco use	***Low risk*:** 0-1 point (probability of thrombotic events: 1.03% of patients/year) ***Intermediate risk*:** 2 points (2.35% of patients/year) ***High risk*:** ≥3 points (3.56% of patients/year) Potential therapeutic implications: (1) observation alone may be adequate in patients with no risk factors; (2) low-dose aspirin should be used in all patients with *JAK2* V617F and/or cardiovascular risk factors; (3) older patients (≥60 y) without additional risk factors may not need a cytoreductive treatment; (4) conversely, a cytoreductive treatment may be considered in younger patients (<60 y) with *JAK2*-mutant ET and concomitant cardiovascular risk factors, even in the absence of previous thrombosis
	IPSET (International Prognostic Score for ET: predicts survival) (Barbui et al., 2015)	
	Risk factors (weight): • Age ≥60 y (2 points) • Previous thrombosis (1 point) • WBC count >11 x 10^9/L (1 point)	***Low risk*:** 0 (median survival not reached) ***Intermediate risk*:** 1-2 points (median survival, 24.5 y) ***High risk*:** 3-4 points (median survival, 13.8 y)
Primary myelofibrosis	**IPSS (Cervantes et al., 2009)**	
	Risk factors (weight): • Age >65 y (1 point) • Constitutional symptoms (1 point) • Hb <10 g/dL (1 point) • WBC count >25 x 10^9/L (1 point) • Circulating blasts ≥1% (1 point)	***Low risk*:** 0 (median survival, 11.3 y) ***Intermediate-1 risk*:** 1 point (7.9 y) ***Intermediate-2 risk*:** 2 points (4.0 y) ***High risk*:** ≥3 points (2.3 y) IPSS estimates survival at the time of diagnosis
	DIPSS (Passamonti, Cervantes, et al., 2010)	
	Risk factors (weight): • Age >65 y (1 point) • Constitutional symptoms (1 point • Hb <10 g/dL (2 points) • WBC count >25 x 10^9/L (1 point) • Circulating blasts ≥1% (1 point)	***Low risk*:** 0 (median survival, >20 y) ***Intermediate-1 risk*:** 1-2 points (14.2 y) ***Intermediate-2 risk*:** 3-4 points (4.0 y) ***High risk*:** 5-6 points (1.5 y) DIPSS can be applied anytime during clinical course
	DIPSS-plus (Gangat et al., 2011)	
	Risk factors (weight): • DIPSS score (DIPPS low=0, DIPPS int-1=1 point, DIPPS int-2=2 points, DIPSS high=3 points) • RBC transfusion need (1 point) • PLT count <100 x 10^9/L (1 point) • Unfavorable karyotype* (1 point) *(18,27/7q,i(17)q,25/5q,12p2,inv(3),11q23 rearrangements)	***Low risk*:** 0 (median survival, 15 y) ***Intermediate-1 risk*:** 1 point (6.6 y) ***Intermediate-2 risk*:** 2-3 points (2.9 y) ***High risk*:** 4-6 points (1.3 y) DIPSS-plus can be applied anytime during clinical course

Hb – hemoglobin; PLT – platelets; RBC – red blood cells; WBC – white blood cells;

DIPSS – Dynamic International Prognostic Scoring System; IPSET – International Prognostic System for ET; IPSS – International Prognostic Scoring System.

In a recent international study involving PMF patients, it was reported that *CALR1ASXL12* patients had the longest survival (median 10.4 years) and *CALR2ASXL11* patients the shortest (median 2.3 years). *CALR1ASXL11* and *CALR2ASXL12* patients had similar survival, grouped together in an intermediate risk category (median survival 5.8 years) (Tefferi, 2016).

Regarding post-PV/ET MF, in a very recent article Tefferi *et al.* provided evidence to support the use of IPSS/DIPSS/DIPSS-plus, thus maintaining familiarity and uniformity, until a molecularly more robust system is developed (Tefferi et al., 2017). In patients with secondary myelofibrosis, only *SRSF2* is associated with shortened survival (Spivak, 2017).

THERAPY MANAGEMENT AND RESPONSE EVALUATION

Cure is the ultimate objective of cancer therapy. However, current available drug therapy in PV, ET, or PMF does not have curative power and chronicity is the hallmark of PN-MPNs. Globally, the main goal in PN-MPNs therapy consists in prevention of thrombotic events, occurrence of other nonmyeloid neoplasms and fibrotic or AML transformation. Thus, strategies for accurate risk assessment are essential to maximize therapeutic benefits, with the minimal possible iatrogenic harm, and prolong life.

Accordingly, treatment indications in PV and ET are directed towards alleviation of symptoms and prevention of complications, such as thrombosis and/or bleeding events (Figure 1.21) (Tefferi, 2016), to which is necessary to lower hematocrit below 45% and correct leukocytosis, respectively, since thrombosis probability in ET patients under treatment is not influenced by platelet count (more related with bleeding) (Rumi & Cazzola, 2017).

On the other hand, in PMF the principal aim is symptomatic control (ameliorating anemia, reducing splenomegaly and improving systemic symptoms), with the possibility of cure limited to patients undergoing allogeneic stem cell transplant (discussed below) (Tefferi, 2016).

Treatment of PV and the other MPNs have included skeletal radiation therapy (1917), acetylphenylhydrazine (1918), potassium arsenite (1933), lead acetate (1942), nitrogen mustard (1950), triethylene melamine (1952), pyrimethamine (1954), 6-mercaptopurine (1962), uracil mustard (1964), chlorambucil (1965) and dapsone (1966). P32 was introduced in 1940, busulfan 1958, pipobroman 1962, hydroxyurea and melphalan in the 1970s and interferon-α in the 1980s (Tefferi, 2008c).

Phlebotomy prescription, indicated in all patients with PV, has been used since the disease was first described (Tefferi, 2008c). Is the recommended main tool to control hematocrit levels below 45% reducing a the rate of major thrombosis and dead from cardiovascular causes as it was

demonstrated in the Cytoreductive Therapy in PV study (Vannucchi, 2015), with a significant improvement in patients medial survival (<2years increased to >14 years) (Tefferi, 2016). One of the most concerning side effects of phlebotomies is iron deficiency and its complications (Vannucchi, 2015). Patients with PV or ET whose thrombohemorragic risk is high enough, might require treatment with aspirin and/or cytoreductive drugs such as hydroxyurea, for lowering that risk and symptomatic control, but subject to short- and long-term side effects of these drugs (Figure 1.21) (Tefferi, 2016).

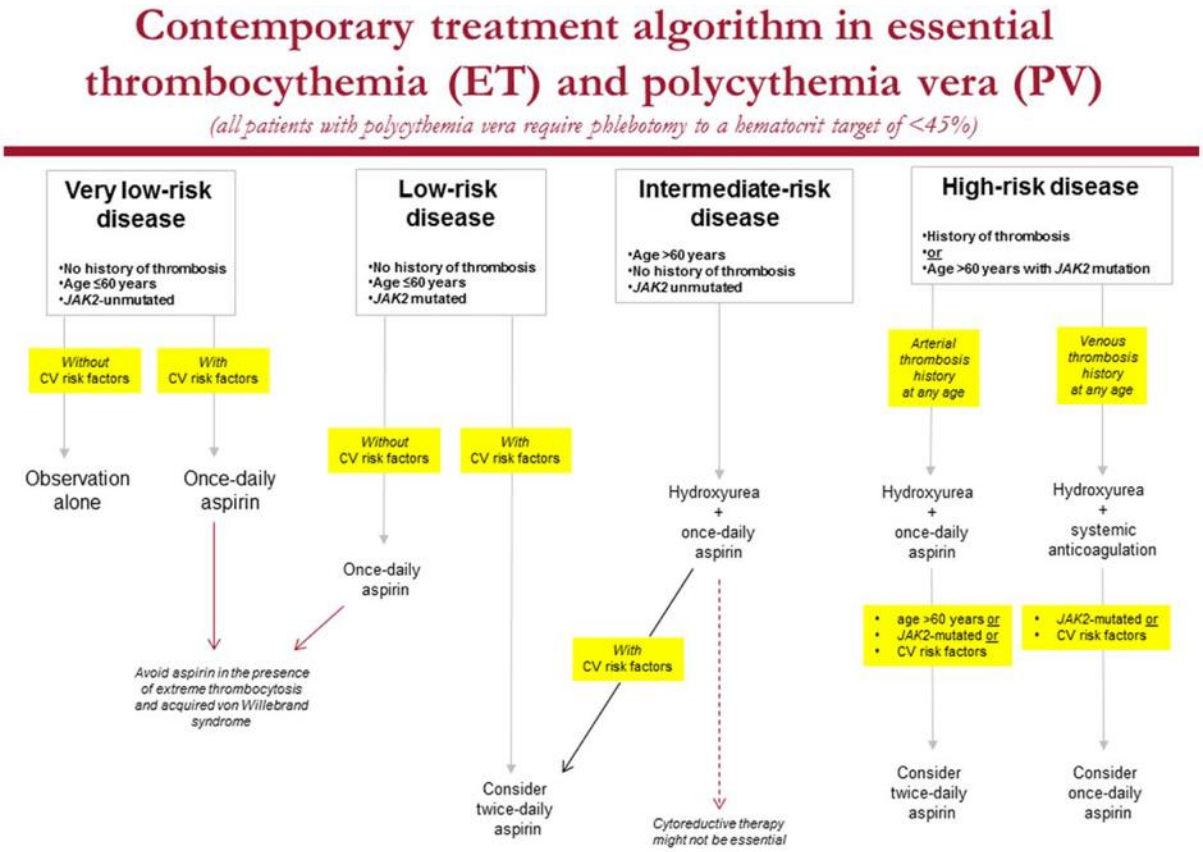


Figure 1.21 – Contemporary treatment flow-chart in ET and PV.
(Adapted from (Tefferi, 2016).

The use of **acetylsalicylic acid** therapy in PV is supported by controlled evidence in the ECLAP (European Collaboration study on Low-dose Aspirin in Polycythemia) study (Barbui et al., 2013; Tefferi, 2016). Since then, low-dose aspirin is standard treatment in PV and also many patients with ET and PMF (Landolfi et al., 2004). In ET it has been suggested that antiplatelet therapy reduces the incidence of venous thrombosis in *JAK2* positive patients and the rate of arterial thrombosis in patients with associated cardiovascular risk factors (Barbui et al., 2013; Tefferi, 2016). Current treatment should not primarily aim at lowering the platelet count for thrombosis prevention. However, platelet-lowering drugs should be considered at platelet counts >1500 × 10^9/L to reduce the risk of

bleeding (Barbui et al., 2013), but with serious caution. In regard to bleeding, the annual incidence of major hemorrhages attributed to aspirin in PV and ET patients ranges between 0.3% and 0.8%, and this rate is higher in ET patients presenting with prefibrotic myelofibrosis (1.39% patient-years) (Barbui et al., 2013).

Regarding **cytoreductive therapy** in PN-MPNs, it is recommended for those patients with persistent or progressive hematologic abnormalities, splenomegaly or other symptoms, at high risk for thrombosis, or for PV patients who cannot undergo phlebotomy or who require them frequently (Vannucchi, 2015). In addition, an expert consensus conference indicated that another aim of cytoreduction in ET and PV should be to keep the leucocytes count within the normal range (Barbui et al., 2013). There is controlled evidence of value for chlorambucil or radiophosphorus in PV, and hydroxyurea in high risk ET. However, the use of chlorambucil and radiophosphorus was complicated by shortened survival because of the increased leukemogenicity of these drugs, as it was demonstrated in a trial developed by the Polycythemia Vera Study Group (1967-1997), compared to patients treated with phlebotomy alone (Berk et al., 1981). Pipobroman, another myelosuppressive drug used in Europe, was also recently implicated as being leukemogenic in PV patients (Tefferi, 2016).

Therefore, while the anti-thrombotic value of cytoreductive therapy in high risk PV and ET is well recognized, the only drug with adequate evidence of efficacy and safety, in this regard, is **hydroxyurea (HU)**, also called hydroxycarbamide, which is currently the first-line drug of choice in intermediate and high risk PV or ET patients (Barbui et al., 2013; Tefferi, 2016).

HU (Figure 1.22) is an antimetabolite/cytotoxic non-alkylating antineoplastic and antiviral agent, capable of inhibiting DNA-synthesis and cell growth, that has been used for a variety of conditions in hematology, oncology, infectious disease and dermatology. It was first synthesized over a century ago in 1869, but it was not until ~60 years later in 1928 that the biological effects of this simple antimetabolite compound on blood cells in rabbits were reported. In the 1960s, a large-scale drug screen revealed its anti-tumor potential. Subsequent studies showed that it could be used to treat several types of solid tumors and myeloproliferative disorders (Tefferi, 2016). It is metabolized in liver (to CO2 and urea), with a half-life of 2-4 hours is being excreted by kidney and lung. This drug is a ribonucleotide reductase inhibitor, blocking the synthesis of DNA and arresting cells in the S phase of the cell cycle, characterized by an easy reversibility of its action. HU has been shown to induce chromosome damage in various organisms and also cytotoxic effects depending on the concentration used, duration of exposure, and sensitivity of the cell lines used in the studies. HU can also cross the placenta, being teratogenic. Thus, the DNA damage, such as the strand breaks caused by inhibition of DNA synthesis, is generally believed to be responsible for its cytotoxicity, anti-neoplastic activity and teratogenic effects. However, the reversible effect of HU on DNA replication suggests that it is a cytostatic agent, and, in addition to the DNA damage, its cytotoxic effects may involve a more complex mechanism (Singh & Xu, 2016).

HU is the most commonly used first-line cytoreductive agent because of its proven efficiency, especially in reducing thrombotic risk (Cortelazzo et al., 1995). In patients with high risk ET, it was associated with a lower risk of arterial thrombosis and progression to myelofibrosis, but a higher risk of

venous thrombosis compared to anagrelide (discussed below) (C. N. Harrison et al., 2005). Furthermore, attempts to achieve hematologic remission with HU have failed to prolong survival (Spivak, 2017).

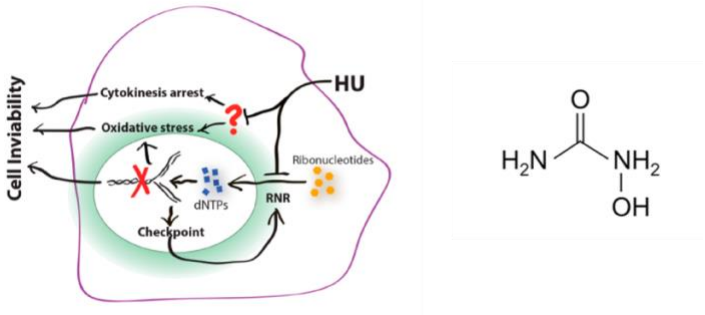


Figure 1.22 – Hydroxyurea (HU) molecule and its cellular effects.
HU inhibits its primary cellular target ribonucleotide reductase (RNR), which decreases the deoxyribonucleotide triphosphate (dNTP) levels and slows the movement of DNA polymerases at the forks (red cross). Slowed forks activate the DNA replication checkpoint, which in turn stimulates RNR to increase the dNTP production for DNA synthesis and fork recovery. Activated checkpoint can also suppress mitosis to prevent aberrant cell division (not shown). Without a functional checkpoint, slowed forks collapse and thus generate DNA damage, which leads to cell inviability. Recent studies suggest that, in addition to RNR, HU may have a secondary target(s) (red question mark) such as metal enzymes and matrix proteases, that are suppressed and may arrest the cells in cytokinesis or generate oxidative stress, which also leads to cell lethality (Adapted from (Singh & Xu, 2016).

HU is recommended to be used with caution in young patients, regarding the data reported by Kiladjian *et al*., showing a risk increment of leukemia in long-term usage (Andıç et al., 2016). However, this effect was not supported by other authors (I. Nielsen & Hasselbalch, 2003).

Some patients have important side effects or an inadequate response to the drug, being classified according to the European LeukemiaNet (ELN) criteria as having intolerance or resistance to HU (Barosi et al., 2013), associated with shorter survival (Vannucchi, 2015). In those patients, data from non-controlled studies support the use of **interferon-alpha** or **busulfan** as second-line drugs of choice. There is adequate long-term safety information on these drugs, which have both been shown to significantly reduce *JAK2* V617F mutant allele burden in ET and PV and *CALR* in ET patients and result in hematologic and complete molecular responses (in 20% of PV patients), although the effect of such biologic activity on risk of thrombosis or survival is unknown (Rumi & Cazzola, 2017; Tefferi, 2016). Moreover, interferon-alpha showed to reduce bone marrow fibrosis in early stages of PMF (Kiladjian et al., 2008; Silver, Vandris, & Goldman, 2011). However, interferon-alpha is the least commonly used agent in all PN-MPNs groups, probably because of its parenteral usage and poor tolerability (immunosuppression, myelotoxicity, and neurotoxicity) (Andıç et al., 2016; Kiladjian et al., 2008; Spivak, 2017). Moreover, it is well known that alkylating agents (given alone or in combination/sequentially) increase the risk of transformation into AML (Santoro et al., 2017; Vannucchi, 2015).

In the ANAHYDRET study, it was shown that **anagrelide** was as effective as HU in PN-MPNs treatment (Gisslinger et al., 2013). Anagrelide is effective in reducing platelet counts in ET and PV

patients who are resistant or intolerant to HU. The platelet-lowering effect of anagrelide results from impaired megakaryocyte maturation and reduced proplatelet formation, both of which are deregulated in ET, although the basis for this effect remains unclear (Espasandin et al., 2015). In high risk ET, ANAHYDRET study showed no difference in the risk of arterial or venous thrombosis or bleeding (Gisslinger et al., 2013). Secondary leukemia has not been reported with anagrelide treatment yet (Andıç et al., 2016), but its use in high risk ET was associated with increased risk of post-ET MF (Tefferi, 2016).

The discovery of the *JAK2* mutations and their relation with the subsequent activation of the JAK-STAT pathway was crucial to the understanding of the pathogenesis of PV, ET and PMF. This knowledge has led to the development of small molecular ***JAK* inhibitors** to target autoimmune disease/immunosuppression (anti-*JAK1*, *JAK3*) and MPNs and leukemia/lymphoma (anti-*JAK2*, *JAK1*), which have been tested in several clinical trials, suggesting an overall reduction in JAK-STAT signaling and pro-inflammatory cytokines (Pardanani et al., 2007; Lasho et al., 2008; Pardanani, 2008). About ten compounds were studied for MPNs, rheumatoid arthritis, psoriasis and inflammatory bowel disease, all of them targeting the ATP-binding site of JAKs and so none is absolutely specific for any JAK (Tefferi, 2008a). Nevertheless, **ruxolitinib** (a *JAK1*, *JAK2* inhibitor, trade name Jakavi®) has been approved by the Food and Drug Administration (FDA) in November 2011, for use in myelofibrosis and tofacitinib (a *JAK1*, J*AK3* inhibitor) has been approved for use in rheumatoid arthritis. The first two randomized controlled trials (Comfort I and II) on the effect of the JAK2 inhibitor ruxolitinib vs placebo and vs best available therapy in intermediate-2 and high risk PMF showed a decrease in spleen size and symptom burden in the experimental arm of both studies and in Comfort I, a survival benefit was observed in the ruxolitinib arm compared to patients on placebo (C. Harrison et al., 2012; Verstovsek et al., 2012).

The treatment options of PMF patients are currently limited (Figure 1.23), giving *JAK2* inhibitors immediate clinical value in the management of symptoms. PMF patients may benefit from *JAK2* inhibition through directly modulating the pro-growth signals of the JAK-STAT pathway, suppression of hematopoietic progenitor cell proliferation, and from downregulating specific pro-inflammatory cytokines produced by the affected clone (Spivak, 2017; Tefferi & Pardanani, 2011).

Ruxolitinib treatment substantially alleviates symptomatic splenomegaly and constitutional symptoms and improve quality of life in a significant proportion of patients with primary or post-PV/ET myelofibrosis (Tefferi, 2008a). Surprisingly, treatment with ruxolitinib is also effective in patients without mutated *JAK2*, suggesting that other, still unknown, underlying mechanism(s) are responsible for the increased JAK/STAT pathway activity in PN-MPNs patients. On the other hand, there is not convincing evidence of reduction in mutated allele burden or progression to AML (Tefferi, 2016).

Ruxolitinib is capable of induce complete molecular response in PMF and recently was also approved for use in HU-intolerant/resistant PV and ET , based on its ability to alleviate constitutional symptoms and reduce spleen size (C. N. Harrison et al., 2017; Rumi & Cazzola, 2017; Vannucchi, 2015; Verstovsek et al., 2017). However, the drug has not been shown to modify the natural history of PV and is associated with significant short and long term side effects that make its utility questionable

and not recommended for use in PV, in the absence of refractory symptomatic splenomegaly or intractable pruritus that fail to respond to HU, busulfan or interferon alfa (Tefferi, 2016).

Whether the presence of additional mutations impairs the effectiveness of ruxolitinib is disputed (Spivak, 2017).

Other promising *JAK* inhibitors investigational drugs for PMF, under study in different phases of clinical trials, include fedratinib, momelotinib, and pacritinib (Tefferi, 2016), but they have so far not shown disease-modifying activity, including reversal of bone marrow fibrosis or induction of complete or partial remissions (Tefferi, 2016).

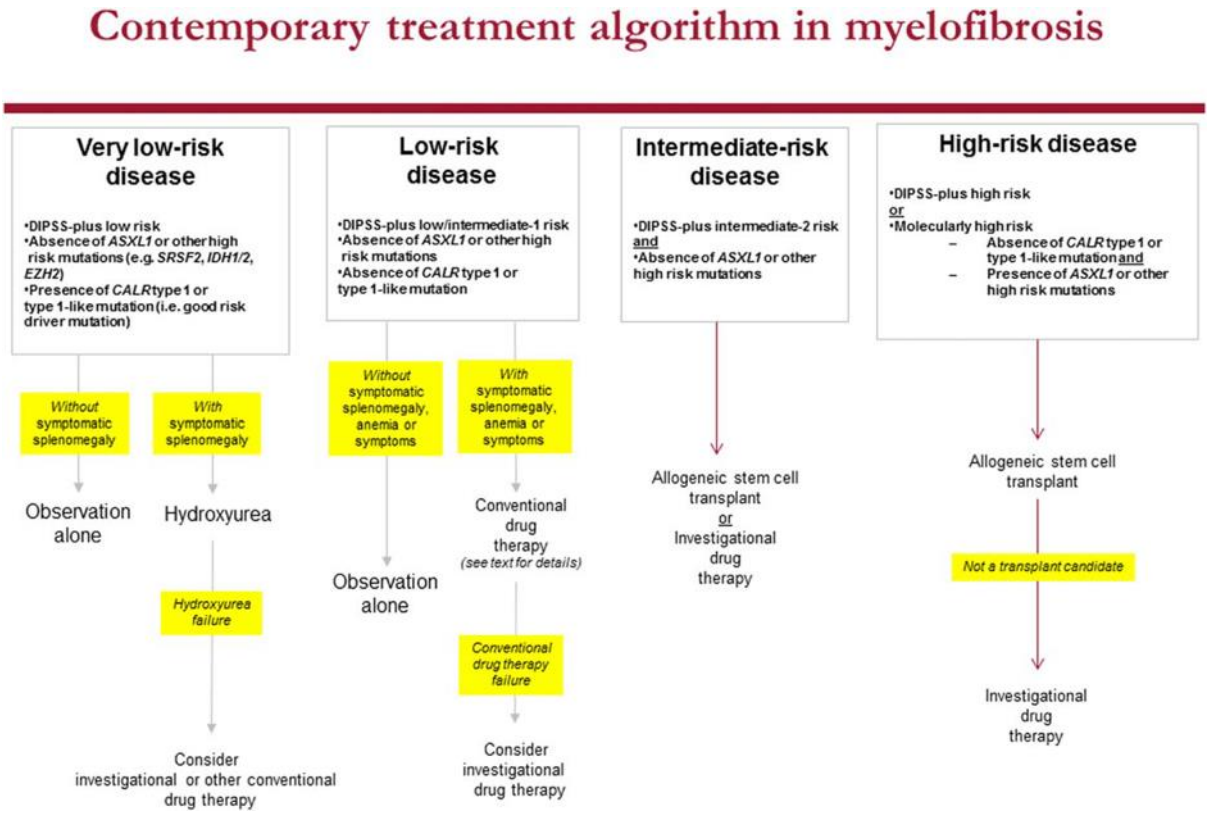


Figure 1.23 – Contemporary treatment flow-chart in myelofibrosis.
(Adapted from (Tefferi, 2016).

The side effects of JAK inhibitors include thrombocytopenia, anemia and neutropenia and gastro-intestinal symptoms, such as diarrhoea, nausea and vomiting. Further, a withdrawal syndrome has been reported during discontinuation of ruxolitinib, characterized by acute relapse of disease symptoms, accelerated splenomegaly and worsening of cytopenias, requiring a balance for each individual patient with enough benefit and the least side effects (Tefferi, 2016).

In terms of treatment, the limitation of JAK inhibitors to disease palliation is now well recognized and there is a dire need for disease-modifying drugs. In this regard, two papers on the **telomerase inhibitor** imetelstat were recently published in the New England Journal of Hematology and showed a

potential disease modifying activity through the induction of clinical, histological and molecular remissions in both ET and PMF. Severe myelosuppression and mostly reversible low grade abnormalities in liver function tests were the two main side effects of treatment with imetelstat (Tefferi, 2016). **Other therapies** that are currently being tested primarily in PMF include immunomodulators, histone deacetylase inhibitors, inhibitors interfering with epigenetic mechanisms and signaling pathways (Bose & Verstovsek, 2017). Ongoing work will answer whether the use of these epigenetic therapies as alterative pathways in combination with JAK inhibitors may be more effective than single agent treatment (McPherson et al., 2017).

Despite some controversies, current drug therapy for PMF is not curative and is unlikely to prolong survival, with **allogeneic stem cell transplant,** introduced during the 1970s and 80s, being the only treatment modality capable of achieving those aims. Therefore, it is recommended in either DIPSS-plus intermediate-2 and high risk (ie, presence of at least three of the eight DIPSS-plus risk factors) or molecularly high risk disease (absence of type 1/type 1-like *CALR* mutation and presence of ASXL1 or related high risk mutation) (Figure 1.23) (Tefferi, 2016). However, it is associated with a substantial transplantation-related mortality (Abelsson et al., 2012; Ballen et al., 2010).

There is no evidence to support the value of specific therapy in asymptomatic patients with DIPSS-plus low or intermediate-1 risk disease, however, such patients might require therapy. First-line drugs of choice in such patients are HU for symptomatic splenomegaly, androgens preparations, prednisone, danazol, thalidomide/prednisone or lenalidomide/prednisone and transfusions for symptomatic anemia, splenectomy (or splenic radiotherapy for nonsurgical candidates) in cases of splenomegaly resistant to conventional drug therapy, field radiotherapy for non hepatosplenic extramedullary hematopoiesis and associated pulmonary hypertension and ruxolitinib (and analgesics) for severe constitutional symptoms and bone pain that are resistant to HU therapy (Tefferi, 2016).

Recommendations for assessing therapeutic response, morphologic remission, improvement of associated symptomatic burden and to facilitate the development of new and more effective drugs for PN-MPNs have been recently revised by the European Leukemia Net (ELN) and the International Working Group-Myeloproliferative Neoplasms Research and Treatment (IWG-MRT) groups, based on several studies that are ongoing (Barosi et al., 2013; Miller et al., 2017; Rumi & Cazzola, 2017; Tefferi, Cervantes, et al., 2013). In PV and ET, definitions of complete and partial remission incorporate clinical, hematological, and histological response assessments, including a standardized symptom evaluation and considering absence of vascular events and signs of disease progression (Barosi et al., 2013). In PMF the current criteria include stricter definitions of red cell transfusion dependency and independency and consideration of the Myeloproliferative Neoplasm Symptom Assessment Form as a tool to quantify meaningful changes in disease-related symptoms. These are organized in six response categories, namely complete remission and partial remission, corresponding to treatment effects that are consistent with disease modification, whereas drug-induced improvements in symptomatic burden are considered as clinical improvement, anemia response, spleen response, or symptoms response. Additional criteria are provided for progressive disease, stable disease, and relapse. To evaluate therapy response, there

are also included recommendations for assessing cytogenetic and molecular remissions, not required for complete remission assignment (Tefferi, Cervantes, et al., 2013). It is necessary to test for the driver mutations, but also for the other possible concomitant mutations (Rumi & Cazzola, 2017).

Despite what was referred above and all the advances that have been achieved in the last few years, many unanswered questions remain. It is essential to well characterize PN-MPNs patients in terms of clinical phenotype and clinical outcome, and to proceed with investigation on molecular mechanisms involved, and their complex interactions, in order to a better clarification of the pathophysiology of these diseases. This may allow the identification, in advance, of subgroups of patients with a specified potencial therapeutic response, and at the same time make possible the elaboration of therapeutic strategies against new targets, more directed and efficient. These aspects represented one of the motivations for the elaboration of this thesis, as will be detailed below in Chapter 2 – Study Aims.

1.2 – INDIVUDUAL SUSCEPTIBILITY: GENETIC POLYMORPHISMS AND ASSOCIATION STUDIES

Genetic variability has been for a long time recognized as a determinant modulator factor for susceptibility and development of disease, including cancer. It is based on the existence of DNA polymorphic variations that occur in the population, estimated between 10^6-10^7, including repetitions, deletions, insertions/duplications, indels, complex rearrangements and single nucleotide polymorphisms. These variations are distinct from mutations, which are associated to disease with a hereditary pattern of transmission, more frequently observed in the population (mutations > 1% *vs* polymorphisms <1%) (Tabor, Risch, & Myers, 2002).

The most common polymorphisms are **single nucleotide polymorphisms (SNPs)** distributed throughout the genome, in exons, introns, intergenic regions and promoter regions. Thus, a SNP located in the coding region may directly affect a particular protein by substitution of an amino acid, a SNP located in intron-exon boundary can influence the process of splicing, while a SNP located in the promoter region may influence the expression of a gene. The vast majority of cases have two alleles, representing a substitution of a base to another. For an individual SNP, one allele is designated as the most common or wild type, and the other as the least common or variant, based on the frequency observed in the general population (Crawford & Nickerson, 2005).

Several SNPs have been identified, that may influence the normal gene "function" and, in turn, confer genetic predisposition to disease and determine therapeutic response.

Although the individual effects of SNPs generally don't have consequence, the genetic effect of combinations of relevant functional SNPs may additionally and synergistically contribute to the increased risk of cancer (Ponder, 2001).

The specific combination of SNPs functional alleles that are in alignment on one of the two homologous chromosomes and are inherited in blocks is called **haplotype** (Crawford & Nickerson, 2005).

An individual is said to be **homozygous** at a given SNP locus if the two observed alleles are the same. **Heterozygosity**, on the other hand, refers to the presence of more than one allele at a given site. The term **loss of heterozygosity (LOH),** commonly used in the context of oncology, refers specifically to the loss of function of an allele, when a second allele is already inactive, through inheritance of the heterozygous genotype.

The **minor allele frequency (MAF)**, also referred to as the variant allele frequency, refers to the frequency of the less common allele at a variable site.

Linkage disequilibrium (LD) measures the correlation between SNP alleles at sites in the same region of the genome, allowing to correlate short regions of the genome, with no need of genotyping all the possible allelic variants to detect an association between de different SNPs involved in a specific disorder, which means that knowledge of the genotype of one SNP predicts the genotype

of another SNP if the association between them exists (Crawford & Nickerson, 2005; Lewis & Knight, 2012).

Until some time ago the methodologies available for **association studies** allowed only the individual evaluation of a small number of polymorphisms, being an important tool in the detection of low penetrance alleles (Easton et al., 2007). However, advances in genotyping and sequencing technologies, coupled with the development of sophisticated statistical methods, have provided investigators novel opportunities to define the role of sequence variation and increase the predictability in the development of common human diseases.

Due to the completion of human *HapMap* project (The International HapMap Consortium, 2003), and others such as *SeattleSNP Variation Discovery Resource and SNP500* Project (Packer et al., 2006) and after the great development of high-throughput sequencing/genotyping methodologies in the last few years, the availability of an increasing amount of SNPs data and the discovery of novel genetic variants has increased the potential of human genetic association studies to identify in a genome-wide scale (**genome-wide association studies - GWAS, also known as whole genome association study – WGAS)** common, but also rare, variants underlying complex diseases such as cancer, including myeloproliferative disorders (Huh et al., 2010; Jouni et al., 2013; D. H. Kim et al., 2011; Merker et al., 2013; Stadler, Gallagher, Thom, & Offit, 2010; Stadler, Thom, et al., 2010; Stadler, Vijai, et al., 2010; Van Bergen et al., 2010; Wray, Goddard, & Visscher, 2007).

GWA studies compare the DNA of participants having varying phenotypes for a particular trait or disease and the resulting significant genetic association may be interpreted as either (1) direct association, in which the genotyped SNP is the true and direct causal variant conferring disease susceptibility; (2) indirect association, in which the genotyped SNP is in linkage disequilibrium (LD) with the true causal variant (Lewis & Knight, 2012).

In these processes, statistical and computational methods play key roles, among which information-based association tests have gained large popularity (C. Wu, Li, & Cui, 2012).

The first successful GWAS was published in 2005. It investigated patients with age-related macular degeneration and found two SNPs with significantly altered allele frequency compared to healthy controls. As of 2007, hundreds or thousands of individuals were tested in a typical GWA study, over 3,000 human GWA studies have examined over 1,800 diseases and traits, and thousands of SNPs associations have been found (C. Wu et al., 2012).

Two strategies can be used when based in detection of SNPs. One based on the study of SNPs in genes putatively or known to be associated with the disease – the **candidate gene approach**, and as in the case of GWAS an agnostic one, whose conclusions rely on the statistical significance of the whole genome associations, in spite of the putatively (if any) role of the gene(s) on which the significant SNPs were found.

At the forefront of these investigations is the use of dense maps of SNPs and the haplotypes derived from these polymorphisms, which are the principal focus on **case-control association studies**,

contributing to the identification of candidate genes associated with increased risk for cancer and other diseases development (Crawford & Nickerson, 2005; Syvänen, 2001; C. Wu et al., 2012).

Therefore, the genetic association studies constitute a major tool to test for the eventual association between a disease status and the genetic variation in a series of individuals, in order to identify candidate genes or genome regions that contribute to that specific disease (Lewis & Knight, 2012). These studies allow to identify genes conferring susceptibility to complex disorders, including neoplasms, to which environmental factors also contribute to susceptibility risk (Lewis & Knight, 2012).

When a SNP allele or genotype is present at a higher frequency in a group of individuals affected with a disease, this can be interpreted as meaning that the tested variant can be involved in increasing the risk for a specific disease (Lewis & Knight, 2012). Generally, multiple SNPs within a single gene are evaluated. Thus, polymorphic genotype frequencies are compared between groups with different phenotypes. The objective is to study which polymorphisms may be related to disease risk or which are in linkage disequilibrium with the causative variants (because recombination tends to occur in hot spots, making neighboring polymorphisms possibly related) (Easton et al., 2007).

Most SNPs do not occur in the coding regions of the genes, thus not translating into an amino acid change in the protein. Although SNPs located at non coding regions may be also relevant, the few non-synonymous SNPs (occurring in the coding regions and translating into an amino acid change) are the subject of investigation because a change in an amino acid is presumed to lead to altered protein function (Crawford & Nickerson, 2005).

In association studies SNPs are the most common tested markers, but other factors can also be tested, as microsatellite markers, insertion/deletions, variable-number tandem repeats (VNTRs), and copy-number variants (CNVs) (Lewis & Knight, 2012).

Despite associations with polymorphisms in candidate genes have already been confirmed and reinforced by meta-analysis studies in many different diseases, such as diabetes, cardiovascular and autoimmune disorders (Berndt et al., 2016; Lohmueller, Pearce, Pike, Lander, & Hirschhorn, 2003), they have been identified many other novel associations in genes that until the moment were not considered as strong enough candidates for the disease under test. A modest increase in risk implies that large well-designed and analyzed studies are required to detect and confirm signals for association, leading to the need of improvement of SNP data analysis methodologies (Lewis & Knight, 2012; C. Wu et al., 2012) .

The case–control study is the simplest study design used to test for association, in which a series of cases affected by the disease of interest are collected together with a series of control individuals that can be selected by different methods: the use of either a series of individuals who have been screened as negative for presence of the disease or randomly ascertained from the population, with unknown disease status, or a group of individuals with different phenotypes for a particular trait. This approach is known as phenotype-first, in which the participants are classified first by their clinical manifestation(s), as opposed to genotype-first (Lewis & Knight, 2012). The specific phenotype used for the cases selection may define the exact hypothesis to be tested, and the application of strict and

rigorous clinical criteria is necessary to ensure a homogeneous set of cases.

Both last control sets form a valid test for association, and have similar power for a rare disease analysis. But when is necessary to evaluate a more common disease, a study with screened unaffected controls will have higher power to detect association compared with a study using population-based controls, and the increase in power is notable for diseases with high prevalence. For some diseases, screening controls for the presence or absence of the disease may be difficult, and using a larger sample of unscreened controls may be more efficient (Lewis & Knight, 2012).

A sample of DNA is obtained from each person, from which millions of genetic variants are read using SNP arrays. If one type of the variant (one allele) is more frequent in people with the disease, the variant is said to be associated with the disease. The associated SNPs are then considered to mark a region of the human genome that may influence the risk of disease (Lewis & Knight, 2012).

Other approaches to genetic association studies include family-based association studies and quantitative trait locus studies.

In conclusion, by association studies carried out, it seems to exist clear evidence that SNPs may confer some significant risk increase in the development of the disease. However, the information obtained in risk analysis can be an asset into a preventive or therapeutic approach to the disease, although this may not be well established in all the situations (Tempfer, Hefler, Schneeberger, & Huber, 2006).

Part of the work developed and presented in this dissertation was based on case-control association studies. The obtained information came once again to demonstrate the usefulness and relevance of this type of studies.

1.3 – CASPASES AND APOPTOSIS

The term "apoptosis" is derived from the Greek words "*απο*" and "*πτωσις*" meaning "dropping off", regarding to the falling of leaves from trees in autumn. Since the description of apoptosis or programmed cell death process in the 1970' by Kerr *et al. (Kerr, Wyllie, & Currie, 1972),* who linked this mechanism to the elimination of potentially malignant cells, hyperplasia and tumor progression, it remains one of the most challenging investigated processes in biologic research (Wong, 2011).

Apoptosis is critically important during various developmental processes and its role in normal physiology is as significant as that of its complementary counterpart, mitosis. It is estimated that to maintain homeostasis in the adult human body, around 10 billion cells are made each day just to balance those dying by apoptosis. And that number can increase significantly when there is increased apoptosis during normal development and aging or during disease (Elmore, 2007).

In contrast to necrosis, apoptosis is a highly selective process, generally characterized by distinct morphological characteristics (Table 1.9) and energy-dependent biochemical mechanisms, in which a cell pursues its live course until the moment that certain stimuli induce death (Elmore, 2007). It constitutes a vital component of various cellular mechanisms, including normal cell turnover, proper development and functioning of the immune system and central nervous system, hormone-dependent atrophy, embryonic development, tissue homeostasis, chemical-induced cell death and removal of damaged cells, which are involved and condition both physiological and pathological states, such as inflammation and cancer (Elmore, 2007; Wong, 2011).

Table 1.9 – Morphological features of apoptosis and necrosis.
(Adapted from (Elmore, 2007).

Apoptosis	Necrosis
Single or small clusters of cells	Several, often contiguous cells
Cell shrinkage and convolution	Cell swelling
Pyknosis and karyorrhexis	Karyolysis, pyknosis, and karyorrhexis
Intact cell membrane	Disrupted cell membrane
Cytoplasm retained in apoptotic bodies	Cytoplasm released
No inflammation	Inflammation usually present

Modifications in apoptotic genes caused by mutations or epigenetic modeling of gene expression and disruption of apoptosis mechanisms have been identified as a cause of the onset of some diseases, such as cancer, AIDS and other immune disorders, cardiovascular diseases, including stroke and other ischemic processes, other inflammatory disorders and neurodegenerative diseases (such as Alzheimer and Parkinson diseases) (S. G. Cho & Choi, 2002; Elmore, 2007).

Cancer is the result of successive genetic changes, during which a normal cell is transformed into a malignant one, associated to a loss of balance between cell division and cell death, with persistence of the malignant cells. Thus, cell growth is the result not only of uncontrolled proliferation but also of reduced cell death. Proteins that signal cell errors or aberrant growth signals arrest the cell cycle so that these errors can be repaired, or alternatively, for apoptosis to be induced. Malfunction in this system leads to the onset of cancer, allowing cells to proliferate when they should be repaired or die. Hence, reduced apoptosis or its resistance plays a vital role in carcinogenesis, existing many mechanisms by which cell evasion can occur: 1) disrupted balance of pro-apoptotic and anti-apoptotic proteins, 2) reduced caspase function and 3) impaired death receptor signaling (Wong, 2011).

The clarification of apoptotic mechanisms in disease conditions is crucial, as it not only gives insights into the pathogenesis of a disease but may also provide clues on possible apoptotic genes or pathways targets for the development of drugs with great therapeutic potential (Elmore, 2007; McIlwain, Berger, & Mak, 2013; Wong, 2011).

Robert Horvitz and Junying Yuan initially established the importance of caspases in apoptosis, in 1993. Caspases are a family of endoproteases that hydrolyze peptide bonds in a reaction that depends on catalytic cysteine residues in the caspase active site, which can result in substrate inactivation or generate active signaling molecules that participate in ordered processes such as apoptosis and inflammation (McIlwain et al., 2013). They can be organized into three functional groups: initiator, executioner and inflammatory caspases (Figure 1.24).

Such as many other protein systems, initially caspases are synthesized as inactive zymogens, pro-caspases. **Initiator caspases 8 and 9** inactive procaspase monomers are activated and gain catalytic activity following signaling events, promoting their aggregation into stable dimers or macromolecular complexes (Boatright & Salvesen, 2003). **Executioner caspases 3, 6, and 7**, also initially as inactive procaspase dimers, are cleaved by initiator caspases, which allows a conformational change that brings the two active sites of the executioner caspase dimer together and creates a functional mature protease (Riedl & Shi, 2004). Once activated, a single executioner caspase can cleave and activate other executioner caspases, leading to a chain reaction and accelerated feedback loop of caspase activation. Some procaspases can also aggregate and autoactivate.

Activation of apoptotic caspases induce inactivation or activation of substrates, and the generation of a cascade of amplified signaling events permitting the cleavage of cellular components, in order to induce the morphological changes leading to rapid cell death (Elmore, 2007; McIlwain et al., 2013; Wong, 2011).

The other caspases that have been identified include the **inflammatory caspases** (caspase 1, 4, 5), caspase 11, which is reported to regulate apoptosis and cytokine maturation during septic shock, caspase 12, which mediates endoplasmic-specific apoptosis and cytotoxicity by amyloid-$\boldsymbol{\beta}$, caspase 13, which is suggested to be a bovine gene, and caspase 14, which is highly expressed in embryonic tissues but not in adult tissues (Elmore, 2007).

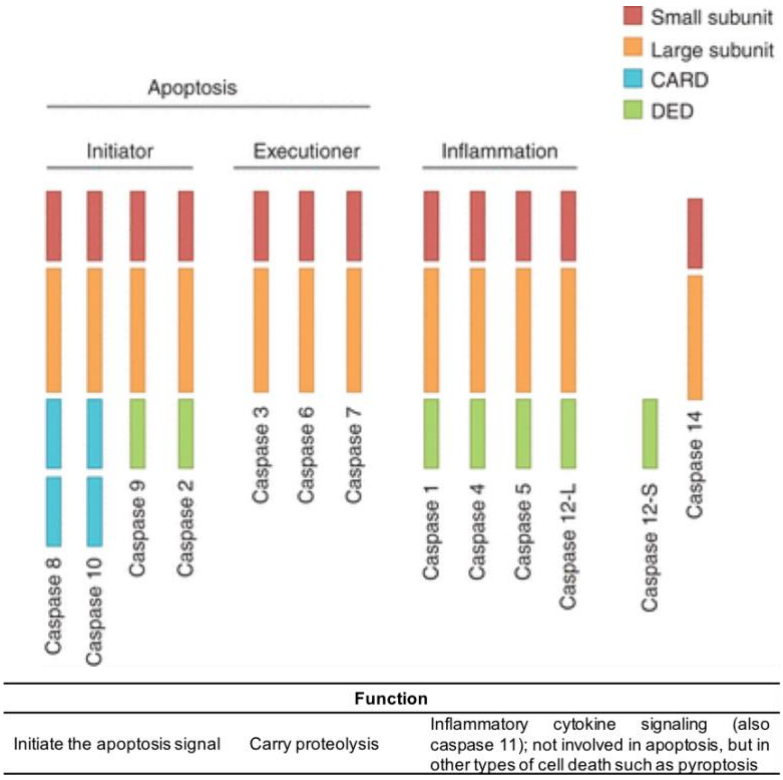


Figure 1.24 – Caspases domain structures and functions.
CARD - Caspase recruitment domain; DED - Death effector domain (Adapted from (Elmore, 2007).

MECHANISMS OF APOPTOSIS

As referred above, caspases are central to the mechanism of apoptosis. It is mediated via different highly complex pathways by which caspases can be activated, distinguished by the adapters and initiator caspases involved, being capable of inducing programmed cell death following exposure to apoptotic insults and the accumulation of excess DNA damage (Malherbe et al., 2016).

The two commonly described initiation pathways are the intrinsic (or mitochondrial) and extrinsic (or death receptor) pathways of apoptosis, represented in Figure 1.25. Other apoptosis initiation pathways include the perforin/granzyme dependent pathway, P53 and the less well-known intrinsic endoplasmic reticulum pathway. The extrinsic, intrinsic and granzyme B pathways lead to a common pathway - the execution phase of apoptosis (Figure 1.25).

The **intrinsic pathway of apoptosis** (Figure 1.25) is also known as mitochondrial apoptosis because it depends on factors released from the mitochondria, existing a growing evidence that this pathway is crucial for tumorigenesis (Brenner & Mak, 2009).

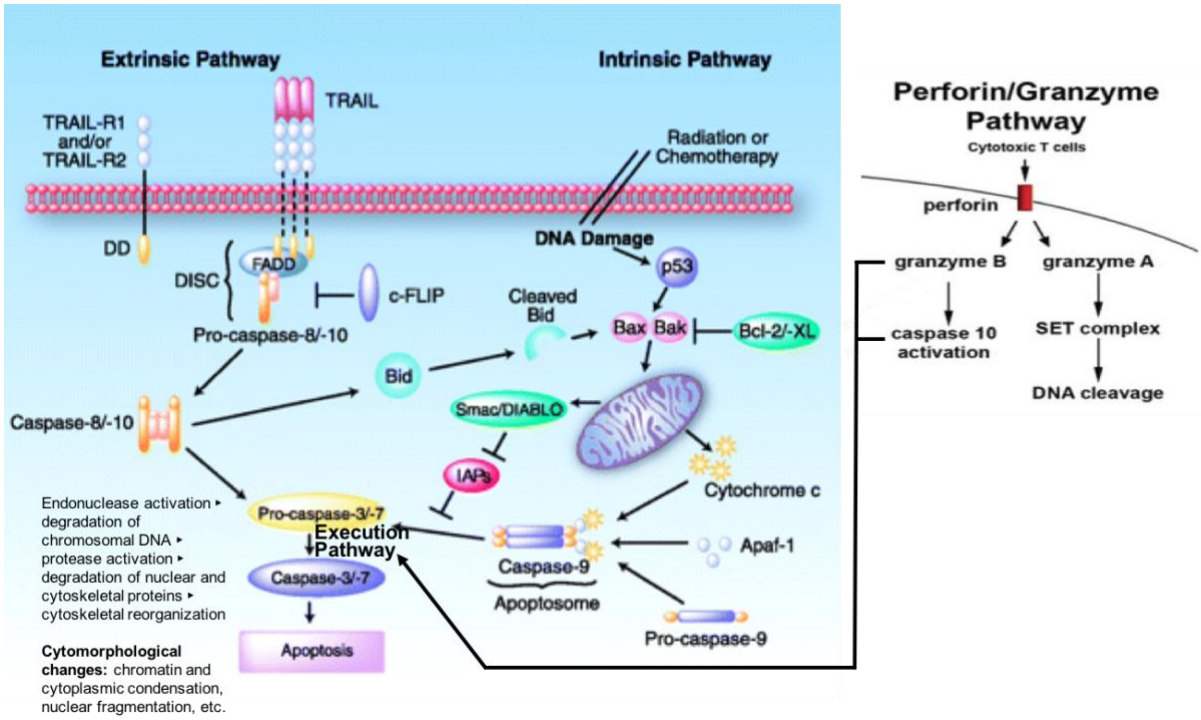


Figure 1.25 – Diagrammatic representation of the extrinsic, intrinsic and perforin/granzyme apoptotic pathways.
(Adapted from (Carlo-Stella et al., 2007; Elmore, 2007).

This pathway is activated by several cellular stresses, including growth factor deprivation, cytoskeletal disruption, DNA damage, accumulation of unfolded proteins, extremely high concentrations of cytosolic Ca^{2+}, severe oxidative stress, hypoxia, hormones and many others (Brenner & Mak, 2009; McIlwain et al., 2013; Wong, 2011). Regardless of the stimuli, intrinsic apoptosis pathway is the result of increased mitochondrial permeability and the release of pro-apoptotic molecules such as cytochrome-c into the cytoplasm. This molecule binds an adaptor protein (apoptotic protease activating factor-1, APAF-1), in an independent way of its mitochondrial electron transport function, as it was demonstrated by Hao *et al.* in 2005 (Hao et al., 2005), and recruit initiator caspase 9 (via CARD-CARD interactions).This leads to the formation of a caspase activating multiprotein complex called the apoptosome, that will cleave and activate other executioner caspases, such as caspase 3 (Brenner & Mak, 2009; McIlwain et al., 2013; Wong, 2011).

This pathway is closely regulated by a group of proteins belonging to the B-cell lymphoma 2 (Bcl-2) family (Wong, 2011). These can be divided in two groups, namely the pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic proteins (e.g. Bcl2, BclxL, Bcl-W, Bfl-1 and Mcl-1) (Wong, 2011). While the anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome-c, the pro-apoptotic proteins act by promoting such release. It is the balance between these pro- and anti-apoptotic proteins that determines whether apoptosis would be initiated (Wong, 2011).

Other molecules that are released from the mitochondrial intermembrane space into the cytoplasm with the capacity of induce caspase activation are apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct IAP Binding protein with Low pI (DIABLO) and Omi/high temperature requirement protein A (HtrA2) (Wong, 2011). These molecules bind to inhibitor of apoptosis proteins (IAPs), subsequently leading to disruption of their interaction with caspase 3 or 9 (Wong, 2011). One of the IAPs that specifically constrain the pro-death actions of caspase 9 via DIABLO, conferring a cytoprotective effect, is survinin. Downregulation has been associated with progressive disease and poor survival in both solid and hematological malignancies, both leukemias and lymphomas (Malherbe et al., 2016).

In the **extrinsic apoptopic pathway** (Figure 1.25), the caspase cascade is also activated by extracellular ligands, via cell surface death receptors. Although several death receptors have been described, the best known are type 1 TNF receptor (TNFR1) and a related protein called Fas (CD95), and their ligands are TNF and Fas ligand (FasL), respectively. Others include death receptor 3 (DR3), TNF-related apoptosis-inducing ligand receptor-1 (TRAIL-R1; also called DR4), and TRAIL-R2 (also called DR5 in humans) (McIlwain et al., 2013; Wong, 2011). These death receptors have an intracellular death domain that recruits adapter proteins such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD). Binding of the death ligand to the death receptor results in the formation of a binding site for an adaptor protein, with the formation of a multiprotein Death Inducing Signaling Complex (DISC) that recruits and activates pro-caspase 8 monomers (McIlwain et al., 2013; Wong, 2011). This could lead to either downstream activation of the intrinsic pathway by inducing mitochondrial stress, or direct activation of executioner caspases (3, 6 and 7) to degrade cellular components, depending on the cell type, based in their content of IAPs, which block executioner caspase function unless suppressed by proteins released from the mitochondria (Jost et al., 2009; Spencer, Gaudet, Albeck, Burke, & Sorger, 2009). In type I cells, caspase 8 initiates apoptosis directly by cleaving and thereby activating executioner caspases, while in type II cells, caspase 8 must first activate the intrinsic apoptotic pathway to induce efficient cell death (Samraj, Keil, Ueffing, Schulze-Osthoff, & Schmitz, 2006).

The **granzyme B dependent pathway** (Figure 1.25) involves the release of this protease, contained in specialized granules released by cytotoxic T lymphocytes or by natural killer cells, into certain target cells. Perforin is also released in those granules, induces oligomerization of cell membrane and facilitates the entry of granzyme B, which will participate in executioner caspases activation, namely caspase 3 (Taylor, Cullen, & Martin, 2008).

Granzyme A is also important in cytotoxic T cell induced apoptosis and activates parallel caspase independent pathways, contributing to apoptosis by blocking the maintenance of DNA and chromatin structure integrity (Elmore, 2007).

The intrinsic, extrinsic and granzyme B pathways converge on the same terminal, in **the execution pathway** (Figure 1.25). This pathway is initiated by the cleavage of caspase 3, which in turn cleaves the inhibitor of the caspase-activated deoxyribonuclease, which is responsible for nuclear

apoptosis. Caspase 3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (8, 9, or 10). In addition, downstream caspases induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins and inhibitory subunits of endonucleases family. They also have an effect on the cytoskeleton, cell cycle and signaling pathways, which together result in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands and cell surface markers for phagocytic cell receptors and finally uptake by phagocytic cells, contributing to the typical morphological changes in apoptosis (Elmore, 2007; Wong, 2011). Although externalization of phosphatidylserine is a well-known recognition ligand for phagocytes on the surface of the apoptotic cell, some studies have shown that other proteins, such as annexin I and calreticulin that interact with phosphatidylserine, are also exposed on the cell surface during apoptotic cell clearance (Elmore, 2007).

Another pathway of induction of apoptosis involves the **P53 nuclear protein**, which is activated in response to DNA errors. P53 is a regulator of cell cycle, inducing arrest of cell cycle, senescence and promoting DNA repair mechanisms. Failing these, p53 activates the intrinsic apoptotic cascade by binding Bak/Bax to induce cytochrome-c release, simultaneously inhibits antiapoptotic Bcl2-related proteins and survinin (Malherbe et al., 2016) and triggers transcription of genes whose products produce reactive oxygen species (Ding et al., 2000; Vousden & Lane, 2007).

The **intrinsic endoplasmic reticulum (ER) pathway** is a third pathway and is less well known, which is believed to be caspase 12-dependent and mitochondria-independent. When the ER is injured by cellular stresses such as hypoxia, free radicals or glucose starvation, there is misfolding of proteins, leading to ER stress and reduced protein synthesis in the cell, and an adaptor protein known as TNF receptor associated factor 2 (TRAF2) dissociates from procaspase 12, resulting in the activation of the latter (Wong, 2011). In turn, caspase 12 activates caspase 9 and ER stress.

CASPASES AND CANCER

Tumor cells can acquire resistance to apoptosis by the expression of anti-apoptotic proteins such as Bcl2 or by the down-regulation or mutation of pro-apoptotic proteins such as Bax, whose expression is regulated by the ***p53*** tumor suppressor gene. Another method of apoptosis suppression in cancer involves evasion of immune surveillance (Elmore, 2007).

Deregulation of caspases and their pathways is involved in the persistence of mutated cells and promote tumorigenesis. However, several studies have shown that the inactivation of individual caspases is not usually sufficient to either interrupt the caspase cascade, or to disturb alternative nonapoptotic cell death mechanisms. Instead, malignant cells appear to more frequently gain a survival advantage by inactivating signaling mediators involved in caspase activation (McIlwain et al., 2013).

Despite the above, the reduced expression of proapoptotic caspases has been reported in a great diversity of neoplasms, and specific inactivating mutations, although not frequent, have been

linked to various tumor types and stages of transformation. Moreover, certain caspase polymorphisms thought to affect caspase abundance or activity have been associated with variable effects on tumorigenesis (McIlwain et al., 2013).

Alterations in caspase biology, including caspase 8 (extrinsic) and caspase 9 (intrinsic), have been implicated in a number of malignancies in humans and animal models.

In **caspase 8** deficient mice, B-lymphocytes have impaired cytokinesis and chromosomal instability. Isolated, caspase 8 polymorphisms have been associated to multiple cancers, including with brain tumors (neuroblastoma) (Cacina et al., 2015; Rihani et al., 2014), non-Hodgkin lymphoma (Lan et al., 2009) and in a published meta-analysis study to breast, lung and gastrointestinal cancers susceptibility (Ji, Li, Cui, & Wang, 2014; McIlwain et al., 2013; Sun et al., 2007). Another study provided evidence that FAS-FASL-CASP8 polymorphisms contributed to a reduced risk of childhood acute lymphoblastic leukemia (Tong et al., 2012).

Both caspase 8 and **caspase 9** are potentially important in regulating megakaryocyte turnover in the MPNs. In megakaryocytes, caspase 9 activity appears necessary for proplatelet formation and caspase 9 loss may enhance the tumorigenic potential of megakaryocytes, promoting their hyperplasia and thrombocytosis (Malherbe et al., 2015; Malherbe et al., 2016). The pathobiological basis underlying these numerical and morphological megakaryocytic abnormalities is thought to result from multiple molecular disruptions promoting proliferation and enhancing survival. Although the mechanism is not well clarified, frameshift lesions targeting *CALR* may disrupt megakaryocyte apoptosis, through its inability to facilitate caspase 8 activation and antiapoptotic protein cleavage (Malherbe et al., 2016). These megakaryocytes have impaired death mechanisms conferred by overexpression of antiapoptotic BclxL and reductions in pro-death BNIP-3. These changes are universal in PN-MPNs, but there are differences between entities. Megakaryocytes in ET have been shown to have a more proliferative profile, whereas in PMF they exhibit greater proapoptotic impairment. These changes occur irrespective of the *JAK2* V617F or *CALR* driver mutations, although those with a *CALR* lesion have greater proapoptotic dysfunction (Malherbe et al., 2015).

Caspase pathway genes are highly polymorphic and its occurrence have been correlated to several disorders. Caspase 9 gene polymorphisms and its downregulation are associated with solid tumors and their malignant progression. Alterations of caspase 9 gene and SNPs have been linked to non-Hodgkin's lymphoma and lung cancer (McIlwain et al., 2013). For instance, a recently published manuscript come to reinforce the role of caspase 9 gene polymorphisms in the severity of prostate cancer (Yilmaz, Yencilek, Yildirim, Yencilek, & Isbir, 2017) and renal cell carcinoma (Marques et al., 2013). In a study developed in papillary thyroid carcinoma patients, caspase 9 was observed to be associated with susceptibility to the disease, while Bcl2 polymorphism evidenced to play a protective role (Y. X. Wang, Zhao, Wang, Liu, & Yu, 2012). Cingeetahm *et al.* also reported its association with AML (Cingeetham et al., 2014).

It is also well known the association of caspase 8 and 9 genes polymorphisms with clinical and molecular phenotypes in breast cancer (Brynychová et al., 2016). Both caspase 8 and 10

polymorphisms seem to be associated with breast cancer and melanoma (Frank et al., 2005; C. Li et al., 2008; MacPherson et al., 2004; McIlwain et al., 2013; H. L. Park et al., 2016).

Caspase 7 polymorphisms have been associated to cervical cancer (Shi et al., 2015) and gastric adenocarcinoma (M. Y. Wang et al., 2013). Park *et al.* also demonstrated that there is association between caspases 7 and 14 genetic polymorphisms and the risk of childhood leukemia (Park et al., 2012).

Polymorphisms in **caspase 3** gene have also been associated with lung cancer (David, O'Shea, & Kundu, 2007; Lin et al., 2016), esophageal squamous cell carcinoma (Z. Zhang et al., 2012) and hepatocellular carcinoma (Deng et al., 2016), as it has been described by several authors.

Another study reported an association of death receptor 4, caspases 3 and 5 genes polymorphisms with increased risk to bladder cancer (Mittal et al., 2011). Caspases 3 and 9 polymorphisms were found to play a protective role in multiple myeloma (Hosgood et al., 2008), further, variants in caspases 3, 8 and 10 were associated with a decreased risk of marginal zone lymphoma and variants in caspases 3 and 10 were associated with a lower risk of chronic lymphocytic leukemia and related subtypes (Lan et al., 2007).

Caspases 1, 4, 5 and 6 have been associated with cancers of colonic or gastric origin and rare **caspase 10** mutations have also been detected in cases of T-cell acute lymphoblastic leukemia and multiple myeloma, as well as in colon, breast, lung, hepatocellular and gastric cancers (McIlwain et al., 2013). On the other hand, no association was found between caspase 10 polymorphisms and susceptibility to lymphoma, myeloma, melanoma, or lung cancer, as it was reported in a meta-analysis study (S. Yan et al., 2012).

Chapters 7 and 9 of this dissertation are dedicated to the study of polymorphisms in caspases genes and their possible association with PN-MPNs susceptibility and clinical outcome, respectively.

1.4 – GENETIC LESION REPAIR: BASE EXCISION REPAIR

Organisms' genome stability is crucial, but every day this equilibrium is subject to the test of constant threats. Our cells are systematically exposed to insults, estimated to account daily for 10^4 events per cell, from various endogenous and environmental damaging agents, capable of modify the structural properties of DNA bases, induce lesion and alter or eliminate fundamental cellular processes, such as DNA replication or transcription, and compromise cell viability (Baute & Depicker, 2008).

DNA is susceptible to several forms of endogenous damage, such as spontaneous hydrolysis (Lindahl, 1993), chemical modification by reactive molecules that are created during normal cellular metabolism (ex: reactive oxygen species – ROS, reactive nitrogen species, alkylation) (Apel & Hirt, 2004; De Bont & van Larebeke, 2004; Marnett, 2000) and physiological DNA processing reactions (ex: replication, DNA repair processes themselves) (Bridges, 2005; McCulloch & Kunkel, 2008; Shimizu et al., 2003).

Reactive oxygen species produced during cell metabolism can react randomly with lipids, proteins and nucleic acids causing oxidative stress and lesions in these macromolecules. The great consequence of oxidative stress, in fact, are the DNA lesions responsible for great genomic instability (Maynard, Schurman, Harboe, de Souza-Pinto, & Bohr, 2009).

Besides the numerous endogenous sources of DNA damage, cellular DNA is also under constant attack from exogenous or environmental DNA-damaging agents. These include physical stresses, such as ultraviolet light (UV) from the sun (Ravanat, Douki, & Cadet, 2001), ionizing radiation (Ward, 1988) and chemical agents (Irigaray & Belpomme, 2010; Wogan, Hecht, Felton, Conney, & Loeb, 2004)

DNA lesions commonly include base and sugar modifications, single- and double-strand breaks, DNA-protein cross-links, and base-free sites. Specific DNA lesions can also induce mutations that cause cancer or other diseases as well as contribute to the aging process (Dexheimer, Antony, Marchand, & Pommier, 2008).

Depending on the type of damage inflicted on the DNA's double helical structure, and to counteract the deleterious effects of DNA damage, a complex set of cellular responses are elicited, through a developed and specialized DNA repair system, that senses the DNA damage, signals its presence, and mediates its repair, in order to restore lost information. The importance of this mechanism is underscored by the prevalence of neurological and cancer susceptibility disorders, such as Ataxia-telangiectasia, Fanconi anemia, and Xeroderma pigmentosum, that are caused by DNA repair deficiencies (McKinnon, 2009).

In hematopoietic system, DNA damage to precursor cells would appear to be essential to malignant transformation and leukemia development, notwithstanding DNA repair systems act to repair the DNA damage, thus maintaining genetic integrity (Batar et al., 2009; Bănescu et al., 2014).

This coordinated system can be subdivided into five distinct mechanisms based on the type of DNA lesion (Baute & Depicker, 2008).

When only one of the two strands of the double helix is affected, the other strand can be used as a template to guide the correction of the damaged strand. There exist a number of excision repair mechanisms that remove the damaged nucleotide and replace it with another, which is complementary to that found in the undamaged DNA strand - base excision repair, mismatch repair and nucleotide excision repair (Figure 1.26).

Double-strand breaks, in which both strands in the double helix are severed and neither strand can then serve as a template for repair, can lead to genome rearrangements and are particularly deleterious to cells. Mechanisms to repair this type of lesions comprise both homologous recombination and non-homologous end-joining pathways (Figure 1.26).

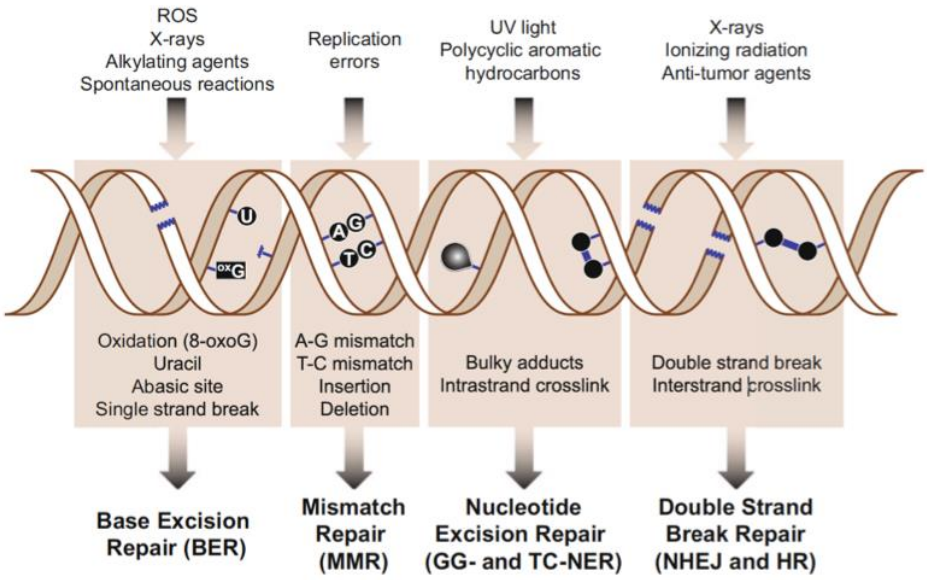


Figure 1.26 – DNA damage agents and repair mechanisms.
GG – Global genomic; TC – Transcription coupled repair (Adapted from (Boland, Luciani, Gasche, & Goel, 2005).

In this dissertation, base excision repair (BER) pathway will be focused in more detail below. The other pathways will be briefly described next.

Mismatch repair pathway is present in essentially all cells to recognize and repair erroneous insertion, deletion, and mis-incorporation of bases that can arise during DNA replication and recombination, as well as repair of other forms of DNA lesion. This system consists of at least two proteins, in which one detects the mismatch, and the other recruits an endonuclease that cleaves the newly synthesized DNA strand close to the region of damage. This is followed by removal of damaged region by an exonuclease, re-synthesis by DNA polymerase, and nick sealing by DNA ligase (Larrea, Lujan, & Kunkel, 2010).

Nucleotide excision repair pathway is a highly evolutionarily conserved repair mechanism, repairing damaged DNA which commonly consists of bulky, helix-distorting damage, such as pyrimidine dimerization caused by UV light. Damaged regions are removed in 12-24 nucleotide-long strands in a three-step process which consists of recognition of damage, excision of damaged DNA by endonucleases, both upstream and downstream of damage site, and re-synthesis of removed DNA region (Reardon & Sancar, 2006).

Non-homologous end joining pathway repairs double-strand breaks in DNA. It is referred to as "non-homologous" because the break ends are directly joined without the need for a homologous template. This pathway typically uses short homologous DNA sequences called microhomologies to guide repair, which are often present in single-stranded overhangs on the ends of double-strand breaks. When the overhangs are perfectly compatible, usually the break is repaired accurately. Imprecise repair leading to loss of nucleotides can also occur, but is much more common when the overhangs are not compatible (Budman & Chu, 2005). This pathway dysfunction can lead to translocations and telomere fusion, both involved in tumorgenesis (Espejel et al., 2002).

In contrast, **homologous repair pathway** requires a homologous sequence to guide repair, in which nucleotide sequences are exchanged between two similar or identical molecules of DNA, to accurately repair harmful breaks that occur on both strands of DNA (double-strand breaks). This pathway also produces new combinations of DNA sequences during meiosis, representing genetic variation. Although it varies widely among different organisms and cell types, most forms involve the same basic steps. After a double-strand break occurs, sections of DNA around the 5’ ends of the break are resected, followed by an overhanging 3’ end of the broken DNA molecule that "invades" a similar or identical DNA molecule that is not broken. Homologous recombination that occurs during DNA repair tends to result in non-crossover products, in effect restoring the damaged DNA molecule as it existed before the double-strand break (Jasin & Rothstein, 2013).

Several polymorphisms in DNA repair genes have been identified, with the capacity of affect protein function and thus DNA damage repair, leading to susceptibility to malignancy, in spite of their low genetic penetrance (Batar et al., 2009; Bolufer et al., 2006; Bănescu et al., 2014; Hoeijmakers, 2001; C. Q. Wang et al., 2014). A nucleotide excision repair gene polymorphism displayed strong association with leukemic transformation and development of non-myeloid malignancies in patients with ET and PV (Hernández-Boluda et al., 2012).

Moreover, the DNA damage theory of aging, supported by several review articles, proposes that aging is a consequence of deficient DNA repair, resulting in the accumulation of naturally occurring DNA lesions. On the other hand, an increased DNA repair facilitates greater longevity (Best, 2009; M. Cho & Suh, 2014; Freitas & de Magalhães, 2011).

BASE EXCISION REPAIR PATHWAY

The base excision repair (BER) pathway is critical in maintaining genomic integrity. It is the predominant pathway for recognizing, excising, and repairing a broad range of small lesions of a single base resulting from oxidation, methylation, deamination alkylation, and hydroxylation, caused by free radicals formed endogenously or after exposure to exogenous agents such as ionizing radiation, which do not significantly distort the overall structure of the DNA helix (Hoeijmakers, 2001; Wilson, Kim, Berquist, & Sigurdson, 2011; Zharkov, 2008).

This pathway recognizes and repairs modified bases, as well as apurinic/apyrimidinic sites (APs) and single-stranded DNA breaks, mostly produced by ROS (Maynard et al., 2009).

DNA bases are very sensitive to ROS oxidation, especially guanine because of their low redox potential, so one of the best characterized ROS-generated lesions is 8-hydroxyguanine (8-oxoG), a highly mutagenic lesion (Maynard et al., 2009).

BER is typically initiated by the series of lesion-specific DNA glycosylases that recognize and remove the specific damaged or inappropriate base, forming AP sites. These are then cleaved by an AP endonuclease and the resulting single-strand break can then be processed by either short-patch or long-patch BER, by which the new strand is correctly synthesized using the complementary strand as a template (Figure 1.27) (Liu et al., 2007).

BER is described as a highly coordinated pathway of consecutive enzymatic reactions, involving several proteins, such as 8-oxoguanine DNA glycosylase (OGG1), earlier mutY homolog (*E. coli*) (MUTYH), apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1), X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) or poly (ADP-ribose) polymerase (PARP1 and PARP4) (Baute & Depicker, 2008), that act to detect the lesion, remove the single damaged base, and fill in the resulting single-stranded gap using the intact complementary strand as template (Hoeijmakers, 2001; Hung, Hall, Brennan, & Boffetta, 2005; Wilson et al., 2011).

The BER pathway presents two sub-pathways depending on the type of damage encountered at the onset as well as throughout the BER process, and the enzymes involved in this response: the shorter and more frequent *Short-patch BER,* where a single nucleotide is replaced, and the *Long-patch BER*, usually to repair a lesion between 2 to 10 nucleotides that are synthesized (Figure 1.27) ((Hoeijmakers, 2001; Y. J. Kim & Wilson, 2012; Maynard et al., 2009; Sancar, Lindsey-Boltz, Unsal-Kaçmaz, & Linn, 2004). Each pathway shares a set of common elements and generally invokes the five following steps (Y. J. Kim & Wilson, 2012):

1) Recognition and removal of an incorrect or damaged substrate base by a DNA glycosylase, to cleave the sugar–nitrogen base bond, to delete the base and create an abasic site (AP site) in the DNA strand. Each DNA glycosylase has its unique specificity, according to the type of lesion to be repaired, and many of them are ubiquitous in microorganisms, mammals, and plants (Baute & Depicker, 2008). There are DNA glycosylases that recognize oxidized/reduced bases, alkylated bases (usually methylated), deaminated bases and mismatch bases (Maynard et al., 2009; Sancar et al., 2004). Their

classification is based on their reaction mechanisms, some act as simple glycosylases catalyzing the base removal reaction forming an AP site, which will involve the action of another enzyme with lyase function (usually APE1) responsible for the cleavage of the chain, while others play the dual glycosylase and AP lyase function;

2) Intermediate abasic site incision by an AP endonuclease or AP lyase;

3) Removal of the remaining sugar fragment by a lyase or phosphodiesterase;

4) Gap filling by a DNA polymerase. When the injured base is removed by a glycosylase/AP lyase, responsible for the cleavage of the 3'-phosphodiester bond of the abasic site, the APE1 endonuclease breaks the 5' bond and recruits the DNA Polymerase β (Polβ) which will fill the gap formed, the chain binding being effected by the Ligase 3/XRCC1 complex. When the AP site is generated by spontaneous hydrolysis, the 5'-phosphate group becomes modified and becomes resistant to Polβ elimination, with repair continuing through the long-patch route;

5) Sealing of the nick by a DNA ligase. After recognition of the lesion, a PCNA/DNA polymerase complex δ/ε (Pol δ / ε) is recruited, responsible for adding more nucleotides to the 3' end of the AP site, forming a loop that is removed by an endonuclease (FEN1), followed by junction of DNA ends by DNA ligase I (Dianov, Sleeth, Dianova, & Allinson, 2003; Q. M. Zhang & Dianov, 2005).

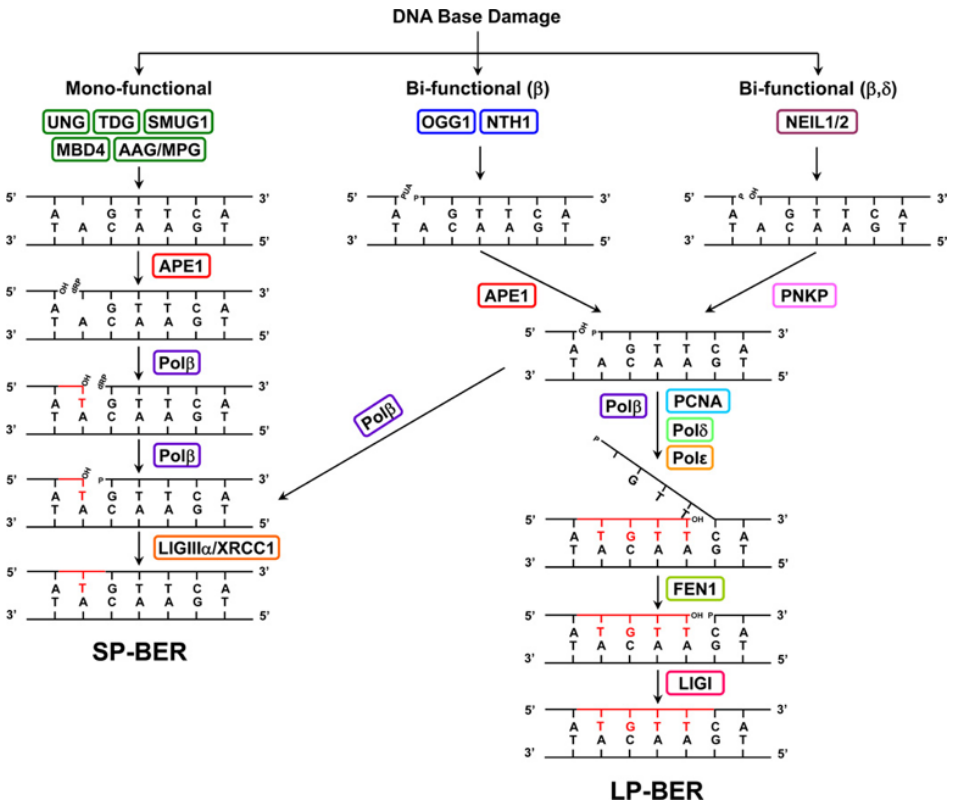


Figure 1.27 – BER pathway.
LP – Long patch; SP – Short patch. Adapted from (Y. J. Kim & Wilson, 2012).

OGG1 is a DNA glycosylase enzyme responsible for the excision of 8-oxoguanine (8-oxoG), a mutagenic base byproduct that occurs as a result of exposure to ROS. OGG1 is a bifunctional glycosylase, as it is able to both cleave the glycosidic bond of the mutagenic lesion and cause a strand break in the DNA backbone (Bjørås, Seeberg, Luna, Pearl, & Barrett, 2002).

OGG1 facilitates removal of these lesions, producing an AP site in the DNA, that is subsequently incised by AP-endonuclease 1 (**APE1**). APE1 is a multifunctional protein involved in stimulation of turnover of several glycosylases by accelerating rate-limiting product release, in base excision repair function in its C-terminal domain, and in the redox regulation of transcription factors in its N-terminal domain, converting certain transcription factors from inactive oxidized to active reduced forms (Parker et al., 2001).

MUTYH glycosylase is also involved in oxidative DNA damage repair. The enzyme excises adenine bases from the DNA backbone at sites where adenine is inappropriately paired with guanine, cytosine, or 8-oxo-7,8-dihydroguanine, a major oxidatively damaged DNA lesion. The protein is localized to the nucleus and mitochondria (Parker et al., 2001).

XRCC1 protein is involved in repair of DNA single-strand breaks, formed by exposure to ionizing radiation and alkylating agents (base excision repair and nucleotide excision repair), interacting with DNA ligase III, polymerase beta and poly(ADP-ribose) polymerase, without individual enzymatic activity (London, 2015). But it also has an essential role in double strand breaks repair (homologous repair pathway) (Sharma et al., 2015).

PARP1 has a role in repair of single-stranded DNA breaks, and in conjunction with BRCA acts on double strands (homologous repair pathway) (Sharma et al., 2015). It modifies various nuclear proteins by poly(ADP-ribosyl)ation and is involved in the regulation of various important cellular processes such as differentiation, proliferation, and tumor transformation. Besides DNA repair, PARP1 also functions as a transcriptional regulator, acts on chromatin remodeling complexes to control DNA accessibility for RNA polymerase, and functions as a transcription factor by binding an octamer motif in promoter elements to regulate gene expression (Ko & Ren, 2012).

PARP4, the largest protein of PARP family, is involved in DNA repair and cell cycle regulation (Perina et al., 2014).

In addition to the enzymes referred to as participants in this repair pathway, **other protein factors** have been identified as modulators of BER pathway activity. These auxiliary proteins may interact with the major pathway and/or DNA proteins, in order to increase the enzymatic activity or the efficiency of the reactions (Fan & Wilson, 2005; Maynard et al., 2009). Among the vast array of proteins is emphasized the action of p53, a tumor suppressor protein, which is presumed to facilitate the binding activity of Polβ to DNA; and MSH2/MSH6 mismatch recognition complex, that seems to promote the repair of the damaged base “marked” to be repaired by BER pathway (Fan & Wilson, 2005).

BER protects against cancer, aging, and neurodegeneration and takes place both in nuclei and mitochondria. More recently, an important role of uracil-DNA glycosylase UNG2 in adaptive immunity

was revealed. Furthermore, other DNA glycosylases may have important roles in epigenetics, thus expanding the repertoire of BER proteins (Krokan & Bjørås, 2013).

Previous reports have identified several polymorphisms identified in the *XRCC1*, *OGG1*, *MUTYH*, *APEX1* and *PARP1* genes as being associated with individual susceptibility to several types of cancer, including breast, lung, colorectal and skin cancers (Hoeijmakers, 2001; Hung et al., 2005; Silva et al., 2007; Wilson et al., 2011; L. Yan, Li, Li, Ji, & Zhang, 2016). However, except for XRCC1 polymorphisms, it seems that there is no association between BER and thyroid cancer (Santos et al., 2012).

Moreover, several polymorphisms in genes of the BER pathway [*APEX1*, *MUTYH*, *OGG1*, *PARP1*, *PARP4* and *XRCC1*] have been identified and studied for their association with the risk of leukemia and disease outcome (Annamaneni et al., 2013; Bolufer et al., 2006; Bănescu et al., 2014).

More considerations related to each one of the several polymorphisms in genes of the BER pathway will be discussed in more detail in Chapters 8 and 9 of this dissertation, dedicated to the presentation of results obtained on the study of polymorphisms in BER genes and their possible association with PN-MPNs susceptibility and clinical outcome, respectively.

1.4 – REFERENCES

Abdel-Wahab, O., Manshouri, T., Patel, J., Harris, K., Yao, J., Hedvat, C., . . . Verstovsek, S. (2010). Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias. *Cancer Res, 70*(2), 447-452. doi:10.1158/0008-5472.CAN-09-3783

Abelsson, J., Merup, M., Birgegård, G., WeisBjerrum, O., Brinch, L., Brune, M., . . . Group, N. M. S. (2012). The outcome of allo-HSCT for 92 patients with myelofibrosis in the Nordic countries. *Bone Marrow Transplant, 47*(3), 380-386. doi:10.1038/bmt.2011.91

Adamson, J. W., Fialkow, P. J., Murphy, S., Prchal, J. F., & Steinmann, L. (1976). Polycythemia vera: stem-cell and probable clonal origin of the disease. *N Engl J Med, 295*(17), 913-916. doi:10.1056/NEJM197610212951702

Anand, S., Stedham, F., Beer, P., Gudgin, E., Ortmann, C. A., Bench, A., . . . Huntly, B. J. (2011). Effects of the JAK2 mutation on the hematopoietic stem and progenitor compartment in human myeloproliferative neoplasms. *Blood, 118*(1), 177-181. doi:10.1182/blood-2010-12-327593

Ancochea, A., Alvarez-Larrán, A., Morales-Indiano, C., García-Pallarols, F., Martínez-Avilés, L., Angona, A., . . . Besses, C. (2014). The role of serum erythropoietin level and JAK2 V617F allele burden in the diagnosis of polycythaemia vera. *Br J Haematol, 167*(3), 411-417. doi:10.1111/bjh.13047

Andıç, N., Ünübol, M., Yağcı, E., Akay, O. M., Yavaşoğlu, İ., Kadıköylü, V. G., & Bolaman, A. Z. (2016). Clinical Features of 294 Turkish Patients with Chronic Myeloproliferative Neoplasms. *Turk J Haematol, 33*(3), 187-195. doi:10.4274/tjh.2015.0041

Annamaneni, S., Gorre, M., Kagita, S., Addepalli, K., Digumarti, R. R., Satti, V., & Battini, M. R. (2013). Association of XRCC1 gene polymorphisms with chronic myeloid leukemia in the population of Andhra Pradesh, India. *Hematology, 18*(3), 163-168. doi:10.1179/1607845412Y.0000000040

Apel, K., & Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol, 55*, 373-399. doi:10.1146/annurev.arplant.55.031903.141701

Arber, D. A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M. J., Le Beau, M. M., . . . Vardiman, J. W. (2016). The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood, 127*(20), 2391-2405. doi:10.1182/blood-2016-03-643544

Babon, J. J., Lucet, I. S., Murphy, J. M., Nicola, N. A., & Varghese, L. N. (2014). The molecular regulation of Janus kinase (JAK) activation. *Biochem J, 462*(1), 1-13. doi:10.1042/BJ20140712

Bai, J., Xue, Y., Ye, L., Yao, J., Zhou, C., Shao, Z., . . . Zheng, Y. (2008). Risk factors of long-term incidences of thrombosis, myelofibrosis and evolution into malignance in polycythemia vera: a single center experience from China. *Int J Hematol, 88*(5), 530-535. doi:10.1007/s12185-008-0188-y

Ballen, K. K., Shrestha, S., Sobocinski, K. A., Zhang, M. J., Bashey, A., Bolwell, B. J., . . . Arora, M. (2010). Outcome of transplantation for myelofibrosis. *Biol Blood Marrow Transplant, 16*(3), 358-367. doi:10.1016/j.bbmt.2009.10.025

Barbui, T., Barosi, G., Birgegard, G., Cervantes, F., Finazzi, G., Griesshammer, M., . . . LeukemiaNet, E. (2011). Philadelphia-negative classical myeloproliferative neoplasms: critical concepts and management recommendations from European LeukemiaNet. *J Clin Oncol, 29*(6), 761-770. doi:10.1200/JCO.2010.31.8436

Barbui, T., Carobbio, A., Cervantes, F., Vannucchi, A. M., Guglielmelli, P., Antonioli, E., . . . Barosi, G. (2010). Thrombosis in primary myelofibrosis: incidence and risk factors. *Blood, 115*(4), 778-782. doi:10.1182/blood-2009-08-238956

Barbui, T., Finazzi, G., Carobbio, A., Thiele, J., Passamonti, F., Rumi, E., . . . Tefferi, A. (2012). Development and validation of an International Prognostic Score of thrombosis in World Health Organization-essential thrombocythemia (IPSET-thrombosis). *Blood, 120*(26), 5128-5133; quiz 5252. doi:10.1182/blood-2012-07-444067

Barbui, T., Finazzi, G., & Falanga, A. (2013). Myeloproliferative neoplasms and thrombosis. *Blood, 122*(13), 2176-2184. doi:10.1182/blood-2013-03-460154

Barbui, T., Vannucchi, A. M., Buxhofer-Ausch, V., De Stefano, V., Betti, S., Rambaldi, A., . . . Tefferi, A. (2015). Practice-relevant revision of IPSET-thrombosis based on 1019 patients with WHO-defined essential thrombocythemia. *Blood Cancer J, 5*, e369. doi:10.1038/bcj.2015.94

Barosi, G., Mesa, R., Finazzi, G., Harrison, C., Kiladjian, J. J., Lengfelder, E., . . . Barbui, T. (2013). Revised response criteria for polycythemia vera and essential thrombocythemia: an ELN and IWG-MRT consensus project. *Blood, 121*(23), 4778-4781. doi:10.1182/blood-2013-01-478891

Batar, B., Güven, M., Bariş, S., Celkan, T., & Yildiz, I. (2009). DNA repair gene XPD and XRCC1 polymorphisms and the risk of childhood acute lymphoblastic leukemia. *Leuk Res, 33*(6), 759-763. doi:10.1016/j.leukres.2008.11.005

Baute, J., & Depicker, A. (2008). Base excision repair and its role in maintaining genome stability. *Crit Rev Biochem Mol Biol, 43*(4), 239-276. doi:10.1080/10409230802309905

Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., . . . Project, C. G. (2005). Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet, 365*(9464), 1054-1061. doi:10.1016/S0140-6736(05)71142-9

Becerra-Díaz, M., Valderrama-Carvajal, H., & Terrazas, L. I. (2011). Signal Transducers and Activators of Transcription (STAT) family members in helminth infections. *Int J Biol Sci, 7*(9), 1371-1381.

Beer, P. A., Delhommeau, F., LeCouédic, J. P., Dawson, M. A., Chen, E., Bareford, D., . . . Green, A. R. (2010). Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. *Blood, 115*(14), 2891-2900. doi:10.1182/blood-2009-08-236596

Bellanné-Chantelot, C., Chaumarel, I., Labopin, M., Bellanger, F., Barbu, V., De Toma, C., . . . Najman, A. (2006). Genetic and clinical implications of the Val617Phe JAK2 mutation in 72 families with myeloproliferative disorders. *Blood, 108*(1), 346-352. doi:10.1182/blood-2005-12-4852

Bench, A. J., Baxter, E. J., & Green, A. R. (2013). Methods for detecting mutations in the human JAK2 gene. *Methods Mol Biol, 967*, 115-131. doi:10.1007/978-1-62703-242-1_8

Berk, P. D., Goldberg, J. D., Silverstein, M. N., Weinfeld, A., Donovan, P. B., Ellis, J. T., . . . Wasserman, L. R. (1981). Increased incidence of acute leukemia in polycythemia vera associated with chlorambucil therapy. *N Engl J Med, 304*(8), 441-447. doi:10.1056/NEJM198102193040801

Berndt, S. I., Camp, N. J., Skibola, C. F., Vijai, J., Wang, Z., Gu, J., . . . Slager, S. L. (2016). Meta-analysis of genome-wide association studies discovers multiple loci for chronic lymphocytic leukemia. *Nat Commun, 7*, 10933. doi:10.1038/ncomms10933

Best, B. P. (2009). Nuclear DNA damage as a direct cause of aging. *Rejuvenation Res, 12*(3), 199-208. doi:10.1089/rej.2009.0847

Bittencourt, R. I., Vassallo, J., Chauffaille, M. e. L., Xavier, S. G., Pagnano, K. B., Nascimento, A. C., . . . Chiattone, C. S. (2012). Philadelphia-negative chronic myeloproliferative neoplasms. *Rev Bras Hematol Hemoter, 34*(2), 140-149. doi:10.5581/1516-8484.20120034

Björkholm, M., Hultcrantz, M., & Derolf, Å. (2014). Leukemic transformation in myeloproliferative neoplasms: therapy-related or unrelated? *Best Pract Res Clin Haematol, 27*(2), 141-153. doi:10.1016/j.beha.2014.07.003

Bjørås, M., Seeberg, E., Luna, L., Pearl, L. H., & Barrett, T. E. (2002). Reciprocal "flipping" underlies substrate recognition and catalytic activation by the human 8-oxo-guanine DNA glycosylase. *J Mol Biol, 317*(2), 171-177. doi:10.1006/jmbi.2002.5400

Boatright, K. M., & Salvesen, G. S. (2003). Mechanisms of caspase activation. *Curr Opin Cell Biol, 15*(6), 725-731.

Boland, C. R., Luciani, M. G., Gasche, C., & Goel, A. (2005). Infection, inflammation, and gastrointestinal cancer. *Gut, 54*(9), 1321-1331. doi:10.1136/gut.2004.060079

Bolufer, P., Barragan, E., Collado, M., Cervera, J., López, J. A., & Sanz, M. A. (2006). Influence of genetic polymorphisms on the risk of developing leukemia and on disease progression. *Leuk Res, 30*(12), 1471-1491. doi:10.1016/j.leukres.2006.01.016

Bose, P., & Verstovsek, S. (2017). Developmental Therapeutics in Myeloproliferative Neoplasms. *Clin Lymphoma Myeloma Leuk, 17S*, S43-S52. doi:10.1016/j.clml.2017.02.014

Brenner, D., & Mak, T. W. (2009). Mitochondrial cell death effectors. *Curr Opin Cell Biol, 21*(6), 871-877. doi:10.1016/j.ceb.2009.09.004

Bridges, B. A. (2005). Error-prone DNA repair and translesion synthesis: focus on the replication fork. *DNA Repair (Amst), 4*(5), 618-619, 634. doi:10.1016/j.dnarep.2004.11.005

Brynychová, V., Václavíková, R., Kubáčková, K., Mrhalová, M., Kodet, R., Rauš, K., . . . Souček, P. (2016). [Clinical and Functional Importance of Selected CASP8 and CASP9 Polymorphisms in Breast Carcinoma]. *Klin Onkol, 29*(6), 445-453.

Budman, J., & Chu, G. (2005). Processing of DNA for nonhomologous end-joining by cell-free extract. *EMBO J, 24*(4), 849-860. doi:10.1038/sj.emboj.7600563

Butcher, C. M., Hahn, U., To, L. B., Gecz, J., Wilkins, E. J., Scott, H. S., . . . D'Andrea, R. J. (2008). Two novel JAK2 exon 12 mutations in JAK2V617F-negative polycythaemia vera patients. *Leukemia, 22*(4), 870-873. doi:10.1038/sj.leu.2404971

Bănescu, C., Trifa, A. P., Demian, S., Benedek Lazar, E., Dima, D., Duicu, C., & Dobreanu, M. (2014). Polymorphism of XRCC1, XRCC3, and XPD genes and risk of chronic myeloid leukemia. *Biomed Res Int, 2014*, 213790. doi:10.1155/2014/213790

Cacina, C., Pence, S., Turan, S., Genc, F., Ozdemir, H., Kafadar, A., . . . Yaylim, I. (2015). Analysis of CASP8 D302H Gene Variants in Patients with Primary Brain Tumors. *In Vivo, 29*(5), 601-604.

Campbell, P. J., MacLean, C., Beer, P. A., Buck, G., Wheatley, K., Kiladjian, J. J., . . . Green, A. R. (2012). Correlation of blood counts with vascular complications in essential thrombocythemia: analysis of the prospective PT1 cohort. *Blood, 120*(7), 1409-1411. doi:10.1182/blood-2012-04-424911

Campregher, P. V., Santos, F. P., Perini, G. F., & Hamerschlak, N. (2012). Molecular biology of Philadelphia-negative myeloproliferative neoplasms. *Rev Bras Hematol Hemoter, 34*(2), 150-155. doi:10.5581/1516-8484.20120035

Carlo-Stella, C., Lavazza, C., Locatelli, A., Viganò, L., Gianni, A. M., & Gianni, L. (2007). Targeting TRAIL agonistic receptors for cancer therapy. *Clin Cancer Res, 13*(8), 2313-2317. doi:10.1158/1078-0432.CCR-06-2774

Carobbio, A., Finazzi, G., Guerini, V., Spinelli, O., Delaini, F., Marchioli, R., . . . Barbui, T. (2007). Leukocytosis is a risk factor for thrombosis in essential thrombocythemia: interaction with treatment, standard risk factors, and Jak2 mutation status. *Blood, 109*(6), 2310-2313. doi:10.1182/blood-2006-09-046342

Cazzola, M., & Kralovics, R. (2014). From Janus kinase 2 to calreticulin: the clinically relevant genomic landscape of myeloproliferative neoplasms. *Blood, 123*(24), 3714-3719. doi:10.1182/blood-2014-03-530865

Cerquozzi, S., & Tefferi, A. (2015). Blast transformation and fibrotic progression in polycythemia vera and essential thrombocythemia: a literature review of incidence and risk factors. *Blood Cancer J, 5*, e366. doi:10.1038/bcj.2015.95

Cervantes, F., Dupriez, B., Pereira, A., Passamonti, F., Reilly, J. T., Morra, E., . . . Tefferi, A. (2009). New prognostic scoring system for primary myelofibrosis based on a study of the International Working Group for Myelofibrosis Research and Treatment. *Blood, 113*(13), 2895-2901. doi:10.1182/blood-2008-07-170449

Chen, E., & Mullally, A. (2014). How does JAK2V617F contribute to the pathogenesis of myeloproliferative neoplasms? *Hematology Am Soc Hematol Educ Program, 2014*(1), 268-276. doi:10.1182/asheducation-2014.1.268

Cho, M., & Suh, Y. (2014). Genome maintenance and human longevity. *Curr Opin Genet Dev, 26*, 105-115. doi:10.1016/j.gde.2014.07.002

Cho, S. G., & Choi, E. J. (2002). Apoptotic signaling pathways: caspases and stress-activated protein kinases. *J Biochem Mol Biol, 35*(1), 24-27.

Cingeetham, A., Vuree, S., Dunna, N. R., Gorre, M., Nanchari, S. R., Edathara, P. M., . . . Satti, V. (2014). Association of caspase9 promoter polymorphisms with the susceptibility of AML in south Indian subjects. *Tumour Biol, 35*(9), 8813-8822. doi:10.1007/s13277-014-2096-5

Cortelazzo, S., Finazzi, G., Ruggeri, M., Vestri, O., Galli, M., Rodeghiero, F., & Barbui, T. (1995). Hydroxyurea for patients with essential thrombocythemia and a high risk of thrombosis. *N Engl J Med, 332*(17), 1132-1136. doi:10.1056/NEJM199504273321704

Crawford, D. C., & Nickerson, D. A. (2005). Definition and clinical importance of haplotypes. *Annu Rev Med, 56*, 303-320. doi:10.1146/annurev.med.56.082103.104540

Cross, N. C. (2011). Genetic and epigenetic complexity in myeloproliferative neoplasms. *Hematology Am Soc Hematol Educ Program, 2011*, 208-214. doi:10.1182/asheducation-2011.1.208

da Silva Silveira, V., Canalle, R., Scrideli, C. A., Queiroz, R. G., Bettiol, H., Valera, E. T., & Tone, L. G. (2009). Polymorphisms of xenobiotic metabolizing enzymes and DNA repair genes and outcome in childhood acute lymphoblastic leukemia. *Leuk Res, 33*(7), 898-901. doi:10.1016/j.leukres.2008.12.006

Dahabreh, I. J., Zoi, K., Giannouli, S., Zoi, C., Loukopoulos, D., & Voulgarelis, M. (2009). Is JAK2 V617F mutation more than a diagnostic index? A meta-analysis of clinical outcomes in essential thrombocythemia. *Leuk Res, 33*(1), 67-73. doi:10.1016/j.leukres.2008.06.006

Dameshek, W. (1951). Some speculations on the myeloproliferative syndromes. *Blood, 6*(4), 372-375.

David, S. S., O'Shea, V. L., & Kundu, S. (2007). Base-excision repair of oxidative DNA damage. *Nature, 447*(7147), 941-950. doi:10.1038/nature05978

De Bont, R., & van Larebeke, N. (2004). Endogenous DNA damage in humans: a review of quantitative data. *Mutagenesis, 19*(3), 169-185.

De Stefano, V., Za, T., Rossi, E., Vannucchi, A. M., Ruggeri, M., Elli, E., . . . Party, G. C. M. N. W. (2010). Leukocytosis is a risk factor for recurrent arterial thrombosis in young patients with polycythemia vera and essential thrombocythemia. *Am J Hematol, 85*(2), 97-100. doi:10.1002/ajh.21593

De Stefano, V., Za, T., Rossi, E., Vannucchi, A. M., Ruggeri, M., Elli, E., . . . Party, G. C.-W. (2008). Recurrent thrombosis in patients with polycythemia vera and essential thrombocythemia: incidence, risk factors, and effect of treatments. *Haematologica, 93*(3), 372-380. doi:10.3324/haematol.12053

Delhommeau, F., Jeziorowska, D., Marzac, C., & Casadevall, N. (2010). Molecular aspects of myeloproliferative neoplasms. *Int J Hematol, 91*(2), 165-173. doi:10.1007/s12185-010-0530-z

Deng, B., Liu, F., Luo, L., Wei, Y., Li, B., & Yang, H. (2016). CASP 3 genetic polymorphisms and risk of Hepatocellular carcinoma: a case-control study in a Chinese population. *Tumour Biol, 37*(7), 8985-8991. doi:10.1007/s13277-015-4779-y

Dexheimer, T. S., Antony, S., Marchand, C., & Pommier, Y. (2008). Tyrosyl-DNA phosphodiesterase as a target for anticancer therapy. *Anticancer Agents Med Chem, 8*(4), 381-389.

Dianov, G. L., Sleeth, K. M., Dianova, I. I., & Allinson, S. L. (2003). Repair of abasic sites in DNA. *Mutat Res, 531*(1-2), 157-163.

Ding, H. F., Lin, Y. L., McGill, G., Juo, P., Zhu, H., Blenis, J., . . . Fisher, D. E. (2000). Essential role for caspase-8 in transcription-independent apoptosis triggered by p53. *J Biol Chem, 275*(49), 38905-38911. doi:10.1074/jbc.M004714200

Duletić, A. N., Dekanić, A., Hadzisejdić, I., Kusen, I., Matusan-Ilijas, K., Grohovac, D., . . . Jonjić, N. (2012). JAK2-v617F mutation is associated with clinical and laboratory features of myeloproliferative neoplasms. *Coll Antropol, 36*(3), 859-865.

Easton, D. F., Pooley, K. A., Dunning, A. M., Pharoah, P. D., Thompson, D., Ballinger, D. G., . . . Group, A. M. (2007). Genome-wide association study identifies novel breast cancer susceptibility loci. *Nature, 447*(7148), 1087-1093. doi:10.1038/nature05887

Ebid, G. T., Ghareeb, M., Salaheldin, O., & Kamel, M. M. (2015). Prevalence of the frequency of JAK2 (V617F) mutation in different myeloproliferative disorders in Egyptian patients. *Int J Clin Exp Pathol, 8*(9), 11555-11559.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol, 35*(4), 495-516. doi:10.1080/01926230701320337

Espasandin, Y. R., Glembotsky, A. C., Grodzielski, M., Lev, P. R., Goette, N. P., Molinas, F. C., . . . Heller, P. G. (2015). Anagrelide platelet-lowering effect is due to inhibition of both megakaryocyte maturation and proplatelet formation: insight into potential mechanisms. *J Thromb Haemost, 13*(4), 631-642. doi:10.1111/jth.12850

Espejel, S., Franco, S., Rodríguez-Perales, S., Bouffler, S. D., Cigudosa, J. C., & Blasco, M. A. (2002). Mammalian Ku86 mediates chromosomal fusions and apoptosis caused by critically short telomeres. *EMBO J, 21*(9), 2207-2219. doi:10.1093/emboj/21.9.2207

Espert, L., Dusanter-Fourt, I., & Chelbi-Alix, M. K. (2005). [Negative regulation of the JAK/STAT: pathway implication in tumorigenesis]. *Bull Cancer, 92*(10), 845-857.

Fan, J., & Wilson, D. M. (2005). Protein-protein interactions and posttranslational modifications in mammalian base excision repair. *Free Radic Biol Med, 38*(9), 1121-1138. doi:10.1016/j.freeradbiomed.2005.01.012

Fialkow, P. J. (1977). Glucose-6-phosphate dehydrogenase (G-6-PD) markers in Burkitt lymphoma and other malignancies. *Haematol Blood Transfus, 20*, 297-305.

Fialkow, P. J., Faguet, G. B., Jacobson, R. J., Vaidya, K., & Murphy, S. (1981). Evidence that essential thrombocythemia is a clonal disorder with origin in a multipotent stem cell. *Blood, 58*(5), 916-919.

Finazzi, G., Carobbio, A., Thiele, J., Passamonti, F., Rumi, E., Ruggeri, M., . . . Barbui, T. (2012). Incidence and risk factors for bleeding in 1104 patients with essential thrombocythemia or prefibrotic myelofibrosis diagnosed according to the 2008 WHO criteria. *Leukemia, 26*(4), 716-719. doi:10.1038/leu.2011.258

Frank, B., Bermejo, J. L., Hemminki, K., Klaes, R., Bugert, P., Wappenschmidt, B., . . . Burwinkel, B. (2005). Re: Association of a common variant of the CASP8 gene with reduced risk of breast cancer. *J Natl Cancer Inst, 97*(13), 1012; author reply 1012-1013. doi:10.1093/jnci/dji178

Freitas, A. A., & de Magalhães, J. P. (2011). A review and appraisal of the DNA damage theory of ageing. *Mutat Res, 728*(1-2), 12-22. doi:10.1016/j.mrrev.2011.05.001

Gangat, N., Caramazza, D., Vaidya, R., George, G., Begna, K., Schwager, S., . . . Tefferi, A. (2011). DIPSS plus: a refined Dynamic International Prognostic Scoring System for primary myelofibrosis that incorporates prognostic information from karyotype, platelet count, and transfusion status. *J Clin Oncol, 29*(4), 392-397. doi:10.1200/JCO.2010.32.2446

Gangat, N., Strand, J., Lasho, T. L., Finke, C. M., Knudson, R. A., Pardanani, A., . . . Tefferi, A. (2008). Cytogenetic studies at diagnosis in polycythemia vera: clinical and JAK2V617F allele burden correlates. *Eur J Haematol, 80*(3), 197-200. doi:10.1111/j.1600-0609.2007.01003.x

Gangat, N., Tefferi, A., Thanarajasingam, G., Patnaik, M., Schwager, S., Ketterling, R., & Wolanskyj, A. P. (2009). Cytogenetic abnormalities in essential thrombocythemia: prevalence and prognostic significance. *Eur J Haematol, 83*(1), 17-21. doi:10.1111/j.1600-0609.2009.01246.x

Gisslinger, H., Gotic, M., Holowiecki, J., Penka, M., Thiele, J., Kvasnicka, H. M., . . . Group, A. S. (2013). Anagrelide compared with hydroxyurea in WHO-classified essential thrombocythemia: the ANAHYDRET Study, a randomized controlled trial. *Blood, 121*(10), 1720-1728. doi:10.1182/blood-2012-07-443770

Godfrey, A. L., Chen, E., Massie, C. E., Silber, Y., Pagano, F., Bellosillo, B., . . . Green, A. R. (2016). STAT1 activation in association with JAK2 exon 12 mutations. *Haematologica, 101*(1), e15-19. doi:10.3324/haematol.2015.128546

Gong, J. Z., Cook, J. R., Greiner, T. C., Hedvat, C., Hill, C. E., Lim, M. S., . . . Pathology, A. f. M. (2013). Laboratory practice guidelines for detecting and reporting JAK2 and MPL mutations in myeloproliferative neoplasms: a report of the Association for Molecular Pathology. *J Mol Diagn, 15*(6), 733-744. doi:10.1016/j.jmoldx.2013.07.002

Green, D. R., & Llambi, F. (2015). Cell Death Signaling. *Cold Spring Harb Perspect Biol, 7*(12). doi:10.1101/cshperspect.a006080

Guglielmelli, P., Lasho, T. L., Rotunno, G., Score, J., Mannarelli, C., Pancrazzi, A., . . . Tefferi, A. (2014). The number of prognostically detrimental mutations and prognosis in primary myelofibrosis: an international study of 797 patients. *Leukemia, 28*(9), 1804-1810. doi:10.1038/leu.2014.76

Guo, B. B., Allcock, R. J., Mirzai, B., Malherbe, J. A., Choudry, F. A., Frontini, M., . . . Erber, W. N. (2017). Megakaryocytes in Myeloproliferative Neoplasms Have Unique Somatic Mutations. *Am J Pathol, 187*(7), 1512-1522. doi:10.1016/j.ajpath.2017.03.009

Ha, J. S., Kim, Y. K., Jung, S. I., Jung, H. R., & Chung, I. S. (2012). Correlations between Janus kinase 2 V617F allele burdens and clinicohematologic parameters in myeloproliferative neoplasms. *Ann Lab Med, 32*(6), 385-391. doi:10.3343/alm.2012.32.6.385

Hao, Z., Duncan, G. S., Chang, C. C., Elia, A., Fang, M., Wakeham, A., . . . Mak, T. W. (2005). Specific ablation of the apoptotic functions of cytochrome C reveals a differential requirement for cytochrome C and Apaf-1 in apoptosis. *Cell, 121*(4), 579-591. doi:10.1016/j.cell.2005.03.016

Harrison, C., Kiladjian, J. J., Al-Ali, H. K., Gisslinger, H., Waltzman, R., Stalbovskaya, V., . . . Barosi, G. (2012). JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. *N Engl J Med, 366*(9), 787-798. doi:10.1056/NEJMoa1110556

Harrison, C. N., Campbell, P. J., Buck, G., Wheatley, K., East, C. L., Bareford, D., . . . Study, U. K. M. R. C. P. T. (2005). Hydroxyurea compared with anagrelide in high-risk essential thrombocythemia. *N Engl J Med, 353*(1), 33-45. doi:10.1056/NEJMoa043800

Harrison, C. N., Mead, A. J., Panchal, A., Fox, S., Yap, C., Gbandi, E., . . . McMullin, M. F. (2017). Ruxolitinib versus best available therapy for ET intolerant or resistant to hydroxycarbamide in a randomized trial. *Blood*. doi:10.1182/blood-2017-05-785790

Hermouet, S., Bigot-Corbel, E., & Gardie, B. (2015). Pathogenesis of Myeloproliferative Neoplasms: Role and Mechanisms of Chronic Inflammation. *Mediators Inflamm, 2015*, 145293. doi:10.1155/2015/145293

Hernández-Boluda, J. C., Pereira, A., Cervantes, F., Alvarez-Larrán, A., Collado, M., Such, E., . . . Guillem, V. (2012). A polymorphism in the XPD gene predisposes to leukemic transformation and new nonmyeloid malignancies in essential thrombocythemia and polycythemia vera. *Blood, 119*(22), 5221-5228. doi:10.1182/blood-2012-02-411215

Hinds, D. A., Barnholt, K. E., Mesa, R. A., Kiefer, A. K., Do, C. B., Eriksson, N., . . . Gotlib, J. (2016). Germ line variants predispose to both JAK2 V617F clonal hematopoiesis and myeloproliferative neoplasms. *Blood, 128*(8), 1121-1128. doi:10.1182/blood-2015-06-652941

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature, 411*(6835), 366-374. doi:10.1038/35077232

Hosgood, H. D., Baris, D., Zhang, Y., Zhu, Y., Zheng, T., Yeager, M., . . . Lan, Q. (2008). Caspase polymorphisms and genetic susceptibility to multiple myeloma. *Hematol Oncol, 26*(3), 148-151. doi:10.1002/hon.852

Huh, J., Tiu, R. V., Gondek, L. P., O'Keefe, C. L., Jasek, M., Makishima, H., . . . Maciejewski, J. P. (2010). Characterization of chromosome arm 20q abnormalities in myeloid malignancies using genome-wide single nucleotide polymorphism array analysis. *Genes Chromosomes Cancer, 49*(4), 390-399. doi:10.1002/gcc.20748

Hultcrantz, M., Kristinsson, S. Y., Andersson, T. M., Landgren, O., Eloranta, S., Derolf, A. R., . . . Björkholm, M. (2012). Patterns of survival among patients with myeloproliferative neoplasms diagnosed in Sweden from 1973 to 2008: a population-based study. *J Clin Oncol, 30*(24), 2995-3001. doi:10.1200/JCO.2012.42.1925

Hultcrantz, M., Wilkes, S. R., Kristinsson, S. Y., Andersson, T. M., Derolf, Å., Eloranta, S., . . . Björkholm, M. (2015). Risk and Cause of Death in Patients Diagnosed With Myeloproliferative Neoplasms in Sweden Between 1973 and 2005: A Population-Based Study. *J Clin Oncol, 33*(20), 2288-2295. doi:10.1200/JCO.2014.57.6652

Hung, R. J., Hall, J., Brennan, P., & Boffetta, P. (2005). Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. *Am J Epidemiol, 162*(10), 925-942. doi:10.1093/aje/kwi318

Hussein, K., Huang, J., Lasho, T., Pardanani, A., Mesa, R. A., Williamson, C. M., . . . Tefferi, A. (2009). Karyotype complements the International Prognostic Scoring System for primary myelofibrosis. *Eur J Haematol, 82*(4), 255-259. doi:10.1111/j.1600-0609.2009.01216.x

Irigaray, P., & Belpomme, D. (2010). Basic properties and molecular mechanisms of exogenous chemical carcinogens. *Carcinogenesis, 31*(2), 135-148. doi:10.1093/carcin/bgp252

Ishii, T., Bruno, E., Hoffman, R., & Xu, M. (2006). Involvement of various hematopoietic-cell lineages by the JAK2V617F mutation in polycythemia vera. *Blood, 108*(9), 3128-3134. doi:10.1182/blood-2006-04-017392

Jacobson, L. O., Goldwasser, E., Fried, W., & Plazk, L. (1957). Role of the kidney in erythropoiesis. *Nature, 179*(4560), 633-634.

Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P. V., Mar, B. G., . . . Ebert, B. L. (2014). Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med, 371*(26), 2488-2498. doi:10.1056/NEJMoa1408617

James, C., Ugo, V., Le Couédic, J. P., Staerk, J., Delhommeau, F., Lacout, C., . . . Vainchenker, W. (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature, 434*(7037), 1144-1148. doi:10.1038/nature03546

Jan Jacques, M., Mihaela, T., Adrian, T., Emilia, N. M., Anca, L., Ana Maria, V., . . . Violeta, P. (2016). 2016 WHO Clinical Molecular and Pathological Criteria for Classification and Staging of Myeloproliferative Neoplasms (MPN) Caused by MPN Driver Mutations in the JAK2, MPL and CALR Genes in the Context of New 2016 WHO Classification: Prognostic and Therapeutic Implications. *Maedica (Buchar), 11*(1), 5-25.

Jankowska, A. M., Szpurka, H., Tiu, R. V., Makishima, H., Afable, M., Huh, J., . . . Maciejewski, J. P. (2009). Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. *Blood, 113*(25), 6403-6410. doi:10.1182/blood-2009-02-205690

Jasin, M., & Rothstein, R. (2013). Repair of strand breaks by homologous recombination. *Cold Spring Harb Perspect Biol, 5*(11), a012740. doi:10.1101/cshperspect.a012740

Ji, G. H., Li, M., Cui, Y., & Wang, J. F. (2014). The relationship of CASP 8 polymorphism and cancer susceptibility: a meta-analysis. *Cell Mol Biol (Noisy-le-grand), 60*(6), 20-28

Jiang, J., Zhang, X., Yang, H., & Wang, W. (2009). Polymorphisms of DNA repair genes: ADPRT, XRCC1, and XPD and cancer risk in genetic epidemiology. *Methods Mol Biol, 471*, 305-333. doi:10.1007/978-1-59745-416-2_16

Jones, A. V., Chase, A., Silver, R. T., Oscier, D., Zoi, K., Wang, Y. L., . . . Cross, N. C. (2009). JAK2 haplotype is a major risk factor for the development of myeloproliferative neoplasms. *Nat Genet, 41*(4), 446-449. doi:10.1038/ng.334

Jones, A. V., & Cross, N. C. (2013). Inherited predisposition to myeloproliferative neoplasms. *Ther Adv Hematol, 4*(4), 237-253. doi:10.1177/2040620713489144

Joshi, D., Korgaonkar, S., Shanmukhaiah, C., & Vundinti, B. R. (2015). Association of XPD (Lys751Gln) and XRCC1 (Arg280His) gene polymorphisms in myelodysplastic syndrome. *Ann Hematol*. doi:10.1007/s00277-015-2528-3

Jost, P. J., Grabow, S., Gray, D., McKenzie, M. D., Nachbur, U., Huang, D. C., . . . Kaufmann, T. (2009). XIAP discriminates between type I and type II FAS-induced apoptosis. *Nature, 460*(7258), 1035-1039. doi:10.1038/nature08229

Jouni, H., Shameer, K., Asmann, Y. W., Hazin, R., de Andrade, M., & Kullo, I. J. (2013). Clinical Correlates of Autosomal Chromosomal Abnormalities in an Electronic Medical Record-Linked Genome-Wide Association Study: A Case Series. *J Investig Med, 1*(4).

Kang, M. G., Choi, H. W., Lee, J. H., Choi, Y. J., Choi, H. J., Shin, J. H., . . . Shin, M. G. (2016). Coexistence of JAK2 and CALR mutations and their clinical implications in patients with essential thrombocythemia. *Oncotarget, 7*(35), 57036-57049. doi:10.18632/oncotarget.10958

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer, 26*(4), 239-257.

Kieslinger, M., Woldman, I., Moriggl, R., Hofmann, J., Marine, J. C., Ihle, J. N., . . . Decker, T. (2000). Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. *Genes Dev, 14*(2), 232-244.

Kiladjian, J. J., Cassinat, B., Chevret, S., Turlure, P., Cambier, N., Roussel, M., . . . Fenaux, P. (2008). Pegylated interferon-alfa-2a induces complete hematologic and molecular responses with low toxicity in polycythemia vera. *Blood, 112*(8), 3065-3072. doi:10.1182/blood-2008-03-143537

Kilpivaara, O., & Levine, R. L. (2008). JAK2 and MPL mutations in myeloproliferative neoplasms: discovery and science. *Leukemia, 22*(10), 1813-1817. doi:10.1038/leu.2008.229

Kim, D. H., Lee, S. T., Won, H. H., Kim, S., Kim, M. J., Kim, H. J., . . . Lipton, J. H. (2011). A genome-wide association study identifies novel loci associated with susceptibility to chronic myeloid leukemia. *Blood, 117*(25), 6906-6911. doi:10.1182/blood-2011-01-329797

Kim, Y. J., & Wilson, D. M. (2012). Overview of base excision repair biochemistry. *Curr Mol Pharmacol, 5*(1), 3-13.

Klampfl, T., Gisslinger, H., Harutyunyan, A. S., Nivarthi, H., Rumi, E., Milosevic, J. D., . . . Kralovics, R. (2013). Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med, 369*(25), 2379-2390. doi:10.1056/NEJMoa1311347

Ko, H. L., & Ren, E. C. (2012). Functional Aspects of PARP1 in DNA Repair and Transcription. *Biomolecules, 2*(4), 524-548. doi:10.3390/biom2040524

Koopmans, S. M., Schouten, H. C., & van Marion, A. M. (2015). BCR-ABL negative myeloproliferative neoplasia: a review of involved molecular mechanisms. *Histol Histopathol, 30*(2), 151-161.

Kralovics, R., Buser, A. S., Teo, S. S., Coers, J., Tichelli, A., van der Maas, A. P., & Skoda, R. C. (2003). Comparison of molecular markers in a cohort of patients with chronic myeloproliferative disorders. *Blood, 102*(5), 1869-1871. doi:10.1182/blood-2003-03-0744

Kralovics, R., Passamonti, F., Buser, A. S., Teo, S. S., Tiedt, R., Passweg, J. R., . . . Skoda, R. C. (2005). A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med, 352*(17), 1779-1790. doi:10.1056/NEJMoa051113

Krokan, H. E., & Bjørås, M. (2013). Base excision repair. *Cold Spring Harb Perspect Biol, 5*(4), a012583. doi:10.1101/cshperspect.a012583

Lan, Q., Morton, L. M., Armstrong, B., Hartge, P., Menashe, I., Zheng, T., . . . Wang, S. S. (2009). Genetic variation in caspase genes and risk of non-Hodgkin lymphoma: a pooled analysis of 3 population-based case-control studies. *Blood, 114*(2), 264-267. doi:10.1182/blood-2009-01-198697

Lan, Q., Zheng, T., Chanock, S., Zhang, Y., Shen, M., Wang, S. S., . . . Rothman, N. (2007). Genetic variants in caspase genes and susceptibility to non-Hodgkin lymphoma. *Carcinogenesis, 28*(4), 823-827. doi:10.1093/carcin/bgl196

Landgren, O., Goldin, L. R., Kristinsson, S. Y., Helgadottir, E. A., Samuelsson, J., & Björkholm, M. (2008). Increased risks of polycythemia vera, essential thrombocythemia, and myelofibrosis among 24,577 first-degree relatives of 11,039 patients with myeloproliferative neoplasms in Sweden. *Blood, 112*(6), 2199-2204. doi:10.1182/blood-2008-03-143602

Landolfi, R., Marchioli, R., Kutti, J., Gisslinger, H., Tognoni, G., Patrono, C., . . . Investigators, E. C. o. L.-D. A. i. P. V. (2004). Efficacy and safety of low-dose aspirin in polycythemia vera. *N Engl J Med, 350*(2), 114-124. doi:10.1056/NEJMoa035572

Langabeer, S. E., Andrikovics, H., Asp, J., Bellosillo, B., Carillo, S., Haslam, K., . . . MPN&MPNr-EuroNet. (2015). Molecular diagnostics of myeloproliferative neoplasms. *Eur J Haematol, 95*(4), 270-279. doi:10.1111/ejh.12578

Larrea, A. A., Lujan, S. A., & Kunkel, T. A. (2010). SnapShot: DNA mismatch repair. *Cell, 141*(4), 730.e731. doi:10.1016/j.cell.2010.05.002

Larsen, T. S., Pallisgaard, N., Møller, M. B., & Hasselbalch, H. C. (2007). The JAK2 V617F allele burden in essential thrombocythemia, polycythemia vera and primary myelofibrosis--impact on disease phenotype. *Eur J Haematol, 79*(6), 508-515. doi:10.1111/j.1600-0609.2007.00960.x

Levine, R. L. (2009). Mechanisms of mutations in myeloproliferative neoplasms. *Best Pract Res Clin Haematol, 22*(4), 489-494. doi:10.1016/j.beha.2009.08.006

Levine, R. L., Pardanani, A., Tefferi, A., & Gilliland, D. G. (2007). Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders. *Nat Rev Cancer, 7*(9), 673-683. doi:10.1038/nrc2210

Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J., . . . Gilliland, D. G. (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell, 7*(4), 387-397. doi:10.1016/j.ccr.2005.03.023

Lewis, C. M., & Knight, J. (2012). Introduction to genetic association studies. *Cold Spring Harb Protoc, 2012*(3), 297-306. doi:10.1101/pdb.top068163

Li, C., Zhao, H., Hu, Z., Liu, Z., Wang, L. E., Gershenwald, J. E., . . . Wei, Q. (2008). Genetic variants and haplotypes of the caspase-8 and caspase-10 genes contribute to susceptibility to cutaneous melanoma. *Hum Mutat, 29*(12), 1443-1451. doi:10.1002/humu.20803

Li, J., Kent, D. G., Godfrey, A. L., Manning, H., Nangalia, J., Aziz, A., . . . Green, A. R. (2014). JAK2V617F homozygosity drives a phenotypic switch in myeloproliferative neoplasms, but is insufficient to sustain disease. *Blood, 123*(20), 3139-3151. doi:10.1182/blood-2013-06-510222

Li, Y., Hetet, G., Maurer, A. M., Chait, Y., Dhermy, D., & Briere, J. (1994). Spontaneous megakaryocyte colony formation in myeloproliferative disorders is not neutralizable by antibodies against IL3, IL6 and GM-CSF. *Br J Haematol, 87*(3), 471-476.

Lin, J., Zhang, Y., Wang, H., Chang, J., Wei, L., Cao, L., . . . Zhang, X. (2016). Genetic Polymorphisms in the Apoptosis-Associated Gene CASP3 and the Risk of Lung Cancer in Chinese Population. *PLoS One, 11*(10), e0164358. doi:10.1371/journal.pone.0164358

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature, 362*(6422), 709-715. doi:10.1038/362709a0

Liu, Y., Prasad, R., Beard, W. A., Kedar, P. S., Hou, E. W., Shock, D. D., & Wilson, S. H. (2007). Coordination of steps in single-nucleotide base excision repair mediated by apurinic/apyrimidinic endonuclease 1 and DNA polymerase beta. *J Biol Chem, 282*(18), 13532-13541. doi:10.1074/jbc.M611295200

Lohmueller, K. E., Pearce, C. L., Pike, M., Lander, E. S., & Hirschhorn, J. N. (2003). Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet, 33*(2), 177-182. doi:10.1038/ng1071

London, R. E. (2015). The structural basis of XRCC1-mediated DNA repair. *DNA Repair (Amst), 30*, 90-103. doi:10.1016/j.dnarep.2015.02.005

Lussana, F., Caberlon, S., Pagani, C., Kamphuisen, P. W., Büller, H. R., & Cattaneo, M. (2009). Association of V617F Jak2 mutation with the risk of thrombosis among patients with essential thrombocythaemia or idiopathic myelofibrosis: a systematic review. *Thromb Res, 124*(4), 409-417. doi:10.1016/j.thromres.2009.02.004

MacNerney, M., Godley, L., Le Beau, M. (2017). Therapy-related myeloid neoplasms: when genetics and environment collide. *Nature Reviews 17(9)*, 513-527. doi:10.1038/nrc.2017.60

MacPherson, G., Healey, C. S., Teare, M. D., Balasubramanian, S. P., Reed, M. W., Pharoah, P. D., . . . Cox, A. (2004). Association of a common variant of the CASP8 gene with reduced risk of breast cancer. *J Natl Cancer Inst, 96*(24), 1866-1869. doi:10.1093/jnci/dji001

Malherbe, J. A., Fuller, K. A., Arshad, A., Nangalia, J., Romeo, G., Hall, S. L., . . . Erber, W. N. (2015). Megakaryocytic hyperplasia in myeloproliferative neoplasms is driven by disordered proliferative, apoptotic and epigenetic mechanisms. *J Clin Pathol*. doi:10.1136/jclinpath-2015-203177

Malherbe, J. A., Fuller, K. A., Mirzai, B., Kavanagh, S., So, C. C., Ip, H. W., . . . Erber, W. N. (2016). Dysregulation of the intrinsic apoptotic pathway mediates megakaryocytic hyperplasia in myeloproliferative neoplasms. *J Clin Pathol*. doi:10.1136/jclinpath-2016-203625

Marnett, L. J. (2000). Oxyradicals and DNA damage. *Carcinogenesis, 21*(3), 361-370.

Marques, I., Teixeira, A. L., Ferreira, M., Assis, J., Lobo, F., Maurício, J., & Medeiros, R. (2013). Influence of survivin (BIRC5) and caspase-9 (CASP9) functional polymorphisms in renal cell carcinoma development: a study in a southern European population. *Mol Biol Rep, 40*(8), 4819-4826. doi:10.1007/s11033-013-2578-3

Masarova, L., Bose, P., Daver, N., Pemmaraju, N., Newberry, K. J., Manshouri, T., . . . Verstovsek, S. (2017). Patients with post-essential thrombocythemia and post-polycythemia vera differ from patients with primary myelofibrosis. *Leuk Res, 59*, 110-116. doi:10.1016/j.leukres.2017.06.001

Maxwell, A. P., Lappin, T. R., Johnston, C. F., Bridges, J. M., & McGeown, M. G. (1990). Erythropoietin production in kidney tubular cells. *Br J Haematol, 74*(4), 535-539

Maynard, S., Schurman, S. H., Harboe, C., de Souza-Pinto, N. C., & Bohr, V. A. (2009). Base excision repair of oxidative DNA damage and association with cancer and aging. *Carcinogenesis, 30*(1), 2-10. doi:10.1093/carcin/bgn250

McCulloch, S. D., & Kunkel, T. A. (2008). The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases. *Cell Res, 18*(1), 148-161. doi:10.1038/cr.2008.4

McIlwain, D. R., Berger, T., & Mak, T. W. (2013). Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol, 5*(4), a008656. doi:10.1101/cshperspect.a008656

McKinnon, P. J. (2009). DNA repair deficiency and neurological disease. *Nat Rev Neurosci, 10*(2), 100-112. doi:10.1038/nrn2559

McLornan, D., Percy, M., & McMullin, M. F. (2006). JAK2 V617F: a single mutation in the myeloproliferative group of disorders. *Ulster Med J, 75*(2), 112-119.

McPherson, S., McMullin, M. F., & Mills, K. (2017). Epigenetics in Myeloproliferative Neoplasms. *J Cell Mol Med, 21*(9), 1660-1667. doi:10.1111/jcmm.13095

Mehta, J., Wang, H., Iqbal, S. U., & Mesa, R. (2014). Epidemiology of myeloproliferative neoplasms in the United States. *Leuk Lymphoma, 55*(3), 595-600. doi:10.3109/10428194.2013.813500

Merker, J. D., Roskin, K. M., Ng, D., Pan, C., Fisk, D. G., King, J. J., . . . Gotlib, J. (2013). Comprehensive whole-genome sequencing of an early-stage primary myelofibrosis patient defines low mutational burden and non-recurrent candidate genes. *Haematologica, 98*(11), 1689-1696. doi:10.3324/haematol.2013.092379

Mesa, R., Miller, C. B., Thyne, M., Mangan, J., Goldberger, S., Fazal, S., . . . Mascarenhas, J. O. (2016). Myeloproliferative neoplasms (MPNs) have a significant impact on patients' overall health and productivity: the MPN Landmark survey. *BMC Cancer, 16*(1), 167. doi:10.1186/s12885-016-2208-2

Meyer, S. C., & Levine, R. L. (2014). Molecular pathways: molecular basis for sensitivity and resistance to JAK kinase inhibitors. *Clin Cancer Res, 20*(8), 2051-2059. doi:10.1158/1078-0432.CCR-13-0279

Michiels, J. J. (2005). Clinical, pathological and molecular features of the chronic myeloproliferative disorders: MPD 2005 and beyond. *Hematology, 10 Suppl 1*, 215-223. doi:10.1080/10245330512331390456

Michiels, J. J., Berneman, Z., Schroyens, W., Finazzi, G., Budde, U., & van Vliet, H. H. (2006). The paradox of platelet activation and impaired function: platelet-von Willebrand factor interactions, and the etiology of thrombotic and hemorrhagic manifestations in essential thrombocythemia and polycythemia vera. *Semin Thromb Hemost, 32*(6), 589-604. doi:10.1055/s-2006-949664

Miller, C. B., Komrokji, R. S., Mesa, R. A., Sun, W., Montgomery, M., & Verstovsek, S. (2017). Practical Measures of Clinical Benefit With Ruxolitinib Therapy: An Exploratory Analysis of COMFORT-I. *Clin Lymphoma Myeloma Leuk, 17*(8), 479-487. doi:10.1016/j.clml.2017.05.015

Milosevic, J. D., Puda, A., Malcovati, L., Berg, T., Hofbauer, M., Stukalov, A., . . . Kralovics, R. (2012). Clinical significance of genetic aberrations in secondary acute myeloid leukemia. *Am J Hematol, 87*(11), 1010-1016. doi:10.1002/ajh.23309

Mittal, R. D., Srivastava, P., Mittal, T., Verma, A., Jaiswal, P. K., Singh, V., . . . Mandhani, A. (2011). Association of death receptor 4, Caspase 3 and 5 gene polymorphism with increased risk to bladder cancer in North Indians. *Eur J Surg Oncol, 37*(8), 727-733. doi:10.1016/j.ejso.2011.05.013

Moulard, O., Mehta, J., Fryzek, J., Olivares, R., Iqbal, U., & Mesa, R. A. (2014). Epidemiology of myelofibrosis, essential thrombocythemia, and polycythemia vera in the European Union. *Eur J Haematol, 92*(4), 289-297. doi:10.1111/ejh.12256

Nangalia, J., & Green, T. R. (2014). The evolving genomic landscape of myeloproliferative neoplasms. *Hematology Am Soc Hematol Educ Program, 2014*(1), 287-296. doi:10.1182/asheducation-2014.1.287

Nangalia, J., Massie, C. E., Baxter, E. J., Nice, F. L., Gundem, G., Wedge, D. C., . . . Green, A. R. (2013). Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med, 369*(25), 2391-2405. doi:10.1056/NEJMoa1312542

Nielsen, C., Birgens, H. S., Nordestgaard, B. G., & Bojesen, S. E. (2013). Diagnostic value of JAK2 V617F somatic mutation for myeloproliferative cancer in 49 488 individuals from the general population. *Br J Haematol, 160*(1), 70-79. doi:10.1111/bjh.12099

Nielsen, C., Bojesen, S. E., Nordestgaard, B. G., Kofoed, K. F., & Birgens, H. S. (2014). JAK2V617F somatic mutation in the general population: myeloproliferative neoplasm development and progression rate. *Haematologica, 99*(9), 1448-1455. doi:10.3324/haematol.2014.107631

Nielsen, I., & Hasselbalch, H. C. (2003). Acute leukemia and myelodysplasia in patients with a Philadelphia chromosome negative chronic myeloproliferative disorder treated with hydroxyurea alone or with hydroxyurea after busulphan. *Am J Hematol, 74*(1), 26-31. doi:10.1002/ajh.10375

Nowell, P. C., & Hungerford, D. A. (1960). Chromosome studies on normal and leukemic human leukocytes. *J Natl Cancer Inst, 25*, 85-109.

Numata, A., Shimoda, K., Kamezaki, K., Haro, T., Kakumitsu, H., Shide, K., . . . Harada, M. (2005). Signal transducers and activators of transcription 3 augments the transcriptional activity of CCAAT/enhancer-binding protein alpha in granulocyte colony-stimulating factor signaling pathway. *J Biol Chem, 280*(13), 12621-12629. doi:10.1074/jbc.M408442200

Nussenzveig, R. H., Pham, H. T., Perkins, S. L., Prchal, J. T., Agarwal, A. M., & Salama, M. E. (2016). Increased frequency of co-existing JAK2 exon-12 or MPL exon-10 mutations in patients with low JAK2(V617F) allelic burden. *Leuk Lymphoma, 57*(6), 1429-1435. doi:10.3109/10428194.2015.1091932

Oh, S. T., & Gotlib, J. (2010). JAK2 V617F and beyond: role of genetics and aberrant signaling in the pathogenesis of myeloproliferative neoplasms. *Expert Rev Hematol, 3*(3), 323-337. doi:10.1586/ehm.10.28

Olcaydu, D., Harutyunyan, A., Jäger, R., Berg, T., Gisslinger, B., Pabinger, I., . . . Kralovics, R. (2009). A common JAK2 haplotype confers susceptibility to myeloproliferative neoplasms. *Nat Genet, 41*(4), 450-454. doi:10.1038/ng.341

Packer, B. R., Yeager, M., Burdett, L., Welch, R., Beerman, M., Qi, L., . . . Chanock, S. J. (2006). SNP500Cancer: a public resource for sequence validation, assay development, and frequency analysis for genetic variation in candidate genes. *Nucleic Acids Res, 34*(Database issue), D617-621. doi:10.1093/nar/gkj151

Pakakasama, S., Sirirat, T., Kanchanachumpol, S., Udomsubpayakul, U., Mahasirimongkol, S., Kitpoka, P., . . . Hongeng, S. (2007). Genetic polymorphisms and haplotypes of DNA repair genes in childhood acute lymphoblastic leukemia. *Pediatr Blood Cancer, 48*(1), 16-20. doi:10.1002/pbc.20742

Papadakis, E., Hoffman, R., & Brenner, B. (2010). Thrombohemorrhagic complications of myeloproliferative disorders. *Blood Rev, 24*(6), 227-232. doi:10.1016/j.blre.2010.08.002

Pardanani, A., Fridley, B. L., Lasho, T. L., Gilliland, D. G., & Tefferi, A. (2008). Host genetic variation contributes to phenotypic diversity in myeloproliferative disorders. *Blood, 111*(5), 2785-2789. doi:10.1182/blood-2007-06-095703

Pardanani, A., Lasho, T., McClure, R., Lacy, M., & Tefferi, A. (2006). Discordant distribution of JAK2V617F mutation in siblings with familial myeloproliferative disorders. *Blood, 107*(11), 4572-4573. doi:10.1182/blood-2005-12-4988

Park, C., Han, S., Lee, K. M., Choi, J. Y., Song, N., Jeon, S., . . . Kang, D. (2012). Association between CASP7 and CASP14 genetic polymorphisms and the risk of childhood leukemia. *Hum Immunol, 73*(7), 736-739. doi:10.1016/j.humimm.2012.04.017

Park, C. H., Lee, K. O., Jang, J. H., Jung, C. W., Kim, J. W., Kim, S. H., & Kim, H. J. (2016). High frequency of JAK2 exon 12 mutations in Korean patients with polycythaemia vera: novel mutations and clinical significance. *J Clin Pathol, 69*(8), 737-741. doi:10.1136/jclinpath-2016-203649

Park, H. L., Ziogas, A., Chang, J., Desai, B., Bessonova, L., Garner, C., . . . Anton-Culver, H. (2016). Novel polymorphisms in caspase-8 are associated with breast cancer risk in the California Teachers Study. *BMC Cancer, 16*, 14. doi:10.1186/s12885-015-2036-9

Parker, A., Gu, Y., Mahoney, W., Lee, S. H., Singh, K. K., & Lu, A. L. (2001). Human homolog of the MutY repair protein (hMYH) physically interacts with proteins involved in long patch DNA base excision repair. *J Biol Chem, 276*(8), 5547-5555. doi:10.1074/jbc.M008463200

Passamonti, F., Cervantes, F., Vannucchi, A. M., Morra, E., Rumi, E., Pereira, A., . . . Tefferi, A. (2010). A dynamic prognostic model to predict survival in primary myelofibrosis: a study by the IWG-MRT (International Working Group for Myeloproliferative Neoplasms Research and Treatment). *Blood, 115*(9), 1703-1708. doi:10.1182/blood-2009-09-245837

Passamonti, F., Elena, C., Schnittger, S., Skoda, R. C., Green, A. R., Girodon, F., . . . Cazzola, M. (2011). Molecular and clinical features of the myeloproliferative neoplasm associated with JAK2 exon 12 mutations. *Blood, 117*(10), 2813-2816. doi:10.1182/blood-2010-11-316810

Passamonti, F., Mora, B., & Maffioli, M. (2016). New molecular genetics in the diagnosis and treatment of myeloproliferative neoplasms. *Curr Opin Hematol, 23*(2), 137-143. doi:10.1097/MOH.0000000000000218

Passamonti, F., Rumi, E., Arcaini, L., Boveri, E., Elena, C., Pietra, D., . . . Lazzarino, M. (2008). Prognostic factors for thrombosis, myelofibrosis, and leukemia in essential thrombocythemia: a study of 605 patients. *Haematologica, 93*(11), 1645-1651. doi:10.3324/haematol.13346

Passamonti, F., Rumi, E., Pietra, D., Elena, C., Boveri, E., Arcaini, L., . . . Cazzola, M. (2010). A prospective study of 338 patients with polycythemia vera: the impact of JAK2 (V617F) allele burden and leukocytosis on fibrotic or leukemic disease transformation and vascular complications. *Leukemia, 24*(9), 1574-1579. doi:10.1038/leu.2010.148

Perina, D., Mikoč, A., Ahel, J., Ćetković, H., Žaja, R., & Ahel, I. (2014). Distribution of protein poly(ADP-ribosyl)ation systems across all domains of life. *DNA Repair (Amst), 23*, 4-16. doi:10.1016/j.dnarep.2014.05.003

Pietra, D., Li, S., Brisci, A., Passamonti, F., Rumi, E., Theocharides, A., . . . Cazzola, M. (2008). Somatic mutations of JAK2 exon 12 in patients with JAK2 (V617F)-negative myeloproliferative disorders. *Blood, 111*(3), 1686-1689. doi:10.1182/blood-2007-07-101576

Pikman, Y., Lee, B. H., Mercher, T., McDowell, E., Ebert, B. L., Gozo, M., . . . Levine, R. L. (2006). MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med, 3*(7), e270. doi:10.1371/journal.pmed.0030270

Ponder, B. A. (2001). Cancer genetics. *Nature, 411*(6835), 336-341. doi:10.1038/35077207

Rampal, R., Ahn, J., Abdel-Wahab, O., Nahas, M., Wang, K., Lipson, D., . . . Levine, R. L. (2014). Genomic and functional analysis of leukemic transformation of myeloproliferative neoplasms. *Proc Natl Acad Sci U S A, 111*(50), E5401-5410. doi:10.1073/pnas.1407792111

Ravanat, J. L., Douki, T., & Cadet, J. (2001). Direct and indirect effects of UV radiation on DNA and its components. *J Photochem Photobiol B, 63*(1-3), 88-102.

Rawlings, J. S., Rosler, K. M., & Harrison, D. A. (2004). The JAK/STAT signaling pathway. *J Cell Sci, 117*(Pt 8), 1281-1283. doi:10.1242/jcs.00963

Reardon, J. T., & Sancar, A. (2006). Purification and characterization of Escherichia coli and human nucleotide excision repair enzyme systems. *Methods Enzymol, 408*, 189-213. doi:10.1016/S0076-6879(06)08012-8

Remy, I., Wilson, I. A., & Michnick, S. W. (1999). Erythropoietin receptor activation by a ligand-induced conformation change. *Science, 283*(5404), 990-993

Reuther, G. W. (2016). Myeloproliferative Neoplasms: Molecular Drivers and Therapeutics. *Prog Mol Biol Transl Sci, 144*, 437-484. doi:10.1016/bs.pmbts.2016.09.004

Rice, K. L., Lin, X., Wolniak, K., Ebert, B. L., Berkofsky-Fessler, W., Buzzai, M., . . . Zhang, W. (2011). Analysis of genomic aberrations and gene expression profiling identifies novel lesions and pathways in myeloproliferative neoplasms. *Blood Cancer J, 1*(11), e40. doi:10.1038/bcj.2011.39

Riedl, S. J., & Shi, Y. (2004). Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol, 5*(11), 897-907. doi:10.1038/nrm1496

Rihani, A., De Wilde, B., Zeka, F., Laureys, G., Francotte, N., Tonini, G. P., . . . Van Maerken, T. (2014). CASP8 SNP D302H (rs1045485) is associated with worse survival in MYCN-amplified neuroblastoma patients. *PLoS One, 9*(12), e114696. doi:10.1371/journal.pone.0114696

Rotunno, G., Mannarelli, C., Guglielmelli, P., Pacilli, A., Pancrazzi, A., Pieri, L., . . . Investigators, A. I. p. l. R. s. C. G. I. M. M. (2014). Impact of calreticulin mutations on clinical and hematological phenotype and outcome in essential thrombocythemia. *Blood, 123*(10), 1552-1555. doi:10.1182/blood-2013-11-538983

Rueff, J., & Rodrigues, A. S. (2016). Cancer Drug Resistance: A Brief Overview from a Genetic Viewpoint. *Methods Mol Biol, 1395*, 1-18. doi:10.1007/978-1-4939-3347-1_1

Rumi, E., & Cazzola, M. (2017). Diagnosis, risk stratification, and response evaluation in classical myeloproliferative neoplasms. *Blood, 129*(6), 680-692. doi:10.1182/blood-2016-10-695957

Rumi, E., Passamonti, F., Della Porta, M. G., Elena, C., Arcaini, L., Vanelli, L., . . . Lazzarino, M. (2007). Familial chronic myeloproliferative disorders: clinical phenotype and evidence of disease anticipation. *J Clin Oncol, 25*(35), 5630-5635. doi:10.1200/JCO.2007.12.6896

Rumi, E., Passamonti, F., Picone, C., Della Porta, M. G., Pascutto, C., Cazzola, M., & Lazzarino, M. (2008). Disease anticipation in familial myeloproliferative neoplasms. *Blood, 112*(6), 2587-2588; author reply 2588-2589. doi:10.1182/blood-2008-05-160739

Rumi, E., Pietra, D., Ferretti, V., Klampfl, T., Harutyunyan, A. S., Milosevic, J. D., . . . Investigators, A. I. p. l. R. s. C. G. I. M. M. (2014). JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. *Blood, 123*(10), 1544-1551. doi:10.1182/blood-2013-11-539098

Samraj, A. K., Keil, E., Ueffing, N., Schulze-Osthoff, K., & Schmitz, I. (2006). Loss of caspase-9 provides genetic evidence for the type I/II concept of CD95-mediated apoptosis. *J Biol Chem, 281*(40), 29652-29659. doi:10.1074/jbc.M603487200

Sancar, A., Lindsey-Boltz, L. A., Unsal-Kaçmaz, K., & Linn, S. (2004). Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem, 73*, 39-85. doi:10.1146/annurev.biochem.73.011303.073723

Santoro, C., Sperduti, I., Latagliata, R., Baldacci, E., Anaclerico, B., Avvisati, G., . . . Mazzucconi, M. G. (2017). Role of treatment on the development of secondary malignancies in patients with essential thrombocythemia. *Cancer Med, 6*(6), 1233-1239. doi:10.1002/cam4.1081

Santos, L. S., Branco, S. C., Silva, S. N., Azevedo, A. P., Gil, O. M., Manita, I., . . . Gaspar, J. F. (2012). Polymorphisms in base excision repair genes and thyroid cancer risk. *Oncol Rep, 28*(5), 1859-1868. doi:10.3892/or.2012.1975

Scott, L. M. (2011). The JAK2 exon 12 mutations: a comprehensive review. *Am J Hematol, 86*(8), 668-676. doi:10.1002/ajh.22063

Scott, L. M., Beer, P. A., Bench, A. J., Erber, W. N., & Green, A. R. (2007). Prevalance of JAK2 V617F and exon 12 mutations in polycythaemia vera. *Br J Haematol, 139*(3), 511-512. doi:10.1111/j.1365-2141.2007.06806.x

Scott, L. M., Tong, W., Levine, R. L., Scott, M. A., Beer, P. A., Stratton, M. R., . . . Green, A. R. (2007). JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. *N Engl J Med, 356*(5), 459-468. doi:10.1056/NEJMoa065202

Seedhouse, C., Bainton, R., Lewis, M., Harding, A., Russell, N., & Das-Gupta, E. (2002). The genotype distribution of the XRCC1 gene indicates a role for base excision repair in the development of therapy-related acute myeloblastic leukemia. *Blood, 100*(10), 3761-3766. doi:10.1182/blood-2002-04-1152

Sharma, S., Javadekar, S. M., Pandey, M., Srivastava, M., Kumari, R., & Raghavan, S. C. (2015). Homology and enzymatic requirements of microhomology-dependent alternative end joining. *Cell Death Dis, 6*, e1697. doi:10.1038/cddis.2015.58

Shi, T. Y., He, J., Wang, M. Y., Zhu, M. L., Yu, K. D., Shao, Z. M., . . . Wei, Q. (2015). CASP7 variants modify susceptibility to cervical cancer in Chinese women. *Sci Rep, 5*, 9225. doi:10.1038/srep09225

Shimizu, M., Gruz, P., Kamiya, H., Kim, S. R., Pisani, F. M., Masutani, C., . . . Nohmi, T. (2003). Erroneous incorporation of oxidized DNA precursors by Y-family DNA polymerases. *EMBO Rep, 4*(3), 269-273. doi:10.1038/sj.embor.embor765

Silva, S. N., Moita, R., Azevedo, A. P., Gouveia, R., Manita, I., Pina, J. E., . . . Gaspar, J. (2007). Menopausal age and XRCC1 gene polymorphisms: role in breast cancer risk. *Cancer Detect Prev, 31*(4), 303-309. doi:10.1016/j.cdp.2007.07.001

Silver, R. T., Vandris, K., & Goldman, J. J. (2011). Recombinant interferon-α may retard progression of early primary myelofibrosis: a preliminary report. *Blood, 117*(24), 6669-6672. doi:10.1182/blood-2010-11-320069

Singh, A., & Xu, Y. J. (2016). The Cell Killing Mechanisms of Hydroxyurea. *Genes (Basel), 7*(11). doi:10.3390/genes7110099

Skoda, R. C., Duek, A., & Grisouard, J. (2015). Pathogenesis of myeloproliferative neoplasms. *Exp Hematol, 43*(8), 599-608. doi:10.1016/j.exphem.2015.06.007

Spencer, S. L., Gaudet, S., Albeck, J. G., Burke, J. M., & Sorger, P. K. (2009). Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. *Nature, 459*(7245), 428-432. doi:10.1038/nature08012

Spivak, J. L. (2017). Myeloproliferative Neoplasms. *N Engl J Med, 376*(22), 2168-2181. doi:10.1056/NEJMra1406186

Srour, S. A., Devesa, S. S., Morton, L. M., Check, D. P., Curtis, R. E., Linet, M. S., & Dores, G. M. (2016). Incidence and patient survival of myeloproliferative neoplasms and myelodysplastic/myeloproliferative neoplasms in the United States, 2001-12. *Br J Haematol, 174*(3), 382-396. doi:10.1111/bjh.14061

Stadler, Z. K., Gallagher, D. J., Thom, P., & Offit, K. (2010). Genome-wide association studies of cancer: principles and potential utility. *Oncology (Williston Park), 24*(7), 629-637.

Stadler, Z. K., Thom, P., Robson, M. E., Weitzel, J. N., Kauff, N. D., Hurley, K. E., . . . Offit, K. (2010). Genome-wide association studies of cancer. *J Clin Oncol, 28*(27), 4255-4267. doi:10.1200/JCO.2009.25.7816

Stadler, Z. K., Vijai, J., Thom, P., Kirchhoff, T., Hansen, N. A., Kauff, N. D., . . . Offit, K. (2010). Genome-wide association studies of cancer predisposition. *Hematol Oncol Clin North Am, 24*(5), 973-996. doi:10.1016/j.hoc.2010.06.009

Steensma, D. P., McClure, R. F., Karp, J. E., Tefferi, A., Lasho, T. L., Powell, H. L., . . . Kaufmann, S. H. (2006). JAK2 V617F is a rare finding in de novo acute myeloid leukemia, but STAT3 activation is common and remains unexplained. *Leukemia, 20*(6), 971-978. doi:10.1038/sj.leu.2404206

Sun, T., Gao, Y., Tan, W., Ma, S., Shi, Y., Yao, J., . . . Lin, D. (2007). A six-nucleotide insertion-deletion polymorphism in the CASP8 promoter is associated with susceptibility to multiple cancers. *Nat Genet, 39*(5), 605-613. doi:10.1038/ng2030

Swerdlow, S. H., Campo, E., Harris, N. L., Jaffe, E. S., Pileri, S. A., Stein, H., . . . Vardiman, J. W. (2008). *WHO Classification of Tumours of Haematopioetic and Lymphoid Tissues*. Lyon: World Health Organization.

Syvänen, A. C. (2001). Accessing genetic variation: genotyping single nucleotide polymorphisms. *Nat Rev Genet, 2*(12), 930-942. doi:10.1038/35103535

Tabor, H. K., Risch, N. J., & Myers, R. M. (2002). Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nat Rev Genet, 3*(5), 391-397. doi:10.1038/nrg796

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol, 9*(3), 231-241. doi:10.1038/nrm2312

Tefferi, A. (2008a). Essential thrombocythemia, polycythemia vera, and myelofibrosis: current management and the prospect of targeted therapy. *Am J Hematol, 83*(6), 491-497. doi:10.1002/ajh.21183

Tefferi, A. (2008b). JAK and MPL mutations in myeloid malignancies. *Leuk Lymphoma, 49*(3), 388-397. doi:10.1080/10428190801895360

Tefferi, A. (2008c). The history of myeloproliferative disorders: before and after Dameshek. *Leukemia, 22*(1), 3-13. doi:10.1038/sj.leu.2404946

Tefferi, A. (2012). Myeloproliferative neoplasms 2012: the John M. Bennett 80th birthday anniversary lecture. *Leuk Res, 36*(12), 1481-1489. doi:10.1016/j.leukres.2012.08.011

Tefferi, A. (2016). Myeloproliferative neoplasms: A decade of discoveries and treatment advances. *Am J Hematol, 91*(1), 50-58. doi:10.1002/ajh.24221

Tefferi, A., & Barbui, T. (2017). Polycythemia vera and essential thrombocythemia: 2017 update on diagnosis, risk-stratification, and management. *Am J Hematol, 92*(1), 94-108. doi:10.1002/ajh.24607

Tefferi, A., Cervantes, F., Mesa, R., Passamonti, F., Verstovsek, S., Vannucchi, A. M., . . . Barosi, G. (2013). Revised response criteria for myelofibrosis: International Working Group-Myeloproliferative Neoplasms Research and Treatment (IWG-MRT) and European LeukemiaNet (ELN) consensus report. *Blood, 122*(8), 1395-1398. doi:10.1182/blood-2013-03-488098

Tefferi, A., Guglielmelli, P., Larson, D. R., Finke, C., Wassie, E. A., Pieri, L., . . . Vannucchi, A. M. (2014). Long-term survival and blast transformation in molecularly annotated essential thrombocythemia, polycythemia vera, and myelofibrosis. *Blood, 124*(16), 2507-2513; quiz 2615. doi:10.1182/blood-2014-05-579136

Tefferi, A., Lasho, T. L., Tischer, A., Wassie, E. A., Finke, C. M., Belachew, A. A., . . . Pardanani, A. D. (2014). The prognostic advantage of calreticulin mutations in myelofibrosis might be confined to type 1 or type 1-like CALR variants. *Blood, 124*(15), 2465-2466. doi:10.1182/blood-2014-07-588426

Tefferi, A., Lim, K. H., Abdel-Wahab, O., Lasho, T. L., Patel, J., Patnaik, M. M., . . . Levine, R. L. (2009). Detection of mutant TET2 in myeloid malignancies other than myeloproliferative neoplasms: CMML, MDS, MDS/MPN and AML. *Leukemia, 23*(7), 1343-1345. doi:10.1038/leu.2009.59

Tefferi, A., & Pardanani, A. (2011). JAK inhibitors in myeloproliferative neoplasms: rationale, current data and perspective. *Blood Rev, 25*(5), 229-237. doi:10.1016/j.blre.2011.06.002

Tefferi, A., & Pardanani, A. (2015). Myeloproliferative Neoplasms: A Contemporary Review. *JAMA Oncol, 1*(1), 97-105. doi:10.1001/jamaoncol.2015.89

Tefferi, A., Rumi, E., Finazzi, G., Gisslinger, H., Vannucchi, A. M., Rodeghiero, F., . . . Barbui, T. (2013). Survival and prognosis among 1545 patients with contemporary polycythemia vera: an international study. *Leukemia, 27*(9), 1874-1881. doi:10.1038/leu.2013.163

Tefferi, A., Saeed, L., Hanson, C. A., Ketterling, R. P., Pardanani, A., & Gangat, N. (2017). Application of current prognostic models for primary myelofibrosis in the setting of post polycythemia vera or post-essential thrombocythemia myelofibrosis. *Leukemia*. doi:10.1038/leu.2017.268

Tefferi, A., & Vannucchi, A. M. (2017). Genetic Risk Assessment in Myeloproliferative Neoplasms. *Mayo Clin Proc, 92*(8), 1283-1290. doi:10.1016/j.mayocp.2017.06.002

Tefferi, A., & Vardiman, J. W. (2008). Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia, 22*(1), 14-22. doi:10.1038/sj.leu.2404955

Tempfer, C. B., Hefler, L. A., Schneeberger, C., & Huber, J. C. (2006). How valid is single nucleotide polymorphism (SNP) diagnosis for the individual risk assessment of breast cancer? *Gynecol Endocrinol, 22*(3), 155-159. doi:10.1080/09513590600629175

Theocharides, A., Boissinot, M., Girodon, F., Garand, R., Teo, S. S., Lippert, E., . . . Skoda, R. C. (2007). Leukemic blasts in transformed JAK2-V617F-positive myeloproliferative disorders are frequently negative for the JAK2-V617F mutation. *Blood, 110*(1), 375-379. doi:10.1182/blood-2006-12-062125

Titmarsh, G. J., Duncombe, A. S., McMullin, M. F., O'Rorke, M., Mesa, R., De Vocht, F., . . . Anderson, L. A. (2014). How common are myeloproliferative neoplasms? A systematic review and meta-analysis. *Am J Hematol, 89*(6), 581-587.

Tong, N., Zhang, L., Sheng, X., Wang, M., Zhang, Z., Fang, Y., . . . Li, J. (2012). Functional polymorphisms in FAS, FASL and CASP8 genes and risk of childhood acute lymphoblastic leukemia: a case-control study. *Leuk Lymphoma, 53*(7), 1360-1366. doi:10.3109/10428194.2011.654117

Vainchenker, W., & Kralovics, R. (2017). Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms. *Blood, 129*(6), 667-679. doi:10.1182/blood-2016-10-695940

Valentino, L., & Pierre, J. (2006). JAK/STAT signal transduction: regulators and implication in hematological malignancies. *Biochem Pharmacol, 71*(6), 713-721. doi:10.1016/j.bcp.2005.12.017

Van Bergen, C. A., Rutten, C. E., Van Der Meijden, E. D., Van Luxemburg-Heijs, S. A., Lurvink, E. G., Houwing-Duistermaat, J. J., . . . Griffioen, M. (2010). High-throughput characterization of 10 new minor histocompatibility antigens by whole genome association scanning. *Cancer Res, 70*(22), 9073-9083. doi:10.1158/0008-5472.CAN-10-1832

Vannucchi, A. M. (2015). Ruxolitinib versus standard therapy for the treatment of polycythemia vera. *N Engl J Med, 372*(17), 1670-1671. doi:10.1056/NEJMc1502524

Vannucchi, A. M., Antonioli, E., Guglielmelli, P., Rambaldi, A., Barosi, G., Marchioli, R., . . . Barbui, T. (2007). Clinical profile of homozygous JAK2 617V>F mutation in patients with polycythemia vera or essential thrombocythemia. *Blood, 110*(3), 840-846. doi:10.1182/blood-2006-12-064287

Vannucchi, A. M., Pieri, L., & Guglielmelli, P. (2011). JAK2 Allele Burden in the Myeloproliferative Neoplasms: Effects on Phenotype, Prognosis and Change with Treatment. *Ther Adv Hematol, 2*(1), 21-32. doi:10.1177/2040620710394474

Vannucchi, A. M., Rotunno, G., Bartalucci, N., Raugei, G., Carrai, V., Balliu, M., . . . Guglielmelli, P. (2014). Calreticulin mutation-specific immunostaining in myeloproliferative neoplasms: pathogenetic insight and diagnostic value. *Leukemia, 28*(9), 1811-1818. doi:10.1038/leu.2014.100

Vardiman, J. W., Harris, N. L., & Brunning, R. D. (2002). The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood, 100*(7), 2292-2302. doi:10.1182/blood-2002-04-1199

Verstovsek, S., Mesa, R. A., Gotlib, J., Levy, R. S., Gupta, V., DiPersio, J. F., . . . Kantarjian, H. M. (2012). A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. *N Engl J Med, 366*(9), 799-807. doi:10.1056/NEJMoa1110557

Verstovsek, S., Passamonti, F., Rambaldi, A., Barosi, G., Rumi, E., Gattoni, E., . . . Vannucchi, A. M. (2017). Ruxolitinib for essential thrombocythemia refractory to or intolerant of hydroxyurea: long-term phase 2 study results. *Blood*. doi:10.1182/blood-2017-02-765032

Vorechovsky, I., Jones, A. V., & Cross, N. C. (2013). Why do we see JAK2 exon 12 mutations in myeloproliferative neoplasms? *Leukemia, 27*(9), 1930-1932. doi:10.1038/leu.2013.85

Vousden, K. H., & Lane, D. P. (2007). p53 in health and disease. *Nat Rev Mol Cell Biol, 8*(4), 275-283. doi:10.1038/nrm2147

Wang, C. Q., Krishnan, V., Tay, L. S., Chin, D. W., Koh, C. P., Chooi, J. Y., . . . Osato, M. (2014). Disruption of Runx1 and Runx3 leads to bone marrow failure and leukemia predisposition due to transcriptional and DNA repair defects. *Cell Rep, 8*(3), 767-782. doi:10.1016/j.celrep.2014.06.046

Wang, M. Y., Zhu, M. L., He, J., Shi, T. Y., Li, Q. X., Wang, Y. N., . . . Wei, Q. Y. (2013). Potentially functional polymorphisms in the CASP7 gene contribute to gastric adenocarcinoma susceptibility in an eastern Chinese population. *PLoS One, 8*(9), e74041. doi:10.1371/journal.pone.0074041

Wang, Y. X., Zhao, L., Wang, X. Y., Liu, C. M., & Yu, S. G. (2012). Role of Caspase 8, Caspase 9 and Bcl-2 polymorphisms in papillary thyroid carcinoma risk in Han Chinese population. *Med Oncol, 29*(4), 2445-2451. doi:10.1007/s12032-011-0121-8

Ward, J. F. (1988). DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. *Prog Nucleic Acid Res Mol Biol, 35*, 95-125.

Williams, D. M., Kim, A. H., Rogers, O., Spivak, J. L., & Moliterno, A. R. (2007). Phenotypic variations and new mutations in JAK2 V617F-negative polycythemia vera, erythrocytosis, and idiopathic myelofibrosis. *Exp Hematol, 35*(11), 1641-1646. doi:10.1016/j.exphem.2007.08.010

Wilson, D. M., Kim, D., Berquist, B. R., & Sigurdson, A. J. (2011). Variation in base excision repair capacity. *Mutat Res, 711*(1-2), 100-112. doi:10.1016/j.mrfmmm.2010.12.004

Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., & Ihle, J. N. (1993). JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell, 74*(2), 227-236.

Wogan, G. N., Hecht, S. S., Felton, J. S., Conney, A. H., & Loeb, L. A. (2004). Environmental and chemical carcinogenesis. *Semin Cancer Biol, 14*(6), 473-486. doi:10.1016/j.semcancer.2004.06.010

Wong, R. S. (2011). Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res, 30*, 87. doi:10.1186/1756-9966-30-87

Wray, N. R., Goddard, M. E., & Visscher, P. M. (2007). Prediction of individual genetic risk to disease from genome-wide association studies. *Genome Res, 17*(10), 1520-1528. doi:10.1101/gr.6665407

Wu, C., Li, S., & Cui, Y. (2012). Genetic association studies: an information content perspective. *Curr Genomics, 13*(7), 566-573. doi:10.2174/138920212803251382

Wu, Z., Zhang, X., Xu, X., Chen, Y., Hu, T., Kang, Z., . . . Guan, M. (2014). The mutation profile of JAK2 and CALR in Chinese Han patients with Philadelphia chromosome-negative myeloproliferative neoplasms. *J Hematol Oncol, 7*, 48. doi:10.1186/s13045-014-0048-6

Yan, L., Li, Q., Li, X., Ji, H., & Zhang, L. (2016). Association Studies Between XRCC1, XRCC2, XRCC3 Polymorphisms and Differentiated Thyroid Carcinoma. *Cell Physiol Biochem, 38*(3), 1075-1084. doi:10.1159/000443058

Yan, S., Li, Y. Z., Zhu, J. W., Liu, C. L., Wang, P., & Liu, Y. L. (2012). Role of CASP-10 gene polymorphisms in cancer susceptibility: a HuGE review and meta-analysis. *Genet Mol Res, 11*(4), 3998-4007. doi:10.4238/2012.November.26.1

Yao, H., Ma, Y., Hong, Z., Zhao, L., Monaghan, S. A., Hu, M. C., & Huang, L. J. (2017). Activating JAK2 mutants reveal cytokine receptor coupling differences that impact outcomes in myeloproliferative neoplasm. *Leukemia*. doi:10.1038/leu.2017.1

Yilmaz, S. G., Yencilek, F., Yildirim, A., Yencilek, E., & Isbir, T. (2017). Effects of Caspase 9 Gene Polymorphism in Patients with Prostate Cancer. *In Vivo, 31*(2), 205-208. doi:10.21873/invivo.11046

Zhang, Q. M., & Dianov, G. L. (2005). DNA repair fidelity of base excision repair pathways in human cell extracts. *DNA Repair (Amst), 4*(2), 263-270. doi:10.1016/j.dnarep.2004.10.004

Zhang, Z., Yu, X., Guo, Y., Song, W., Yu, D., & Zhang, X. (2012). Genetic variant in CASP3 affects promoter activity and risk of esophageal squamous cell carcinoma. *Cancer Sci, 103*(3), 555-560. doi:10.1111/j.1349-7006.2011.02173.x

Zharkov, D. O. (2008). Base excision DNA repair. *Cell Mol Life Sci, 65*(10), 1544-1565. doi:10.1007/s00018-008-7543-2.

CHAPTER 2:
STUDY AIMS AND STRUCTURE

2.1 – STUDY AIMS

2.2 – STUDY STRUCTURE

CHAPTER 2: STUDY AIMS AND STRUCTURE

2.1 – STUDY AIMS

As previously mentioned, the molecular factors that set up and prepare clonal proliferation in PN-MPNs remain an important diagnostic criterion according to WHO. Besides, there is evidence that the phenotypic diversity of PN-NMPs results from the combination of several factors, namely somatic mutations, some of them already identified and characterized, inherited genetic variability, postgenetic regulation, and individual conditioners. Identification of the *JAK2* V617F mutation developed the knowledge of PN-MPNs, contributing to and influencing the definition of the phenotype and prognostic impact.

According to the literature, several SNPs have been identified (ex: polymorphisms associated with DNA repair pathways and apoptosis), influencing genetic transcription and/or cell function, which may confer genetic predisposition to disease, although less specific than known variations. Not all mutations involved in cancer may lead to cancer. This can occur due to different variants within the same gene or between variants in different genes. The latter must be considered, as the number of passenger mutations in a tumour may modulate the effect of driver mutations, thus acting as putative modifier genes. Additionally, epimutations that can silence tumour-suppressor genes must be taken into account, which highlights the concept that, probably more important than the genes, are their expression levels.

Independently of what mutation is involved in the development of MPNs, or even if the molecular trigger is not known, one of the major points is that hyperactive JAK/STAT signaling pathway appears to be a constant. This pathway induces a proliferative activity, but is also characterized for having an anti-apoptotic effect, through the upregulation of anti-apoptotic proteins involved in the regulation of the caspases that integrate apoptosis pathway. Particularly in PN-MPNs, it appears that the deregulation of apoptosis is involved in the pathophysiology of these diseases.

DNA repair is a ubiquitous process in all living systems. Its universality reflects the constant pressure that leads to altered genome integrity resulting from the intrinsic instability of the genetic material and limitations on the fidelity of DNA replication. Several agents induce different types of lesions in the DNA and according to the lesion they will create and activate a set of proteins responsible for the efficient repair of the lesion. Endogenously produced metabolites are also capable of acting as carcinogenic agents, in particular ROS, which may be formed by different pathways, or exogenously the exposure to genotoxic compounds. These processes can induce specific types of DNA lesion, whose repair mechanisms are related to the BER pathway.

On the other hand, therapeutic exposure and enviroment factos may lead to modifications at the genome level, leading to changes in cell cycle regulation. The universality of the apoptosis and DNA repair processes reflects the constant challenge to genome integrity and cell function, given the inherent instability of DNA, the natural limitations in DNA synthesis, and interferences in cell cycle. The susceptibility to PN-MPNs, therapeutic response and clinical outcome, influencing the prognosis, may be mediated, in part, by the genetic variability of genes involved in these pathways. The vast majority of the proteins described as being involved are polymorphic enzymes, so changes in the genes encoding them may compromise their performance, thus contributing to changes in repairability and interfering with mechanisms of programmed cell death and concomitantly create genomic instability into cells. Moreover, despite the development of more efficient drugs in the last years, some patients with PN-MPNs still progress to conditions more aggressive and difficult to treat such as myelodysplasia, myelofibrosis and acute leukemia.

Concerning the study of the association of polymorphisms with PN-MPNs, the data is scarce. The use of population-based association studies, known as case-control studies, through the investigation of candidate genes, is a very useful tool that allows the evaluation of the potential association of polymorphic genes to a given disease in a population.

On the other hand, in our country in the last few years several efforts have been done to create registries and databases that allow us to organize the relevant data about these patients and characterize them, but the amount of Portuguese epidemiological data is scarce and there is still a long way to go in this direction.

Thus, the aims of the present study that conducted to this dissertation consisted of:

1) Contribute to a database of patients with PN-MPNs, referred to Hematology Consultation from Hospital São Francisco Xavier, Centro Hospitalar Lisboa Ocidental (CHLO);

2) Characterize these patients versus a healthy population in terms of PN-MPNs epidemiology and prevalence of *JAK2* mutation, and evaluate in the patients population their clinical phenotype and clinical outcome, which can be to a certain extent the reflexion of Portuguese state of the art in what concerns to this group of disorders;

3) Present the case of some patients which stood out for some particularity;

4) Carry out case-control studies in the referred population, with the selection of candidate genes that code for proteins with eventual potential involvement in PN-MPNs susceptibility: apoptosis and the BER pathway;

5) To evaluate among the patients under treatment the role of the presence of *JAK2* V617F mutation, other risk factors and polymorphisms in genes involved in apoptosis and BER pathway, in terms of disease progression to mielofibrosis/AML, predisposition to the development of new nonmyeloid neoplasms and the occurrence of thrombotic events.

2.2 – STUDY STRUCTURE

This study has been developed, since the beginning of 2009, in the Centre for Toxicogenomics and Human Health, Genetics, Oncology and Human Toxicology (ToxOmics), NOVA Medical School/Faculdade de Ciências Médicas from Universidade Nova de Lisboa, in collaboration with the Clinical Pathology Department, from Hospital de São Francisco Xavier, Centro Hospitalar de Lisboa Ocidental (HSFX, CHLO), which is actively involved in the diagnosis and characterization of this group of disorders. The study also has the collaboration of the Clinical Hematology Department of the HSFX, namely in the selection and follow-up of patients, in the therapeutic institution and in the correlation of laboratory results with clinical data.

The author has fully participated in the conception of the research project, selection and clinical/laboratorial evaluation of the patients, execution of the experimental work and interpretation of the results.

Initially, it was thought to apply this study to MPNs both *BCR-ABL1* positive and negative, but it turned out to be limited to the study of PN-MPNs only, namely PV, ET and PMF. *BCR-ABL1* CML had been investigated and was genetically well characterized in some of its aspects by our group and the results are published (Dinis et al., 2012; Gromicho et al., 2016; Gromicho et al., 2013; Gromicho et al., 2011; Rodrigues et al., 2012)*. The mechanisms of PN-MPNs pathogenesis had begun to be explored more recently and information about this theme was scarce. Moreover, regarding therapy management and clinical outcome, imatinib and the other tyrosine kinase inhibitors discovery brought to *BCR-ABL1* CML patients better quality of life, better disease control and increased survival, almost curative for some patients. Whereas, for PN-MPNs the used drugs were not so successful, with the bone marrow transplant being the only option to MF, with many patients evidencing disease progression to clinical presentations more difficult to control, remaining a great challenge.

The present study involved 133 Caucasian Portuguese PN-MPNs patients and 281 age- and sex-matched control subjects (at least two for each patient), selected since January 2009 to July 2016 within the Portuguese population recruited at the Departments of Clinical Hematology and of Clinical Pathology, HSFX, CHLO, where those individuals were admitted, followed up and treated.

Patients and healthy controls were selected and a questionnaire was administered to each one of the participants (Appendix 1), properly authorized and signed for informed consent, after clarification on the nature and objectives of the study by trained interviewers, prior to blood withdrawal and in agreement with the Declaration of Helsinki. This questionnaire aimed to characterize epidemiologically these disorders and to evaluate some important risk factors associated with susceptibility to their development and clinical outcome. The anonymity of the patient and control populations was guaranteed.

Complete inquiries and respective blood samples were than transferred to the Centre for Toxicogenomics and Human Health, Genetics, Oncology and Human Toxicology (ToxOmics), for DNA extraction and analysis.

During the accomplishment of this work, a database has been prepared and organized to compile epidemiological, clinical and laboratory data and the obtained results.

All clinical and hematologic data were obtained from registries of patients, whose hematological data was diagnosed with the contribution of the author, and patients were selected on the basis of diagnostic criteria for this group of disorders.

In order to accomplish the aims of this study, we proceed to the selection and identification of genetic polymorphisms, from public databases, potentially related to PN-MPNs individual genetic susceptibility, clinical outcome and prognostic impact, namely polymorphisms of genes involved in apoptosis and BER pathway.

With regard to apoptosis, nine polymorphisms of four caspases genes were selected and genotyped, namely rs1045485 and rs1035142 (*CASP8*), rs1052576, rs2308950, rs1820204 and rs1052571 (*CASP9*), rs2227309 and rs2227310 (*CASP7*) and rs13006529 (*CASP10*).

Concerning BER pathway, the following eight polymorphisms of six genes were selected and genotyped, namely rs1799782 (*XRCC1_194*), rs25487 (*XRCC1_399*), rs1052133 (*OGG1*), rs1136410 (*PARP1*), rs13428 and rs1050112 (*PARP4*), rs1130409 (*APEX1*) and rs3219489 (*MUTYH*).

Regarding the methodology to be used for analysis, all polymorphisms were evaluated using Real-time Polymerase Chain Reaction (PCR), and TaqMan genotyping assays specific to each SNP under study, according to the manufacturer's instructions and optimizing aspects previously achieved trough the evaluation of genetic polymorphisms in the area of breast cancer, in which the working group presented a vast experience. Statistical analysis of obtained data was performed using SPSS software version 22.0 (SPSS Inc.).

More details of each step of this study will be provided in the following Chapters.

The validation of the obtained results and the disclosure of the work that was carried out has been done through the publication of manuscripts in international indexed journals with peer-review and through their presentation in conferences dedicated to Genetics, Hematology and Laboratory fields.

The current study was conducted with approval by the Institutional Ethics' Commissions of the involved institutions, namely CHLO (approved the 6th April 2009) and Nova Medical School (approved the 27th November 2015) (Appendix 2 and 3), and since the beginning of the study by the directive boards of the Clinical Pathology and the Clinical Hematology Departments from CHLO.

The study was supported by funding from the Center for Toxicogenomics and Human Health (ToxOmics), from Fundação para a Ciência e Tecnologia (FCT), Portugal (grant no. UID/BIM/00009/2013).

This dissertation is divided into ten Chapters, in which the first one is dedicated to a brief theorical introduction about PN-MPNs, individual susceptibility, genetic polymorphisms and association studies, caspases and BER pathway. The following Chapters refer to the description of all the work

that has been developed in the context of this thesis. Epidemiological and clinical characterization of the subjects involved in this study is discussed in Chapter 3, followed by the description of three interesting and rare clinical cases involving patients from the studied population, from Chapter 4 to 6. Chapters 7 and 8 are dedicated to each specific mechanistic pathway (caspases and BER) and their association with individual susceptibility for this group of disorders. Finally, in Chapter 9 it is discussed the potential role of genetic polymorphisms in both caspases and BER pathway, and other factors in PN-MPNs survival and prognostic impact and in clinical outcome of patients under treatment, in terms of progression to secondary MF/AML, development of new primary nonmyeloid malignancies and thrombotic events occurrence.

The main reflexions and conclusions of this work, which were exposed throughout the several Chapters of this dissertation, are summarized in the last Chapter “Conclusions and final remarks” of the data set obtained.

* Dinis J, Silva V, Gromicho M, Martins C, Laires A, Tavares P, Rendeiro P, Torres F, Rueff J, Rodrigues A. DNA damage response in imatinib resistant chronic myeloid leukemia K562 cells. Leuk Lymphoma 2012; 53(10):2004-14. DOI: 10.3109/10428194.2012.681654

Gromicho M, Rueff J, Rodrigues A. Dynamics of expression of drug transporters: methods for appraisal. Methods mol biol 2016; 1395:75-85. DOI: 10.1007/978-1-4939-3347-1_6

Gromicho M, Magalhães M, Torres F, Dinis J, Fernandes A, Rendeiro P, Tavares P, Laires A, Rueff J, Rodrigues A. Instability of mRNA expression signatures of drug transporters in chronic myeloid leukemia patients resistant to imatinib. Oncol Reports 2013;29(2):741-50: DOI: 10.3892/or.2012.2153

Gromicho M, Dinis J, Magalhães M, Fernandes A, Tavares P, Laires A, Rueff J, Rodrigues A. Development of imatinib and dasatinib resistance: dynamics of expression of drug transporters ABCB1, ABCC1, ABCG2, MVP, and SLC22A1. Leuk Lymphoma 2011; 52(10): 1980-90. DOI: 10.3109/10428194.2011.584005

Rodrigues A, Dinis J, Gromicho M, Martins C, Laires A, Rueff J. Genomics and cancer drug resistance. Curr Pharm Biotechnol 2012; 13(5):651-73. PMID: 22122479

CHAPTER 3:
PREVALENCE OF *JAK2* V617F MUTATION IN PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS IN A PORTUGUESE POPULATION

Published in Azevedo *et al.*, 2017, Biomedical Reports 7:370-376
DOI: 10.3892/br.2017.977

ABSTRACT

Myeloproliferative neoplasms (MPNs) result from the malignant transformation of a hematopoietic stem-cell (HSC), leading to abnormal amplification and proliferation of myeloid lineages. Identification of the Janus kinase 2 (JAK2) V617F mutation developed the knowledge of Philadelphia-negative (PN)-MPNs, contributing to and influencing the definition of the phenotype and prognostic impact.

Considering the lack of Portuguese epidemiological data, the present study intends to characterize the prevalence of the JAK2 mutation in a PN-MPN versus a control Portuguese population.

Caucasian Portuguese PN-MPN patients (n=133) and 281 matched control subjects were investigated. No significant differences were identified between the case and control groups concerning age distribution or smoking habits. Pathology distribution was as follows: 60.2% with essential thrombocythemia (ET), 29.3% with polycythemia vera (PV) and 10.5% with primary myelofibrosis (PMF). A total of 75.0% of patients were positive for the presence of the JAK2 V617F mutation. In addition, the prevalence of PV was 87.2%, ET was 73.4% and PMF was 50.0%.

The JAK2 V617F mutation is observed in various MPN phenotypes, and has an increased incidence in ET patients and a decreased incidence in PV patients. These data may contribute to improving the knowledge of the pathophysiology of these disorders, and to a more rational and efficient selection of therapeutic strategies to be adopted, notably because an important amount of patients are JAK2 V617F negative.

CHAPTER 3: PREVALENCE OF JAK2 V617F MUTATION IN PHILADELPHIA NEGATIVE MYELOPROLIFERATIVE NEOPLASMS IN A PORTUGUESE POPULATION

3.1 – INTRODUCTION

Myeloproliferative neoplasms (MPNs) are clonal disorders resulting from malignant transformation of hematopoietic stem cells, leading to abnormal amplification and proliferation of one or more myeloid lineages. According to the World Health Organization (WHO) 2008 classification and the 2016 revision (Arber et al., 2016; Swerdlow et al., 2008), the classic MPNs encompass chronic myelogenous leukemia and the *BCR/ABL*-negative disorders (Philadelphia-negative MPNs; PN-MPNs), such as polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). Less frequent MPNs are chronic neutrophilic leukemia, chronic eosinophilic leukemia and other unclassifiable entities (Arber et al., 2016; Swerdlow et al., 2008).

The PN-MPNs have a combined annual incidence rate of 0.84 for PV, 1.03 for ET and 0.47 for PMF, per 100,000 respectively (Moulard et al., 2014; Titmarsh et al., 2014).

The presence of clonal hematopoiesis and cytokine hypersensitivity are fundamental for distinguishing PN-MPNs from reactive conditions. In routine practice, clonality is usually determined by the presence of an acquired mutation or cytogenetic abnormality, although additional clinical, laboratory and morphological information is important in the diagnosis of each specific subtype (Cross, 2011; Duletić et al., 2012; Tefferi & Pardanani, 2015).

Clinically, PV is characterized by excessive production of erythrocytes, increased red cell mass and extramedullary hematopoiesis, leading to splenomegaly. In ET there is a high platelet count, often associated with thrombotic and hemorrhagic events; however, bone marrow fibrosis is the hallmark of PMF, resulting in a variable count of myeloid series cells and hepatosplenomegaly (Arber et al., 2016; Swerdlow et al., 2008; Tefferi & Vardiman, 2008).

Major genetic insights into the pathogenesis of the PN-MPNs include identification of the somatic point gain-of-function mutations in the Janus kinase 2 (*JAK2*) gene (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Scott, Tong, et al., 2007), myeloproliferative leukemia (*MPL*) virus oncogene (more frequently termed *W515L/K*), and recently calreticulin (*CALR*) mutations, which contributed to an improved understanding of the pathophysiology of these disorders, their diagnostic tools and therapeutic management (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Scott, Tong, et al., 2007). According to the available studies, the frequencies of these mutations are ~95, 0, and 0% in PV, 60, 3, and 20% in ET, and 60, 7, and 25% in PMF, respectively (Cazzola & Kralovics, 2014; Hinds et al., 2016; Levine, 2009; Nangalia et al., 2013;

Oh & Gotlib, 2010; Tefferi & Pardanani, 2015). Although it is possible to identify one of these mutations in the majority of the *BCR/ABL*-negative disorder patients, there are unidentified genetic defects in approximately 10-15% of cases, predominantly of ET and PMF and, furthermore, those mutations cannot fully explain the phenotypic heterogeneity of PN-MPNs nor the susceptibility of progression to myelofibrosis, acute myeloid leukaemia (AML) or myelodysplastic syndromes (MDS) (Hinds et al., 2016). In addition, the cellular and molecular mechanisms involved in the pathophysiology of MPNs have not yet been fully clarified (Beer et al., 2010; Björkholm, Hultcrantz, & Derolf, 2014; Bolufer et al., 2006; Campregher, Santos, Perini, & Hamerschlak, 2012; Delhommeau, Jeziorowska, Marzac, & Casadevall, 2010; Hinds et al., 2016; Kilpivaara & Levine, 2008; Rice et al., 2011; Rueff & Rodrigues, 2016).

It is well known that hematopoietic cytokine receptor signaling is largely mediated by JAKs, a family of tyrosine kinases, and their downstream transcription factors, termed signal transducer and activator of transcription (STAT). JAK2 is essential for normal hematopoiesis, as demonstrated by defects in erythropoiesis observed in JAK2-deficient mice (Ebid, Ghareeb, Salaheldin, & Kamel, 2015). It is composed of two main domains, one is an enzymatically active kinase domain (JAK homology 1; JH1) and the other consists of a catalytically inactive pseudokinase domain (JH2), which exerts an inhibitory affect that generally inhibits the kinase activity of JAK2 (Anand et al., 2011; Ebid et al., 2015; Jatiani, Baker, Silverman, & Reddy, 2010).

In MPN patients, even in the absence of the *JAK2* mutation, the other genetic changes result in activation of the JAK2 signaling pathway (Reuther, 2016).

The most common mutation of *JAK2* consists of a substitution of valine with phenylalanine at position 617 in the JH2 domain (*JAK2* V617F) in exon 14, which affects the inhibitory function of the pseudokinase JH2 domain, inducing an increased activity in myeloid progenitor cells, leading to proliferation and excessive production of mature cells (Anand et al., 2011; Chen & Mullally, 2014; Ebid et al., 2015; Green & Llambi, 2015; Steensma et al., 2006). *JAK2* V617F and exon 12 mutations signal through the same C-terminal tyrosine kinase of JAK2, but result in very different phenotypic readouts. *JAK2* exon 12 mutations are tightly associated with PV in patients and mouse models (Scott, Tong, et al., 2007). The reasons for these different abnormal phenotypic outcomes remain unclear and are likely to be complex (Godfrey et al., 2016; Passamonti et al., 2011) .

For the *JAK2* V617F mutation to affect hematopoietic progenitor cells, the presence of receptors for erythropoietin, thrombopoietin or granulocyte-colony stimulating factor (CSF) is essential, leading to enhanced functional activity and increased sensitivity to cytokines and hematopoietic growth factors, such as interleukin 3 (IL-3), stem cell factor (SCF), granulocyte-macrophage CSF and insulin-like growth factor-1 (Ebid et al., 2015).

Previous data demonstrate the contribution and influence of the presence of the *JAK2* V617F mutation and the respective gene dosage in the definition of phenotype and prognostic impact in PN-MPNs (Nielsen, Bojesen, Nordestgaard, Kofoed, & Birgens, 2014). For example, the V617F allele burden tends to be higher in PV and PMF, and is associated with the presence of acquired uniparental

disomy (UPD), whereas a lower allele burden is generally observed in ET patients (Chen & Mullally, 2014; Duletić et al., 2012; Ha, Kim, Jung, Jung, & Chung, 2012; Hinds et al., 2016; Larsen, Pallisgaard, Møller, & Hasselbalch, 2007).

In PV and ET, risk factors for survival include older age, leukocytosis and thrombosis, whereas in ET, the *JAK2* V617F mutation is associated with increased risk of thrombosis, and is incorporated into the International Prognostic Score for Thrombosis in ET-thrombosis score (Barbui et al., 2015; Baxter et al., 2005). Accumulation of *JAK2* mutated allele accompanies the transformation of PV and ET to secondary myelofibrosis (Vannucchi, Pieri, & Guglielmelli, 2011). Furthermore, the presence of two or more mutations predict a worse survival and is associated with shortened leukemia-free survival (Guglielmelli et al., 2014).

Furthermore, *JAK2* V617F is not specific for a particular PN-MPN, nor does its absence exclude MPNs. Indeed, this has been reported in certain cases of MDS/MPN, in rare cases of AML (in combination with other well-defined genetic abnormalities, such as BCR-ABL1), and in association with certain solid tumors (Ebid et al., 2015; Nielsen, Birgens, Nordestgaard, Kjaer, & Bojesen, 2011; Steensma et al., 2006; Swerdlow et al., 2008; Thomas, Snowden, Zeidler, & Danson, 2015; Vainchenker & Constantinescu, 2013).

A wider characterization of molecular genetic features in PN-MPNs may contribute to an improved knowledge and understanding of the physiopathology of these disorders, allowing achievement of novel specific diagnostic, prognostic and therapeutic tools (Delhommeau et al., 2010; Mambet, Matei, Necula, & Diaconu, 2016). The identification of *JAK2/MPL* mutations in the majority of patients led to the development of JAK kinase inhibitors. Although ruxolitinib was recently approved for use in hydroxyurea-resistant PV, its role in routine clinical practice remains controversial (Hobbs, Rozelle, & Mullally, 2017; Tefferi, 2016; Tefferi & Barbui, 2017; Tefferi & Pardanani, 2015; Vannucchi & Harrison, 2017). For myelofibrosis patients, stem cell transplant is the current treatment of choice for genetically or clinically high-risk disease. For all other patients that require treatment, the currently available drugs, including JAK inhibitors, are palliative, as they improve patient symptoms and reduce splenomegaly, but have not been identified as disease modifying, nor do they significantly reduce the mutant allele burden (Stahl & Zeidan, 2017; Tefferi, 2016).

The present study describes a hospital based case-control study to evaluate the prevalence of the *JAK2* mutation in PN-MPNs patients, as well as in healthy individuals without clinical disease, in a Caucasian Portuguese population.

3.2 – MATERIALS AND METHODS

3.2.1 – STUDY SUBJECTS

The present case-control study involved 133 Caucasian Portuguese PN-MPN patients (80 with ET, 39 with PV and 14 with PMF) and 281 age- and sex-matched control subjects selected since January 2009 to July 2016 within the Portuguese population recruited at the Departments of Clinical Hematology and of Clinical Pathology, Hospital de São Francisco Xavier, Centro Hospitalar de Lisboa Ocidental (CHLO; Lisbon, Portugal), a public general hospital that provides healthcare to the western population of Lisbon, where those patients were admitted, followed up and treated. The diagnostic criteria for all patients were those defined by the World Health Organization in the 2008 and updated 2016 guidelines (Arber et al., 2016; Swerdlow et al., 2008). For all cases, at least two control individuals (n=281), without neoplastic pathology, matched for age (±2 years), gender and ethnicity were recruited, with no personal or family history of PN-MPNs, no previous or current malignant disease, nor history of blood transfusions. All study subjects were Portuguese, with Portuguese ascendants. Information on demographic characteristics, family history of cancer, lifestyle habits (e.g. smoking) and exposure to ionizing radiation was collected via a questionnaire administered by trained interviewers. With respect to smoking habits, former smokers were considered as non-smokers if they gave up smoking either 2 years before PN-MPN diagnosis or, for controls, 2 years before the date of inclusion in the study. The response rate was >95% for the cases and control subjects. The anonymity of the patients and control population was guaranteed, and written informed consent was obtained from all those involved, prior to blood withdrawal, in agreement with the Declaration of Helsinki. The current study was conducted with approval by the institutional ethics' boards of the involved institutions - CHLO and Nova Medical School (were the practical work was performed), both in Lisbon, Portugal.

The general characteristics for PN-MPNs patients at the time of diagnosis and the control populations are summarized in Tables 3.1 and 3.2. All clinical and hematologic data were obtained from registries, and were selected on the basis of diagnostic criteria for this type of disease.

3.2.2 – DNA EXTRACTION

Peripheral blood samples (7-8 ml) of all patients and controls were collected by qualified personnel into 10 ml EDTA tubes and maintained thereafter at -80ºC. Genomic DNA was obtained from each blood sample (250µl) using a commercially available kit, accomplished by cell lysis followed by ethanol precipitation and recovery of the DNA by elution in a buffer solution (QIAamp® DNA mini kit (Cat. No. 51306); Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. All DNA samples were stored at -20°C until analysis.

3.2.3 – GENOTYPING

The *JAK2* V617F mutational status was determined via quantitative real time polymerase chain reaction (qPCR; Applied Biosystems 7300 Real-Time PCR System), and TaqMan® single nucleotide polymorphism genotyping assay (rs77375493 - Cat. No. C_101301592_10, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Amplification was carried out within 96-well microplates containing 10ng of genomic DNA, 1xSNP genotyping assay mix and 1xTaqman Universal PCR Master mix per well (final volume, 10 µl/well). Initial enzyme activation (10 min, at 95°C) was ensued by 40 cycles of denaturation (15 sec at 92°C) and probe annealing/extension (1 min at 60°C). Allelic discrimination was then performed by measuring fluorescence emitted by both VIC and FAM dyes in each well (60 sec) and computing the results into the System SDS software version. The methodology was performed according to the manufacturer's protocol with minor modifications included in previous reports from our group and using primers from the Taqman kit (Bastos et al., 2009; Conde et al., 2009; Gomes et al., 2010; Silva et al., 2009; Silva et al., 2007), with a minor modification where the final volume of the reaction was adjusted to 10 µl. Genotype determination was performed in 20% of samples in independent experiments (60 randomly selected individuals for the control group and 30 for the case group), and all of the inconclusive samples were reanalyzed.

3.2.4 – STATISTICAL ANALYSIS

The analysis of Hardy-Weinberg frequencies for all alleles in the control and patient populations was conducted using exact probability tests available in SNPStat website software (http://bioinfo.iconcologia.net/SNPstats) (Solé, Guinó, Valls, Iniesta, & Moreno, 2006). Differences in genotype frequency, smoking status, age ranges and gender distributions between PN-MPN cancer patients and control subjects were evaluated using the χ^2 test ($P<0.05$ was considered to indicate a statistically significant difference).

3.3 – RESULTS

The present study included 133 PN-MPN patients and 281 age- and sex-matched control subjects. The baseline characteristics (sex, age and smoking habits) of the case and control populations are presented in Table 3.1. The case group included 72 (54.1%) female and 61 (45.9%) male patients, with an overall mean age of 68 years (Table 3.1). No significant differences were identified between the case and control groups concerning age distribution or smoking habits (Table 3.1).

Table 3.1 – General characteristics of the PN-MPNs case (n=133) and control populations (n=281).

Characteristic	Cases, *n* (%)	Controls, *n* (%)	P-value
Gender			
Male	61 (45.9)	133 (47.3)	0.8
Female	72 (54.1)	148 (52.7)	
Age, years[a]			
30-49	16 (12.0)	43 (15.3)	0.6
50-69	50 (37.6)	107 (38.1)	
≥70	67 (50.4)	131 (46.6)	
Smoking habits			
Never	104 (78.2)	213 (76.1)	0.6
Current	29 (21.8)	67 (23.9)	
Alcohol habits			
Never	103 (77.4)	191 (68.2)	**<0.0001**
Social	20 (15.0)	25 (8.9)	
Regular	10 (7.5)	64 (22.9)	
JAK2 V617F mutation			
Yes	99 (75.0)		
Essential thrombocythemia	58 (73.4)		**0.020**
Polycythemia vera	34 (87.2)		
Primary myelofibrosis	7 (50.0)		
No	33 (25.0)	281 (100.0)	

[a] Age at diagnosis for the patients and age of the control population subjects at the time of diagnosis of the matched case.

According to the diagnostic criteria, the patient distributions were as follows: 80 (60.2%) with ET, 39 (29.3%) with PV and 14 (10.5%) with PMF. The majority of the patients with ET were female (60.0%), while in PV and PMF, males predominated (51.3 and 64.3%, respectively; Table 3.2).

Patient and control populations were stratified according to the presence of the *JAK2* V617F mutation, demonstrating that 75.0% of patients and none of the controls were positive for *JAK2* V617F (Table 3.1). The prevalence of the *JAK2* V617F mutation for patients with PV was 87.2%, for ET 73.4% and for PMF 50.0% (Table 3.2). The allelic distribution of the PN-MPN cases, stratified by diagnosis, is presented in Table 3.2. General characteristics of patients are presented in Table 3.2, according to the type of PN-MPN.

Table 3.2 – *JAK2* V617F mutation allelic distribution in the PN-MPNs cases (n=133).

Diagnosis	Janus kinase 2		
	Val/val (%)	Val/phe (%)	Phe/phe (%)
Essential thrombocythemia	21 (26.6)	56 (70.9)	2 (2.5)
Polycythemia vera	5 (12.8)	31 (79.5)	3 (7.7)
Primary myelofibrosis	7 (50.0)	5 (35.7)	2 (14.3)

Phe – Phenilalanin; Val – Valin.

3.4 – DISCUSSION AND CONCLUSIONS

In recent years, investigation on MPNs revealed that there are three driver mutations (*JAK2, MPL and CALR*) essential as clonal markers, activating the cytokine receptor JAK2 signaling pathway and the downstream effectors (Reuther, 2016; Rumi & Cazzola, 2017).

However, it is known that the different driver mutations involved in the pathogenesis of MPNs leads to different clinical effects, and that a single mutation may be associated with distinct phenotypes and clinical outcomes. This finding may be due to the association with other commutated non-MPN-driver genes (for example, *additional sex combs like 1, transcriptional regulator, enhancer of zeste 2 polycomb repressive complex 2 subunit, tet methylcytosine dioxygenase 2, isocitrate dehydrogenase (NADP$^+$)1/2, cytosolic, splicing factor 3b subunit 1, serine and arginine rich splicing factor 2*) (Rumi & Cazzola, 2017).

It is unequivocal that the *JAK2* V617F mutation is found in various phenotypes; however, it is seemingly also associated with other malignancies (generally non-hematological types) (Nielsen et al., 2011).

The role of the JAK/STAT signaling pathway in the pathogenesis of MPNs and other cancers is questionable when considering the example of rare families presenting with germline mutations leading to weak JAK activation. The mutations originate a hereditary thrombocytosis, but hematopoiesis is polyclonal and there is no development of hematological malignancies or solid tumors, indicating that JAK/STAT activation alone does not drive malignant disease (Thomas et al., 2015).

The *JAK2* V617F mutation was screened in the patients and healthy control subjects, to evaluate the eventual presence and prevalence of the *JAK2* mutation in healthy individuals in our control population, reflecting healthy Portuguese general population status, as it is the most prevalent among the driver mutations for PN-MPNs. The fact that no *JAK2* V617F mutation was identified in healthy control subjects during the present study does not dismiss the possible weak effect of this mutation in driving MPNs. Although the absence of the *JAK2* mutation does not exclude MPN, its presence is not specific for any specific PN-MPN and phenotypic expression may depend on various factors.

However, as the *JAK2* V617F mutation is found in the great majority of MPNs indicates that it is probably the primary abnormality driving myeloproliferative cells, although it is not definitely clear whether it has to be homozygous in all cases; it may become homozygous as a result of the loss of heterozygosity (LOH) or UPD (Chen & Mullally, 2014; Jones et al., 2005). Furthermore, it cannot be ruled out whether an inherited mutation in one of the alleles may be accompanied by an epigenomic inactivation of the other otherwise normal alleles rendering the cell biological homozygous. What seems clear, however, and the present data contributes to this conclusion, is that the role of the *JAK2* V617F mutation in the pathogenicity of the different MPNs may differ amongst different MPNs requiring the *JAK2* V617F mutation more often than others (e.g. ET vs. PV), which would indicate other oncogenic

mutations that may be relevant for certain cases others than *JAK2* V617F (Chen & Mullally, 2014; Hinds et al., 2016; Jones et al., 2005; Lundberg et al., 2014).

Besides mutations and other molecular abnormalities, various factors, such as gene burden and individual genetic background, may influence the predisposition for developing an MPN, as well as their heterogeneity (Hinds et al., 2016; Rumi & Cazzola, 2017). Although *JAK2* V617F homozygous subclones are present in PV and ET patients, the expansion of a dominant homozygous subclone occurs almost exclusively in PV patients (~80% in PV and 50% in ET) (Chen & Mullally, 2014; Rumi & Cazzola, 2017), due to either additional genetic or epigenetic events or non-cell-autonomous selective pressures, such as low levels of circulating erythropoietin in the context of elevated hematocrit (Chen & Mullally, 2014).

However, the WHO classification of classical MPNs allows that the diagnosis is established on the basis of other criteria, even if the criteria concerning the driver mutations is not met, and that diagnosis is established on the basis of other criteria. Indeed, these disorders are primarily defined on the basis of clinical, pathologic and morphologic/histologic features, with the possibility of diagnosing MPN without any evidence of a driver mutation (Arber et al., 2016; Swerdlow et al., 2008).

The present study revealed a higher incidence of the *JAK2* V617F mutation in ET patients and a comparatively lower incidence in the PV patients, when compared with the published data for each disease (Duletić et al., 2012; Levine, 2009; Nangalia et al., 2013; Tefferi & Pardanani, 2015). The discrepancy between published data and the present results is greater in the case of ET, than in PV. This may be due to the small size of the investigated population and to the larger number of ET patients that was included. The majority of patients presented with ET (80 of a total of 133 patients), leading to a more consistent and representative result, reflecting Portuguese reality, when compared with PV. This indicates that the population of the present study has a different pattern concerning the presence of this mutation, when compared with other already studied populations from other countries, highlighting the importance of developing future studies in larger and diversified populations. representing a tendency of how relevant could it be for future studies in larger and different populations.

As described by Rumi and Cazzola (Rumi & Cazzola, 2017), patients with the wild type *JAK2* V617F or exon 12 mutation are extremely rare. However, the current results revealed a prevalence of 12.8% of patients with the wild type genotype. This finding supports the fact that the *JAK2* mutation acting alone may not be sufficient to develop the PV phenotype. However, larger studies are required to confirm this hypothesis.

Subsequent to performing a literature review, almost all of the patients diagnosed with PV negative for the *JAK2* V617F mutation were exon 12-positive (96 vs. ~3%, respectively) (Arber et al., 2016; Butcher et al., 2008; Pardanani, Lasho, Finke, Hanson, & Tefferi, 2007; Park et al., 2016; Passamonti et al., 2011; Passamonti et al., 2010; Rumi et al., 2014; Scott, 2011; Scott, Beer, Bench, Erber, & Green, 2007; Scott, Tong, et al., 2007). In the current study, from the five PV patients that were exon 14-negative, two underwent exon 12 molecular evaluation, and only one was positive. For certain patients, particularly those selected at the beginning of the study, it was no longer possible to

obtain blood samples to proceed with the study.

CALR and *MPL* mutations were not assessed in the ET and MF patients that were *JAK2*-negative when the diagnosis was no doubtful , and therefore when the diagnosis was certain on the basis of clinical, pathologic and morphologic/histologic features. The presence of these mutations may thus not be a necessarily met criteria according to the WHO classification.

The patients and controls included in the present study were recruited from 2009 until 2016. The majority of the patients were evaluated according to the WHO 2008 diagnostic criteria, which was revised in 2016 (Table 3.1). Although bone marrow biopsies may not be a met criterion (for ET and PV) in the two versions of the WHO classification, in our institution this is a type of routine examination for these patients, as it is considered to be important for predicting the prognosis.

Published epidemiology data are scarce (Moulard et al., 2014; Titmarsh et al., 2014). The gender distribution observed in the current population was consistent with previous data regarding this group of disorders (Azevedo et al., 2017). Furthermore, although certain published studies consider smoking as a contributing factor for PN-MPNs (Hasselbalch, 2015; Lindholm Sørensen & Hasselbalch, 2015), the present study did not reveal a significant association, potentially due to the small number of smoking individuals that was included.

Additional studies involving larger populations are required to further clarify the potential value of different genotypes as predictive biomarkers of susceptibility to PN-MPNs. An improved understanding of the pathophysiological mechanisms will enable the development of drugs that are more directly and specifically targeted, with high efficacy and fewer adverse effects, thus contributing to the compliance of patients with treatments.

The discovery of *JAK2* was a very important milestone for the studies that followed and for what we know today, but the ongoing identification of other mutations in MPNs will make possible the signaling of new drug targets and prognostic biomarkers that will for certain improve clinical practice and patients' outcome. All in all, it remains to be fully ascertained whether *JAK2* mutations may considered as 'driver mutations' for MPNs, or if they can act as 'passenger mutations' which may change place with the former and have 'driver' functions (Rueff & Rodrigues, 2016).

3.5 – REFERENCES

Anand, S., Stedham, F., Beer, P., Gudgin, E., Ortmann, C. A., Bench, A., . . . Huntly, B. J. (2011). Effects of the JAK2 mutation on the hematopoietic stem and progenitor compartment in human myeloproliferative neoplasms. *Blood, 118*(1), 177-181. doi:10.1182/blood-2010-12-327593

Arber, D. A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M. J., Le Beau, M. M., . . . Vardiman, J. W. (2016). The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood, 127*(20), 2391-2405. doi:10.1182/blood-2016-03-643544

Azevedo, A. P., Silva, S. N., De Lima, J. P., Reichert, A., Lima, F., Júnior, E., & Rueff, J. (2017). DNA repair genes polymorphisms and genetic susceptibility to Philadelphia-negative myeloproliferative neoplasms in a Portuguese population: The role of base excision repair genes polymorphisms. *Oncol Lett, 13*(6), 4641-4650. doi:10.3892/ol.2017.6065

Barbui, T., Vannucchi, A. M., Buxhofer-Ausch, V., De Stefano, V., Betti, S., Rambaldi, A., . . . Tefferi, A. (2015). Practice-relevant revision of IPSET-thrombosis based on 1019 patients with WHO-defined essential thrombocythemia. *Blood Cancer J, 5*, e369. doi:10.1038/bcj.2015.94

Bastos, H. N., Antão, M. R., Silva, S. N., Azevedo, A. P., Manita, I., Teixeira, V., . . . Gaspar, J. F. (2009). Association of polymorphisms in genes of the homologous recombination DNA repair pathway and thyroid cancer risk. *Thyroid, 19*(10), 1067-1075. doi:10.1089/thy.2009.0099

Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., . . . Project, C. G. (2005). Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet, 365*(9464), 1054-1061. doi:10.1016/S0140-6736(05)71142-9

Beer, P. A., Delhommeau, F., LeCouédic, J. P., Dawson, M. A., Chen, E., Bareford, D., . . . Green, A. R. (2010). Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. *Blood, 115*(14), 2891-2900. doi:10.1182/blood-2009-08-236596

Björkholm, M., Hultcrantz, M., & Derolf, Å. (2014). Leukemic transformation in myeloproliferative neoplasms: therapy-related or unrelated? *Best Pract Res Clin Haematol, 27*(2), 141-153. doi:10.1016/j.beha.2014.07.003

Bolufer, P., Barragan, E., Collado, M., Cervera, J., López, J. A., & Sanz, M. A. (2006). Influence of genetic polymorphisms on the risk of developing leukemia and on disease progression. *Leuk Res, 30*(12), 1471-1491. doi:10.1016/j.leukres.2006.01.016

Butcher, C. M., Hahn, U., To, L. B., Gecz, J., Wilkins, E. J., Scott, H. S., . . . D'Andrea, R. J. (2008). Two novel JAK2 exon 12 mutations in JAK2V617F-negative polycythaemia vera patients. *Leukemia, 22*(4), 870-873. doi:10.1038/sj.leu.2404971

Campregher, P. V., Santos, F. P., Perini, G. F., & Hamerschlak, N. (2012). Molecular biology of Philadelphia-negative myeloproliferative neoplasms. *Rev Bras Hematol Hemoter, 34*(2), 150-155. doi:10.5581/1516-8484.20120035

Cazzola, M., & Kralovics, R. (2014). From Janus kinase 2 to calreticulin: the clinically relevant genomic landscape of myeloproliferative neoplasms. *Blood, 123*(24), 3714-3719. doi:10.1182/blood-2014-03-530865

Chen, E., & Mullally, A. (2014). How does JAK2V617F contribute to the pathogenesis of myeloproliferative neoplasms? *Hematology Am Soc Hematol Educ Program, 2014*(1), 268-276. doi:10.1182/asheducation-2014.1.268

Conde, J., Silva, S. N., Azevedo, A. P., Teixeira, V., Pina, J. E., Rueff, J., & Gaspar, J. F. (2009). Association of common variants in mismatch repair genes and breast cancer susceptibility: a multigene study. *BMC Cancer, 9*, 344. doi:10.1186/1471-2407-9-344

Cross, N. C. (2011). Genetic and epigenetic complexity in myeloproliferative neoplasms. *Hematology Am Soc Hematol Educ Program, 2011*, 208-214. doi:10.1182/asheducation-2011.1.208

Delhommeau, F., Jeziorowska, D., Marzac, C., & Casadevall, N. (2010). Molecular aspects of myeloproliferative neoplasms. *Int J Hematol, 91*(2), 165-173. doi:10.1007/s12185-010-0530-z

Duletić, A. N., Dekanić, A., Hadzisejdić, I., Kusen, I., Matusan-Ilijas, K., Grohovac, D., . . . Jonjić, N. (2012). JAK2-v617F mutation is associated with clinical and laboratory features of myeloproliferative neoplasms. *Coll Antropol, 36*(3), 859-865.

Ebid, G. T., Ghareeb, M., Salaheldin, O., & Kamel, M. M. (2015). Prevalence of the frequency of JAK2 (V617F) mutation in different myeloproliferative disorders in Egyptian patients. *Int J Clin Exp Pathol, 8*(9), 11555-11559.

Godfrey, A. L., Chen, E., Massie, C. E., Silber, Y., Pagano, F., Bellosillo, B., . . . Green, A. R. (2016). STAT1 activation in association with JAK2 exon 12 mutations. *Haematologica, 101*(1), e15-19. doi:10.3324/haematol.2015.128546

Gomes, B. C., Silva, S. N., Azevedo, A. P., Manita, I., Gil, O. M., Ferreira, T. C., . . . Gaspar, J. F. (2010). The role of common variants of non-homologous end-joining repair genes XRCC4, LIG4 and Ku80 in thyroid cancer risk. *Oncol Rep, 24*(4), 1079-1085.

Green, D. R., & Llambi, F. (2015). Cell Death Signaling. *Cold Spring Harb Perspect Biol, 7*(12). doi:10.1101/cshperspect.a006080

Guglielmelli, P., Lasho, T. L., Rotunno, G., Score, J., Mannarelli, C., Pancrazzi, A., . . . Tefferi, A. (2014). The number of prognostically detrimental mutations and prognosis in primary myelofibrosis: an international study of 797 patients. *Leukemia, 28*(9), 1804-1810. doi:10.1038/leu.2014.76

Ha, J. S., Kim, Y. K., Jung, S. I., Jung, H. R., & Chung, I. S. (2012). Correlations between Janus kinase 2 V617F allele burdens and clinicohematologic parameters in myeloproliferative neoplasms. *Ann Lab Med, 32*(6), 385-391. doi:10.3343/alm.2012.32.6.385

Hasselbalch, H. C. (2015). Smoking as a contributing factor for development of polycythemia vera and related neoplasms. *Leuk Res*. doi:10.1016/j.leukres.2015.09.002

Hinds, D. A., Barnholt, K. E., Mesa, R. A., Kiefer, A. K., Do, C. B., Eriksson, N., . . . Gotlib, J. (2016). Germ line variants predispose to both JAK2 V617F clonal hematopoiesis and myeloproliferative neoplasms. *Blood, 128*(8), 1121-1128. doi:10.1182/blood-2015-06-652941

Hobbs, G. S., Rozelle, S., & Mullally, A. (2017). The Development and Use of Janus Kinase 2 Inhibitors for the Treatment of Myeloproliferative Neoplasms. *Hematol Oncol Clin North Am, 31*(4), 613-626. doi:10.1016/j.hoc.2017.04.002

James, C., Ugo, V., Le Couédic, J. P., Staerk, J., Delhommeau, F., Lacout, C., . . . Vainchenker, W. (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature, 434*(7037), 1144-1148. doi:10.1038/nature03546

Jatiani, S. S., Baker, S. J., Silverman, L. R., & Reddy, E. P. (2010). Jak/STAT pathways in cytokine signaling and myeloproliferative disorders: approaches for targeted therapies. *Genes Cancer, 1*(10), 979-993. doi:10.1177/1947601910397187

Jones, A. V., Kreil, S., Zoi, K., Waghorn, K., Curtis, C., Zhang, L., . . . Cross, N. C. (2005). Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. *Blood, 106*(6), 2162-2168. doi:10.1182/blood-2005-03-1320

Kilpivaara, O., & Levine, R. L. (2008). JAK2 and MPL mutations in myeloproliferative neoplasms: discovery and science. *Leukemia, 22*(10), 1813-1817. doi:10.1038/leu.2008.229

Kralovics, R., Passamonti, F., Buser, A. S., Teo, S. S., Tiedt, R., Passweg, J. R., . . . Skoda, R. C. (2005). A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med, 352*(17), 1779-1790. doi:10.1056/NEJMoa051113

Larsen, T. S., Pallisgaard, N., Møller, M. B., & Hasselbalch, H. C. (2007). The JAK2 V617F allele burden in essential thrombocythemia, polycythemia vera and primary myelofibrosis--impact on disease phenotype. *Eur J Haematol, 79*(6), 508-515. doi:10.1111/j.1600-0609.2007.00960.x

Levine, R. L. (2009). Mechanisms of mutations in myeloproliferative neoplasms. *Best Pract Res Clin Haematol, 22*(4), 489-494. doi:10.1016/j.beha.2009.08.006

Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J., . . . Gilliland, D. G. (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell, 7*(4), 387-397. doi:10.1016/j.ccr.2005.03.023

Lindholm Sørensen, A., & Hasselbalch, H. C. (2015). Smoking and philadelphia-negative chronic myeloproliferative neoplasms. *Eur J Haematol*. doi:10.1111/ejh.12684

Lundberg, P., Takizawa, H., Kubovcakova, L., Guo, G., Hao-Shen, H., Dirnhofer, S., . . . Skoda, R. C. (2014). Myeloproliferative neoplasms can be initiated from a single hematopoietic stem cell expressing JAK2-V617F. *J Exp Med, 211*(11), 2213-2230. doi:10.1084/jem.20131371

Mambet, C., Matei, L., Necula, L. G., & Diaconu, C. C. (2016). A link between the driver mutations and dysregulated apoptosis in BCR-ABL1 negative myeloproliferative neoplasms. *J Immunoassay Immunochem, 37*(4), 331-345. doi:10.1080/15321819.2016.1152276

Moulard, O., Mehta, J., Fryzek, J., Olivares, R., Iqbal, U., & Mesa, R. A. (2014). Epidemiology of myelofibrosis, essential thrombocythemia, and polycythemia vera in the European Union. *Eur J Haematol, 92*(4), 289-297. doi:10.1111/ejh.12256

Nangalia, J., Massie, C. E., Baxter, E. J., Nice, F. L., Gundem, G., Wedge, D. C., . . . Green, A. R. (2013). Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med, 369*(25), 2391-2405. doi:10.1056/NEJMoa1312542

Nielsen, C., Birgens, H. S., Nordestgaard, B. G., Kjaer, L., & Bojesen, S. E. (2011). The JAK2 V617F somatic mutation, mortality and cancer risk in the general population. *Haematologica, 96*(3), 450-453. doi:10.3324/haematol.2010.033191

Nielsen, C., Bojesen, S. E., Nordestgaard, B. G., Kofoed, K. F., & Birgens, H. S. (2014). JAK2V617F somatic mutation in the general population: myeloproliferative neoplasm development and progression rate. *Haematologica, 99*(9), 1448-1455. doi:10.3324/haematol.2014.107631

Oh, S. T., & Gotlib, J. (2010). JAK2 V617F and beyond: role of genetics and aberrant signaling in the pathogenesis of myeloproliferative neoplasms. *Expert Rev Hematol, 3*(3), 323-337. doi:10.1586/ehm.10.28

Pardanani, A., Lasho, T. L., Finke, C., Hanson, C. A., & Tefferi, A. (2007). Prevalence and clinicopathologic correlates of JAK2 exon 12 mutations in JAK2V617F-negative polycythemia vera. *Leukemia, 21*(9), 1960-1963. doi:10.1038/sj.leu.2404810

Park, C. H., Lee, K. O., Jang, J. H., Jung, C. W., Kim, J. W., Kim, S. H., & Kim, H. J. (2016). High frequency of JAK2 exon 12 mutations in Korean patients with polycythaemia vera: novel mutations and clinical significance. *J Clin Pathol, 69*(8), 737-741. doi:10.1136/jclinpath-2016-203649

Passamonti, F., Elena, C., Schnittger, S., Skoda, R. C., Green, A. R., Girodon, F., . . . Cazzola, M. (2011). Molecular and clinical features of the myeloproliferative neoplasm associated with JAK2 exon 12 mutations. *Blood, 117*(10), 2813-2816. doi:10.1182/blood-2010-11-316810

Passamonti, F., Rumi, E., Pietra, D., Elena, C., Boveri, E., Arcaini, L., . . . Cazzola, M. (2010). A prospective study of 338 patients with polycythemia vera: the impact of JAK2 (V617F) allele burden and leukocytosis on fibrotic or leukemic disease transformation and vascular complications. *Leukemia, 24*(9), 1574-1579. doi:10.1038/leu.2010.148

Reuther, G. W. (2016). Myeloproliferative Neoplasms: Molecular Drivers and Therapeutics. *Prog Mol Biol Transl Sci, 144*, 437-484. doi:10.1016/bs.pmbts.2016.09.004

Rice, K. L., Lin, X., Wolniak, K., Ebert, B. L., Berkofsky-Fessler, W., Buzzai, M., . . . Zhang, W. (2011). Analysis of genomic aberrations and gene expression profiling identifies novel lesions and pathways in myeloproliferative neoplasms. *Blood Cancer J, 1*(11), e40. doi:10.1038/bcj.2011.39

Rueff, J., & Rodrigues, A. S. (2016). Cancer Drug Resistance: A Brief Overview from a Genetic Viewpoint. *Methods Mol Biol, 1395*, 1-18. doi:10.1007/978-1-4939-3347-1_1

Rumi, E., & Cazzola, M. (2017). Diagnosis, risk stratification, and response evaluation in classical myeloproliferative neoplasms. *Blood, 129*(6), 680-692. doi:10.1182/blood-2016-10-695957

Rumi, E., Pietra, D., Ferretti, V., Klampfl, T., Harutyunyan, A. S., Milosevic, J. D., . . . Investigators, A. I. p. l. R. s. C. G. I. M. M. (2014). JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. *Blood, 123*(10), 1544-1551. doi:10.1182/blood-2013-11-539098

Scott, L. M. (2011). The JAK2 exon 12 mutations: a comprehensive review. *Am J Hematol, 86*(8), 668-676. doi:10.1002/ajh.22063

Scott, L. M., Beer, P. A., Bench, A. J., Erber, W. N., & Green, A. R. (2007). Prevalance of JAK2 V617F and exon 12 mutations in polycythaemia vera. *Br J Haematol, 139*(3), 511-512. doi:10.1111/j.1365-2141.2007.06806.x

Scott, L. M., Tong, W., Levine, R. L., Scott, M. A., Beer, P. A., Stratton, M. R., . . . Green, A. R. (2007). JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. *N Engl J Med, 356*(5), 459-468. doi:10.1056/NEJMoa065202

Silva, S. N., Azevedo, A. P., Teixeira, V., Pina, J. E., Rueff, J., & Gaspar, J. F. (2009). The role of GSTA2 polymorphisms and haplotypes in breast cancer susceptibility: a case-control study in the Portuguese population. *Oncol Rep, 22*(3), 593-598.

Silva, S. N., Moita, R., Azevedo, A. P., Gouveia, R., Manita, I., Pina, J. E., . . . Gaspar, J. (2007). Menopausal age and XRCC1 gene polymorphisms: role in breast cancer risk. *Cancer Detect Prev, 31*(4), 303-309. doi:10.1016/j.cdp.2007.07.001

Solé, X., Guinó, E., Valls, J., Iniesta, R., & Moreno, V. (2006). SNPStats: a web tool for the analysis of association studies. *Bioinformatics, 22*(15), 1928-1929. doi:10.1093/bioinformatics/btl268

Stahl, M., & Zeidan, A. M. (2017). Management of myelofibrosis: JAK inhibition and beyond. *Expert Rev Hematol, 10*(5), 459-477. doi:10.1080/17474086.2017.1317590

Steensma, D. P., McClure, R. F., Karp, J. E., Tefferi, A., Lasho, T. L., Powell, H. L., . . . Kaufmann, S. H. (2006). JAK2 V617F is a rare finding in de novo acute myeloid leukemia, but STAT3 activation is common and remains unexplained. *Leukemia, 20*(6), 971-978. doi:10.1038/sj.leu.2404206

Swerdlow, S. H., Campo, E., Harris, N. L., Jaffe, E. S., Pileri, S. A., Stein, H., . . . Vardiman, J. W. (2008). *WHO Classification of Tumours of Haematopioetic and Lymphoid Tissues*. Lyon: World Health Organization.

Tefferi, A. (2016). Myeloproliferative neoplasms: A decade of discoveries and treatment advances. *Am J Hematol, 91*(1), 50-58. doi:10.1002/ajh.24221

Tefferi, A., & Barbui, T. (2017). Polycythemia vera and essential thrombocythemia: 2017 update on diagnosis, risk-stratification, and management. *Am J Hematol, 92*(1), 94-108. doi:10.1002/ajh.24607

Tefferi, A., & Pardanani, A. (2015). Myeloproliferative Neoplasms: A Contemporary Review. *JAMA Oncol, 1*(1), 97-105. doi:10.1001/jamaoncol.2015.89

Tefferi, A., & Vardiman, J. W. (2008). Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia, 22*(1), 14-22. doi:10.1038/sj.leu.2404955

Thomas, S. J., Snowden, J. A., Zeidler, M. P., & Danson, S. J. (2015). The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours. *Br J Cancer, 113*(3), 365-371. doi:10.1038/bjc.2015.233

Titmarsh, G. J., Duncombe, A. S., McMullin, M. F., O'Rorke, M., Mesa, R., De Vocht, F., . . . Anderson, L. A. (2014). How common are myeloproliferative neoplasms? A systematic review and meta-analysis. *Am J Hematol, 89*(6), 581-587.

Vainchenker, W., & Constantinescu, S. N. (2013). JAK/STAT signaling in hematological malignancies. *Oncogene, 32*(21), 2601-2613. doi:10.1038/onc.2012.347

Vannucchi, A. M., & Harrison, C. N. (2017). Emerging treatments for classical myeloproliferative neoplasms. *Blood, 129*(6), 693-703. doi:10.1182/blood-2016-10-695965

Vannucchi, A. M., Pieri, L., & Guglielmelli, P. (2011). JAK2 Allele Burden in the Myeloproliferative Neoplasms: Effects on Phenotype, Prognosis and Change with Treatment. *Ther Adv Hematol, 2*(1), 21-32. doi:10.1177/2040620710394474

CHAPTER 4:
ATYPICAL HEMATOLOGICAL PRESENTATION IN A CASE OF POLYCYTHEMIA VERA WITH A NEW VARIANT MUTATION DETECTED IN EXON 12 - C.1605G>T(P.MET535ILE)

Published in Pita & Azevedo *et al.*, 2017, BMJ – Journal of Clinical Pathology Oct11 (Epub)
DOI: 10.1136/jclinpath.2017.204556

Abstract - Apresentação hematológica atípica num caso de policitémia vera com mutação variante detetada no exão 12 – c.1605G>T (P.Met535Ile).
(2017) Published in Blood Transfusion 15 (Suplement 2):s415

ABSTRACT

One of the major genetic insights into the pathogenesis of Polycythemia Vera included the identification of the somatic point gain-of-function mutations in Janus kinase 2 gene – first JAK2 V617F on exon 14, present in 95-97% of the cases, and later on exon 12.

In the literature we can find some reported studies where different exon 12 mutations are identified.

Unlike patients with JAK2 V617F mutation in exon 14, the mutation at exon 12 is not usually associated with an increase in the 3 hematopoietic series (erythrocytosis, leukocytosis and thrombocytosis). It appears to be associated with a distinct syndrome, mostly characterized by isolated and more marked erythrocytosis, independently of the mutational variant.

We report here the case of a patient JAK2 exon 12 positive, presenting a novel mutation – c. 1605G>T (p.Met535Ile) - associated with c.1612C>T (p.His538Tyr) mutation previously described, evidencing an atypical

clinical phenotype characterized by the presence of thrombocytosis, instead of erythrocytosis which is the usual main feature associated with JAK2 exon 12 mutations.

CHAPTER 4: ATYPICAL HEMATOLOGICAL PRESENTATION IN A CASE OF POLYCYTHEMIA VERA WITH A NEW VARIANT MUTATION DETECTED IN EXON 12 – C.1605G>T(P.MET535ILE)

4.1 – INTRODUCTION

According to the World Health Organization (WHO) 2008 Classification of Tumours of Haematopoietic and Lymphoid Tissues and 2016 revision (Arber et al., 2016), Philadelphia-negative Myeloproliferative Neoplasms (PN-MPNs) can be classified into major entities, such as Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF).

One of the major genetic insights into the pathogenesis of the PN-MPNs included the identification of somatic mutations in Janus kinase 2 gene (*JAK2*; exon 14 V617F gain-of-function mutation first in 2005; then later in 2007 exon 12 mutation), leading to the activation of the JAK/STAT signaling pathway (signal transducer and activator of transcription), culminating in exacerbated cellular proliferation, resistance to apoptosis and evolution to MPNs (Kralovics et al., 2005; Levine et al., 2005; Scott, Tong, et al., 2007). Nevertheless, JAK/STAT activation in MPNs is a nonspecific occurrence and there is still much information to be unveiled (Nielsen, Birgens, Nordestgaard, & Bojesen, 2013; Tefferi & Pardanani, 2015).

According to the available published literature, frequencies of *JAK2* V617F mutations are approximately 95-97% in PV and 60% in ET and PMF (Hinds et al., 2016; James et al., 2005; Scott, Tong, et al., 2007; Tefferi & Pardanani, 2015).

PV is characterized by excessive production of erythrocytes, increased red cell mass and risk of thrombosis or haemorrhage (Arber et al., 2016).

According to the literature, unlike patients with mutation in exon 14 of the *JAK2* gene, the mutation at exon 12 is not usually associated with an increase in the 3 hematopoietic series (erythrocytosis, leukocytosis and thrombocytosis). it appears to be associated with a distinct syndrome, mostly characterized by isolated and more marked erythrocytosis, independently of the mutational variant (Godfrey et al., 2016; Passamonti et al., 2011; Scott, 2011)**.**

Moreover, it seems that mitotic recombination, which leads to homozygosity, is more likely to occur in PV patients with mutation in exon 14 of the *JAK2* gene than in those with exon 12 mutations (Pietra et al., 2008).

We report here the case of the only patient *JAK2* exon 12 positive, presenting a novel mutation – c. 1605G>T (p.Met535Ile) and an atypical hematological phenotype. This patient was initially part of a study which included 39 patients with PV. Among those patients, five were *JAK2* V617F negative,

two of which further undertook search for the exon 12 mutation and only one, the patient within discussion, was positive for the aforementioned mutation (Azevedo et al., 2017).

4.2 – PATIENT AND METHODS

A 64-year-old woman, caucasian, who was accompanied by a General Practioner (GP) doctor for benign morbidities, namely type 2 diabetes, hypertension and hyperuricemia was referred to an Haematology consultation in January 2012. The reason for the referral was persistent alarming alterations in her complete blood counts which were not associated with any signs of malignant disease. She had a history of thrombocytosis over the former two years combined with recent erythrocytosis as well as microcytic and hypochromic erythrocytes (Table 4.1).

At the time of the first Haematology appointment, in January of 2012, the patient revealed no symptomatology of PV disorder such as headache, dizziness, visual disturbances, aquagenic pruritus, early satiety and constitutional symptoms, physical examination did not reveal splenomegaly.

Diagnostic tests were performed including complete blood counts (Table 4.1), erythropoietin levels (<1 mUI/mL), search for V617F mutation in exon 14 of the *JAK2* gene (by PCR; negative).

Following the initial results, the clinic-laboratorial picture was suggestive of an MPN, not further classifiable. However, the presence of iron deficiency, lowering haemoglobin levels, and the very low erythropoietin level, led us to consider the hypothesis of prodromal prepolycythaemic phase of PV as a possible diagnosis. Subsequently, the patient started treatment with phlebotomies and, after the worsening of thrombocytosis, phlebotomies were supplemented with hydroxyurea (Table 4.1).

The patient was taking acetylsalicylic acid (150 mg/day) since referral. Afterwards, an abdominal ultrasound was performed (May 2012) showing non-quantified splenomegaly.

Treatment was maintained and the patient went clinically and laboratorial stable during the following years.

In 2015, a search for the mutation in exon 12 of the *JAK2* gene (by PCR) was positive in peripheral blood, revealing a mutation c.1612C>T (p.His538Tyr) and a variant c.1605G> T (p.Met535Ile) (Figure 4.1). Afterwards, in 2016 a bone marrow aspirate and biopsy were performed showing hypercellularity with trilineage growth (panmyelosis) including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic mature megakaryocytes (differences in size and nuclear lobulation) (Figure 4.2), present in intersticial and paratrabecular spaces, confirming the PV diagnosis. Also, iron deposits were absent. Search for the mutation in exon 12 was also performed in medullary blood, revealing the same mutations previously identified in peripheral blood, but in a larger cellular population.

Currently the patient is still under the same prescription, with a complete haematological remission. Occasional adjustments, according to the laboratorial goals (Hematocrit<45%; Leucocytes ≤ $10x10^9$/L and Platelets<$400x10^9$/L), are made.

Table 4.1 – Complete blood count and treatment prescription over time.

CBC	21/2/2011	13/3/2012	17/4/2012	15/5/2012
Erythrocytes ($x10^{12}$/L)	5.13	6.15	5.79	5.35
Haemoglobin (g/L)	149	159	147	134
Hematocrit (%)	45.1	50.1	45.6	42.1
VGM (fL)	87.9	81.5	78.9	78.7
HGM (pg)	29.2	25.8	25.4	25.2
Leucocytes ($\times10^9$/L)	14.3	9.9	10.6	8.5
Neutrophils ($\times10^9$/L)	12.17	6.74	7.30	5.53
Lymphocytes ($\times10^9$/L)	1.49	2.40	2.34	2.24
Monocytes ($\times10^9$/L)	0.47	0.42	0.54	0.42
Eosinophils ($\times10^9$/L)	0.13	0.25	0.34	0.25
Basophils ($\times10^9$/L)	0.04	0.10	0.07	0.07
Platelets ($\times10^9$/L)	801	728	806	517
TREATMENT		P: 400 ml	P: 350 ml HU: 500 mg/7 d/w	HU: 500 mg/5 d/w

CBC – Complete Blood Count; HU – Hydroxyurea; P – Phlebotomy; d – days; w – week

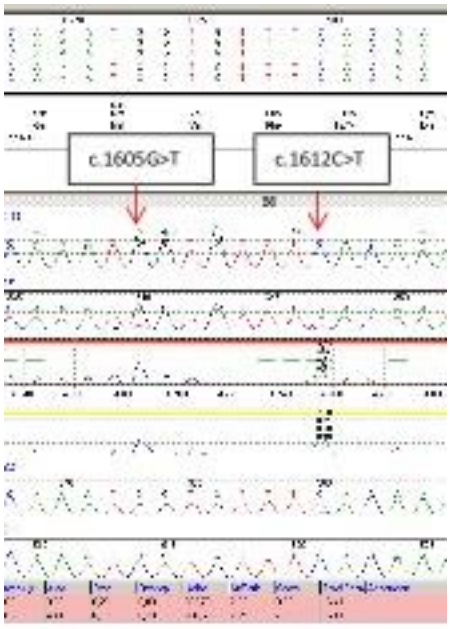


Figure 4.1 – Exon 12 mutation of the *JAK2* gene analysis in peripheral blood.
It revealed c.1612C>T (p.His538Tyr) mutation and a novel variant c.1605G> T (p.Met535Ile). This result was later confirmed in medullary blood.

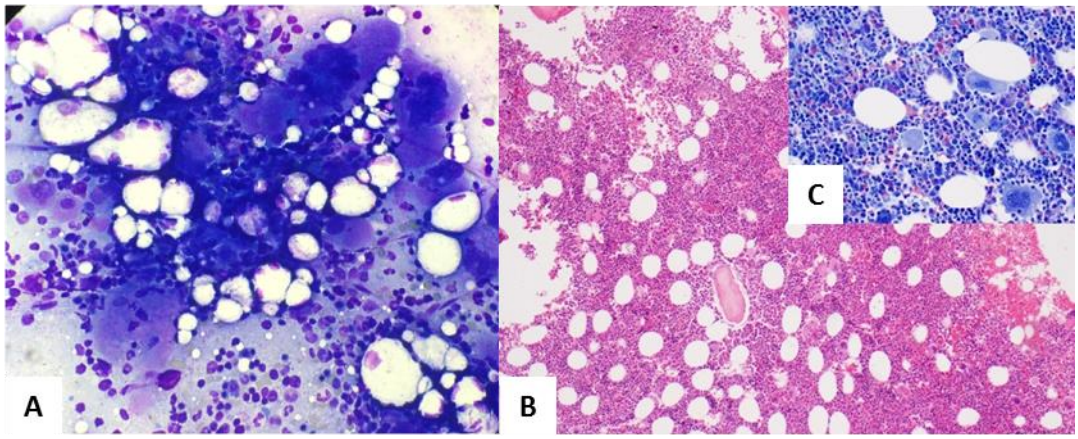


Figure 4.2 – Bone marrow aspirate a) and biopsy b) and c) showing trilinear hyperplasia and pleomorphic mature megakaryocytes.
A) May-Grünwald-Giemsa stain, x40; **B)** Hematoxylin eosin stain, x40; **C)** Giemsa stain, x200.

4.3 – DISCUSSION

PV is a rare neoplasm with an incidence of 0.4 to 2.8 per 100.000 cases per year per person, which can strike an individual at any age, with a mean of presentation of 65 years (Rumi & Cazzola, 2017) and an equal incidence between gender (McMullin et al., 2005).

Typically, PV initially presents a pre-polycythemic stage, characterized by borderline erythrocytosis and thrombocytosis, evolving to a polycythemic phase marked with hyperplasia of the three series and eventually post-PV myelofibrosis (Passamonti et al., 2011).

About 95-97% of patients with PV are carriers of a single V617F mutation in exon 14 of the *JAK2* gene. This mutation, along with other driver mutations connected with clonal expansion of hematopoietic cells, might also represent a feature of the aging hematopoietic system in individuals without a malignant disease (Jaiswal et al., 2014; Nielsen et al., 2013).

This share of the population usually presents higher erythrocyte, platelet and leucocyte counts. Moreover, it has been observed that individuals without malignant disease and who are positive to the *JAK2* V617F somatic mutation were 44/28 risk fold more likely to develop an haematological cancer (Nielsen et al., 2013).

The V617F somatic mutation causes the substitution of phenylalanine for valine at position 617 in the *JAK2* gene, which encodes a cytoplasmic tyrosine kinase. The mutation, which occurs in the JAK homology 2 (JH2) negative regulatory domain, increases JAK2 kinase activity and causes cytokine-independent growth of cell lines and cultured bone marrow cells (Kralovics et al., 2005; Levine et al., 2005).

Among the cases negative for V617F, a significant fraction presents mutations in the exon 12 of the *JAK2* gene (about 3% of PV patients) (Arber et al., 2016; Butcher et al., 2008; Pardanani, Lasho,

Finke, Hanson, & Tefferi, 2007; Park et al., 2016; Passamonti et al., 2011; Passamonti et al., 2010; Rumi et al., 2014; Scott, 2011; Scott, Beer, Bench, Erber, & Green, 2007; Scott, Tong, et al., 2007). At least 17 different mutations have been described (Bench et al., 2013) in patients who present exon 12 mutations and, unlike those who are *JAK2* V617F-positive, they are not commonly homozygous for the *JAK2* mutations (Godfrey et al., 2016; Scott, 2011; Scott, Tong, et al., 2007).

Like V617F, exon 12 mutations are associated with activation of JAK2, STAT5 and ERK1/2 (Godfrey et al., 2016; Scott, Tong, et al., 2007).

According to Passamonti et al (2010), *JAK2* exon 12 mutations were detected in 4% of the cases, among 338 genotyped patients (Passamonti et al., 2010). 3% of patients have one of four different mutations in exon 12 (Arber et al., 2016; Butcher et al., 2008; Rumi et al., 2014; Scott, Beer, et al., 2007). Some studies revealed a high incidence of *JAK2* exon 12 mutations in oriental patients, compared to reports in the Western literature (Table 4.2) (Berndt et al., 2016; Krähling et al., 2014; Park et al., 2016; Williams, Kim, Rogers, Spivak, & Moliterno, 2007; Wu et al., 2014). However, there are exceptions as it was demonstrated in a Chinese study, in which the mutational profile was tested in 1648 PN-MPNs patients, revealing that *JAK2* exon 12 mutation was found in 1,7% of PV patients (M. Y. Li et al., 2017).

In the literature there is also report of the co-existence of *JAK2* V617F and exon 12 mutations, as two separate clones (Nussenzveig et al., 2016; Passamonti et al., 2011).

Patients with a *JAK2* exon 12 mutation present at a younger age than those *JAK2* V617F positive, with approximately 40% of cases being reported at, or below 50 years of age (Langabeer et al., 2015; Nussenzveig et al., 2016).

Compared to PV patients with *JAK2* V617F mutation, patients with exon 12 mutations do not usually present panmyelosis (Arber et al., 2016). PN-MPNs *JAK2* exon 12 positive patients appears to be associated with a distinct syndrome, mainly characterized by isolated erythrocytosis (marked erythrocytosis) with higher hemoglobin concentrations, lower white cells and platelets counts (minimal thrombocytosis) (Park et al., 2016), and isolated bone marrow erythroid hyperplasia (Godfrey et al., 2016), independently of the mutational variant (Godfrey et al., 2016; Passamonti et al., 2011).

The fact that exon 12 mutations are more frequently associated with erythrocytosis is compatible with their absence in ET, but possible presence in PMF or AML secondary to PV (Scott, 2011)

However, despite the phenotypic differences, the clinical course seems similar between *JAK2* V617F and *JAK2* exon 12-positive patients, with similar incidences of thrombosis, myelofibrosis, leukemia and death (Passamonti et al., 2011).

Concerning the case reported by the authors, the first *JAK2* exon 12 mutation c.1612C>T (p.His538Tyr) is registered in the COSMIC database (COSM1462563) and has already been described in other patients by Wu et al (2014) (Wu et al., 2014). The variant c.1605G> T (p.Met535Ile) has not yet been described in the literature, as far as we know (Park et al., 2016; Pietra et al., 2008). However, a mutation in this same position (c.1605G> C) was previously described by Wu et al (2014) (Wu et al.,

2014), which results in the same amino acid exchange, indicating that this variant is most likely a pathogenic variant.

Information about whether the Met535leI and His538Tyr substitutions occurred in mutually exclusive disease clones or within the same cell is not available, because single colony genotyping was not performed.

The patient's reported main feature, unlike most patients who are PV exon 12 mutations-positive, was thrombocytosis. According to literature, even though these mutations are mostly associated with erythrocytosis, up to 25% percent of patients present with elevated counts of other lineages (Scott, 2011).

Moreover, the higher platelet count may be due to the fact that this patient harbors two lesions, both of which on their own have been associated with erythrocytosis phenotypes. Perhaps the excess

signaling from either a single clone with two lesions, or two hematopoietic stem cell clones with different exon 12 mutations, is driving more JAK2 signaling (and therefore more thrombocytosis) than we typically observe with the exon 12 lesions reported (Tables 4.1 and 4.2) (Williams et al., 2007; Kim et al., 2016; Pardanani et al., 2007; Park et al., 2016; Scott, Tong, et al., 2007; Siemiatkowska, Bieniaszewska, Hellmann, & Limon, 2010; Wu et al., 2014).

Williams et al identified *JAK2* exon 12 lesions in 30% of *JAK2* V617F negative PV patients, and while the clinical phenotype of *JAK2* exon 12 lesions in the MPNs was predominantly erythroid, in those patients there was significant disease spectrum overlap between *JAK2* V617F and *JAK2* exon 12 mutations, but on average the *JAK2* exon 12 patients had significantly lower white blood cell and platelet counts than *JAK2* V617F patients (Williams et al., 2007).

Scott et al. Have found similar results in their patients (Scott, Tong, et al., 2007).

On the other hand, Pardanani et al identified 6 of a total 220 cases with PV that were *JAK2* V617F negative (prevalence of 3%). Although erythroid hyperplasia was the predominant histologic feature on bone marrow examination, most PV patients harboring exon 12 mutations revealed megakaryocyte abnormalities and reticulin fibrosis, overlapping with what happens in some *JAK2* V617F positive PV cases. Therefore, a distinct genotype-phenotype association could not be established (Pardanani et al., 2007).

Pietra *et al.* Identified 17 exon 12 positive patients among 37 PV *JAK2* V617F negative patients, and in this study there is evidence of low white blood cell count, but an increase of mean value of platelet count, compared to the other studies (Pietra et al., 2008).

In the same way, Wu *et al.* described 10 Chinese patients with diverse exon 12 mutations, among 80 PV cases (prevalence of 13%), in which were also evident lower white blood cell and platelet counts, compared to what is characteristic of *JAK2* V617F patients (Wu et al., 2014).

In a study developed by Park *et al.*, with 42 PV Korean patients, 12% exon 12 positive, genotype-phenotype correlations demonstrated lower white blood cell and platelet counts in exon 12 mutations than V617F (Park et al., 2016).

Meanwhile, in another study also performed in 111 Korean patients, Kim et al. Identified 7 of them with exon 12 mutations, with an identical phenotype (Kim et al., 2016).

The basis for the different phenotype of our unique patient is not known (J. Li et al., 2014). Scott *et al*. suggest that genetic factors might be involved, such as the nature of the mutation or other additional MPN-associated mutations (Scott, 2011). An enhanced STAT1 activation might cause superior megakaryopoiesis and, therefore, possibly be a contributing factor to the understanding of the small share of PV patients with exon 12 mutation who behave differently. On the other hand, the absence of thrombocytosis in the majority of patients may be related to stat1-independent mechanisms that might cause, for instance, increase platelet destruction (Godfrey et al., 2016).

Sequencing granulocyte DNA was the first method used to detect the *JAK2* V617F mutation in exon 14. Afterwards, pcr-based methods were developed and, due to their superior sensitivity, the frequency of this mutation in MPNs patients increased. After using an allele-specific PCR assay it was found that almost all PV patients were *JAK2* V617F positive.

Evidence of the existence of patients that were *JAK2* V617F-negative led to the development of further studies, contributing to a better elucidation of MPNs pathogenesis in the following years. In 2007, *JAK2* exons were sequenced in granulocyte DNA acquired from *JAK2* V617F negative patients and exon 12 mutations were found, further clarifying this subject (Scott, 2011). The rarity of patients with *JAK2* exon 12 mutations, the variation on the type of mutation and of the low mutant allele burden, make selection of an appropriate assay for the diagnostic very challenging, requiring very sensitive assays (Bench et al., 2013; Langabeer et al., 2015).

Table 4.2 – Clinical and demographic features of other published exon 12 cases.

Study	JAK2 exon12 prevalence % (cases exon12/PV)	Novel mutations	Age at diagnosis (years) Gender (Female/Male)	Duration of disease (years)	Erythrocytes ($x10^{12}$/L)	Haemoglobin (g/L)	Leucocytes ($x10^{9}$/L)	Platelets ($x10^{9}$/L)
Scott* *et al* 2007, United Kingdom	ND		40.5 (17-59) 3/3	--	--	211.3 (179-234)	9.3 (4.4-14.4)	328 (204-450)
Williams *et al.* 2007, USA	1.9 (3/157)	R541_E543delinsK H538_K539delinsL	43 (18-61) 1/2	20 (1-54)	--	--	8.5 (6.7-11.0)	314 (60-579)
Pardanani *et al.* 2007, USA	2.2 (5/220)	No	38.4 (18-73) 2/3	13.7 (3-40)	--	201.6 (183.0-240.0)	9.0 (7.7-10.3)	305.4 (152-476)
Pietra *et al.* 2008, Italy	-- (17/37PVV617F neg)	V536_I546Dup11 F537_I546dup10+F547L	55.3 (35-80) 11/6	--	--	194.4 (167-238)	7.4 (4.2-11.8)	404.2 (102-956)
Wu *et al.* 2014, China	13 (10/80)	M532V/E543G N533D M535I/H538Y/K549I E543G D544N	42.4 (23-60) 4/6	--	5.1 (3.86-5.83)	166.0 (112.0-216.0)	9.2 (5.85-14.46)	245 (85-494)
Park *et al.* 2016, Korea	12 (5/42)	H538_R541delinsLII F537_K539delinsVL	43 (30.0-72.7) 2/3	--	--	200 (135-226)	9.6 (5.6-41.0)	194 (131-476)
Kim *et al.* 2016, Korea	6.3 (7/111)	No	66 (46-76) 5/2	--	6.9 (5.83-8.50)	183 (137-211)	8.2 (6.17-22.32)	281 (58-310)

ND – Not determined

*In the study of Scott et al., prevalence of exon 12 was not possible to calculate due to the fact that 8 of the patients were not randomly tested among PV patients.

4.4 – REFERENCES

Arber, D. A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M. J., Le Beau, M. M., . . . Vardiman, J. W. (2016). The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood, 127*(20), 2391-2405. doi:10.1182/blood-2016-03-643544

Azevedo, A. P., Silva, S. N., De Lima, J. P., Reichert, A., Lima, F., Júnior, E., & Rueff, J. (2017). DNA repair genes polymorphisms and genetic susceptibility to Philadelphia-negative myeloproliferative neoplasms in a Portuguese population: The role of base excision repair genes polymorphisms. *Oncol Lett, 13*(6), 4641-4650. doi:10.3892/ol.2017.6065

Bench, A. J., White, H. E., Foroni, L., Godfrey, A. L., Gerrard, G., Akiki, S., . . . Haematology, B. C. f. S. i. (2013). Molecular diagnosis of the myeloproliferative neoplasms: UK guidelines for the detection of JAK2 V617F and other relevant mutations. *Br J Haematol, 160*(1), 25-34. doi:10.1111/bjh.12075

Berndt, S. I., Camp, N. J., Skibola, C. F., Vijai, J., Wang, Z., Gu, J., . . . Slager, S. L. (2016). Meta-analysis of genome-wide association studies discovers multiple loci for chronic lymphocytic leukemia. *Nat Commun, 7*, 10933. doi:10.1038/ncomms10933

Butcher, C. M., Hahn, U., To, L. B., Gecz, J., Wilkins, E. J., Scott, H. S., . . . D'Andrea, R. J. (2008). Two novel JAK2 exon 12 mutations in JAK2 V617F-negative polycythaemia vera patients. *Leukemia, 22*(4), 870-873. doi:10.1038/sj.leu.2404971

Godfrey, A. L., Chen, E., Massie, C. E., Silber, Y., Pagano, F., Bellosillo, B., . . . Green, A. R. (2016). STAT1 activation in association with JAK2 exon 12 mutations. *Haematologica, 101*(1), e15-19. doi:10.3324/haematol.2015.128546

Hinds, D. A., Barnholt, K. E., Mesa, R. A., Kiefer, A. K., Do, C. B., Eriksson, N., . . . Gotlib, J. (2016). Germ line variants predispose to both JAK2 V617F clonal hematopoiesis and myeloproliferative neoplasms. *Blood, 128*(8), 1121-1128. doi:10.1182/blood-2015-06-652941

Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P. V., Mar, B. G., . . . Ebert, B. L. (2014). Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med, 371*(26), 2488-2498. doi:10.1056/NEJMoa1408617

James, C., Ugo, V., Le Couédic, J. P., Staerk, J., Delhommeau, F., Lacout, C., . . . Vainchenker, W. (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature, 434*(7037), 1144-1148. doi:10.1038/nature03546

Kim, Y., Park, J., Jo, I., Lee, G. D., Kim, J., Kwon, A., . . . Kim, M. (2016). Genetic-pathologic characterization of myeloproliferative neoplasms. *Exp Mol Med, 48*, e247. doi:10.1038/emm.2016.55

Kralovics, R., Passamonti, F., Buser, A. S., Teo, S. S., Tiedt, R., Passweg, J. R., . . . Skoda, R. C. (2005). A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med, 352*(17), 1779-1790. doi:10.1056/NEJMoa051113

Krähling, T., Balassa, K., Meggyesi, N., Bors, A., Csomor, J., Bátai, Á., . . . Andrikovics, H. (2014). [Complex molecular genetic algorithm in the diagnosis of myeloproliferative neoplasms]. *Orv Hetil, 155*(52), 2074-2081. doi:10.1556/OH.2014.30051

Langabeer, S. E., Andrikovics, H., Asp, J., Bellosillo, B., Carillo, S., Haslam, K., . . . MPN&MPNr-EuroNet. (2015). Molecular diagnostics of myeloproliferative neoplasms. *Eur J Haematol, 95*(4), 270-279. doi:10.1111/ejh.12578

Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J., . . . Gilliland, D. G. (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell, 7*(4), 387-397. doi:10.1016/j.ccr.2005.03.023

Li, J., Kent, D. G., Godfrey, A. L., Manning, H., Nangalia, J., Aziz, A., . . . Green, A. R. (2014). JAK2 V617F homozygosity drives a phenotypic switch in myeloproliferative neoplasms, but is insufficient to sustain disease. *Blood, 123*(20), 3139-3151. doi:10.1182/blood-2013-06-510222

Li, M. Y., Chao, H. Y., Sun, A. N., Qiu, H. Y., Jin, Z. M., Tang, X. W., . . . Wu, D. P. (2017). [Clinical significance of JAK2、CALR and MPL gene mutations in 1 648 Philadelphia chromosome negative myeloproliferative neoplasms patients from a single center]. *Zhonghua Xue Ye Xue Za Zhi, 38*(4), 295-300.

McMullin, M. F., Bareford, D., Campbell, P., Green, A. R., Harrison, C., Hunt, B., . . . Haematology, G. H. T. F. o. t. B. C. f. S. i. (2005). Guidelines for the diagnosis, investigation and management of polycythaemia/erythrocytosis. *Br J Haematol, 130*(2), 174-195. doi:10.1111/j.1365-2141.2005.05535.x

Nielsen, C., Birgens, H. S., Nordestgaard, B. G., & Bojesen, S. E. (2013). Diagnostic value of JAK2 V617F somatic mutation for myeloproliferative cancer in 49 488 individuals from the general population. *Br J Haematol, 160*(1), 70-79. doi:10.1111/bjh.12099

Nussenzveig, R. H., Pham, H. T., Perkins, S. L., Prchal, J. T., Agarwal, A. M., & Salama, M. E. (2016). Increased frequency of co-existing JAK2 exon-12 or MPL exon-10 mutations in patients with low JAK2(V617F) allelic burden. *Leuk Lymphoma, 57*(6), 1429-1435. doi:10.3109/10428194.2015.1091932

Pardanani, A., Lasho, T. L., Finke, C., Hanson, C. A., & Tefferi, A. (2007). Prevalence and clinicopathologic correlates of JAK2 exon 12 mutations in JAK2V617F-negative polycythemia vera. *Leukemia, 21*(9), 1960-1963. doi:10.1038/sj.leu.2404810

Park, C. H., Lee, K. O., Jang, J. H., Jung, C. W., Kim, J. W., Kim, S. H., & Kim, H. J. (2016). High frequency of JAK2 exon 12 mutations in Korean patients with polycythaemia vera: novel mutations and clinical significance. *J Clin Pathol, 69*(8), 737-741. doi:10.1136/jclinpath-2016-203649

Passamonti, F., Elena, C., Schnittger, S., Skoda, R. C., Green, A. R., Girodon, F., . . . Cazzola, M. (2011). Molecular and clinical features of the myeloproliferative neoplasm associated with JAK2 exon 12 mutations. *Blood, 117*(10), 2813-2816. doi:10.1182/blood-2010-11-316810

Passamonti, F., Rumi, E., Pietra, D., Elena, C., Boveri, E., Arcaini, L., . . . Cazzola, M. (2010). A prospective study of 338 patients with polycythemia vera: the impact of JAK2 (V617F) allele burden and leukocytosis on fibrotic or leukemic disease transformation and vascular complications. *Leukemia, 24*(9), 1574-1579. doi:10.1038/leu.2010.148

Pietra, D., Li, S., Brisci, A., Passamonti, F., Rumi, E., Theocharides, A., . . . Cazzola, M. (2008). Somatic mutations of JAK2 exon 12 in patients with JAK2 (V617F)-negative myeloproliferative disorders. *Blood, 111*(3), 1686-1689. doi:10.1182/blood-2007-07-101576

Rumi, E., & Cazzola, M. (2017). Diagnosis, risk stratification, and response evaluation in classical myeloproliferative neoplasms. *Blood, 129*(6), 680-692. doi:10.1182/blood-2016-10-695957

Rumi, E., Pietra, D., Ferretti, V., Klampfl, T., Harutyunyan, A. S., Milosevic, J. D., . . . Investigators, A. I. p. l. R. s. C. G. I. M. M. (2014). JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. *Blood, 123*(10), 1544-1551. doi:10.1182/blood-2013-11-539098

Scott, L. M. (2011). The JAK2 exon 12 mutations: a comprehensive review. *Am J Hematol, 86*(8), 668-676. doi:10.1002/ajh.22063

Scott, L. M., Beer, P. A., Bench, A. J., Erber, W. N., & Green, A. R. (2007). Prevalance of JAK2 V617F and exon 12 mutations in polycythaemia vera. *Br J Haematol, 139*(3), 511-512. doi:10.1111/j.1365-2141.2007.06806.x

Scott, L. M., Tong, W., Levine, R. L., Scott, M. A., Beer, P. A., Stratton, M. R., . . . Green, A. R. (2007). JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. *N Engl J Med, 356*(5), 459-468. doi:10.1056/NEJMoa065202

Siemiatkowska, A., Bieniaszewska, M., Hellmann, A., & Limon, J. (2010). JAK2 and MPL gene mutations in V617F-negative myeloproliferative neoplasms. *Leuk Res, 34*(3), 387-389. doi:10.1016/j.leukres.2009.06.017

Tefferi, A., & Pardanani, A. (2015). Myeloproliferative Neoplasms: A Contemporary Review. *JAMA Oncol, 1*(1), 97-105. doi:10.1001/jamaoncol.2015.89

Williams, D. M., Kim, A. H., Rogers, O., Spivak, J. L., & Moliterno, A. R. (2007). Phenotypic variations and new mutations in JAK2 V617F-negative polycythemia vera, erythrocytosis, and idiopathic myelofibrosis. *Exp Hematol, 35*(11), 1641-1646. doi:10.1016/j.exphem.2007.08.010

Wu, Z., Zhang, X., Xu, X., Chen, Y., Hu, T., Kang, Z., . . . Guan, M. (2014). The mutation profile of JAK2 and CALR in Chinese Han patients with Philadelphia chromosome-negative myeloproliferative neoplasms. *J Hematol Oncol, 7*, 48. doi:10.1186/s13045-014-0048-6

CHAPTER 5:
CONCOMITANT PRESENCE OF *JAK2* V617F MUTATION AND *BCR-ABL* TRANSLOCATION IN TWO PATIENTS: A NEW ENTITY OR A VARIANT OF MYELOPROLIFERATIVE NEOPLASMS

Submitted for publication in Molecular Medicine Reports

ABSTRACT

Myeloproliferative neoplasms (MPNs) are classically divided into BCR-ABL positive Chronic Myeloid Leukemia (CML) and BCR-ABL negative MPNs, including Essential Thrombocythemia (ET). One of the major diagnostic criteria for ET is the absence of the Philadelphia chromosome (Ph), meaning that when present it would be almost indicative of CML. ET and CML are considered to be mutually exclusive, however, exceptionally there are rare situations in which ET patients present positive BCR-ABL without features of CML.

Although from the literature review the frequency of coexistence of JAK2 V617F mutation and BCR-ABL translocation in myeloproliferative neoplasms is low, it might be higher than expected.

We present the case report of two patients with the initial diagnosis of ET in the presence of JAK2 V617F mutation and BCR-ABL translocation by FISH, with an atypical pattern. Both patients present an heterozygous BCR-ABL translocation and absence of identification of p190 and p210 transcripts, seemingly a der(9) in a background of an ET JAK2 V617F mutation.

CHAPTER 5: CONCOMITANT PRESENCE OF JAK2V617F MUTATION AND BCR-ABL TRANSLOCATION IN TWO PATIENTS: A NEW ENTITY OR A VARIANT OF MYELOPROLIFERATIVE NEOPLASMS

5.1 – INTRODUCTION

According to World Health Organization (WHO) 2008 Classification of Tumours of Haematopoietic and Lymphoid Tissues and 2016 revision (Arber et al., 2016), Myeloproliferative Neoplasms (MPNs) can be classified into two major groups, Chronic Myeloid Leukemia (CML) and Philadelphia-negative MPNs (PN-MPNs), such as Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF). These disorders are more frequently found in elderly patients, mostly in men (Arber et al., 2016).

One of the major genetic insights into the pathogenesis of the PN-MPNs included the identification of the somatic point gain-of-function mutations in Janus kinase 2 gene (*JAK2*), leading to the activation of the JAK/STAT signaling pathway (signal transducer and activator of transcription), culminating in exacerbated cellular proliferation, resistance to apoptosis and evolution to MPNs (Baxter et al., 2005; Kralovics et al., 2005; Levine et al., 2005). On the other hand, the identification of Philadelphia chromosome (Ph), a translocation involving chromosomes 9 and 22 that results in the formation of the *BCR-ABL* fusion gene, constitutes the defining leukemogenic event in CML (Nowell, 1962; Rowley, 1973). ET is characterized by a high platelet count, often associated with thrombotic and hemorrhagic events, and the presence of *JAK2* mutation in about 50-60% of cases (Hinds et al., 2016; Levine, 2009; Tefferi & Pardanani, 2015).

As far as we know from literature revision, the frequency of concurrent presence of *JAK2* V617F mutation and *BCR-ABL* translocation in a single individual with a MPN is a rare event, independently of what phenotype expresses earlier, PN-MPN or CML (Pagnano et al., 2016; Qin, Yang, Li, & Wang, 2014; Ursuleac et al., 2013; Zhou, Knoche, Engle, Fisher, & Oh, 2015).

Although ET and CML are considered to be mutually exclusive, rare cases of concomitant presence of *BCR-ABL* translocation positive CML and *JAK2* V617F mutation positive ET have been reported in the literature (Pagnano et al., 2016; Qin et al., 2014).

We report here the case of two patients initially included in a data base population of 58 patients with the diagnosis of ET in the presence of *JAK2* V617F mutation, with the suspicion of coexistence with *BCR-ABL* translocation.

5.2 – MATERIALS AND METHODS

CASE STUDY 1:

A 75-year-old man with a medical history of dyslipidemia, hypertension, acute myocardial infarction, and Ischemic stroke in August 2013. In December 2013 this patient was hospitalized with his second ischemic stroke. Although he had confirmed poor adherence to the prescribed therapy for cardiovascular risk patients, in January 2014 he was referred to the Hematology consultation for maintained thrombocytosis and leukocytosis, since at least August 2013 (as there was no previous laboratory data available).

Evaluation revealed platelet count of 1405x10^9/L, leukocytosis (15x10^9/L), with normal formula, and without immature precursor cells as well as normal hemoglobin (Table 5.1).

Table 5.1 – Results over time and therapy prescribed for case study 1.

	2014				2016	2017
	January	**February**	**June**	**September**	**August**	**January**
Platelets (×10^9/L)	1405	698	375	547	199	1596
Haemoglobin (g/dL)	14.3	13.4	12.3	12.8	13.9	7.9
Leucocytes (×10^9/L)	15.1	6.9	6.2	6.3	3.9	18.9
***JAK2* V617F mutation**	Positive (25%)	--	--	--	Positive	--
***BCR/ABL* t(9;22)** (FISH)	Positive 16% (atypical patern)	--	--	Positive 21% (atypical patern)	--	--
	⬆ Hydroxyurea Aspirin					

Abdominal ultrasound confirmed absent splenomegaly and Bone marrow (BM) aspirate showed megakaryocytic hyperplasia and enlarged megakaryocytes, with no abnormalities of the myeloid and erythroid series (Figure 5.1). BM biopsy showed a hypercellular marrow (80%), megakaryocytic and granulocytic (slight) hyperplasia (Figure 5.2).

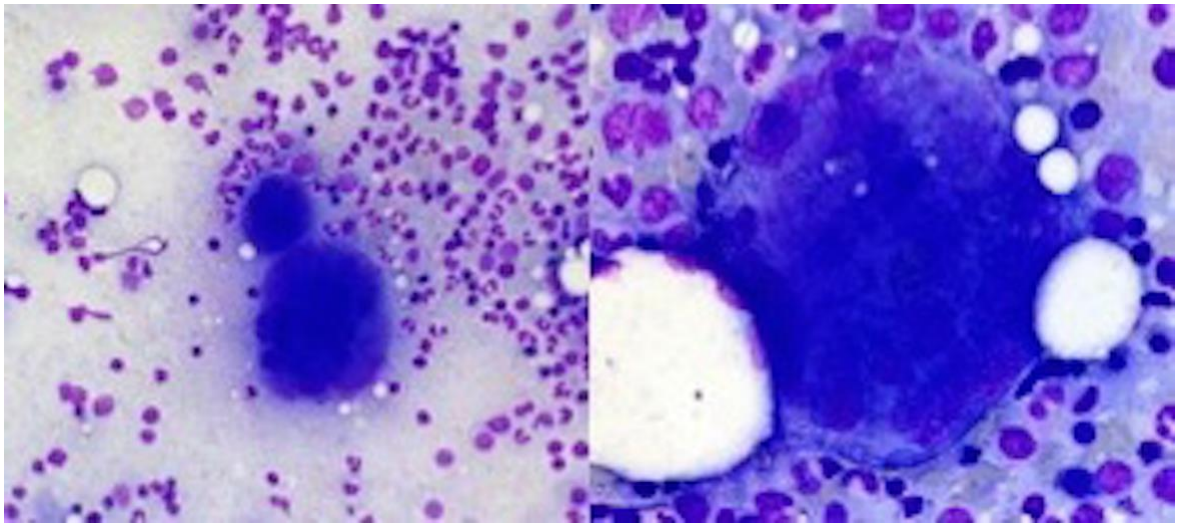


Figure 5.1 – Case study 1: bone marrow aspirate.
It showed megakaryocytic hyperplasia and enlarged megakaryocytes, with no abnormalities of the myeloid and erythroid series (May-Grünwald-Giemsa stain, x40 and x 100).

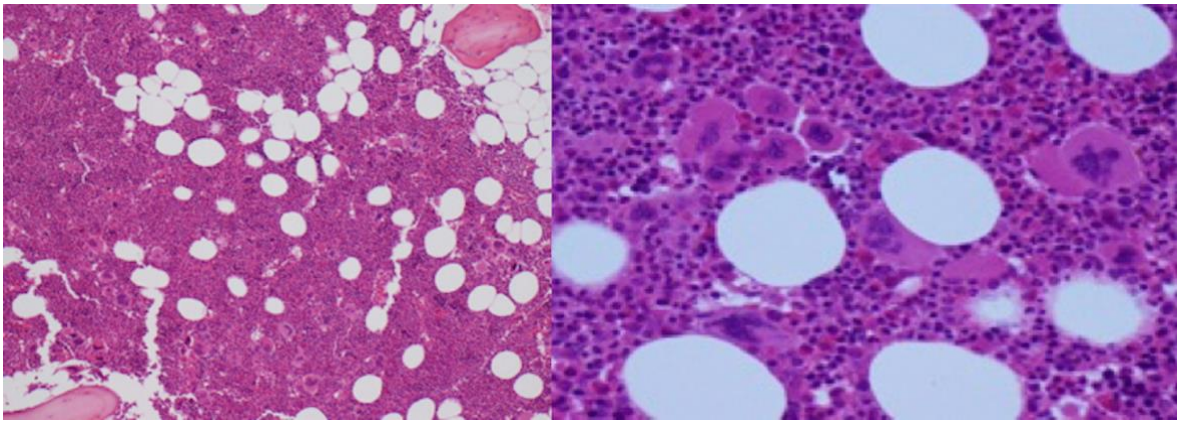


Figure 5.2 – Case study 1: bone marrow biopsy.
It showed a hypercellular marrow (80%), megakaryocytic and granulocytic (slight) hyperplasia (Hematoxylin eosin stain, x40).

Molecular biology (Figure 5.3) and cytogenetic tests were performed in peripheral blood and the results revealed positivity for the *JAK2* V617F mutation and a karyotype of 45,X,-Y[5]/46,XY[15]. The fluorescence *in situ* hybridization (FISH) was positive for the *BCR-ABL* translocation in 16% with an atypical pattern. The *BCR-ABL* transcript was not detected by the conventional reverse transcriptase-polymerase chain reaction (RT-PCR) method (specific for p190 and p210 transcripts).

This high risk patient received a daily hydroxyurea (HU), and low dose aspirin regimen as secondary thrombotic prevention. A good response to treatment was achieved, with normalization of leukocytes and platelets reduction of greater than 50% after one month and normalization of platelets after five months of therapy (Table 5.1). This patient had very poor compliance to the therapy and hospital check-ups, so Tyrosine Kinase Inhibitor (TKI) that was planned to be introduced, was never started since the patient did not come to collect the medication at the hospital. His clinical and laboratory situation has worsened and the patient died by the beginning of 2017, from infectious complications.

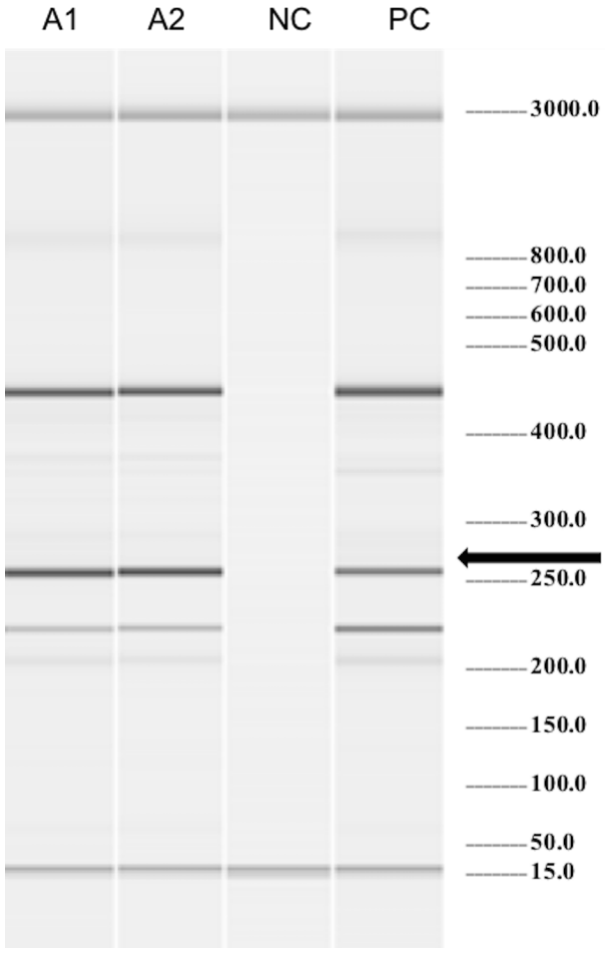


Figure 5.3 – Case study 1: molecular biology test.
It revealed positivity for the *JAK2* V617F mutation (black arrow) in peripheral blood (A1 and A2 – patient; NC – negative control; PC – positive control).

CASE STUDY 2:

A 76 years old man, with previous history of cardiovascular risk factors, namely Diabetes mellitus, dyslipidemia and Ischemic cardiomiopathy submitted to cardiac bypass due to myocardial infarction in 2001.

Presented with isolated thrombocytosis (1022 x10^9/L) in 2005, which led the patient to the Hematology department to study the etiology behind the maintained increased level of platelet count (Table 5.2).

Table 5.2 – Results over time and therapy prescribed for case study 2.

	2005	**2009**	**2013**	**2016**	**2017**	
	March	**March**	**September**	**February**	**January**	**August**
Platelets (×10^9/L)	1022	478	684	909	413	252
Haemoglobin (g/dL)	14.6	14.7	12.2	13.1	14.1	11.8
Leucocytes (×10^9/L)	9.4	6.9	22.4	30.1	38.4	7.3
***JAK2* V617F mutation**	--	--	--	--	Positive	--
***BCR/ABL* t(9;22)** (FISH)	--	--	--	--	Positive 17% (atypical patern)	--
		⬆			⬆	⬆
		Hydroxyurea			Hydroxyurea TKI	~~TKI~~

In a patient with previous history of thrombotic event, it was imperative to understand the etiology of such abnormal changes in blood analysis, since it might have been in close relation to the previous cardiac event described.

At this time, high platelet count was asymptomatic, and there was neither clinical nor analytical blood data for detecting an associated inflammatory process or any recent surgeries explaining this finding.

Abdominal ultrasound showed normal spleen morphology, and there were no Howell-Jolly bodies nor pitted erythrocytes found in blood smear analysis, that could be interpreted as reactive thrombocytosis due to functional hyposplenism.

Blood sideremia and iron stores were between normal ranges, and no history of hemorrhage was present. Excluded secondary causes of thrombocytosis and based on an indolent clinical course,

a primary cause was admitted. The MPNs are the most common responsible entities and so cytogenetics and molecular biology tests on *JAK2* V617F mutation and *BCR-ABL* t(9;22) were performed in peripheral blood. *JAK2* V617F was positive and, once again, the fluorescence *in situ* hybridization (FISH) test was positive for the *BCR-ABL* translocation in 17% with an atypical pattern (Figure 5.4), but *BCR-ABL* transcript was not detected by the conventional (RT-PCR) method. No metaphases were observed in the karyotype for evaluation. Having this data discussed, and taking into account the presence of these mutations, the diagnosis of ET *JAK2* V617F and *BCR-ABL* positive was admitted. The patient started on HU 500mg (alternate day progressing to 1g/alternate day) and TKI (Imatinib 400mg/day). A few months later, TKI was suspended and the patient remained under treatment with HU, actually with well controlled disease.

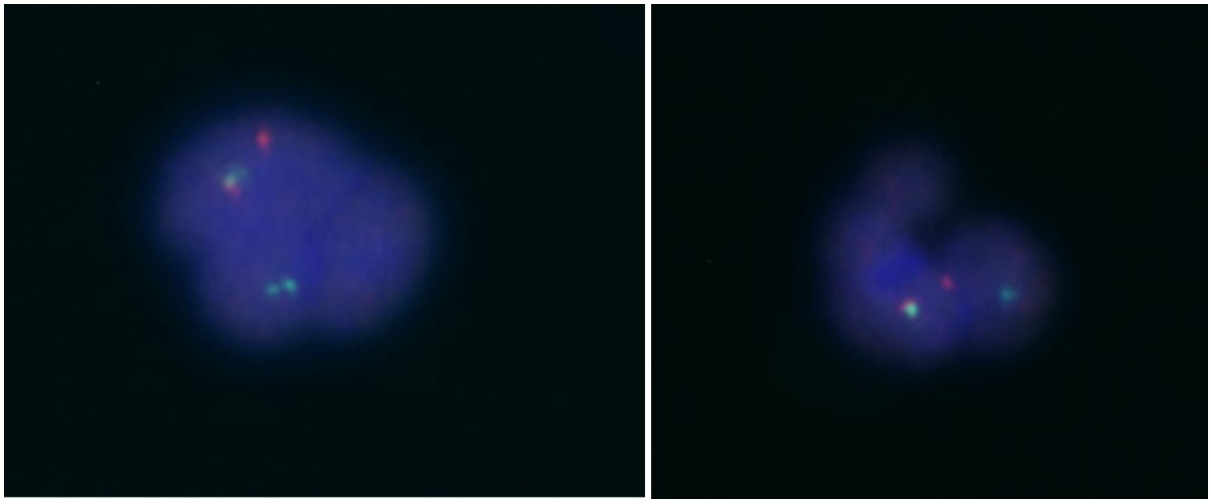


Figure 5.4 – Case study 2: FISH test.
It revealed positivity for the the *BCR-ABL* translocation with an atypical pattern, in which a unique fusion signal is detected.

Regarding the methodology used for genetic study, extraction of whole DNA from peripheral blood was accomplished by cell lysis followed by ethanol precipitation and recovery of the DNA by elution in a buffer solution (QIAamp® DNA Mini kit; Qiagen GmbH, Hilden, Germany). The presence/absence of *JAK2V617F* mutation was determined by amplification refractory mutation system (ARMS)-PCR (in-house), based on amplification of a genomic fragment which includes the region corresponding to amino acid 617 of the JAK2 protein, and on the differential detection on agarose gel of the normal or mutated alleles through the use of allele-specific primers. The test result is qualitative and the test sensitivity is 1%. Quantification of *JAK2* was obtained by high resolution melting PCR (HRM-PCR) (LightCycler® 480 Instrument, Roche), with a sensitivity of about 10% of mutated cells. Conventional RT-PCR was performed for the identification of *BCR-ABL* transcripts (specific for p190

and p210), after RNA extraction, according to the methodology described by Dongen *et al*.(van Dongen et al., 1999). Results are analyzed on agarose gel electrophoresis. FISH analysis was done on 100 nuclei after hybridization with specific probes for t(9;22) *BCR-ABL* (Vysis LSI *BCR-ABL* Dual Color, Dual Fusion Translocation Probe).

Patients anonymity and consent was guaranteed, in agreement with the Declaration of Helsinki. The institutional ethic board approved this report.

5.3 – DISCUSSION

Several authors have investigated the relationship between *JAK2* V617F and *BCR-ABL* anomalies and many theories have been postulated in the last years, especially after the identification of *JAK2* V617F mutation in 2005.

The Janus kinase 2 gene (*JAK2*; cytogenetic location: 9p24.1) provides instructions for making a protein that promotes the growth and division (proliferation) of cells. This protein is part of a signaling pathway called the JAK/STAT pathway, which transmits chemical signals from outside the cell to the cell's nucleus. The JAK2 protein is especially important for controlling the production of blood cells from hematopoietic stem cells. These stem cells are located within the bone marrow and have the potential to develop into red blood cells, white blood cells, and platelets.

The Philadelphia chromosome (chromosome 22) results from the reciprocal translocation of genetic material between chromosome 9 and chromosome 22, and contains the fusion gene *BCR-ABL*, which codes for a tyrosine kinase signaling protein that causes the cells to divide uncontrollably (particularly CML cells).

From 2007 to 2015, at least 42 patients with this double mutated phenotype were reported in the literature (Hummel et al., 2012; Michiels, Ten Kate, De Raeve, & Gadisseur, 2015; Qin et al., 2014; Ursuleac et al., 2013). Moreover, the italian group of Pieri *et al* (Pieri et al., 2011) studied 314 patients with CML and identified 8 cases (2.55%) with concomitant *JAK2* V617F mutation. Pagnano *et al* (Pagnano et al., 2016) detected only one case with *JAK2* V617F mutation among 55 cases of CML analyzed.

Among these different studies reported, several patterns were described: 1) initially diagnosed with CML and treated with imatinib that proceeded to a *JAK2* V617F myeloproliferative phenotype; 2) initially diagnosed with CML coexisting with *JAK2* V617F mutation positive PV, ET or PMF; or 3) initially diagnosed with *JAK2* V617F mutation positive PN-MPN, ET more rarely, evolving years later to CML (Qin et al., 2014). Commonly, men above 50 years old were the most frequently affected (Qin et al., 2014).

A question still has to be clarified: which is the first anomaly to occur? Several working groups reported that in some cases of PN-MPNs that evolved to CML, *JAK2* V617F mutation was the first leukemogenic event and *BCR-ABL* t(9;22) the second positive clone (Qin et al., 2014). Moreover, it was also speculated that *JAK2* V617F mutations are present in hematopoietic stem cells, with an additional *BCR-ABL* translocation being subsequently acquired in a sub-clone (Heller et al., 2001; Qin et al., 2014). However, other groups didn't confirm these results, and postulate that the two anomalies are present since the beginning of the process (Qin et al., 2014). Indeed, about the amount of cellular clones involved, there are reports that state that two different clones are involved, with the phenotypic expression depending on which one of the aberrations is "dominating", as a result of therapy targeted to the other anomaly (Bee, Gan, Nadarajan, Latiff, & Menaka, 2010; Kwong, Chiu, Liang, Chan, & Chan, 1996; Qin et al., 2014; Zhou et al., 2015). On the other hand, there are some authors evidencing that all the myeloid cells bear *JAK2* V617F mutation, including granulocytic and erythroid colonies, while *BCR-ABL* translocation is confined to a small compartment of myeloid progenitor cells, only in granulocytic colonies (Qin et al., 2014). In contrast, other reports showed the simultaneous presence of both *BCR-ABL* transcript and *JAK2* V617F mutation in the majority of granulocytic and erythroid colonies at the time CML diagnosis was established, corroborating the hypothesis that only one cellular clone is bearing concomitantly the two anomalies (Qin et al., 2014; Zhou et al., 2015).

Therefore, the phenotypic heterogeneity can be the result of the expression of a pre-existing mutated clone previously "silent" or of the accumulation of several genetic events conferring genetic instability and leading to a "new" anomaly (Ursuleac et al., 2013).

As far as we know from literature revision, there are no other reports of positivity for *JAK2* mutations, other than V617F, with the concomitant presence of *BCR-ABL* translocation.

One of the diagnostic criteria for ET, is the absence of the Ph chromosome. BCR-ABL positive ET without features of CML in blood and bone marrow is a rare entity and constitute less than 5% of ET diagnosis. Some authors have proposed to consider those cases as CML associated with a rather poor prognosis because of the high tendency to progress to myelofibrosis and blastic transformation after a few to several years (Kwong et al., 1996; LeBrun et al., 1991; Michiels et al., 2015).

An important difference between BCR-ABL positive ET and BCR-ABL positive CML at time of presentation is the absence of splenomegaly in the first situation (Michiels et al., 2015).

The bone marrow in BCR-ABL positive ET is featured by predominant smaller than normal and hypo/mononucleated megakaryocytes caused by BCR-ABL gene and protein induced maturation defect of the hematopoietic stem cells. This contrasts with clustered enlarged megakaryocytes in BCR-ABL negative ET due to growth advantage and proliferation of constitutively activated *JAK2* or *MPL* somatic mutated megakaryocytes (Michiels et al., 2015).

The first patient reported had diagnostic features that matched CML and ET. However, his overall clinical presentation including bone marrow features was more commonly suggestive of ET. Since the t(9;22) was positive in FISH, according to the results, there should have been found a positive result in molecular biology tests as well. Moreover, no Ph chromosome was detected by karyotype.

The second patient reported was more suggestive of ET and did not have typical clinical, nor morphologic findings for CML. The t(9;22) was also positive only by FISH, with a negative result in molecular biology tests. In this case it was not possible to evaluate karyotype due to metaphases absence.

In both patients, search for *JAK2* V617F mutation and *BCR-ABL* translocation was concomitant, making very difficult to know if both mutations were present from the beginning or the order of appearance of each one of them. The fact that the study has been performed before therapy institution, excludes the possible inhibitory effect of it over one of the altered clones, making the other more expressive.

Given the above, several questions have to be raised: are these genomic alterations found in these two patients and their atypical pattern really true and clinically significant or are they false positive results? May those be new/distinct clinical entities? Should we consider Ph positive ET as distinct entity, separate from Ph negative ET and Ph positive CML?

As mentioned above, studies describing cases initially diagnosed with *JAK2* V617F mutation positive PN-MPN, evolving later to CML, were rarely ET patients (Qin et al., 2014), in contrast to our report.

Although, the concomitant presence of these two anomalies in these patients didn't seemed to exclude the diagnosis of ET, at diagnosis or in some point of their clinical course, both patients evidenced a distinct clinical (thrombocytosis with associated leukocytosis) or morpho-histological (megakaryocytic) phenotype from what was expected for ET with isolated *JAK2* V617F positive or Ph positive CML, but apparently not influencing the course of the disease.

Both patients showed an atypical pattern for *BCR-ABL* translocation search by FISH, said to be atypical because only one fusion signal was observed, instead of the two signals expected, with a percentage of *BCR-ABL* translocation of approximately 20%. RT-PCR was performed using only a single primer pair, failing the identification of p190 and p210 transcripts of *BCR-ABL* fusion gene, and making the presence of BCR-ABL tyrosine kinase activity questionable. Real time PCR was a distinct possible technique to be used for the identification and quantification of *BCR-ABL* p210 (mainly b3a2 and b2a2 types) transcripts, however it was not performed.

Since no *BCR-ABL* transcripts were detected by RT-PCR, one hypothesis is that the unique fusion signal detected by FISH could correspond only to der(9), and not to Ph chromosome with associated tyrosine kinase activity (on chromosome 22). Confirmation could be achieved doing FISH in metaphases, which was only possible in the first case, since the second patient had no metaphases to allow it. However, this was not done and this way, we were not able to be sure of the localization of breakpoints and consequent fusion.

On the other hand, a missense on the primer site or the probe pairing region could explain such RT-PCR result, but there is a vast experience with the used primers, internationally designed and certified.

Regarding clarification of the possible mechanism of association of *JAK2* V617F mutation and a "true" *BCR-ABL* translocation involved in our patients, it would be useful to analyze *JAK2* V617F mutation and *BCR-ABL* gene in each colony of BFU-E or CFU-C.

Given the above, probably these cases correspond to two patients with a variant ET, in which we possibly can hypothesize that the presence of der(9) chromosome might be involved in those phenotypic differences. As far as we are aware, no other studies describing these two "truly" genomic alterations have found a *BCR-ABL* aberrant pattern similar to our cases. However, Larsen *et al* (Larsen, Hasselbalch, Pallisgaard, & Kerndrup, 2007) described the case of four patients *JAK2* V617F positive with associated distinct karyotypic aberrations (including der(9;18)), presenting with a distinct clinical and prognostic profile. Likewise, another study also reported the association of der(9) chromosome and acute lymphoblastic leukemia (Specchia et al., 2003), with prognostic impact.

Moreover, WHO does not currently address the classification of MPNs that have more than one genetic abnormality, but it is well established that the presence of additional co-operating mutations in myeloid genes (along with other important risk factors) has a straight relationship with phenotype and clinical outcome definition (Rumi & Cazzola, 2017; Tefferi, 2016). Cytogenetic analysis allows to identify subgroups of patients with a distinct phenotype and prognostic profile, and should be performed in conjunction with *JAK2* mutation analysis PN-MPNs patients (Larsen et al., 2007).

Furthermore, the concomitant presence of two molecular markers is well defined for certain diseases, and raises several issues, including the best therapeutic strategy to adopt. But, therapeutic decisions should not be based only on molecular biology test results (Heller et al., 2001).

CML can express on the background of a *JAK2* V617F positive PN-MPN, and treatment with TKI might reveal/make more expressive the PN-phenotype. It is of great importance to recognize and investigate the association of both anomalies, especially in CML patients who have an unusual clinical/laboratorial course, with hemoglobin and/or platelets count increase, or when they do not respond to therapy, making the diagnosis of other MPNs to have practical therapeutic consequences.

It seems that for these complex patients the most efficient therapeutic choice is to associate a TKI with a *JAK2* inhibitor (Qin et al., 2014; Zhou et al., 2015).

5.4 – REFERENCES

Arber, D. A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M. J., Le Beau, M. M., . . . Vardiman, J. W. (2016). The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood, 127*(20), 2391-2405. doi:10.1182/blood-2016-03-643544

Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., . . . Project, C. G. (2005). Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet, 365*(9464), 1054-1061. doi:10.1016/S0140-6736(05)71142-9

Bee, P. C., Gan, G. G., Nadarajan, V. S., Latiff, N. A., & Menaka, N. (2010). A man with concomitant polycythaemia vera and chronic myeloid leukemia: the dynamics of the two disorders. *Int J Hematol, 91*(1), 136-139. doi:10.1007/s12185-009-0471-6

Heller, P., Kornblihtt, L. I., Cuello, M. T., Larripa, I., Najfeld, V., & Molinas, F. C. (2001). BCR-ABL transcripts may be detected in essential thrombocythemia but lack clinical significance. *Blood, 98*(6), 1990.

Hinds, D. A., Barnholt, K. E., Mesa, R. A., Kiefer, A. K., Do, C. B., Eriksson, N., . . . Gotlib, J. (2016). Germ line variants predispose to both JAK2 V617F clonal hematopoiesis and myeloproliferative neoplasms. *Blood, 128*(8), 1121-1128. doi:10.1182/blood-2015-06-652941

Hummel, J. M., Kletecka, M. C., Sanks, J. K., Chiselite, M. D., Roulston, D., Smith, L. B., . . . Lim, M. S. (2012). Concomitant BCR-ABL1 translocation and JAK2(V617F) mutation in three patients with myeloproliferative neoplasms. *Diagn Mol Pathol, 21*(3), 176-183. doi:10.1097/PDM.0b013e318246975e

Kralovics, R., Passamonti, F., Buser, A. S., Teo, S. S., Tiedt, R., Passweg, J. R., . . . Skoda, R. C. (2005). A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med, 352*(17), 1779-1790. doi:10.1056/NEJMoa051113

Kwong, Y. L., Chiu, E. K., Liang, R. H., Chan, V., & Chan, T. K. (1996). Essential thrombocythemia with BCR/ABL rearrangement. *Cancer Genet Cytogenet, 89*(1), 74-76.

Larsen, T. S., Hasselbalch, H. C., Pallisgaard, N., & Kerndrup, G. B. (2007). A der(18)t(9;18)(p13;p11) and a der(9;18)(p10;q10) in polycythemia vera associated with a hyperproliferative phenotype in transformation to postpolycythemic myelofibrosis. *Cancer Genet Cytogenet, 172*(2), 107-112. doi:10.1016/j.cancergencyto.2006.09.015

LeBrun, D. P., Pinkerton, P. H., Sheridan, B. L., Chen-Lai, J., Dubé, I. D., & Poldre, P. A. (1991). Essential thrombocythemia with the Philadelphia chromosome and BCR-ABL gene rearrangement. An entity distinct from chronic myeloid leukemia and Philadelphia chromosome-negative essential thrombocythemia. *Cancer Genet Cytogenet, 54*(1), 21-25.

Levine, R. L. (2009). Mechanisms of mutations in myeloproliferative neoplasms. *Best Pract Res Clin Haematol, 22*(4), 489-494. doi:10.1016/j.beha.2009.08.006

Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J., . . . Gilliland, D. G. (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell, 7*(4), 387-397. doi:10.1016/j.ccr.2005.03.023

Michiels, J. J., Ten Kate, F. W. J., De Raeve, H., & Gadisseur, A. (2015). Bone Marrow Features and Natural History of BCR/ABL-Positive Thrombocythemia and Chronic Myeloid Leukemia Compared to BCR/ABLNegative Thrombocythemia in Essential Thrombocythemia and Polycythemia Vera. *Journal of Hematology and Thromboembolic Diseases, 3*(2).

Nowell, P. C. (1962). The minute chromosome (Phl) in chronic granulocytic leukemia. *Blut, 8*, 65-66.

Pagnano, K. B., Delamain, M. T., Magnus, M. M., Vassallo, J., DE Souza, C. A., DE Almeida, D., & Lorand-Metze, I. (2016). Concomitant essential thrombocythemia with JAK2 V617F mutation in a patient with chronic myeloid leukemia with major molecular response with imatinib and long-term follow-up. *Oncol Lett, 12*(1), 485-487. doi:10.3892/ol.2016.4631

Pieri, L., Spolverini, A., Scappini, B., Occhini, U., Birtolo, S., Bosi, A., . . . Vannucchi, A. M. (2011). Concomitant occurrence of BCR-ABL and JAK2V617F mutation. *Blood, 118*(12), 3445-3446. doi:10.1182/blood-2011-07-365007

Qin, Y. W., Yang, Y. N., Li, S., & Wang, C. (2014). Coexistence of JAK2V617F Mutation and BCR-ABL Translocation in a Pregnant Woman with Essential Thrombocythemia. *Indian J Hematol Blood Transfus, 30*(Suppl 1), 331-334. doi:10.1007/s12288-014-0385-1

Rowley, J. D. (1973). Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature, 243*(5405), 290-293.

Rumi, E., & Cazzola, M. (2017). Diagnosis, risk stratification, and response evaluation in classical myeloproliferative neoplasms. *Blood, 129*(6), 680-692. doi:10.1182/blood-2016-10-695957

Specchia, G., Albano, F., Anelli, L., Storlazzi, C. T., Zagaria, A., Mancini, M., . . . Rocchi, M. (2003). Deletions on der(9) chromosome in adult Ph-positive acute lymphoblastic leukemia occur with a frequency similar to that observed in chronic myeloid leukemia. *Leukemia, 17*(3), 528-531. doi:10.1038/sj.leu.2402829

Tefferi, A. (2016). Myeloproliferative neoplasms: A decade of discoveries and treatment advances. *Am J Hematol, 91*(1), 50-58. doi:10.1002/ajh.24221

Tefferi, A., & Pardanani, A. (2015). Myeloproliferative Neoplasms: A Contemporary Review. *JAMA Oncol, 1*(1), 97-105. doi:10.1001/jamaoncol.2015.89

Ursuleac, I., Colita, A., Adam, T., Jardan, C., Ilea, A., & Coriu, D. (2013). The concomitant occurrence of JAK2V617F mutation and BCR/ABL transcript with phenotypic expression - an overlapping myeloproliferative disorder or two distinct diseases? - case report. *J Med Life, 6*(1), 34-37.

van Dongen, J. J., Macintyre, E. A., Gabert, J. A., Delabesse, E., Rossi, V., Saglio, G., . . . Biondi, A. (1999). Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. *Leukemia, 13*(12), 1901-1928.

Zhou, A., Knoche, E. M., Engle, E. K., Fisher, D. A., & Oh, S. T. (2015). Concomitant JAK2 V617F-positive polycythemia vera and BCR-ABL-positive chronic myelogenous leukemia treated with ruxolitinib and dasatinib. *Blood Cancer J, 5*, e351. doi:10.1038/bcj.2015.77

CHAPTER 6:
BCR-ABL V280G MUTATION POTENTIAL ROLE IN IMATINIB RESISTANCE: FIRST CASE REPORT

Published in Azevedo *et al*., 2017, Clinical Medicine Insights: Oncology 11:1-5
DOI: 10.1177/1179554917702870

and in Reichert *et al.,* 2015, Casos Clínicos em Leucemia Mielóide Crónica – a experiência portuguesa, Edição Novartis Farma – Produtos Farmacêuticos, S.A.

ABSTRACT

The identification of BCR-ABL expression as the defining leukemogenic event in Chronic Myeloid Leukemia (CML) and the introduction of BCR-ABL tyrosine kinase inhibitors in 2001 have revolutionized disease management, leading to a reduction in mortality rates and accordingly in an increase of the estimated prevalence of CML.

Based on medical records and clinical follow-up, the authors present the case of a Philadelphia chromosome-positive CML patient who developed resistance to imatinib. qRT-PCR testing revealed a V280G BCR-ABL mutation.

This is the first report describing a new BCR-ABL kinase domain mutation - V280G, which might be associated with resistance to imatinib. Approximately 15% to 30% of patients treated with imatinib discontinue treatment due to resistance or intolerance. More than 90 BCR-ABL mutations were detected so far, conferring variable degrees of drug resistance, with consequent clinical, therapeutic and prognostic impact.

CHAPTER 6: BCR-ABL V280G MUTATION POTENTIAL ROLE IN IMATINIB RESISTANCE: FIRST CASE REPORT

6.1 – INTRODUCTION

The identification of *BCR-ABL* expression as the defining leukemogenic event in Chronic Myeloid Leukemia (CML) and the introduction of *BCR-ABL* tyrosine kinase inhibitors (TKIs) in 2001 has marked a paradigm shift in the management of the disease, leading to a reduction in mortality rates and accordingly to an increase of the estimated prevalence of CML (Radich, Shah, & Mauro, 2014; Savona, 2014).

Imatinib was initially the standard of care for the first-line treatment of CML patients in chronic-phase, due to its high long-term response rates and favorable tolerability profile compared to previous standard therapies (Kantarjian, Cortes, La Rosée, & Hochhaus, 2010; Savona, 2014; Weisberg, Manley, Cowan-Jacob, Hochhaus, & Griffin, 2007).

Approximately 15% to 30% (2% to 4% annually) of patients treated with imatinib discontinue treatment after 6 years due to resistance or intolerance, particularly in the accelerated and blast phase (Kantarjian et al., 2010; Nicolini et al., 2006; Radich et al., 2014; Weisberg et al., 2007).

Inadequate response to TKI therapy is associated with poor long-term outcome. More than 90 *BCR-ABL* mutations were detected so far, most frequently the T315I and E255K mutations, conferring variable degrees of drug resistance (Nicolini et al., 2006; Soverini et al., 2006).

Below we report the case of a CML patient who developed resistance to imatinib, presenting a new kinase domain mutation - V280G – that has never been described in the literature.

The patient's anonymity and consent was guaranteed, in agreement with the Declaration of Helsinki. The institutional ethic board approved this report.

6.2 – CASE REPORT

A 75 years old female patient, leucodermic, referred to the Oncology consultation, was diagnosed with chronic phase CML in April 2003 after routine tests, compatible bone marrow study and cytogenetics with a classic Philadelphia chromosome involving the reciprocal translocation of chromosomes 9 and 22 (*BCR-ABL* transcripts not evaluated). Complete blood count results, *BCR-ABL* transcript levels evaluation and treatment options over time are presented in Tables 6.1 and 6.2). Past medical history was not relevant and physical examination did not reveal splenomegaly, nor constitutional symptoms. Abdominal ultrasound showed a spleen with 10,0×5,9 cm. She was given an

intermediate-risk Sokal score (Sokal et al., 1984) (0,84) and Hasford score (Hasford et al., 1998) (931,5), but low-risk EUTOS score (Hasford et al., 2011) (40). At that time, she started hydroxyurea 500mg/day and alpha-interferon 3 million units/5 times per week, with a complete hematological response (CHR) three months later. Repetition of the bone marrow study in December 2003 showed a complete cytogenetic response (CCyR) and one year after diagnosis *BCR-ABL* was positive (not quantified).

Table 6.1 – Complete blood count results over time.

	April 2003	**January 2005**	**May 2013**
Haemoglobin (g/L)	117	121	137
Leucocytes (×10^9/L)	29.9	24.0	8.59
Blasts (×10^9/L; %)	0.32; 1	0	0
Myelocytes (×10^9/L)	0.32	1.53	0
Metamyelocytes (×10^9/L)	2.6	0.65	0
Neutrophils (×10^9/L)	18.51	13.30	5.25
Lymphocytes (×10^9/L)	6.17	2.83	2.70
Monocytes (×10^9/L)	0.32	0.65	0.59
Eosinophils (×10^9/L)	1.62	0.65	0.03
Basophils (×10^9/L)	0	4.36	0.02
Platelets (×10^9/L)	370	1321	282

Table 6.2 – BCR-ABL transcript levels evaluation and treatment over time.

	2003	**2004**	**2005**	**2007**	**2010**	**2011**	**2012**	**2013**
***BCR-ABL* p210 (%)**	Not evaluated	Positive (not quantified)	No information	4.3	0.98 to 2.07	2.63 to 0.53	2.74	8.0 to 0.0
Treatment	HU 500mg/day Alfa-IF 3 million units/5x/week		Imatinib 300mg/day			Imatinib 400mg/day		Nilotinib 400mg/2x/ day

HU – Hydroxyurea; Alfa-IF – alfa-interferon

The patient continued initial therapy until January 2005, when she revealed signs of disease progression and therapeutic failure. Abdominal ultrasound did not reveal splenomegaly. She was started on imatinib at a dose of 300mg/day, obtaining a partial cytogenetic response (*BCR-ABL* p210 – 4.3% on the International Scale (IS)) in June 2007. A marrow study revealed some degree of fibrosis, with no disease infiltration.

In January 2010, *BCR-ABL* p210 was positive (maximum 0.98%) and an increase (maximum 2.07%) was detected by December 2010, which motivated her referral to our Hematology consultation by February 2011. Although the bone marrow maintained in remission, the *BCR-ABL* p210 transcript number was persistently increased (maximum 2.63%), which led us to increase the imatinib dose from 300mg to 400mg/day.

After six months of imatinib dose increase, the number of *BCR-ABL* p210 transcripts decreased (0.53%) and, given the good hematological and molecular responses, the imatinib dose was maintained.

By October 2012, although the patient maintained a CHR, a major molecular response (MMR) was never achieved, with a progressive increase of the *BCR-ABL* transcripts (2.74%). As a result of the failure to obtain an adequate molecular response, resistance to imatinib was investigated. We performed a nested qRT-PCR and bidirectional sequencing (as previously described (Gromicho et al., 2013)) to carry-out a *BCR-ABL* kinase domain mutational analysis. This study showed a mutation on the amino acid 280, resulting in the substitution of valine (V) by glycine (G), present in all transcripts (c.839T>G V280G mutation) (Figure 6.1), and negative in DNA samples from the gum mucosa.

On February 2013 *BCR-ABL* p210 was positive (8.0%). Imatinib was discontinued due to resistance and the patient was started on nilotinib 400mg/twice a day.

In May 2013, the patient revealed a good clinical and laboratorial response to therapy, with CCyR and CMR.

Currently, the patient maintains the same nilotinib dose, without evidence of loss of hematological or molecular responses and with a good tolerability.

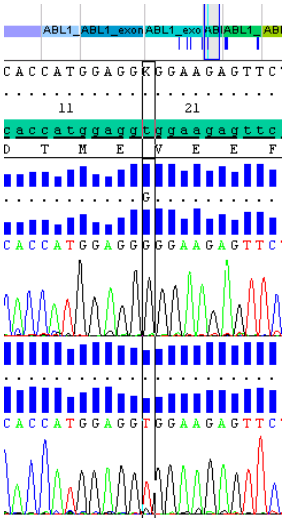


Figure 6.1 – BCR-ABL tyrosine kinase domain mutation analysis in peripheral blood.
It revealed c.839T>G (V280G mutation), in which GTG->GGG (valine to glycine).

6.3 – DISCUSSION AND CONCLUSIONS

TKI therapy with imatinib, dasatinib or nilotinib has resulted in a remarkable improvement in clinical outcomes for CML patients diagnosed in chronic phase. The optimization of monitoring methods and the identification of factors associated with response and long-term outcomes has thus been a major clinical research focus, contributing to recent updates to clinical practice guidelines (Savona, 2014).

In the case presented here, the patient had been previously diagnosed and followed in another hospital center and there was no registry in the process file about the reason why it was decided to start treatment with a subtherapeutic imatinib dose. Although not consensual in the literature (Hayakawa et al., 2013; Liu, Li, Pao, & Michor, 2015; Santos et al., 2010; Tang et al., 2011; Van Obbergh et al., 2016; Vine et al., 2014), this probably contributed to a worse molecular response and to imatinib treatment failure, even after increase of the dose in 2011, when the patient was referred to our consultation. At the moment, it is not specified which of the three TKIs currently approved for the first-line treatment of chronic phase CML is preferred, and although no evidence from clinical trials is available to support the superiority of second-generation TKIs over imatinib with respect to survival outcomes, they induce the achievement of faster and deeper molecular responses, associated with better long-term outcomes, less progression to advanced phases, and the possibility of treatment discontinuation (Cortes et al., 2016; Hochhaus et al., 2016; McDougall, Ramsey, & Radich, 2016; Sundar & Radich, 2016).

The IRIS trial has demonstrated that imatinib induces high durable responses and improves survival in a large proportion of patients with newly diagnosed chronic phase, compared to interferon (Druker et al., 2006; Sundar & Radich, 2016). On the other hand, DASISION and ENESTnd studies showed that dasatinib and nilotinib induce superior cytogenetic and molecular responses and lower rates of progression to accelerated or blast phase, when compared to imatinib (Cortes et al., 2016; Hochhaus et al., 2016).

Although imatinib is still recommended as a reasonable first-line option for patients with newly diagnosed chronic phase, the selection of first-line TKI depends on the risk score (Sokal and Hasford), physician's experience, toxicity profile, patient's age, tolerance and adherence to therapy as well as comorbidities (Sundar & Radich, 2016).

Allogeneic HCT, which was the treatment of choice for CML before the advent of TKI therapy, is now generally reserved for patients in chronic phase resistant to multiple or unable to tolerate TKIs, those who have the T315I mutation and who are not suitable for prolonged ponatinib therapy, or for those progressing to accelerated or blast phases (Sundar & Radich, 2016).

Because most CML patients treated with imatinib achieve CCyR (CCyR roughly corresponds to a *BCR-ABL* level of <1% IS), both the National Comprehensive Cancer Network (NCCN) and the European Leukemia Net (ELN) guidelines emphasize adequate monitoring and measurement of residual disease through sensitive molecular methods such as quantification of *BCR-ABL* transcript levels and real-time quantitative PCR (qRT-PCR), to ensure that patients are meeting the treatment

milestones (Baccarani et al., 2013; Cortes et al., 2016; Deininger, 2015; O'Brien et al., 2014; Soverini, de Benedittis, Mancini, & Martinelli, 2015).

Although most of these patients respond to first-line TKI therapy, the use of TKIs is hindered by the development of resistance or intolerance in some patients, resulting in a loss of response or discontinuation of treatment. This is most commonly associated with the acquisition of resistance-conferring kinase domain point mutations within *BCR-ABL*, which prevent the binding of imatinib to the kinase domain, additional chromosome abnormalities to the Philadelphia chromosome, and/or mutations in genes such as *ASXL1*, *TET2*, *RUNX1*, *DNMT3A*, *EZH2*, and *TP53* among others (Radich et al., 2014; Soverini et al., 2015; Soverini et al., 2011). According to ELN 2013 criteria it is recommended in these cases to perform a mutational analysis (Cortes et al., 2016; Soverini et al., 2014). The recommended methodology is direct sequencing, although it may be preceded by screening with other techniques, such as denaturing-high performance liquid chromatography (Deininger, 2015; Soverini et al., 2011).

Next-generation deep sequencing, complemented with bioinformatics support, mass spectrometry and digital PCR are emerging promising techniques to ensure reliable detection of *BCR-ABL* mutations, allowing early therapy switch and selection of the most appropriate therapy (Machova Polakova et al., 2015; Soverini et al., 2014).

Current data support that for CML patients who fail treatment goals, with primary resistance or intolerance to imatinib, hematologic disease recurrence, or emergent *BCR-ABL* kinase domain mutations, imatinib dose escalation may not be sufficient to control the disease and substitution with another TKI will be necessary (Baccarani et al., 2013; Jabbour, Saglio, Hughes, & Kantarjian, 2012; Kantarjian et al., 2010; Soverini et al., 2014; Soverini et al., 2011; Sundar & Radich, 2016). Patients should be carefully evaluated for alternative treatment options, including dasatinib, nilotinib, bosutinib, and ponatinib, as well as the non-TKI salvage agent omacetaxine mepesuccinate. Treatment selection is based on factors such as the patient's disease state, prior therapies, type of mutation, comorbidities, treatment toxicity, and therapy goals (Kantarjian et al., 2010; Radich et al., 2014).

A literature review shows that pre-existing mutations at baseline confer a more aggressive disease phenotype and patients with advanced stages of the disease often do not respond to therapy or relapse (Nicolini et al., 2006; Soverini et al., 2006; Sundar & Radich, 2016).

There is no reference in the literature on the association between CML/other pathology and the c.839T>G V280G mutation, as detected in this case. Gruber *et al.* have used the V280G variant in their study about the biological significance of aberrant AID expression in a murine model of *BCR-ABL1* driven B-cell lineage Acute Lymphoblastic Leukemia (Gruber, Chang, Sposto, & Müschen, 2010), but this was based on the results presented in a previous publication of Soverini *et al* (2006) (Soverini et al., 2006). However, Soverini *et al.* described in 2011 (Soverini et al., 2011) a mutation in the same codon position, involving different amino acids, associated with imatinib resistance in CML patients. The mutation presented in this case report causes a substitution of a valine by a glycine at amino acid 280, in the kinase active domain, being the only alteration found in this patient after *BCR-ABL* mutation

analysis. A DNA sample of gum mucosa was negative for this type of mutation, which allowed us to conclude that acquired V280G variant was indeed a characteristic of the leukemic clone, rather than a polymorphism. This finding led us to hypothesize that this mutation might be associated with *de novo* resistance to treatment with imatinib. The favorable clinical and hematological parameters to nilotinib also support this statement. However, studies in a larger population and of functional character should be performed in order to evaluate the prevalence of this mutation and its association with imatinib resistance and to determine whether this mutation can be grouped with other similar mutations to better indicate nilotinib as a first-choice treatment. It will also be useful to have functional analyses to better characterize this mutation and show if it affects the ability of imatinib to bind or inhibit *BCR-ABL*.

Regular monitoring of *BCR-ABL* levels, effective management of toxicities, and patient education on adherence to TKI therapy are essential to provide optimal treatment.

The possibility of resistance to treatment should promote a rational development of alternative, synergistic and potentially curative strategies.

The more complete knowledge about the disease and its mutational characterization will allow us to control the disease course.

6.4 – REFERENCES

Baccarani, M., Deininger, M. W., Rosti, G., Hochhaus, A., Soverini, S., Apperley, J. F., . . . Hehlmann, R. (2013). European LeukemiaNet recommendations for the management of chronic myeloid leukemia: 2013. *Blood, 122*(6), 872-884. doi:10.1182/blood-2013-05-501569

Cortes, J. E., Saglio, G., Kantarjian, H. M., Baccarani, M., Mayer, J., Boqué, C., . . . Hochhaus, A. (2016). Final 5-Year Study Results of DASISION: The Dasatinib Versus Imatinib Study in Treatment-Naïve Chronic Myeloid Leukemia Patients Trial. *J Clin Oncol*. doi:10.1200/JCO.2015.64.8899

Deininger, M. W. (2015). Molecular monitoring in CML and the prospects for treatment-free remissions. *Hematology Am Soc Hematol Educ Program, 2015*, 257-263. doi:10.1182/asheducation-2015.1.257

Druker, B. J., Guilhot, F., O'Brien, S. G., Gathmann, I., Kantarjian, H., Gattermann, N., . . . Investigators, I. (2006). Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med, 355*(23), 2408-2417. doi:10.1056/NEJMoa062867

Gromicho, M., Magalhães, M., Torres, F., Dinis, J., Fernandes, A. R., Rendeiro, P., . . . Sebastião Rodrigues, A. (2013). Instability of mRNA expression signatures of drug transporters in chronic myeloid leukemia patients resistant to imatinib. *Oncol Rep, 29*(2), 741-750. doi:10.3892/or.2012.2153

Gruber, T. A., Chang, M. S., Sposto, R., & Müschen, M. (2010). Activation-induced cytidine deaminase accelerates clonal evolution in BCR-ABL1-driven B-cell lineage acute lymphoblastic leukemia. *Cancer Res, 70*(19), 7411-7420. doi:10.1158/0008-5472.CAN-10-1438

Hasford, J., Baccarani, M., Hoffmann, V., Guilhot, J., Saussele, S., Rosti, G., . . . Hehlmann, R. (2011). Predicting complete cytogenetic response and subsequent progression-free survival in 2060 patients with CML on imatinib treatment: the EUTOS score. *Blood, 118*(3), 686-692. doi:10.1182/blood-2010-12-319038

Hasford, J., Pfirrmann, M., Hehlmann, R., Allan, N. C., Baccarani, M., Kluin-Nelemans, J. C., . . . Ansari, H. (1998). A new prognostic score for survival of patients with chronic myeloid leukemia treated with interferon alfa. Writing Committee for the Collaborative CML Prognostic Factors Project Group. *J Natl Cancer Inst, 90*(11), 850-858.

Hayakawa, H., Ichihara, E., Ohashi, K., Ninomiya, T., Yasugi, M., Takata, S., . . . Kiura, K. (2013). Lower gefitinib dose led to earlier resistance acquisition before emergence of T790M mutation in epidermal growth factor receptor-mutated lung cancer model. *Cancer Sci, 104*(11), 1440-1446. doi:10.1111/cas.12284

Hochhaus, A., Saglio, G., Hughes, T. P., Larson, R. A., Kim, D. W., Issaragrisil, S., . . . Kantarjian, H. M. (2016). Long-term benefits and risks of frontline nilotinib vs imatinib for chronic myeloid leukemia in chronic phase: 5-year update of the randomized ENESTnd trial. *Leukemia, 30*(5), 1044-1054. doi:10.1038/leu.2016.5

Jabbour, E., Saglio, G., Hughes, T. P., & Kantarjian, H. (2012). Suboptimal responses in chronic myeloid leukemia: implications and management strategies. *Cancer, 118*(5), 1181-1191. doi:10.1002/cncr.26391

Kantarjian, H. M., Cortes, J., La Rosée, P., & Hochhaus, A. (2010). Optimizing therapy for patients with chronic myelogenous leukemia in chronic phase. *Cancer, 116*(6), 1419-1430. doi:10.1002/cncr.24928

Liu, L. L., Li, F., Pao, W., & Michor, F. (2015). Dose-Dependent Mutation Rates Determine Optimum Erlotinib Dosing Strategies for EGFR Mutant Non-Small Cell Lung Cancer Patients. *PLoS One, 10*(11), e0141665. doi:10.1371/journal.pone.0141665

Machova Polakova, K., Kulvait, V., Benesova, A., Linhartova, J., Klamova, H., Jaruskova, M., . . . Soverini, S. (2015). Next-generation deep sequencing improves detection of BCR-ABL1 kinase domain mutations emerging under tyrosine kinase inhibitor treatment of chronic myeloid leukemia patients in chronic phase. *J Cancer Res Clin Oncol, 141*(5), 887-899. doi:10.1007/s00432-014-1845-6

McDougall, J., Ramsey, S. D., & Radich, J. (2016). What Happens When Imatinib Goes Generic? *J Natl Compr Canc Netw, 14*(2), 128-131.

Nicolini, F. E., Corm, S., Lê, Q. H., Sorel, N., Hayette, S., Bories, D., . . . Roche-Lestienne, C. (2006). Mutation status and clinical outcome of 89 imatinib mesylate-resistant chronic myelogenous leukemia patients: a retrospective analysis from the French intergroup of CML (Fi(phi)-LMC GROUP). *Leukemia, 20*(6), 1061-1066. doi:10.1038/sj.leu.2404236

O'Brien, S., Radich, J. P., Abboud, C. N., Akhtari, M., Altman, J. K., Berman, E., . . . Sundar, H. (2014). Chronic myelogenous leukemia, version 1.2015. *J Natl Compr Canc Netw, 12*(11), 1590-1610.

Radich, J. P., Shah, N. P., & Mauro, M. J. (2014). Integrating current treatment options for TKI-resistant chronic myeloid leukemia. *Clin Adv Hematol Oncol, 12*(7 Suppl 13), 3-17, 11.

Santos, F. P., Kantarjian, H., Fava, C., O'Brien, S., Garcia-Manero, G., Ravandi, F., . . . Cortes, J. (2010). Clinical impact of dose reductions and interruptions of second-generation tyrosine kinase inhibitors in patients with chronic myeloid leukaemia. *Br J Haematol, 150*(3), 303-312. doi:10.1111/j.1365-2141.2010.08245.x

Savona, M. R. (2014). Molecular monitoring and minimal residual disease in the management of chronic myelogenous leukemia. *J Community Support Oncol, 12*(5), 171-178.

Sokal, J. E., Cox, E. B., Baccarani, M., Tura, S., Gomez, G. A., Robertson, J. E., . . . Cervantes, F. (1984). Prognostic discrimination in "good-risk" chronic granulocytic leukemia. *Blood, 63*(4), 789-799.

Soverini, S., Branford, S., Nicolini, F. E., Talpaz, M., Deininger, M. W., Martinelli, G., . . . Shah, N. P. (2014). Implications of BCR-ABL1 kinase domain-mediated resistance in chronic myeloid leukemia. *Leuk Res, 38*(1), 10-20. doi:10.1016/j.leukres.2013.09.011

Soverini, S., Colarossi, S., Gnani, A., Rosti, G., Castagnetti, F., Poerio, A., . . . Leukemia, G. W. P. o. C. M. (2006). Contribution of ABL kinase domain mutations to imatinib resistance in different subsets of Philadelphia-positive patients: by the GIMEMA Working Party on Chronic Myeloid Leukemia. *Clin Cancer Res, 12*(24), 7374-7379. doi:10.1158/1078-0432.CCR-06-1516

Soverini, S., de Benedittis, C., Mancini, M., & Martinelli, G. (2015). Mutations in the BCR-ABL1 Kinase Domain and Elsewhere in Chronic Myeloid Leukemia. *Clin Lymphoma Myeloma Leuk, 15 Suppl*, S120-128. doi:10.1016/j.clml.2015.02.035

Soverini, S., Hochhaus, A., Nicolini, F. E., Gruber, F., Lange, T., Saglio, G., . . . Martinelli, G. (2011). BCR-ABL kinase domain mutation analysis in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors: recommendations from an expert panel on behalf of European LeukemiaNet. *Blood, 118*(5), 1208-1215. doi:10.1182/blood-2010-12-326405

Sundar, H., & Radich, J. (2016). Optimizing patient care in chronic phase Chronic Myelogenous Leukemia: a multidisciplinary approach. *Journal of the National Comprehensive Cancer Network, 14*(1), s-1-s6.

Tang, C., Schafranek, L., Watkins, D. B., Parker, W. T., Moore, S., Prime, J. A., . . . Hughes, T. P. (2011). Tyrosine kinase inhibitor resistance in chronic myeloid leukemia cell lines: investigating resistance pathways. *Leuk Lymphoma, 52*(11), 2139-2147. doi:10.3109/10428194.2011.591013

Van Obbergh, F., Knoops, L., Devos, T., Beguin, Y., Graux, C., Benghiat, F., . . . Wallemacq, P. (2016). The clinical relevance of imatinib plasma trough concentrations in chronic myeloid leukemia. A Belgian study. *Clin Biochem*. doi:10.1016/j.clinbiochem.2016.12.006

Vine, J., Cohen, S. B., Ruchlemer, R., Goldschmidt, N., Levin, M., Libster, D., . . . Rund, D. (2014). Polymorphisms in the human organic cation transporter and the multidrug resistance gene: correlation with imatinib levels and clinical course in patients with chronic myeloid leukemia. *Leuk Lymphoma, 55*(11), 2525-2531. doi:10.3109/10428194.2014.893307

Weisberg, E., Manley, P. W., Cowan-Jacob, S. W., Hochhaus, A., & Griffin, J. D. (2007). Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia. *Nat Rev Cancer, 7*(5), 345-356. doi:10.1038/nrc2126.

CHAPTER 7:
THE ROLE OF CASPASE GENES POLYMORPHISMS IN GENETIC SUSCEPTIBILITY TO PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS IN A PORTUGUESE POPULATION

Accepted for publication in Pathology and Oncology Research (2017)

ABSTRACT

Our main aim was to evaluate the role of caspases' genes SNPs in Philadelphia-chromosome negative chronic myeloproliferative neoplasms (PN-MPNs) susceptibility.

We carried-out a case-control study in 133 Caucasian Portuguese PN-MPNs patients and 281 matched controls, studying SNPs in apoptosis related caspases: rs1045485 and rs1035142 (CASP8), rs1052576, rs2308950, rs1132312 and rs1052571 (CASP9), rs2227309 and rs2227310 (CASP7) and rs13006529 (CASP10).

After stratification by pathology diagnosis for essential thrombocythemia (ET), female gender or JAK2 positive, there is a significant increased risk to those carrying at least one variant allele for CASP9 (C653T) polymorphism (OR 2.203 CI 95% [1.163 – 4.176], P=0.015; OR 4.370 CI95% [1.608 – 11.873], P=0.004; and OR 2.886 CI 95% [1.303 – 6.393], P=0.009, respectively). However, when considered individually, none of the studied caspases polymorphisms was associated with PN-MPNs risk.

Our results do not reveal a significant involvement of caspase genes polymorphisms on the individually susceptibility towards PN-MPNs as a whole. However, for essential thrombocythemia (ET), female gender or JAK2 positive, there is a significant increased risk to those carrying at least one variant allele for CASP9. Although larger studies are required to confirm these results and to provide conclusive evidence of association between these and other caspases variants and PN-MPNs susceptibility, these new data may contribute to a best knowledge of the pathophysiology of these disorders and, in the future, to a more rational and efficient choice of therapeutic strategies to be adopted in PN-MPNs treatment.

CHAPTER 7: THE ROLE OF CASPASE GENES POLYMORPHISMS AND GENETIC SUSCEPTIBILITY TO PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS IN A PORTUGUESE POPULATION

7.1 – INTRODUCTION

According to the World Health Organization (WHO-IARC) classification myeloproliferative neoplasms (MPNs) encompass various conditions including chronic myelogenous leukemia (CML) and the most common Philadelphia-negative myeloproliferative neoplasms (PN-MPNs), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) (Arber et al., 2016; Swerdlow & SH, 2008).

Genetic insights into the pathogenesis of the PN-MPNs include the discovery of the somatic point gain-of-function mutations in the Janus kinase 2 gene (*JAK2*; exon 14 V617F and exon 12 mutations) (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Scott et al., 2007), the myeloproliferative leukemia virus oncogene (*MPL*; more frequently *W515*), and recently calreticulin (*CALR*) mutations, which modified the understanding of these diseases, their diagnosis and management (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Scott et al., 2007; Tognon, Nunes, & Castro, 2013). Frequencies of these mutations are approximately 95%, 0%, and 0% in PV, 60%, 3%, and 20% in ET, and 60%, 7%, and 25% in PMF, respectively (Nangalia et al., 2013; Tefferi & Pardanani, 2015). However, the cellular and molecular mechanisms involved in the pathophysiology of MPNs have not yet been fully clarified. Genetic alterations still await identification in around 40% of ET and PMF cases and, moreover, these mutations cannot fully explain the phenotypic heterogeneity of PN-MPNs. One possible reason is that the outcome of a mutation can depend upon other genetic variants in the genome (Rice et al., 2011; Rueff & Rodrigues, 2016). Indeed, the possible role of silencing of *SOCS* (suppressors of cytokine signalling) by mutations or epigenomic silencing may result in the loss of negative regulators of JAK/STAT pathways. SOCS proteins bind to phosphotyrosine residues of JAK and may act as tumor suppressor genes, unless mutated or epigenetically silenced which may occur in PN-MPNs even if no mutation is present in the *JAK2* gene (Valentino & Pierre, 2006).

Besides modifier genes such as the *SOCS* genes whose proteins inhibit STAT phosphorylation by binding and inhibiting JAKs, polymorphic variants of several genes together with environmental exposure/dietary exposure and immune system characteristics, could predispose to the susceptibility to these disorders (Beer et al., 2010; Björkholm, Hultcrantz, & Derolf, 2014; Bolufer et al., 2006; Delhommeau, Jeziorowska, Marzac, & Casadevall, 2010; Kilpivaara & Levine, 2008). Thus, Single

Nucleotide Polymorphisms (SNP's) at various loci may be important for individual susceptibility risk to PN-MPNs, although less specific, but prognostically relevant (Campregher, Santos, Perini, & Hamerschlak, 2012; Delhommeau et al., 2010; Levine, 2009; Nangalia et al., 2013; Tefferi & Pardanani, 2015).

Apoptosis is a programmed cell death process, acting as a defense mechanism against damaged or stressed cells, to prevent accumulation of non-functional or damaged cells in the tissues. Identification of apoptotic mechanisms is critical and disturbed apoptosis pathways may lead to an accumulation of mutations that may eventually lead to cancer (Goldar, Khaniani, Derakhshan, & Baradaran, 2015; Kiraz, Adan, Kartal Yandim, & Baran, 2016).

The hematopoietic system is particularly sensitive to deregulation of the apoptotic process as these cells undergo a high turnover rate, requiring a tight balance between apoptosis and proliferation. Accordingly, apoptosis is frequently deregulated in hematologic malignancies (Zaman, Wang, & Gandhi, 2014).

Activation of apoptosis occurs through two major routes: the intrinsic and the extrinsic ones. The intrinsic or mitochondrial route may be initiated by various apoptogenic stimuli, such as agents that cause DNA damage, rupture of microtubules, and deficiency or absence of cell growth factors. The extrinsic route is activated by death receptors of the tumor necrosis factor (TNF) family (Green & Llambi, 2015; Kiraz et al., 2016; Tognon et al., 2013). Caspases play a key regulatory role in both intrinsic and extrinsic pathways. Three groups of mammalian caspases exists on the basis of specific functions in different pathways, including developmental, inflammatory, and apoptotic pathways (Goldar et al., 2015; Green & Llambi, 2015). The executioner caspases act in various substrates in the cytoplasm and nucleus, resulting in cellular death.

Deregulation of pro- and anti-apoptotic genes express as cell resistance to apoptosis, culminating with the accumulation of myeloid cells and the establishing of neoplasms (Nunes et al., 2013; Olsson & Zhivotovsky, 2011; Tognon et al., 2011; Tognon et al., 2012; Tognon et al., 2013; Tognon et al., 2016).

A wider characterization of molecular genetic features in PN-MPNs may contribute to a better understanding of the pathogenesis of these diseases and provide new specific diagnostic, prognostic, and therapeutic tools (Delhommeau et al., 2010; Mambet, Matei, Necula, & Diaconu, 2016). However, to date no studies have associated polymorphisms in caspases genes and risk for PN-MPNs.

Thus, the present work describes a hospital based case-control study in a Caucasian Portuguese population in order to evaluate the potential modifying role of nine apoptosis related caspases genes polymorphisms on the individual susceptibility to PN-MPNs.

7.2 – MATERIALS AND METHODS

7.2.1 – STUDY SUBJECTS

The description given in Chapter 3, section 3.2.1 applies in this new chapter.

General characteristics for PN-MPNs patients and control populations are summarized in Table 7.1.

7.2.2 – DNA EXTRACTION

The methodology used is described in Chapter 3, section 3.2.2.

7.2.3 – SNP SELECTION (CASPASES)

Publicly available on-line databases such as NCBI (http://www.ncbi.nlm.nih.gov/projects/snp/), GeneCards (http://www.genecards.org) and SNP500Cancer (http://variantgps.nci.nih.gov/cgfseq/pages/snp500.do) were used to search for SNP's reported to date on genes coding for mediators of apoptosis, to be included in this work. The eligible SNP's in the present study had to be located in a coding region giving rise to an amino acid change (non-synonymous SNP's) and exhibit a minor allele frequency (MAF) >0.1 in Caucasian populations (Table 7.2).

7.2.4 – GENOTYPING

The polymorphisms rs2227309 and rs2227310 (*CASP7*), rs1045485 and rs1035142 (*CASP8*), rs2308950, rs1132312 and rs1052571 (*CASP9*) and rs13006529 (*CASP10*) and *JAK2* V617F mutation were genotyped using the same methodology described in Chapter 3, section 3.2.3.

The SNP genotyping assay information for caspases genes polymorphisms is summarized in Table 7.2.

7.2.5 – STATISTICAL ANALYSIS

The analysis of Hardy-Weinberg frequencies for all alleles in the control and patients' populations and the differences in genotype frequency, smoking/alcohol consumption status, age class and gender distributions between PN-MPNs cancer patients and controls were evaluated using the same statistical approach described in Chapter 3, section 3.2.4.

The crude and adjusted odds ratio (OR) and the corresponding 95% confidence intervals (CI) were calculated using unconditional multiple logistic regression. The model for adjusted OR included terms for gender, age at diagnosis (30-49, 50-69 and ≥70 years), smoking habits (smokers/non-smokers), and alcohol habits (never, social and regular consumption) with male sex, lower age group and non-smokers/non-alcohol consumers being considered as the reference groups for each of these variables. For the purpose of these calculations, age at diagnosis for controls was the age at the time of diagnosis for the matched case. All analyses were performed using the Statistical Package for the Social Sciences for Windows 22.0 version (SPSS, Inc.) (Table 7.3, 7.4 and 7.5). Since this is not a conclusive final study but an exploratory one on the role of apoptosis related caspases polymorphisms in PN-MPNs and the data to be obtained should be looked at as proof of concept, the Bonferroni adjustment was deemed as not necessary as it is too conservative.

7.3 – RESULTS

7.3.1 – CHARACTERIZATION OF POPULATIONS

This study included 133 PN-MPNs patients and 281 age- and sex-matched controls. The baseline characteristics (sex, age alcohol consumption and smoking habits) of both case and control populations are listed in Table 7.1. The case group included 72 (54.1%) females and 61 (45.9%) male patients, with an overall mean age of 68 years, in agreement with the gender distribution usually observed in this type of pathology. No significant differences were found between the case and control groups concerning age distribution or smoking habits (see Table 7.1). However, alcohol consumption is significantly increased in patients when compared with control group (P<0.0001) (see Table 7.1).

According to diagnosis criteria patients' distribution was as follows: 80 (60.2%) with ET, 39 (29.3%) with PV and 14 (10.5%) with PMF (Table 7.1).

Table 7.1 – General characteristics for the PN-MPNs case and control populations and gender distribution for the PN-MPNs cases.
PN-MPNs cases *n*=133; Control population *n*=281.

Characteristics	Cases, *n* (%)	Controls, *n* (%)	*P* value
Gender			
Male	61 (45.9)	133 (47.3)	0.8
Female	72 (54.1)	148 (52.7)	
Age [a,b]			
30-49	16 (12.0)	43 (15.3)	0.6
50-69	50 (37.6)	107 (38.1)	
≥70	67 (50.4)	131 (46.6)	
Smoking habits			
Never	104 (78.2)	213 (76.1)	0.6
Current	29 (21.8)	67 (23.9)	
Alcohol habits			
Never	103 (77.4)	191 (68.2)	**<0.0001**
Social	20 (15.0)	25 (8.9)	
Regular	10 (7.5)	64 (22.9)	
Diagnosis	n Male n (%); Female n (%)		
ET	80 32 (40.0); 48 (60.0)		
PV	39 20 (51.3); 19 (48.7)		
PMF	14 9 (64.3); 5 (35.7)		
JAK2 V617F mutation			
Yes	99 (75.0)		**0.020**
ET	58 (73.4)		
PV	34 (87.2)		
PMF	7 (50.0)		
No	33 (25.0)		

[a] Age of diagnosis for cases. [b] Age of control population at the time of diagnosis for the matched case.

7.3.2 – SNP'S GENOTYPING

The characteristics of each SNP under study are described in Table 7.2, while the genotype frequencies determined for all of them are shown in Table 7.3. All of the SNP's studied were in agreement with expectation of the Hardy-Weinberg law (P>0.05, exact probability test), except for *CASP8*_rs1045485 and for *CASP9*_rs2308950 (P=0.006 and P=0.034 respectively, exact probability test).

The results obtained revealed that after stratification by pathology diagnosis our results showed (Table 7.3) a significant increased risk for patients diagnosed with ET presenting at least one variant allele (T) of *CASP9*_rs1132312 polymorphism: for heterozygous individuals (OR 2.300 CI 95% [1.180 – 4.484], *P*=0.014) as well as for the combination of heterozygous with homozygous for variant allele

(OR 2.203 CI 95% [1.163 – 4.176], *P*=0.015**)**. The same effect was found, after stratification by gender, in women (OR 4.370 CI95% [1.608 – 11.873], *P*=0.004) considering the presence of at least one variant allele. According to our results, the increased risk was verified in all sub-groups, as can be seen in Table 7.3, although when considered individually, none of the polymorphisms studied were associated with PN-MPNs risk (Table 7.4). No significant difference was found between the case and control groups concerning age distribution, gender, smoking habits or genotype frequencies (Table 7.4). The relevance of *JAK2* mutation in PN-MPNs is well known. Thus, the population was also stratified according to the presence of *JAK2* mutation in patients, showing that there is also a significant increased risk for patients diagnosed with ET when at least one variant allele (T) for *CASP9*_rs1132312 polymorphism is present (OR 2.886 CI 95% [1.303 – 6.393], *P*=0.009) (Table 7.3).

Table 7.2 – Selected SNPs and detailed information on the corresponding base and amino acid exchanges as well as minor allele frequency.

Gene	dsSNP	Codon	Nucleotide exchange	Minor allele frequency, MAF (%)[a]
CASP7 (Chr 10)	rs2227309	249	G→A (Arg/ Lys)	28.0
	rs2227310	255	C→G (Asp/Glu)	29.0
CASP8 (Chr 2)	rs1045485	270	G→C (Asp/His)	13.0
	rs1035142	---	G→T 3'UTR	49.0
CASP9 (Chr 1)	rs2308950	173	G→A (Arg/His)	4.0
	rs1132312	136	C→T (Phe/Phe)	49.6
	rs1052571	28	C→T (Ala/Val)	49.6
CASP10 (Chr 2)	rs13006529	522	A → T (Ile/Leu)	44.0

[a] According to http://www.ncbi.nlm.nih.gov/snp/

Table 7.3 – Genotype distribution and myeloproliferative risk.
For the *CASP7*Lys249Arg, *CASP7*Asp255Glu, *CASP8*Asp302His, *CASP8*Tyr12STOP, *CASP9*Arg173His, *CASP9*Phe136Phe, *CASP9*Val28Ala, and *CASP10*Ile522Leu polymorphisms in the MNPs case (n=133) and control (n=281) populations.

Genetic Polymorphism	Controls, *n* (%)	Cases, *n* (%)	*P* value[a]	OR crude (95% CI)	OR adjusted (95% CI)[b]
CASP7 (Lys249Arg; rs2227309)					
G/G	154 (55.2)	77 (57.9)		1 (Reference)	1 (Reference)
G/A	109 (39.1)	50 (37.6)	0.811	0.917 (0.595 – 1.414)	0.899 (0.578 – 1.398)
A/A	16 (5.7)	6 (4.5)		0.750 (0.282 – 1.993)	0.855 (0.309 – 2.364)
G/A + A/A	125 (44.8)	56 (42.1)		0.896 (0.590 – 1.360)	0.894 (0.583 – 1.370)
CASP7 (Asp255Glu; rs2227310)					
C/C	154 (55.8)	73 (55.3)		1 (Reference)	1 (Reference)
C/G	106 (38.4)	52 (39.4)	0.968	1.035 (0.671 – 1.596)	1.032 (0.662 – 1.608)
G/G	16 (5.8)	7 (5.3)		0.923 (0.364 – 2.341)	1.014 (0.386 – 2.669)
C/G + G/G	122 (44.2)	59 (44.7)		1.020 (0.672 – 1.549)	1.030 (0.671 – 1.580)
CASP8 (Asp270His; rs1045485)					
G/G	220 (78.9)	101 (76.5)		1 (Reference)	1 (Reference)
G/C	51 (18.3)	26 (19.7)	0.819	1.110 (0.655 – 1.882)	1.092 (0.634 – 1.881)
C/C	8 (2.9)	5 (3.8)		1.361 (0.435 – 4.265)	1.194 (0.372 – 3.829)
G/C + C/C	59 (21.1)	31 (23.5)		1.144 (0.698 – 1.877)	1.083 (0.643 – 1.823)
CASP8 (3'UTR; rs1035142)					
G/G	97 (34.8)	50 (37.6)		1 (Reference)	1 (Reference)
G/T	137 (49.1)	61 (45.9)	0.816	0.864 (0.548 – 1.362)	0.861 (0.540 – 1.375)
T/T	45 (16.1)	22 (16.5)		0.948 (0.514 – 1.752)	1.072 (0.568 – 2.025
G/T + T/T	182 (65.2)	83 (62.4)		0.885 (0.576 – 1.358)	0.912 (0.581 – 1.432)
CASP9 (Arg173His; rs2308950)					
G/G	276 (98.9)	129 (97.0)		1 (Reference)	1 (Reference)
G/A	3 (1.1)	3 (2.3)	0.224	2.140 (0.426 – 10.746)	1.704 (0.335 – 8.661)
A/A	0 (0.0)	1 (0.8)		ND	ND
G/A + A/A	3 (1.1)	4 (3.0)		2.853 (0.629 – 12.932)	2.170 (0.472 – 9.983)
CASP9 (Phe136Phe; rs1132312)					
C/C	87 (31.2)	31 (23.3)		1 (Reference)	1 (Reference)
C/T	128 (45.9)	74 (55.6)	0.146	1.622 (0.984 – 2.675)	1.669 (1.000 – 2.783)
T/T	64 (22.9)	28 (21.1)		1.228 (0.671 – 2.247)	1.299 (0.699 – 2.413)
C//T + T/T	192 (68.8)	102 (76.7)		1.491 (0.927 – 2.398)	1.548 (0.952 – 2.517)
CASP9 (Ala28Val; rs1052571)					
C/C	70 (25.1)	25 (18.8)		1 (Reference)	1 (Reference)
C/T	129 (46.2)	74 (55.6)	0.176	1.606 (0.937 – 2.753)	1.580 (0.911 – 2.743)
T/T	80 (28.7)	34 (25.6)		1.190 (0.648 – 2.186)	1.150 (0.618 – 2.142)
C/T + T/T	209 (74.9)	108 (81.2)		1.447 (0.861 – 2.415)	1.415 (0.838 – 2.390)
CASP10 (Ile522Leu; rs13006529)					
A/A	82 (29.4)	36 (29.8)		1 (Reference)	1 (Reference)
A/T	123 (44.1)	58 (47.9)	0.647	1.074 (0.651 – 1.773)	1.113 (0.675 – 1.834)
T/T	74 (26.5)	27 (22.3)		0.831 (0.461 – 1.499)	0.893 (0.496 – 1.608)
A/T + T/T	197 (70.6)	85 (70.2)		0.983 (0.616 – 1.568)	0.973 (0.602 – 1.572)

[a] P-value determined by $\chi 2$ test. [b] ORs were adjusted for age (30–49, 50–69, >70 years), smoking status (never and former, and current) and alcohol consumption (never, social and regular drinkers).
ND – Non Determined.

Table 7.4 – ORs (95% CI) for *CASP9* (Phe36Phe) polymorphisms and PN-MPNs association.

Pathology Stratification	N	*CASP9* (Phe136Phe; rs1132312)	*P* value[a]	OR crude (95% CI)	OR Adjusted (95% CI)[b]
ET	80	C/C C/T T/T C//T + T/T	**0.050**	1 (Reference) **2.233 (1.157 – 4.309)⅄** 1.942 (0.912 – 4.133) **2.136 (1.138 – 4.010)◆**	1 (Reference) **2.300 (1.180 – 4.484)⅄⅄** 2.009 (0.932 – 4.330) **2.203 (1.163 – 4.176) ◆◆**
ET, females	48	C/C C/T T/T C//T + T/T	**0.009**	1 (Reference) **4.403 (1.599 – 12.126)†** **3.920 (1.256 – 12.231)×** **4.257 (1.586 – 11.425)***	1 (Reference) **4.663 (1.667 – 13.045)††** **3.777 (1.198 – 11.911)××** **4.370 (1.608 – 11.873)****
ET, *JAK2* positive	58	C/C C/T T/T C//T + T/T	**0.021**	1 (Reference) **3.059 (1.356 – 6.896)‡** 2.379 (0.942 **–** 6.009) **2.832 (1.288 – 6.229)+**	1 (Reference) **3.104 (1.366 – 7.052)‡‡** 2.447 (0.960 **–** 6.239) **2.886 (1.303 – 6.393)++**

⅄PCrude = 0.017; ⅄⅄PAdjusted = 0.014 (P-values are adjusted by unconditional multiplicative logistic regression).
◆PCrude = 0.018; ◆◆PAdjusted = 0.015 (P-values are adjusted by unconditional multiplicative logistic regression).
†PCrude = 0.004; ††PAdjusted = 0.003 (P-values are adjusted by unconditional multiplicative logistic regression).
×PCrude = 0.019; ××PAdjusted = 0.023 (P-values are adjusted by unconditional multiplicative logistic regression).
*PCrude = 0.004; **PAdjusted = 0.004 (P-values are adjusted by unconditional multiplicative logistic regression).
‡PCrude = 0.007; ‡‡PAdjusted = 0.007 (P-values are adjusted by unconditional multiplicative logistic regression)
+PCrude = 0.010; ++PAdjusted = 0.009 (P-values are adjusted by unconditional multiplicative logistic regression)

[a] P-value determined by $\chi 2$ test.
[b] ORs were adjusted for age (30–49, 50–69, >70 years), smoking status (never and former) and alcohol consumption (never, social and regular drinkers).

7.3.3 – HAPLOGROUP ASSOCIATION

A key point that should be explored in studies such as this, is the effect of the combination of all genotypes since the real situation is the effect of the variants altogether. That was achieved using the SNPStat software and the results yielded fifty-one different combinations (data not shown). It should be noted that of all genetic variants under study, only the SNPs of *CASP8* and *CASP7* genes were in linkage disequilibrium (LD). According to the results obtained (Table 7.5), we could only establish a positive haplotype for *CASP9* gene correlated with a decreased risk for PN-MPN diseases in individuals.

Concerning the number of SNPs of the different genes under study, and grouping the effector caspase genes as a whole, we stablished a new haplogroup (Table 7.6). The result obtained substantiate the decreased risk for PN-MPN in our population, as observed for *CASP9* haplogroup. To our knowledge, this is the first study where this kind of association is described.

Table 7.5 – Haplotype association response for SNPs of *CASP9* gene.
These genetic variants did not reveal the presence of LD.

Haplotype association response				
CASP9			**OR (95% CI)**	***P* Value**
rs2308950	rs1132312	rs1052571		
G	C	T	1.00 (Reference)	
G	T	C	1.15 (0.79 – 1.68)	0.46
G	**C**	**C**	**0.24 (0.11 – 0.52)**	**$3e^{-4}$**
G	**T**	**T**	**0.41 (0.25 – 0.70)**	**0.001**

Table 7.6 – Haplogroup association response for SNPs present in all effector caspases studied.

Haplogroup association response							
CASP8		***CASP9***			***CASP10***	***OR (95% CI)***	***P Value***
rs1045485	rs1035142	rs2308950	rs1132312	rs1052571	rs13006529		
G	G	G	C	T	A	1 (Reference)	
G	**G**	**G**	**T**	**T**	**A**	**0.18 (0.03 - 0.96)**	**0.046**
G	**T**	**G**	**C**	**C**	**T**	**0.08 (0.01 - 0.82)**	**0.034**

7.4 – DISCUSSION AND CONCLUSIONS

Polymorphisms in apoptosis related genes may contribute to individual susceptibility to MPNs and, hence, modify disease risk. However, to the best of our knowledge, no clinical association studies have been performed thus far to evaluate the role of caspases genes polymorphisms on PN-MPNs susceptibility.

The present study revealed an increased incidence of *JAK2* V617F mutation in ET patients and a decreased incidence in PV patients, compared with the literature (Nangalia et al., 2013; Tefferi & Pardanani, 2015), probably due to the small population studied. Moreover, the cases included were incident cases diagnosed in a hospital hematological consultation.

This study was intended to ascertain the possible role of genetic polymorphisms *CASP8* (Asp302His and 3'UTR_G/T), *CASP9* (Arg221Gln, Arg173His, Phe136Phe and Ala28Val), *CASP7* (Lys249Arg and Asp255Glu) and *CASP10* (Ile522Leu), on the individual susceptibility for PN-MPNs. Caspases are main components of apoptosis pathway. This specific group of cysteine aspartate proteases are a family of intracellular proteins involved in the initiation and execution of apoptotic processes, responsible for the dismantling and destruction of the cell components (Green & Llambi, 2015; Riedl & Salvesen, 2007). These proteins are produced as proenzymes (zymogens) and can be

activated by proteolytic cleavage in response to various apoptotic stimuli. Each caspase is cleaved to produce a large and a small subunits, forming an active tetrameric form from two molecules of pro-enzyme (Cho & Choi, 2002).

There are 14 different caspases can be classified as initiator, effector and cytokine activators (Kiraz et al., 2016). The initiator caspases (caspase-2, 8, 9, 10) activate the effector caspases (caspase 3, 6, 7 and 14), which are capable of degrading direct multiple substrates leading to deregulation of vital cellular processes and cellular death (Kiraz et al., 2016; Ng, Porter, & Jänicke, 1999; Oliver & Vallette, 2005; Philchenkov, Zavelevich, Kroczak, & Los, 2004; Riedl & Salvesen, 2007), and also the cytokine activator caspases (caspase 1, 4, 5, 11, 12 and 13).

While initiator caspases are self-activated, effector caspases activation is dependent of initiator caspases via internal cleavages. Furthermore, most of caspase family members are functional in cellular proliferation, survival, and inflammation, whereas some of them are essential for apoptosis (Kiraz et al., 2016).

All stimuli that lead to apoptosis appear to initiate a sequence of events that culminate in the activation of caspases, but they do it in different ways. Three pathways are associated with activation of caspases: (1) the intrinsic or mitochondrial pathway, which is initiated by cellular stress signals such as DNA damage (induced by genotoxic agents or defects in DNA repair), endoplasmic reticulum (ER) stress (induced by the accumulation of unfolded proteins), rupture of microtubules, this pathway converge on mitochondria, resulting in permeabilization of the outer membrane and subsequent cytochrome c release (Green & Kroemer, 2004; Petit, Oliver, & Vallette, 2009); (2) the extrinsic pathway, which is initiated by the activation of death membrane receptors of the tumor necrosis factor (TNF) family, induced by ligands; and (3) the pathway involving granzyme B (Creagh, Conroy, & Martin, 2003; Green & Llambi, 2015; Kiraz et al., 2016; Peter & Krammer, 2003; Tognon et al., 2013; Wang, 2001).

Various proteins promote pro- or anti- apoptotic activity in the cell. The ratio of the pro-and anti-apoptotic proteins plays an important role in the regulation of cell death, and disruption in the balance between these proteins has been established to contribute to carcinogenesis by reducing apoptosis in malignant cells (Ding et al., 2000; Goldar et al., 2015; Olsson & Zhivotovsky, 2011). In particular in MPNs, the deregulation of apoptosis is involved in the pathophysiology of these diseases (Malherbe et al., 2016; Testa, 2004; Tognon et al., 2013).

The present study involved four different genes and nine polymorphisms from the caspase cascade, and our results didn't confirm the correlation between different SNPs in PN-MPN diseases as a whole. However, the results reveal that for ET patients alone, or after stratification by female gender or when applied to *JAK2* positive cases, there is a significant increased risk when cases of these sub-groups carried at least one variant allele for *CASP9*_rs1132312 (C653T) polymorphism. Previous studies have described the involvement of *CASP9* gene polymorphisms in the pathogenesis of various types of cancer, such as non-Hodgkin's lymphoma (Kelly et al., 2010; Lan et al., 2009), lung cancer (Lin et al., 2012; Park et al., 2006), breast cancer (Theodoropoulos et al., 2012), and gastric cancer (Liamarkopoulos et al., 2011).

By definition, MPNs are a group of clonal disorders derived from the proliferation of one or more myeloid lineages, in which megakaryocytes are the 'key-cells' for diagnostic histological features (Florena et al., 2009; Malherbe et al., 2016). Previous studies have correlated the uncontrolled proliferation of megakaryocytes to dysregulation of pro-apoptotic and anti-apoptotic mechanisms (Florena et al., 2009; Malherbe et al., 2016).

Caspase 9 is important in regulating megakaryocyte turnover in MPNs. Malherbe and colleagues (Malherbe et al., 2016) showed that disruptions targeting the intrinsic apoptotic cascade (caspase-9 action) apparently promote megakaryocyte accumulation and thrombocytosis in MPNs. Considering that our results revealed an increased risk for ET in patients who present at least one variant allele, we might anticipate that polymorphisms in *CASP9* gene might be responsible for a high proliferation inducing and increased risk for ET.

With regard to the analyses of haplogroups' association response, our results didn't establish a global haplogroup. However, the correlation for SNPs of *CASP9* gene showed a decreased risk for two haplogroups (GCC and GTT), as shown in Table 7.5. Interestingly, a similar effect was obtained when effector caspases where grouped. However, the mechanism by which the studied haplogroups lead to a decreased risk for MPNs remains unknown. These results should suggest that polymorphisms may exert independent or interactive effects on the development of MPNs.

With regard to smoking habits, although some published studies refer smoking as a contributing factor for PN-MPNs (Hasselbalch, 2015; Lindholm Sørensen & Hasselbalch, 2015), this study did not reveal an important association, probably due to the small number of smoking individuals included.

Additional studies involving larger populations should be pursued to further clarify the potential value of the different apoptosis related caspases genotypes as predictive biomarkers of susceptibility to PN-MPNs and also allow for the study of gene-environment and gene-gene interactions as well as stratified analysis according to histological subtype and disease stage.

A better understanding of the pathophysiological mechanisms will allow the development of more directly and specifically targeted drugs, with high efficacy, fewer adverse effects, contributing to compliance of the patients with treatments.

7.5 – REFERENCES

Arber, D. A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M. J., Le Beau, M. M., . . . Vardiman, J. W. (2016). The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood, 127*(20), 2391-2405. doi:10.1182/blood-2016-03-643544

Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., . . . Project, C. G. (2005). Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet, 365*(9464), 1054-1061. doi:10.1016/S0140-6736(05)71142-9

Beer, P. A., Delhommeau, F., LeCouédic, J. P., Dawson, M. A., Chen, E., Bareford, D., . . . Green, A. R. (2010). Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. *Blood, 115*(14), 2891-2900. doi:10.1182/blood-2009-08-236596

Björkholm, M., Hultcrantz, M., & Derolf, Å. (2014). Leukemic transformation in myeloproliferative neoplasms: therapy-related or unrelated? *Best Pract Res Clin Haematol, 27*(2), 141-153. doi:10.1016/j.beha.2014.07.003

Bolufer, P., Barragan, E., Collado, M., Cervera, J., López, J. A., & Sanz, M. A. (2006). Influence of genetic polymorphisms on the risk of developing leukemia and on disease progression. *Leuk Res, 30*(12), 1471-1491. doi:10.1016/j.leukres.2006.01.016

Campregher, P. V., Santos, F. P., Perini, G. F., & Hamerschlak, N. (2012). Molecular biology of Philadelphia-negative myeloproliferative neoplasms. *Rev Bras Hematol Hemoter, 34*(2), 150-155. doi:10.5581/1516-8484.20120035

Cho, S. G., & Choi, E. J. (2002). Apoptotic signaling pathways: caspases and stress-activated protein kinases. *J Biochem Mol Biol, 35*(1), 24-27.

Creagh, E. M., Conroy, H., & Martin, S. J. (2003). Caspase-activation pathways in apoptosis and immunity. *Immunol Rev, 193*, 10-21.

Delhommeau, F., Jeziorowska, D., Marzac, C., & Casadevall, N. (2010). Molecular aspects of myeloproliferative neoplasms. *Int J Hematol, 91*(2), 165-173. doi:10.1007/s12185-010-0530-z

Ding, H. F., Lin, Y. L., McGill, G., Juo, P., Zhu, H., Blenis, J., . . . Fisher, D. E. (2000). Essential role for caspase-8 in transcription-independent apoptosis triggered by p53. *J Biol Chem, 275*(49), 38905-38911. doi:10.1074/jbc.M004714200

Florena, A. M., Tripodo, C., Di Bernardo, A., Iannitto, E., Guarnotta, C., Porcasi, R., . . . Franco, V. (2009). Different immunophenotypical apoptotic profiles characterise megakaryocytes of essential thrombocythaemia and primary myelofibrosis. *J Clin Pathol, 62*(4), 331-338. doi:10.1136/jcp.2007.054353

Goldar, S., Khaniani, M. S., Derakhshan, S. M., & Baradaran, B. (2015). Molecular mechanisms of apoptosis and roles in cancer development and treatment. *Asian Pac J Cancer Prev, 16*(6), 2129-2144.

Green, D. R., & Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. *Science, 305*(5684), 626-629. doi:10.1126/science.1099320

Green, D. R., & Llambi, F. (2015). Cell Death Signaling. *Cold Spring Harb Perspect Biol, 7*(12). doi:10.1101/cshperspect.a006080

Hasselbalch, H. C. (2015). Smoking as a contributing factor for development of polycythemia vera and related neoplasms. *Leuk Res*. doi:10.1016/j.leukres.2015.09.002

James, C., Ugo, V., Le Couédic, J. P., Staerk, J., Delhommeau, F., Lacout, C., . . . Vainchenker, W. (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature, 434*(7037), 1144-1148. doi:10.1038/nature03546

Kelly, J. L., Novak, A. J., Fredericksen, Z. S., Liebow, M., Ansell, S. M., Dogan, A., . . . Cerhan, J. R. (2010). Germline variation in apoptosis pathway genes and risk of non-Hodgkin's lymphoma. *Cancer Epidemiol Biomarkers Prev, 19*(11), 2847-2858. doi:10.1158/1055-9965.EPI-10-0581

Kilpivaara, O., & Levine, R. L. (2008). JAK2 and MPL mutations in myeloproliferative neoplasms: discovery and science. *Leukemia, 22*(10), 1813-1817. doi:10.1038/leu.2008.229

Kiraz, Y., Adan, A., Kartal Yandim, M., & Baran, Y. (2016). Major apoptotic mechanisms and genes involved in apoptosis. *Tumour Biol, 37*(7), 8471-8486. doi:10.1007/s13277-016-5035-9

Kralovics, R., Passamonti, F., Buser, A. S., Teo, S. S., Tiedt, R., Passweg, J. R., . . . Skoda, R. C. (2005). A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med, 352*(17), 1779-1790. doi:10.1056/NEJMoa051113

Lan, Q., Morton, L. M., Armstrong, B., Hartge, P., Menashe, I., Zheng, T., . . . Wang, S. S. (2009). Genetic variation in caspase genes and risk of non-Hodgkin lymphoma: a pooled analysis of 3 population-based case-control studies. *Blood, 114*(2), 264-267. doi:10.1182/blood-2009-01-198697

Levine, R. L. (2009). Mechanisms of mutations in myeloproliferative neoplasms. *Best Pract Res Clin Haematol, 22*(4), 489-494. doi:10.1016/j.beha.2009.08.006

Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J., . . . Gilliland, D. G. (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell, 7*(4), 387-397. doi:10.1016/j.ccr.2005.03.023

Liamarkopoulos, E., Gazouli, M., Aravantinos, G., Tzanakis, N., Theodoropoulos, G., Rizos, S., & Nikiteas, N. (2011). Caspase 8 and caspase 9 gene polymorphisms and susceptibility to gastric cancer. *Gastric Cancer, 14*(4), 317-321. doi:10.1007/s10120-011-0045-1

Lin, J., Lu, C., Stewart, D. J., Gu, J., Huang, M., Chang, D. W., . . . Wu, X. (2012). Systematic evaluation of apoptotic pathway gene polymorphisms and lung cancer risk. *Carcinogenesis, 33*(9), 1699-1706. doi:10.1093/carcin/bgs192

Lindholm Sørensen, A., & Hasselbalch, H. C. (2015). Smoking and philadelphia-negative chronic myeloproliferative neoplasms. *Eur J Haematol*. doi:10.1111/ejh.12684

Malherbe, J. A., Fuller, K. A., Mirzai, B., Kavanagh, S., So, C. C., Ip, H. W., . . . Erber, W. N. (2016). Dysregulation of the intrinsic apoptotic pathway mediates megakaryocytic hyperplasia in myeloproliferative neoplasms. *J Clin Pathol*. doi:10.1136/jclinpath-2016-203625

Mambet, C., Matei, L., Necula, L. G., & Diaconu, C. C. (2016). A link between the driver mutations and dysregulated apoptosis in BCR-ABL1 negative myeloproliferative neoplasms. *J Immunoassay Immunochem, 37*(4), 331-345. doi:10.1080/15321819.2016.1152276

Nangalia, J., Massie, C. E., Baxter, E. J., Nice, F. L., Gundem, G., Wedge, D. C., . . . Green, A. R. (2013). Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med, 369*(25), 2391-2405. doi:10.1056/NEJMoa1312542

Ng, P. W., Porter, A. G., & Jänicke, R. U. (1999). Molecular cloning and characterization of two novel pro-apoptotic isoforms of caspase-10. *J Biol Chem, 274*(15), 10301-10308.

Nunes, N. S., Tognon, R., Moura, L. G., Kashima, S., Covas, D. T., Santana, M., . . . Castro, F. A. (2013). Differential expression of apoptomiRs in myeloproliferative neoplasms. *Leuk Lymphoma, 54*(9), 2047-2051. doi:10.3109/10428194.2013.767453

Oliver, L., & Vallette, F. M. (2005). The role of caspases in cell death and differentiation. *Drug Resist Updat, 8*(3), 163-170. doi:10.1016/j.drup.2005.05.001

Olsson, M., & Zhivotovsky, B. (2011). Caspases and cancer. *Cell Death Differ, 18*(9), 1441-1449. doi:10.1038/cdd.2011.30

Park, J. Y., Park, J. M., Jang, J. S., Choi, J. E., Kim, K. M., Cha, S. I., . . . Jung, T. H. (2006). Caspase 9 promoter polymorphisms and risk of primary lung cancer. *Hum Mol Genet, 15*(12), 1963-1971. doi:10.1093/hmg/ddl119

Peter, M. E., & Krammer, P. H. (2003). The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ, 10*(1), 26-35. doi:10.1038/sj.cdd.4401186

Petit, E., Oliver, L., & Vallette, F. M. (2009). The mitochondrial outer membrane protein import machinery: a new player in apoptosis? *Front Biosci (Landmark Ed), 14*, 3563-3570.

Philchenkov, A., Zavelevich, M., Kroczak, T. J., & Los, M. (2004). Caspases and cancer: mechanisms of inactivation and new treatment modalities. *Exp Oncol, 26*(2), 82-97.

Rice, K. L., Lin, X., Wolniak, K., Ebert, B. L., Berkofsky-Fessler, W., Buzzai, M., . . . Zhang, W. (2011). Analysis of genomic aberrations and gene expression profiling identifies novel lesions and pathways in myeloproliferative neoplasms. *Blood Cancer J, 1*(11), e40. doi:10.1038/bcj.2011.39

Riedl, S. J., & Salvesen, G. S. (2007). The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol, 8*(5), 405-413. doi:10.1038/nrm2153

Rueff, J., & Rodrigues, A. S. (2016). Cancer Drug Resistance: A Brief Overview from a Genetic Viewpoint. *Methods Mol Biol, 1395*, 1-18. doi:10.1007/978-1-4939-3347-1_1

Scott, L. M., Tong, W., Levine, R. L., Scott, M. A., Beer, P. A., Stratton, M. R., . . . Green, A. R. (2007). JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. *N Engl J Med, 356*(5), 459-468. doi:10.1056/NEJMoa065202

Swerdlow, & SH, C. E., Harris NL, et al. (2008). *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.* (IARC Ed. 4 edition (October 27, 2008) ed.). Lyon: World Health Organization.

Tefferi, A., & Pardanani, A. (2015). Myeloproliferative Neoplasms: A Contemporary Review. *JAMA Oncol, 1*(1), 97-105. doi:10.1001/jamaoncol.2015.89

Tefferi, A., & Vardiman, J. W. (2008). Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia, 22*(1), 14-22. doi:10.1038/sj.leu.2404955

Testa, U. (2004). Apoptotic mechanisms in the control of erythropoiesis. *Leukemia, 18*(7), 1176-1199. doi:10.1038/sj.leu.2403383

Theodoropoulos, G. E., Michalopoulos, N. V., Pantou, M. P., Kontogianni, P., Gazouli, M., Karantanos, T., . . . Zografos, G. C. (2012). Caspase 9 promoter polymorphisms confer increased susceptibility to breast cancer. *Cancer Genet, 205*(10), 508-512. doi:10.1016/j.cancergen.2012.08.001

Tognon, R., Gasparotto, E. P., Leroy, J. M., Oliveira, G. L., Neves, R. P., Carrara, R. e. C., . . . de Castro, F. A. (2011). Differential expression of apoptosis-related genes from death receptor pathway in chronic myeloproliferative diseases. *J Clin Pathol, 64*(1), 75-82. doi:10.1136/jcp.2010.080895

Tognon, R., Gasparotto, E. P., Neves, R. P., Nunes, N. S., Ferreira, A. F., Palma, P. V., . . . Castro, F. A. (2012). Deregulation of apoptosis-related genes is associated with PRV1 overexpression and JAK2 V617F allele

burden in Essential Thrombocythemia and Myelofibrosis. *J Hematol Oncol, 5*, 2. doi:10.1186/1756-8722-5-2

Tognon, R., Nunes, N. e. S., & Castro, F. A. (2013). Apoptosis deregulation in myeloproliferative neoplasms. *Einstein (Sao Paulo), 11*(4), 540-544.

Tognon, R., Nunes, N. S., Ambrosio, L., Souto, E. X., Perobelli, L., Simões, B. P., . . . Attié de Castro, F. (2016). Apoptosis- and cell cycle-related genes methylation profile in myeloproliferative neoplasms. *Leuk Lymphoma, 57*(5), 1201-1204. doi:10.3109/10428194.2015.1071491

Valentino, L., & Pierre, J. (2006). JAK/STAT signal transduction: regulators and implication in hematological malignancies. *Biochem Pharmacol, 71*(6), 713-721. doi:10.1016/j.bcp.2005.12.017

Wang, X. (2001). The expanding role of mitochondria in apoptosis. *Genes Dev, 15*(22), 2922-2933.

Zaman, S., Wang, R., & Gandhi, V. (2014). Targeting the apoptosis pathway in hematologic malignancies. *Leuk Lymphoma, 55*(9), 1980-1992. doi:10.3109/10428194.2013.855307

CHAPTER 8:
DNA REPAIR GENES POLYMORPHISMS AND GENETIC SUSCEPTIBILITY TO PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS IN A PORTUGUESE POPULATION: THE ROLE OF BASE EXCISION REPAIR GENES POLYMORPHISMS

Published in Azevedo *et al.*, 2017, Oncology Letters 13:4641-4650
DOI: 10.3892/ol.2017.6065

ABSTRACT

The role of base excision repair (BER) genes in Philadelphia-negative (PN)-myeloproliferative neoplasms (MPNs) susceptibility was evaluated by genotyping eight polymorphisms [apurinic/apyrimidinic endodeoxyribonuclease 1, mutY DNA glycosylase, earlier mutY homolog (E. coli) (MUTYH), 8-oxoguanine DNA glycosylase 1, poly (ADP-ribose) polymerase (PARP)1, PARP4 and X-ray repair cross-complementing 1 (XRCC1)] in a case-control study involving 133 Caucasian Portuguese patients. The results did not reveal a correlation

between individual BER polymorphisms and PN-MPNs when considered as a whole. However, stratification for essential thrombocythaemia revealed i) borderline effect/tendency to increased risk when carrying at least one variant allele for XRCC1_399 single-nucleotide polymorphism (SNP); ii) decreased risk for Janus kinase 2-positive patients carrying at least one variant allele for XRCC1_399 SNP; and iii) decreased risk in females carrying at least one variant allele for MUTYH SNP. Combination of alleles demonstrated an increased risk to PN-MPNs for one specific haplogroup. These findings may provide evidence for gene variants in susceptibility to MPNs. Indeed, common variants in DNA repair genes may hamper the capacity to repair DNA, thus increasing cancer susceptibility.

CHAPTER 8: DNA REPAIR GENES POLYMORPHISMS AND GENETIC SUSCEPTIBILITY TO PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS IN A PORTUGUESE POPULATION: THE ROLE OF BASE EXCISION REPAIR GENES POLYMORPHISMS

8.1 – INTRODUCTION

Among myeloproliferative neoplasms (MPNs), and besides chronic myelogenous leukemia, the World Health Organization (WHO) classification includes Philadelphia-negative (PN)-MPNs, namely, polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (PMF) (Arber et al., 2016; Swerdlow et al., 2008).

Major genetic insights into the pathogenesis of PN-MPNs include the identification of somatic point gain-of-function mutations in the Janus kinase 2 (*JAK2*) gene (V617F mutation in exon 14 first in 2005, then in exon 12) (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Scott et al., 2007) and the myeloproliferative leukemia virus oncogene (most frequently W515L/K), in addition to recently identified calreticulin mutations (Chi et al., 2015; Ha & Kim, 2015; Klampfl et al., 2013; Rumi et al., 2014), with implications in the understanding of these diseases, their diagnosis and management. The corresponding frequencies of these mutations are ~95, 0 and 0% in PV; 60, 3 and 20% in ET; and 60, 7 and 25% in PMF, respectively (Nangalia et al., 2013; Tefferi & Pardanani, 2015).

Those mutations, however, could not fully explain the phenotypic heterogeneity of PN-MPNs. Furthermore, genetic defects still await identification in ~40% of ET and PMF cases (Delhommeau, Jeziorowska, Marzac, & Casadevall, 2010; Nangalia et al., 2013; Tefferi & Pardanani, 2015). Single-nucleotide polymorphisms (SNPs) at various loci and additional somatic genetic effects may be important for PN-MPNs phenotype definition and for prognosis evaluation, although less specific than known variations (Campregher, Santos, Perini, & Hamerschlak, 2012; Delhommeau et al., 2010; Levine, 2009; Nangalia et al., 2013; Tefferi & Pardanani, 2015). Not all mutations involved in cancer initiation may lead to cancer. This can occur due to different variants within the same gene or between variants in different genes. The latter must be considered, as the number of passenger mutations in a tumour may modulate the effect of driver mutations, thus acting as putative modifier genes (Rueff & Rodrigues, 2016). Additionally, epimutations that can silence tumour-suppressor genes must be taken into account (Rueff & Rodrigues, 2016), which highlights the concept that, probably more important than the genes, are their levels of expression.

MPNs have high morbidity with thrombohaemorrhagic complications and risk of progression to acute myeloid leukemia (AML), in occasions preceded by a phase of myelofibrosis or myelodysplastic syndromes (MDS) (Mesa et al., 2016). Life expectancy in patients with PV or ET is reduced compared with that in the general population (Passamonti et al., 2004; Wolanskyj, Schwager, McClure, Larson, & Tefferi, 2006).The "true" rate of transformation is not accurate due to selection bias in clinical trials and underreporting in population-based studies (Björkholm, Hultcrantz, & Derolf, 2014). The occurrence of leukemia in ET and PV is associated with a bad prognosis, and has been reported to occur in 5-10% of patients 10 years following the initial diagnosis (Rampal et al., 2014; Swerdlow et al., 2008). It is well known that high doses of alkylating agents and combined cytoreductive treatments undoubtedly increase the risk of malignant transformation (Björkholm et al., 2014). However, there is also an intrinsic propensity in MPNs to progress to AML/MDS, in an extent that is not fully known (Björkholm et al., 2014; Hernández-Boluda et al., 2012). It cannot be ruled out that mutational burden, polymorphic variants of several genes, ambient/dietary exposure and immune system characteristics could be predisposing factors to susceptibility to these disorders (Beer et al., 2010; Bolufer et al., 2006; Delhommeau et al., 2010; Hasselbalch et al., 2014; Kilpivaara & Levine, 2008).

DNA damage to haematopoietic precursor cells would appear to be essential for the development of leukaemia, notwithstanding DNA repair systems act to repair the DNA damage, thus maintaining genetic integrity (Batar, Güven, Bariş, Celkan, & Yildiz, 2009; Bănescu et al., 2014).

Several polymorphisms in DNA repair genes have been identified that may affect protein function and thus DNA damage repair, leading to susceptibility to malignancy, in spite of their low genetic penetrance (Batar et al., 2009; Bolufer et al., 2006; Bănescu et al., 2014; Hoeijmakers, 2001; C. Q. Wang et al., 2014). Previous reports have identified base excision repair (BER) genes polymorphisms associated with breast and thyroid cancer risk (Santos et al., 2012; Silva et al., 2007; Yan, Li, Li, Ji, & Zhang, 2016) among other malignancies, and a nucleotide excision repair gene polymorphism displayed strong association with leukaemic transformation and development of non-myeloid malignancies in patients with ET and PV (Hernández-Boluda et al., 2012).

The BER pathway typically repairs a small region (1-13 nucleotides) around the damaged base, involving apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1), 8-oxoguanine DNA glycosylase 1 (OGG1), poly (ADP-ribose) polymerase (PARP)1 or X-ray repair cross-complementing 1 (XRCC1) (Baute & Depicker, 2008).

Several SNPs in genes of the BER pathway [*APEX1*, mutY DNA glycosylase, earlier mutY homolog (*E. coli*) (*MUTYH*), *OGG1*, *PARP1*, *PARP4* and *XRCC1*] have been identified and studied for their association with the risk of leukaemia and disease outcome (Annamaneni et al., 2013; Bolufer et al., 2006; Bănescu et al., 2014).

A wider characterization of molecular genetic features in PN-MPNs may contribute to a better understanding of the pathogenesis of these diseases and provide new specific diagnostic, prognostic and therapeutic tools (Delhommeau et al., 2010).

Since data on the role of BER gene variants in PN-MPNs are absent in the literature, the present study describes a hospital-based, case-control study in a Caucasian Portuguese population in order to help assessing a possible role of BER genes on the individual susceptibility to PN-MPNs.

8.2 – MATERIALS AND METHODS

8.2.1 – STUDY SUBJECTS

The description given in Chapter 3, section 3.2.1 applies in this new chapter.

General characteristics for PN-MPNs patients and control populations are summarized in Tables 8.2 and 8.3.

8.2.2 – DNA EXTRACTION

The methodology used is described in Chapter 3, section 3.2.2.

8.2.3 – SNP SELECTION (BASE EXCISION REPAIR PATHWAY)

The appropriate SNPs analyzed in the present study were selected concerning their relevance in the DNA repair pathway (Santos et al., 2012; Silva et al., 2007). All SNPs had a minor allele frequency of >0.1 in Caucasian populations (Table 8.1).

Table 8.1 – Selected SNPs and detailed information on the corresponding base and amino acid exchanges, as well as MAF.

Gene	Codon	Exchange, base (amino acid)	MAF (%)[a]
APEX1 (Chr 14)	148	T→G (Asp/Glu)	44.0
MUTYH (Chr 1)	335	G→C (Gln/His)	31.9
OGG1 (Chr 3)	326	C→G (Ser/Cys)	29.9

PARP1 (Chr 1)	762	T→C (Val/Ala)	24.4
PARP4 (Chr 13)	1,280	G→C (Gly/Arg)	45.8
	1,328	C→A (Pro/Thr)	45.8
XRCC1 (Chr 19)	194	C→T (Arg/Trp)	13.1
	399	G→A (Arg/Gln)	26.6

[a]According to http://www.ncbi.nlm.nih.gov/snp/. MAF, minor allele frequency; APEX1, apurinic/apyrimidinic endonuclease 1; MUTYH, mutY DNA glycosylase, earlier mutY homolog (*E. coli*); OGG1, 8-oxoguanine DNA glycosylase 1; PARP, poly (ADP-ribose) polymerase; XRCC1, X-ray repair cross-complementing 1.

8.2.4 – GENOTYPING

The polymorphisms rs1130409 (*APEX1*), rs3219489 (*MUTYH*), rs1052133 (*OGG1*), rs1136410 (*PARP1*), rs13428 and rs1050112 (*PARP4*), and rs1799782 and rs25487 (*XRCC1*) and *JAK2* V617F mutation were genotyped using the same methodology described in Chapter 3, section 3.2.3.

Table 8.1 summarizes the information on the SNP genotyping assay.

8.2.5 – STATISTICAL ANALYSIS

The statistical approach is described in Chapter 7, section 7.2.5.

8.3 – RESULTS

8.3.1 – CHARACTERIZATION OF POPULATIONS

The present study included 133 PN-MPNs patients and 281 age- and gender-matched controls. According to the diagnosis criteria, the patients' distribution was as follows: 80 patients (60.2%) with ET, 39 (29.3%) with PV and 14 (10.5%) with PMF (Table 8.2). The baseline characteristics (gender, age, alcohol consumption and tobacco smoking habits) of the case and control populations are listed in Table 8.2 and Table 8.3.

The case group included 72 (54.1%) females and 61 (45.9%) males, with an overall mean age of 68 years, in agreement with the gender distribution usually observed in this type of pathology (Geyer

et al., 2017; Swerdlow et al., 2008). No significant differences were observed between the control and patient groups concerning age distribution or tobacco smoking habits (Table 8.3). However, alcohol consumption was significantly increased in patients compared with that in controls (P<0.0001) (Table 8.3).

Table 8.2 – Gender distribution for the PN-MPNs cases.
PN-MPNs cases n=133.

Diagnosis	Patients, n	Male, n (%)	Female, n (%)
ET	80	32 (40.0)	48 (60.0)
PV	39	20 (51.3)	19 (48.7)
PMF	14	9 (64.3)	5 (35.7)

ET, essential thrombocythaemia; PV, polycythaemia vera; PMF, primary myelofibrosis.

Table 8.3 – General characteristics of the PN-MPNs case and control populations.
PN-MPNs cases n=133; Control population n=281.

Characteristics	Cases, n (%)	Controls, n (%)	P-value[c]
Gender			0.780
Male	61 (45.9)	133 (47.3)	
Female	72 (54.1)	148 (52.7)	
Age, years[a,b]			0.622
30-49	16 (12.0)	43 (15.3)	
50-69	50 (37.6)	107 (38.1)	
≥70	67 (50.4)	131 (46.6)	
Tobacco smoking habits			0.633
Never	104 (78.2)	213 (76.1)	
Current	29 (21.8)	67 (23.9)	
Alcohol consumption habits			<0.0001
Never	103 (77.4)	191 (68.2)	
Social	20 (15.0)	25 (8.9)	
Regular	10 (7.5)	64 (22.9)	
JAK2 V617F mutation			
Yes	99 (75.0)		0.020
ET	58 (73.4)		
PV	34 (87.2)		
PMF	7 (50.0)		
No	33 (25.0)		

[a]Age at diagnosis for cases. [b]Age of control population at the time of diagnosis for the matched case. [c]P-value determined by χ^2 test. ET, essential thrombocythaemia; PV, polycythaemia vera; PMF, primary myelofibrosis.

8.3.2 – SNP'S GENOTYPING

Of the eight SNPs included in the study, only seven were completely genotyped (Table 8.1), since both SNPs of the *PARP4* gene were in linkage disequilibrium (r^2>0.80), thus behaving as tag SNPs.

The genotype frequencies determined for all polymorphisms under study are shown in Table 8.4. When considered individually, no correlation between polymorphisms of the BER pathway genes and individual susceptibility to PN-MPNs as a whole could be identified. All the SNPs studied were in agreement with the Hardy-Weinberg law (P>0.05, exact probability test), with the exception of *PARP1* Val762Ala (P=0.029, exact probability test).

The genotypic frequencies obtained in the control populations are similar to those reported previously in other Caucasian populations (Conde et al., 2009; Figueroa et al., 2007; Santos et al., 2012; Silva et al., 2007).

As shown in Table 8.4, no significant differences in genotypic frequencies were observed for any of the seven polymorphisms between cases and controls as a whole (P>0.05, χ^2 test). However, when the population was stratified for pathology status, gender and presence of *JAK2* mutation, a tendency for decrease risk was noticed for *MUTYH* Gln335His and *XRCC1* Gln399Arg polymorphisms (Table 8.5).

Upon stratification for pathology status, the results revealed that, for ET, the presence of at least one variant allele for the *XRCC1* Gln399Arg gene polymorphism displayed a borderline effect on the population (crude OR, 0.623; 95% CI, 0.378-1.028; P=0.069) (Table 8.5).

The results demonstrated a decreased risk in the female group with ET diagnosis and with at least one variant allele for *MUTYH* Gln335His gene polymorphism (OR, 0.478; 95% CI, 0.238-0.962) upon adjustment for potential risk factors (Table 8.5).

The relevance of *JAK2* mutation in PN-MPNs is well known (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Scott et al., 2007). Therefore, the population was also stratified according to the presence of *JAK2* mutation in patients, showing that the presence of at least one variant allele for *XRCC1* Gln399Arg gene polymorphism constitutes a decreased risk for ET patients (OR, 0.500; 95% CI, 0.278-0.896) (Table 8.5).

Overall, the results indicate that only XRCC1 Gln399Arg and MUTYH Gln335His gene polymorphisms appeared to be associated with PN-MPNs risk. For the remaining polymorphisms under study, no significant changes in crude or adjusted OR were observed (Tables 8.4 and 8.5).

Table 8.4 – Genotype distribution and myeloproliferative risk.
For the APEX1 Asp148Glu, MUTYH Gln335His, OGG1 Ser326Cys, PARP1 Val762Ala, PARP4 Gly1280Arg, and XRCC1 Gln399Arg and Arg194Trp polymorphisms in the myeloproliferative neoplasms case (n=133) and control (n=281) populations.

Genetic polymorphism	Controls, n (%)	Cases, n (%)	P-value[a]	OR crude (95% CI)	OR adjusted (95% CI)[b]
APEX1 (Asp148Glu)					
Asp/Asp	73 (26.4)	37 (27.8)		1.000 (Reference)	1.000 (Reference)
Asp/Glu	136 (49.1)	64 (48.1)	0.952	0.928 (0.566-1.523)	0.963 (0.580-1.599)
Glu/Glu	68 (24.5)	32 (24.1)		0.928 (0.521-1.653)	0.923 (0.512-1.663)
Asp/Glu + Glu/Glu	204 (73.6)	96 (72.2)		0.928 (0.584-1.477)	0.949 (0.591-1.526)
MUTYH (Gln335His)					
His/His	142 (51.3)	68 (51.1)		1.000 (Reference)	1.000 (Reference)
His/Gln	112 (40.4)	52 (39.1)	0.877	0.970 (0.626-1.502)	0.902 (0.550-1.413)
Gln/Gln	23 (8.3)	13 (9.8)		1.180 (0.654-2.471)	1.075 (0.506-2.283)
His/Gln + Gln/Gln	135 (48.7)	65 (48.9)		1.005 (0.665-1.521)	0.932 (0.609-1.425)
OGG1 (Ser326Cys)					
Ser/Ser	182 (65.7)	83 (62.6)		1.000 (Reference)	1.000 (Reference)
Ser/Cys	83 (30.0)	41 (30.8)	0.545	1.083 (0.687-1.708)	1.075 (0.672-1.720)
Cys/Cys	12 (4.3)	9 (6.8)		1.645 (0.667-4.055)	1.603 (0.631-4.072)
Ser/Cys + Cys/Cys	95 (34.3)	50 (37.6)		1.154 (0.751-1.774)	1.144 (0.735-1.781)
PARP1 (Val762Ala)					
Val/Val	214 (77.0)	104 (78.2)		1.000 (Reference)	1.000 (Reference)
Val/Ala	63 (22.7)	29 (21.8)	0.769	0.947 (0.575-1.559)	1.019 (0.607-1.712)
Ala/Ala	1 (0.4)	0 (0.0)		ND	ND
Val/Ala + Ala/Ala	64 (23.0)	29 (21.8)		0.932 (0.567-1.533)	1.000 (0.596-1.677)
PARP4 (Gly1280Arg)					
Gly/Gly	105 (37.9)	54 (40.6)		1.000 (Reference)	1.000 (Reference)
Gly/Arg	141 (50.9)	61 (45.9)	0.593	0.841 (0.539-1.313)	0.806 (0.510-1.273)
Arg/Arg	31 (11.2)	18 (13.5)		1.129 (0.579-2.200)	1.000 (0.501-1.997)
Gly/Arg + Arg/Arg	172 (62.9)	79 (59.4)		0.893 (0.585-1.363)	0.841 (0.543-1.301)
XRCC1 (Arg194Trp)					
Arg/Arg	236 (85.5)	121 (91.0)		1.000 (Reference)	1.000 (Reference)
Arg/Trp	39 (14.1)	12 (9.0)	0.263	0.600 (0.303-1.188)	0.650 (0.323-1.307)
Trp/Trp	1 (0.4)	0 (0.0)		ND	ND
Arg/Trp + Trp/Trp	40 (14.5)	12 (9.0)		0.585 (0.296-1.156)	0.633 (0.315-1.270)
XRCC1 (Gln399Arg)					
Arg/Arg	113 (40.8)	61 (45.9)		1.000 (Reference)	1.000 (Reference)
Arg/Gln	134 (48.4)	54 (40.6)	0.318	0.747 (0.479-1.163)	0.762 (0.483-1.204)
Gln/Gln	30 (10.8)	18 (13.5)		1.111 (0.573-2.155)	1.044 (0.531-2.052)
Arg/Gln + Gln/Gln	164 (59.2)	72 (54.1)		0.813 (0.536-1.234)	0.818 (0.532-1.255)

[a]P-value determined by χ^2 test. [b]ORs were adjusted for age (30-49, 50-69 and >70 years), tobacco smoking status (never or former and current) and alcohol consumption (never, social and regular drinkers). APEX1, apurinic/apyrimidinic endonuclease 1; MUTYH, mutY DNA glycosylase, earlier mutY homolog (*E. coli*); OGG1, 8-oxoguanine DNA glycosylase 1; PARP, poly (ADP-ribose) polymerase; XRCC1, X-ray repair cross-complementing 1; OR, odds ratio; CI, confidence interval; ND, none determined.

Table 8.5 – ORs (95% CI) for *MUTYH* (gln335his) and *XRCC1* (gln399arg) polymorphisms and PN-MPNs association.

Pathology stratification	Patients, n	SNP	P-value[a]	OR crude (95% CI)	OR adjusted (95% CI)[b]
		*XRCC1*_399 (Gln399Arg; rs25487)			
		Arg/Arg		1.000 (Reference)	1.000 (Reference)
ET	80	Arg/Gln	0.166	0.602 (0.354-1.025)[*]	0.611 (0.355-1.053)[*]
		Gln/Gln		0.717 (0.305-1.690)	0.667 (0.279-1.595)
		Arg/Gln + Gln/Gln		0.623 (0.378-1.028)[*]	0.622 (0.373-1.038)[*]
		MUTYH (Gln335His; rs3219489)			
		His/His		1.000 (Reference)	1.000 (Reference)
ET, females	48	His/Gln	0.015	0.342 (0.152-0.773)[c]	0.325 (0.142-0.744)[d]
		Gln/Gln		1.331 (0.480-3.692)	1.229 (0.435-3.475)
		His/Gln + Gln/Gln		0.507 (0.256-1.003)[*]	0.478 (0.238-0.962)[**]
		*XRCC1*_399 (Gln399Arg; rs25487)			
		Arg/Arg		1.000 (Reference)	1.000 (Reference)
ET, JAK2	58	Arg/Gln	0.044	0.471 (0.255-0.871)[e]	0.490 (0.263-0.915)[f]
		Gln/Gln		0.554 (0.199-1.538)	0.539 (0.192-1.514)
		Arg/Gln + Gln/Gln		0.486 (0.274-0.864)[g]	0.500 (0.278-0.896)[h]

[a]P-value determined by χ^2 test. [b]ORs were adjusted for age (30-49, 50-69 and >70 years), tobacco smoking status (never or former and current) and alcohol consumption (never, social and regular drinkers). [c]P_{crude} = 0.010; [d]$P_{adjusted}$ = 0.008 (P-values are adjusted by unconditional multiplicative logistic regression). [e]P_{crude} = 0.016; [f]$P_{adjusted}$ = 0.025 (P-values are adjusted by unconditional multiplicative logistic regression). [g]P_{crude} = 0.014; [h]$P_{adjusted}$ = 0.020 (P-values are adjusted by unconditional multiplicative logistic regression). [*]Represents results that almost reached the significance threshold, demonstrating a borderline effect. [**]$P_{adjusted}$ = 0.039 (P-values are adjusted by unconditional multiplicative logistic regression). SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval; MUTYH, mutY DNA glycosylase, earlier mutY homolog (*E. coli*); XRCC1, X-ray repair cross-complementing 1; ET, essential thrombocythaemia; JAK2, Janus kinase 2.

8.3.3 – HAPLOGROUP ASSOCIATION

A key point that should be explored in studies such as the present one is the effect of the combination of all genotypes, since the real situation is the effect of the variants altogether. The results provided by the SNPstat software yielded 60 different combinations, the most frequent of which are shown in Table 8.6. Analyzing the results as haplogroup association response, an increased risk to develop a MPN was obtained for one specific combination (OR, 3.91; 95% CI, 1.02-14.95) (Table 8.7).

Table 8.6 – Haplogroup frequencies for the SNPs under study.

Haplogroup estimation							**Global frequency**	**Haplogroup frequencies**	
APEX D148E	*MUTYH* Q335H	*OGG1* S326C	*PARP1* V762A	*PARP4* G1,280R	*XRCC1*_194 R194W	*XRCC1*_399 R399Q		Controls	Cases
D	H	S	V	G	R	R	0.116	0.167	0.068
E	H	S	V	G	R	R	0.084	0.060	0.130
E	H	S	V	G	R	Q	0.069	0.065	0.062
E	H	S	V	R	R	R	0.050	0.058	0.044
D	H	S	V	R	R	Q	0.045	0.036	0.060
D	H	S	V	R	R	R	0.045	0.030	0.060
E	H	C	V	G	R	R	0.033	0.025	0.042
E	Q	S	V	R	R	R	0.032	0.028	0.053

APEX1, apurinic/apyrimidinic endonuclease 1; MUTYH, mutY DNA glycosylase, earlier mutY homolog (*E. coli*); OGG1, 8-oxoguanine DNA glycosylase 1; PARP, poly (ADP-ribose) polymerase; XRCC1, X-ray repair cross-complementing 1.

Table 8.7 – Haplogroup association response for the SNPs under study.

Haplotype association response							**OR (95% CI)**	**P-value**
APEX D148E	*MUTYH* Q335H	*OGG1* S326C	*PARP1* V762A	*PARP4* G1280R	*XRCC1*_194 R194W	*XRCC1*_399 R399Q		
D	H	S	V	G	R	R	1.00 (Reference)	
E	H	S	V	G	R	R	3.17 (0.99-10.17)	0.05
E	H	S	V	G	R	Q	0.92 (0.34-2.53)	0.88
E	H	S	V	R	R	R	1.55 (0.50-4.76)	0.45
D	H	S	V	R	R	Q	2.13 (0.60-7.53)	0.24
D	H	S	V	R	R	R	1.90 (0.41-8.79)	0.41
E	H	C	V	G	R	R	3.41 (0.87-13.36)	0.08
E	Q	S	V	R	R	R	3.91 (1.02-14.95)	0.05*

APEX1, apurinic/apyrimidinic endonuclease 1; MUTYH, mutY DNA glycosylase, earlier mutY homolog (*E. coli*); OGG1, 8-oxoguanine DNA glycosylase 1; PARP, poly (ADP-ribose) polymerase; XRCC1, X-ray repair cross-complementing 1; OR, odds ratio; CI, confidence interval. *P<0.047.

8.4 – DISCUSSION AND CONCLUSIONS

DNA repair deficiencies and genetic or epigenetic changes may decrease the efficiency of DNA repair, thus contributing to individual susceptibility to DNA damaging agents and to cancer risk (Hirotsu et al., 2015; Walter et al., 2015; Wilson, Kim, Berquist, & Sigurdson, 2011). However, to the best of our knowledge, no clinical association studies have been performed to evaluate the role of BER pathway polymorphisms on PN-MPNs susceptibility.

The present study revealed a higher incidence of *JAK2* V617F mutation in ET patients and a lower incidence in PV patients, compared with that reported in the literature (Nangalia et al., 2013;

Tefferi & Pardanani, 2015). This finding is probably due to the small population studied and to the fact that ET was the most frequent diagnosis among the patients included in the current case group.

The present study was intended to ascertain the possible role of the genetic polymorphisms *APEX1* Asp148Glu, *MUTYH* Gln335His, *OGG1* Ser326Cys, *PARP1* Val762Ala, *PARP4* Gly1280Arg, and *XRCC*1 Arg194Trp and Arg399Gln on the individual susceptibility for PN-MPNs. The genotypic frequencies of the different SNPs in the control population are similar to those reported in other Caucasian populations (Conde et al., 2009; Santos et al., 2012; Silva et al., 2007).

The MUTYH protein acts as a BER glycosylase and is mainly involved in the repair of oxidative DNA lesions (Cheadle & Sampson, 2007; Conde et al., 2009; Das et al., 2015; Huang et al., 2007; Picelli et al., 2010; Przybylowska et al., 2013; Qian et al., 2011; Santos et al., 2012; Tao et al., 2008; Wilson et al., 2011; Zhang et al., 2006). MUTYH dysfunction may therefore be of special relevance in human tumourigenesis, since it is the only mechanism for repairing 8-oxo-dG/adenine mismatches (Cheadle & Sampson, 2007). Indeed, two specific germline mutations in this gene, Tyr165Cys and Gly382Asp, have been associated with a colorectal adenoma and carcinoma predisposition syndrome that is now referred to as MUTYH-associated polyposis (Cheadle & Sampson, 2007). However, various others mutations and SNPs have been described to date, for which a specific phenotypic consequence is unknown (Cheadle & Sampson, 2007). An example of one common SNP in the *MUTYH* gene is the nonsynonymous Gln335His variation in codon 12 (Cheadle & Sampson, 2007; Picelli et al., 2010). The enzyme encoded by this variant has been demonstrated to have partially impaired glycosylase activity *in vitro*, and could therefore contribute to cancer susceptibility, being much more frequently detected in Japanese and Chinese populations than in European populations (Santos et al., 2012; Tao et al., 2008). The Gln335His variant allele has been suggested to be associated with increased risk of colorectal cancer (Picelli et al., 2013; Picelli et al., 2010; Tao et al., 2008) and less consistently with lung cancer (Qian et al., 2011). For the latter, however, existing evidence is conflicting or significant only when taking into account gene-gene interactions (Qian et al., 2011). Notably, an almost significant decrease in breast cancer risk (OR, 0.80; 95% CI, 0.59-1.07) was described previously by our group in *MUTYH* Gln335His heterozygotes (Conde et al., 2009). Furthermore, gene-gene interactions among BER polymorphisms were observed in ever tobacco smokers in a bladder cancer susceptibility study (Huang et al., 2007). Those previous results suggest that genetic variation in BER genes may contribute to cancer risk through gene-gene and gene-environmental interactions.

To the best of our knowledge, there are no clinical association studies in which the role of the *MUTYH* Gln335His polymorphism had been evaluated on PN-MPNs susceptibility. The results reported in the present study suggest that, when considering females with ET, a consistent decrease in overall PN-MPNs risk was observed when at least one variant allele carrying *MUTYH* Gln335His is present (Table 8.5).

The XRCC1 nuclear protein serves an important role in assisting and enabling the repair of single-strand breaks by interacting and recruiting to the DNA lesion sites multiple enzymatic components of repair reactions (Lévy et al., 2006). Arg194Trp and Arg399Gln polymorphisms are

among the most extensively studied SNPs in the *XRCC1* gene (Annamaneni et al., 2013; Batar et al., 2009; Bolufer et al., 2006; Bănescu et al., 2014; Ginsberg, Angle, Guyton, & Sonawane, 2011; Huang et al., 2007; Hung, Hall, Brennan, & Boffetta, 2005; Jiang, Zhang, Yang, & Wang, 2009; Miao et al., 2006; Przybylowska et al., 2013; Qian et al., 2011; Santos et al., 2012; Silva et al., 2007; Wilson et al., 2011; Yan et al., 2016; Zhai et al., 2006; Zhang et al., 2006). These two SNPs have been shown to alter the functional activity of the resulting protein *in vitro* and to interfere with cancer susceptibility. The Arg194Trp variant allele has been associated with decreased risk of certain cancers, particularly among tobacco smokers (Hung et al., 2005). Conversely, the Arg399Gln variant allele has been suggested to be associated with decreased DNA repair capacity and higher sensitivity to genotoxicants compared with the Arg194Trp variant allele (Z. Hu, Ma, Chen, Wei, & Shen, 2005). However, previous epidemiological results have been inconsistent and dependent on the cancer type (Chen, Zhou, Yang, & Wu, 2012; Przybylowska et al., 2013; A. Srivastava, Srivastava, Pandey, Choudhuri, & Mittal, 2009). Several interactions such as gene-environment (e.g. alcohol consumption or menopausal age) and gene-gene (e.g. other DNA repair or chemical metabolizing enzymes) have also been reported for both SNPs (Wilson et al., 2011). However, several well-powered studies and meta-analyses have not confirmed these supposed effects (Ginsberg et al., 2011; Wilson et al., 2011). Generally, the results reported to date suggest a modest impact of both *XRCC1* gene polymorphisms on protein activity and cancer susceptibility. However, certain studies have suggested that they may represent a risk factor for hematological malignancies such as leukaemia, according to previous studies on *XRCC1* polymorphisms in association with AML, acute lymphoblastic leukaemia, chronic lymphocytic leukaemia and lymphoma (Batar et al., 2009; Bolufer et al., 2006; Bănescu et al., 2014). The present results support a borderline effect for ET patients and a protective effect in overall PN-MPNs risk when considering ET patients presenting JAK2 mutation, as observed under the presence of at least one variant allele carrying *XRCC1* Arg399Gln (Table 8.5). Although the Arg194Trp allele did not show any association with the risk of developing PN-MPNs, it should be noted that the Arg194Trp variant allele may be associated with higher DNA repair capacity, lower sensitivity to genotoxicants and decreased risk of other cancers, when compared with other variants. In addition, other factors may influence the biological effect of the Arg194Trp polymorphism, such as disease development stage, specific environmental factors and even different genetic background among populations. In order to clarify the role of *XRCC1* polymorphisms in PN-MPNs susceptibility, larger studies and/or a meta-analysis are required.

Regarding the *OGG1* Ser326Cys polymorphism, the current results did not reveal a significant contribution on individual susceptibility towards PN-MPNs (Tables 8.1 and 8.4). The *OGG1* Ser326Cys polymorphism has been demonstrated to impair protein function (Wilson et al., 2011), and therefore, it has been widely evaluated in different case-control studies (Zou et al., 2016). Significant results were

observed in lung (Hung et al., 2005; B. Wei et al., 2011), head and neck (Kumar, Pant, Singh, & Khandelwal, 2011; W. Wang et al., 2012), colorectal (Canbay et al., 2011; Przybylowska et al., 2013) and gallbladder cancer (A. Srivastava et al., 2009; K. Srivastava, Srivastava, & Mittal, 2010). However, several previous meta-analyses (Das, Nath, Bhowmik, Ghosh, & Choudhury, 2016; Gu, Wang, Zhang, & Chen, 2010; W. Wang et al., 2012; B. Wei et al., 2011; Wenjuan et al., 2016) did not reveal any significant association between this *OGG1* polymorphism and other cancers risk.

Human APEX1 is a multifunctional enzyme that holds complementary key roles in cancer. Notably, this enzyme is a crucial component of the BER pathway due to its ability to process AP sites and other 3′ DNA termini that may result, for example, from exposure to ionizing radiation or direct attack by free radicals (Abbotts & Madhusudan, 2010; Dyrkheeva, Lebedeva, & Lavrik, 2016; Hung et al., 2005). Among the 18 identified SNPs for the *APEX1* gene, the most studied one is the T>G transition at codon 148 of exon 5, which leads to a change in amino acid from Asp to Glu (Das et al., 2015). Its potential role on cancer was evaluated in four meta-analyses on breast cancer susceptibility (Peng et al., 2014; Wu, Liu, Zhang, Dong, & Wu, 2012; Zhang et al., 2006; Zhao et al., 2014), two meta-analyses on prostate cancer (X. Li, Zhang, Huai, & Cao, 2014; Zhou et al., 2015) and several studies on other types of cancer (Dai et al., 2015; D. Hu, Lin, Zhang, Zheng, & Niu, 2013; Jin et al., 2014; H. Li et al., 2015). However, the results published remain inconclusive. Concerning the present results, the association of *APEX1* Asp148Glu with PN-MPNs risk did not exhibit any association.

PARP1 is an abundant nuclear protein that can bind to DNA and promote the poly (ADP-ribosylation) of a variety of proteins. PARP1 acts on single- and double-stranded DNA breaks by recruiting DNA repair factors (Talhaoui et al., 2016). It has a major signaling role in DNA damage detection and repair, acting as a molecular nick sensor to initiate the recruitment of XRCC1 and the assembly of the single-strand break repair machinery (H. Wei & Yu, 2016). *PARP1* Val762Ala is one of the most common nonsynonymous SNPs studied in this gene, resulting in an amino acid substitution within the COOH-terminal catalytic domain of the enzyme (Hua et al., 2014; Qin et al., 2014). This variant has been associated with reduced enzymatic activity (Jiang et al., 2009) and limited capacity for interaction with *XRCC1* (Miao et al., 2006). This may result in decreased BER capacity, thus increasing cancer predisposition in *PARP1* Val762Ala carriers. Indeed, this variant form has been associated in various well-powered clinical association studies with increased cancer susceptibility, namely to lung (Jiang et al., 2009) and gastrointestinal tract (Y. Hu et al., 2014; Miao et al., 2006) cancer, while Adel Fahmideh *et al.* (Adel Fahmideh, Schwartzbaum, Frumento, & Feychting, 2014) described a decreased risk for glioma associated with this SNP. Studies regarding other types of cancer such as breast cancer (Zhai et al., 2006; Zhang et al., 2006) failed to demonstrate an association between *PARP1* Val762Ala and cancer susceptibility. The present results do not suggest any association between *PARP1* Val762Ala polymorphism and PN-MPNs risk (Table 8.4).

Concerning the analysis of haplogroups' association response, an increased risk to PN-MPNs could be observed for one specific combination (Table 8.7). This result should, however, be taken with

care when evaluating this parameter risk for PN-MPNs due to its low frequency (3.2%) in the studied population, even though it may represent a tendency on how SNPs in BER genes influence PN-MPNs.

Overall, the present results reveal that the *XRCC1* Gln399Arg and *MUTYH* Gln335His gene polymorphisms appear to be associated with PN-MPNs risk. For all other polymorphisms under study, no significant change was observed (Tables 8.4 and 8.5).

Although certain published studies consider tobacco smoking as a contributing factor for PN-MPNs (Hasselbalch, 2015; Lindholm Sørensen & Hasselbalch, 2015), the present study did not reveal an important association, probably due to the small number of tobacco smokers included.

Additional studies involving larger populations should be conducted to further clarify the potential value of the different BER genotypes as predictive biomarkers of susceptibility to PN-MPNs and to study gene-environment and gene-gene interactions. In addition, stratified analysis according to histological subtype and disease stage should be conducted.

The MUTYH protein acts as a BER glycosylase and is mainly involved in the repair of oxidative DNA lesions (Cheadle & Sampson, 2007; Conde et al., 2009; Das et al., 2015; Huang et al., 2007; Picelli et al., 2010; Przybylowska et al., 2013; Qian et al., 2011; Santos et al., 2012; Tao et al., 2008; Wilson et al., 2011; Zhang et al., 2006). MUTYH dysfunction may therefore be of special relevance in human tumourigenesis, since it is the only mechanism for repairing 8-oxo-dG/adenine mismatches (Cheadle & Sampson, 2007). Indeed, two specific germline mutations in this gene, Tyr165Cys and Gly382Asp, have been associated with a colorectal adenoma and carcinoma predisposition syndrome that is now referred to as MUTYH-associated polyposis (Cheadle & Sampson, 2007). However, various others mutations and SNPs have been described to date, for which a specific phenotypic consequence is unknown (Cheadle & Sampson, 2007). An example of one common SNP in the *MUTYH* gene is the nonsynonymous Gln335His variation in codon 12 (Cheadle & Sampson, 2007; Picelli et al., 2010). The enzyme encoded by this variant has been demonstrated to have partially impaired glycosylase activity *in vitro*, and could therefore contribute to cancer susceptibility, being much more frequently detected in Japanese and Chinese populations than in European populations (Santos et al., 2012; Tao et al., 2008). The Gln335His variant allele has been suggested to be associated with increased risk of colorectal cancer (Picelli et al., 2013; Picelli et al., 2010; Tao et al., 2008) and less consistently with lung cancer (Qian et al., 2011). For the latter, however, existing evidence is conflicting or significant only when taking into account gene-gene interactions (Qian et al., 2011). Notably, an almost significant decrease in breast cancer risk (OR, 0.80; 95% CI, 0.59-1.07) was described previously by our group in *MUTYH* Gln335His heterozygotes (Conde et al., 2009). Furthermore, gene-gene interactions among BER polymorphisms were observed in ever tobacco smokers in a bladder cancer susceptibility study (Huang et al., 2007). Those previous results suggest that genetic variation in BER genes may contribute to cancer risk through gene-gene and gene-environmental interactions.

To the best of our knowledge, there are no clinical association studies in which the role of the *MUTYH* Gln335His polymorphism had been evaluated on PN-MPNs susceptibility. The results reported in the present study suggest that, when considering females with ET, a consistent decrease in overall PN-MPNs risk was observed when at least one variant allele carrying *MUTYH* Gln335His is present (Table 8.5).

The XRCC1 nuclear protein serves an important role in assisting and enabling the repair of single-strand breaks by interacting and recruiting to the DNA lesion sites multiple enzymatic components of repair reactions (Lévy et al., 2006). Arg194Trp and Arg399Gln polymorphisms are among the most extensively studied SNPs in the *XRCC1* gene (Annamaneni et al., 2013; Batar et al., 2009; Bolufer et al., 2006; Bănescu et al., 2014; Ginsberg, Angle, Guyton, & Sonawane, 2011; Huang et al., 2007; Hung, Hall, Brennan, & Boffetta, 2005; Jiang, Zhang, Yang, & Wang, 2009; Miao et al., 2006; Przybylowska et al., 2013; Qian et al., 2011; Santos et al., 2012; Silva et al., 2007; Wilson et al., 2011; Yan et al., 2016; Zhai et al., 2006; Zhang et al., 2006). These two SNPs have been shown to alter the functional activity of the resulting protein *in vitro* and to interfere with cancer susceptibility. The Arg194Trp variant allele has been associated with decreased risk of certain cancers, particularly among tobacco smokers (Hung et al., 2005). Conversely, the Arg399Gln variant allele has been suggested to be associated with decreased DNA repair capacity and higher sensitivity to genotoxicants compared with the Arg194Trp variant allele (Z. Hu, Ma, Chen, Wei, & Shen, 2005). However, previous epidemiological results have been inconsistent and dependent on the cancer type (Chen, Zhou, Yang, & Wu, 2012; Przybylowska et al., 2013; A. Srivastava, Srivastava, Pandey, Choudhuri, & Mittal, 2009). Several interactions such as gene-environment (e.g. alcohol consumption or menopausal age) and gene-gene (e.g. other DNA repair or chemical metabolizing enzymes) have also been reported for both SNPs (Wilson et al., 2011). However, several well-powered studies and meta-analyses have not confirmed these supposed effects (Ginsberg et al., 2011; Wilson et al., 2011). Generally, the results reported to date suggest a modest impact of both *XRCC1* gene polymorphisms on protein activity and cancer susceptibility. However, certain studies have suggested that they may represent a risk factor for haematological malignancies such as leukaemia, according to previous studies on *XRCC1* polymorphisms in association with AML, acute lymphoblastic leukaemia, chronic lymphocytic leukaemia and lymphoma (Batar et al., 2009; Bolufer et al., 2006; Bănescu et al., 2014). The present results support a borderline effect for ET patients and a protective effect in overall PN-MPNs risk when considering ET patients presenting JAK2 mutation, as observed under the presence of at least one variant allele carrying *XRCC1* Arg399Gln (Table 8.5). Although the Arg194Trp allele did not show any association with the risk of developing PN-MPNs, it should be noted that the Arg194Trp variant allele may be associated with higher DNA repair capacity, lower sensitivity to genotoxicants and decreased risk of other cancers, when compared with other variants. In addition, other factors may influence the biological effect of the Arg194Trp polymorphism, such as disease development stage, specific environmental factors and even different genetic background among populations. In order to clarify the

role of *XRCC1* polymorphisms in PN-MPNs susceptibility, larger studies and/or a meta-analysis are required.

Regarding the *OGG1* Ser326Cys polymorphism, the current results did not reveal a significant contribution on individual susceptibility towards PN-MPNs (Tables 8.1 and 8.4). The *OGG1* Ser326Cys polymorphism has been demonstrated to impair protein function (Wilson et al., 2011), and therefore, it has been widely evaluated in different case-control studies (Zou et al., 2016). Significant results were observed in lung (Hung et al., 2005; B. Wei et al., 2011), head and neck (Kumar, Pant, Singh, & Khandelwal, 2011; W. Wang et al., 2012), colorectal (Canbay et al., 2011; Przybylowska et al., 2013) and gallbladder cancer (A. Srivastava et al., 2009; K. Srivastava, Srivastava, & Mittal, 2010). However, several previous meta-analyses (Das, Nath, Bhowmik, Ghosh, & Choudhury, 2016; Gu, Wang, Zhang, & Chen, 2010; W. Wang et al., 2012; B. Wei et al., 2011; Wenjuan et al., 2016) did not reveal any significant association between this *OGG1* polymorphism and other cancers risk.

Human APEX1 is a multifunctional enzyme that holds complementary key roles in cancer. Notably, this enzyme is a crucial component of the BER pathway due to its ability to process AP sites and other 3′ DNA termini that may result, for example, from exposure to ionizing radiation or direct attack by free radicals (Abbotts & Madhusudan, 2010; Dyrkheeva, Lebedeva, & Lavrik, 2016; Hung et al., 2005). Among the 18 identified SNPs for the *APEX1* gene, the most studied one is the T>G transition at codon 148 of exon 5, which leads to a change in amino acid from Asp to Glu (Das et al., 2015). Its potential role on cancer was evaluated in four meta-analyses on breast cancer susceptibility (Peng et al., 2014; Wu, Liu, Zhang, Dong, & Wu, 2012; Zhang et al., 2006; Zhao et al., 2014), two meta-analyses on prostate cancer (X. Li, Zhang, Huai, & Cao, 2014; Zhou et al., 2015) and several studies on other types of cancer (Dai et al., 2015; D. Hu, Lin, Zhang, Zheng, & Niu, 2013; Jin et al., 2014; H. Li et al., 2015). However, the results published remain inconclusive. Concerning the present results, the association of *APEX1* Asp148Glu with PN-MPNs risk did not exhibit any association.

PARP1 is an abundant nuclear protein that can bind to DNA and promote the poly (ADP-ribosylation) of a variety of proteins. PARP1 acts on single- and double-stranded DNA breaks by recruiting DNA repair factors (Talhaoui et al., 2016). It has a major signaling role in DNA damage detection and repair, acting as a molecular nick sensor to initiate the recruitment of XRCC1 and the assembly of the single-strand break repair machinery (H. Wei & Yu, 2016). *PARP1* Val762Ala is one of the most common nonsynonymous SNPs studied in this gene, resulting in an amino acid substitution within the COOH-terminal catalytic domain of the enzyme (Hua et al., 2014; Qin et al., 2014). This variant has been associated with reduced enzymatic activity (Jiang et al., 2009) and limited capacity for interaction with *XRCC1* (Miao et al., 2006). This may result in decreased BER capacity, thus increasing cancer predisposition in *PARP1* Val762Ala carriers. Indeed, this variant form has been associated in various well-powered clinical association studies with increased cancer susceptibility, namely to lung (Jiang et al., 2009) and gastrointestinal tract (Y. Hu et al., 2014; Miao et al., 2006) cancer, while Adel

Fahmideh *et al.* (Adel Fahmideh, Schwartzbaum, Frumento, & Feychting, 2014) described a decreased risk for glioma associated with this SNP. Studies regarding other types of cancer such as breast cancer (Zhai et al., 2006; Zhang et al., 2006) failed to demonstrate an association between *PARP1* Val762Ala and cancer susceptibility. The present results do not suggest any association between *PARP1* Val762Ala polymorphism and PN-MPNs risk (Table 8.4).

Concerning the analysis of haplogroups' association response, an increased risk to PN-MPNs could be observed for one specific combination (Table 8.7). This result should, however, be taken with care when evaluating this parameter risk for PN-MPNs due to its low frequency (3.2%) in the studied population, even though it may represent a tendency on how SNPs in BER genes influence PN-MPNs.

Overall, the present results reveal that the *XRCC1* Gln399Arg and *MUTYH* Gln335His gene polymorphisms appear to be associated with PN-MPNs risk. For all other polymorphisms under study, no significant change was observed (Tables 8.4 and 8.5).

Although certain published studies consider tobacco smoking as a contributing factor for PN-MPNs (Hasselbalch, 2015; Lindholm Sørensen & Hasselbalch, 2015), the present study did not reveal an important association, probably due to the small number of tobacco smokers included.

Additional studies involving larger populations should be conducted to further clarify the potential value of the different BER genotypes as predictive biomarkers of susceptibility to PN-MPNs and to study gene-environment and gene-gene interactions. In addition, stratified analysis according to histological subtype and disease stage should be conducted.

8.5 – REFERENCES

Abbotts, R., & Madhusudan, S. (2010). Human AP endonuclease 1 (APE1): from mechanistic insights to druggable target in cancer. *Cancer Treat Rev, 36*(5), 425-435. doi:10.1016/j.ctrv.2009.12.006

Adel Fahmideh, M., Schwartzbaum, J., Frumento, P., & Feychting, M. (2014). Association between DNA repair gene polymorphisms and risk of glioma: a systematic review and meta-analysis. *Neuro Oncol, 16*(6), 807-814. doi:10.1093/neuonc/nou003

Annamaneni, S., Gorre, M., Kagita, S., Addepalli, K., Digumarti, R. R., Satti, V., & Battini, M. R. (2013). Association of XRCC1 gene polymorphisms with chronic myeloid leukemia in the population of Andhra Pradesh, India. *Hematology, 18*(3), 163-168. doi:10.1179/1607845412Y.0000000040

Arber, D. A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M. J., Le Beau, M. M., . . . Vardiman, J. W. (2016). The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood, 127*(20), 2391-2405. doi:10.1182/blood-2016-03-643544

Bastos, H. N., Antão, M. R., Silva, S. N., Azevedo, A. P., Manita, I., Teixeira, V., . . . Gaspar, J. F. (2009). Association of polymorphisms in genes of the homologous recombination DNA repair pathway and thyroid cancer risk. *Thyroid, 19*(10), 1067-1075. doi:10.1089/thy.2009.0099

Batar, B., Güven, M., Bariş, S., Celkan, T., & Yildiz, I. (2009). DNA repair gene XPD and XRCC1 polymorphisms and the risk of childhood acute lymphoblastic leukemia. *Leuk Res, 33*(6), 759-763. doi:10.1016/j.leukres.2008.11.005

Baute, J., & Depicker, A. (2008). Base excision repair and its role in maintaining genome stability. *Crit Rev Biochem Mol Biol, 43*(4), 239-276. doi:10.1080/10409230802309905

Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., . . . Project, C. G. (2005). Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet, 365*(9464), 1054-1061. doi:10.1016/S0140-6736(05)71142-9

Beer, P. A., Delhommeau, F., LeCouédic, J. P., Dawson, M. A., Chen, E., Bareford, D., . . . Green, A. R. (2010). Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. *Blood, 115*(14), 2891-2900. doi:10.1182/blood-2009-08-236596

Björkholm, M., Hultcrantz, M., & Derolf, Å. (2014). Leukemic transformation in myeloproliferative neoplasms: therapy-related or unrelated? *Best Pract Res Clin Haematol, 27*(2), 141-153. doi:10.1016/j.beha.2014.07.003

Bolufer, P., Barragan, E., Collado, M., Cervera, J., López, J. A., & Sanz, M. A. (2006). Influence of genetic polymorphisms on the risk of developing leukemia and on disease progression. *Leuk Res, 30*(12), 1471-1491. doi:10.1016/j.leukres.2006.01.016

Bănescu, C., Trifa, A. P., Demian, S., Benedek Lazar, E., Dima, D., Duicu, C., & Dobreanu, M. (2014). Polymorphism of XRCC1, XRCC3, and XPD genes and risk of chronic myeloid leukemia. *Biomed Res Int, 2014*, 213790. doi:10.1155/2014/213790

Campregher, P. V., Santos, F. P., Perini, G. F., & Hamerschlak, N. (2012). Molecular biology of Philadelphia-negative myeloproliferative neoplasms. *Rev Bras Hematol Hemoter, 34*(2), 150-155. doi:10.5581/1516-8484.20120035

Canbay, E., Cakmakoglu, B., Zeybek, U., Sozen, S., Cacina, C., Gulluoglu, M., . . . Bugra, D. (2011). Association of APE1 and hOGG1 polymorphisms with colorectal cancer risk in a Turkish population. *Curr Med Res Opin, 27*(7), 1295-1302. doi:10.1185/03007995.2011.573544

Cheadle, J. P., & Sampson, J. R. (2007). MUTYH-associated polyposis--from defect in base excision repair to clinical genetic testing. *DNA Repair (Amst), 6*(3), 274-279. doi:10.1016/j.dnarep.2006.11.001

Chen, B., Zhou, Y., Yang, P., & Wu, X. T. (2012). Polymorphisms of XRCC1 and gastric cancer susceptibility: a meta-analysis. *Mol Biol Rep, 39*(2), 1305-1313. doi:10.1007/s11033-011-0863-6

Chi, J., Manoloukos, M., Pierides, C., Nicolaidou, V., Nicolaou, K., Kleopa, M., . . . Costeas, P. (2015). Calreticulin mutations in myeloproliferative neoplasms and new methodology for their detection and monitoring. *Ann Hematol, 94*(3), 399-408. doi:10.1007/s00277-014-2232-8

Conde, J., Silva, S. N., Azevedo, A. P., Teixeira, V., Pina, J. E., Rueff, J., & Gaspar, J. F. (2009). Association of common variants in mismatch repair genes and breast cancer susceptibility: a multigene study. *BMC Cancer, 9*, 344. doi:10.1186/1471-2407-9-344

Dai, Z. J., Shao, Y. P., Kang, H. F., Tang, W., Xu, D., Zhao, Y., . . . Wang, X. J. (2015). Relationship between apurinic endonuclease 1 Asp148Glu polymorphism and gastrointestinal cancer risk: An updated meta-analysis. *World J Gastroenterol, 21*(16), 5081-5089. doi:10.3748/wjg.v21.i16.5081

Das, S., Bhowmik, A., Bhattacharjee, A., Choudhury, B., Naiding, M., Laskar, A. K., . . . Choudhury, Y. (2015). XPD, APE1, and MUTYH polymorphisms increase head and neck cancer risk: effect of gene-gene and gene-environment interactions. *Tumour Biol, 36*(10), 7569-7579. doi:10.1007/s13277-015-3472-5

Das, S., Nath, S., Bhowmik, A., Ghosh, S. K., & Choudhury, Y. (2016). Association between OGG1 Ser326Cys polymorphism and risk of upper aero-digestive tract and gastrointestinal cancers: a meta-analysis. *Springerplus, 5*, 227. doi:10.1186/s40064-016-1858-5

Delhommeau, F., Jeziorowska, D., Marzac, C., & Casadevall, N. (2010). Molecular aspects of myeloproliferative neoplasms. *Int J Hematol, 91*(2), 165-173. doi:10.1007/s12185-010-0530-z

Dyrkheeva, N. S., Lebedeva, N. A., & Lavrik, O. I. (2016). AP Endonuclease 1 as a Key Enzyme in Repair of Apurinic/Apyrimidinic Sites. *Biochemistry (Mosc), 81*(9), 951-967. doi:10.1134/S0006297916090042

Figueroa, J. D., Malats, N., Real, F. X., Silverman, D., Kogevinas, M., Chanock, S., . . . García-Closas, M. (2007). Genetic variation in the base excision repair pathway and bladder cancer risk. *Hum Genet, 121*(2), 233-242. doi:10.1007/s00439-006-0294-y

Geyer, H. L., Kosiorek, H., Dueck, A. C., Scherber, R., Slot, S., Zweegman, S., . . . Mesa, R. A. (2017). Associations between gender, disease features and symptom burden in patients with myeloproliferative neoplasms: an analysis by the MPN QOL International Working Group. *Haematologica, 102*(1), 85-93. doi:10.3324/haematol.2016.149559

Ginsberg, G., Angle, K., Guyton, K., & Sonawane, B. (2011). Polymorphism in the DNA repair enzyme XRCC1: utility of current database and implications for human health risk assessment. *Mutat Res, 727*(1-2), 1-15. doi:10.1016/j.mrrev.2011.02.001

Gomes, B. C., Silva, S. N., Azevedo, A. P., Manita, I., Gil, O. M., Ferreira, T. C., . . . Gaspar, J. F. (2010). The role of common variants of non-homologous end-joining repair genes XRCC4, LIG4 and Ku80 in thyroid cancer risk. *Oncol Rep, 24*(4), 1079-1085.

Gu, D., Wang, M., Zhang, Z., & Chen, J. (2010). Lack of association between the hOGG1 Ser326Cys polymorphism and breast cancer risk: evidence from 11 case-control studies. *Breast Cancer Res Treat, 122*(2), 527-531. doi:10.1007/s10549-009-0723-4

Ha, J. S., & Kim, Y. K. (2015). Calreticulin exon 9 mutations in myeloproliferative neoplasms. *Ann Lab Med, 35*(1), 22-27. doi:10.3343/alm.2015.35.1.22

Hasselbalch, H. C. (2015). Smoking as a contributing factor for development of polycythemia vera and related neoplasms. *Leuk Res*. doi:10.1016/j.leukres.2015.09.002

Hasselbalch, H. C., Thomassen, M., Riley, C. H., Kjær, L., Larsen, T. S., Jensen, M. K., . . . Skov, V. (2014). Whole blood transcriptional profiling reveals deregulation of oxidative and antioxidative defence genes in myelofibrosis and related neoplasms. Potential implications of downregulation of Nrf2 for genomic instability and disease progression. *PLoS One, 9*(11), e112786. doi:10.1371/journal.pone.0112786

Hernández-Boluda, J. C., Pereira, A., Cervantes, F., Alvarez-Larrán, A., Collado, M., Such, E., . . . Guillem, V. (2012). A polymorphism in the XPD gene predisposes to leukemic transformation and new nonmyeloid malignancies in essential thrombocythemia and polycythemia vera. *Blood, 119*(22), 5221-5228. doi:10.1182/blood-2012-02-411215

Hirotsu, Y., Nakagomi, H., Sakamoto, I., Amemiya, K., Oyama, T., Mochizuki, H., & Omata, M. (2015). Multigene panel analysis identified germline mutations of DNA repair genes in breast and ovarian cancer. *Mol Genet Genomic Med, 3*(5), 459-466. doi:10.1002/mgg3.157

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature, 411*(6835), 366-374. doi:10.1038/35077232

Hu, D., Lin, X., Zhang, H., Zheng, X., & Niu, W. (2013). APEX nuclease (multifunctional DNA repair enzyme) 1 gene Asp148Glu polymorphism and cancer risk: a meta-analysis involving 58 articles and 48903 participants. *PLoS One, 8*(12), e83527. doi:10.1371/journal.pone.0083527

Hu, Y., Zhou, M., Li, K., Zhang, K., Kong, X., Zheng, Y., . . . Liu, L. (2014). Two DNA repair gene polymorphisms on the risk of gastrointestinal cancers: a meta-analysis. *Tumour Biol, 35*(3), 1715-1725. doi:10.1007/s13277-013-1320-z

Hu, Z., Ma, H., Chen, F., Wei, Q., & Shen, H. (2005). XRCC1 polymorphisms and cancer risk: a meta-analysis of 38 case-control studies. *Cancer Epidemiol Biomarkers Prev, 14*(7), 1810-1818. doi:10.1158/1055-9965.EPI-04-0793

Hua, R. X., Li, H. P., Liang, Y. B., Zhu, J. H., Zhang, B., Ye, S., . . . Sun, X. Z. (2014). Association between the PARP1 Val762Ala polymorphism and cancer risk: evidence from 43 studies. *PLoS One, 9*(1), e87057. doi:10.1371/journal.pone.0087057

Huang, M., Dinney, C. P., Lin, X., Lin, J., Grossman, H. B., & Wu, X. (2007). High-order interactions among genetic variants in DNA base excision repair pathway genes and smoking in bladder cancer susceptibility. *Cancer Epidemiol Biomarkers Prev, 16*(1), 84-91. doi:10.1158/1055-9965.EPI-06-0712

Hung, R. J., Hall, J., Brennan, P., & Boffetta, P. (2005). Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. *Am J Epidemiol, 162*(10), 925-942. doi:10.1093/aje/kwi318

James, C., Ugo, V., Le Couédic, J. P., Staerk, J., Delhommeau, F., Lacout, C., . . . Vainchenker, W. (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature, 434*(7037), 1144-1148. doi:10.1038/nature03546

Jiang, J., Zhang, X., Yang, H., & Wang, W. (2009). Polymorphisms of DNA repair genes: ADPRT, XRCC1, and XPD and cancer risk in genetic epidemiology. *Methods Mol Biol, 471*, 305-333. doi:10.1007/978-1-59745-416-2_16

Jin, F., Qian, C., Qing, Y., Zhang, Z., Wang, G., Shan, J., . . . Wang, D. (2014). Genetic polymorphism of APE1 rs1130409 can contribute to the risk of lung cancer. *Tumour Biol, 35*(7), 6665-6671. doi:10.1007/s13277-014-1829-9

Kilpivaara, O., & Levine, R. L. (2008). JAK2 and MPL mutations in myeloproliferative neoplasms: discovery and science. *Leukemia, 22*(10), 1813-1817. doi:10.1038/leu.2008.229

Klampfl, T., Gisslinger, H., Harutyunyan, A. S., Nivarthi, H., Rumi, E., Milosevic, J. D., . . . Kralovics, R. (2013). Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med, 369*(25), 2379-2390. doi:10.1056/NEJMoa1311347

Kralovics, R., Passamonti, F., Buser, A. S., Teo, S. S., Tiedt, R., Passweg, J. R., . . . Skoda, R. C. (2005). A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med, 352*(17), 1779-1790. doi:10.1056/NEJMoa051113

Kumar, A., Pant, M. C., Singh, H. S., & Khandelwal, S. (2011). Role of OGG1 Ser326Cys polymorphism and 8-oxoguanine DNA damage in risk assessment of squamous cell carcinoma of head and neck in North Indian population. *Mutat Res, 726*(2), 227-233. doi:10.1016/j.mrgentox.2011.09.015

Levine, R. L. (2009). Mechanisms of mutations in myeloproliferative neoplasms. *Best Pract Res Clin Haematol, 22*(4), 489-494. doi:10.1016/j.beha.2009.08.006

Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J., . . . Gilliland, D. G. (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell, 7*(4), 387-397. doi:10.1016/j.ccr.2005.03.023

Li, H., Zou, J., Mi, J., Wei, X., Zhao, D., Zhang, S., & Tian, G. (2015). Association of APE1 Gene Asp148Glu Variant with Digestive Cancer: A Meta-Analysis. *Med Sci Monit, 21*, 2456-2466. doi:10.12659/MSM.893954

Li, X., Zhang, G., Huai, Y. J., & Cao, Z. Q. (2014). Association between APE1 T1349G polymorphism and prostate cancer risk: evidence from a meta-analysis. *Tumour Biol, 35*(10), 10111-10119. doi:10.1007/s13277-014-2115-6

Lindholm Sørensen, A., & Hasselbalch, H. C. (2015). Smoking and philadelphia-negative chronic myeloproliferative neoplasms. *Eur J Haematol*. doi:10.1111/ejh.12684

Lévy, N., Martz, A., Bresson, A., Spenlehauer, C., de Murcia, G., & Ménissier-de Murcia, J. (2006). XRCC1 is phosphorylated by DNA-dependent protein kinase in response to DNA damage. *Nucleic Acids Res, 34*(1), 32-41. doi:10.1093/nar/gkj409

Mesa, R., Miller, C. B., Thyne, M., Mangan, J., Goldberger, S., Fazal, S., . . . Mascarenhas, J. O. (2016). Myeloproliferative neoplasms (MPNs) have a significant impact on patients' overall health and productivity: the MPN Landmark survey. *BMC Cancer, 16*(1), 167. doi:10.1186/s12885-016-2208-2

Miao, X., Zhang, X., Zhang, L., Guo, Y., Hao, B., Tan, W., . . . Lin, D. (2006). Adenosine diphosphate ribosyl transferase and x-ray repair cross-complementing 1 polymorphisms in gastric cardia cancer. *Gastroenterology, 131*(2), 420-427. doi:10.1053/j.gastro.2006.05.050

Nangalia, J., Massie, C. E., Baxter, E. J., Nice, F. L., Gundem, G., Wedge, D. C., . . . Green, A. R. (2013). Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med, 369*(25), 2391-2405. doi:10.1056/NEJMoa1312542

Passamonti, F., Rumi, E., Pungolino, E., Malabarba, L., Bertazzoni, P., Valentini, M., . . . Lazzarino, M. (2004). Life expectancy and prognostic factors for survival in patients with polycythemia vera and essential thrombocythemia. *Am J Med, 117*(10), 755-761. doi:10.1016/j.amjmed.2004.06.032

Peng, Q., Lu, Y., Lao, X., Chen, Z., Li, R., Sui, J., . . . Li, S. (2014). Association between OGG1 Ser326Cys and APEX1 Asp148Glu polymorphisms and breast cancer risk: a meta-analysis. *Diagn Pathol, 9*, 108. doi:10.1186/1746-1596-9-108

Picelli, S., Lorenzo Bermejo, J., Chang-Claude, J., Hoffmeister, M., Fernández-Rozadilla, C., Carracedo, A., . . . Association, M. o. E. C.-G. O. G. o. t. S. G. (2013). Meta-analysis of mismatch repair polymorphisms within the cogent consortium for colorectal cancer susceptibility. *PLoS One, 8*(9), e72091. doi:10.1371/journal.pone.0072091

Picelli, S., Zajac, P., Zhou, X. L., Edler, D., Lenander, C., Dalén, J., . . . Lindblom, A. (2010). Common variants in human CRC genes as low-risk alleles. *Eur J Cancer, 46*(6), 1041-1048. doi:10.1016/j.ejca.2010.01.013

Przybylowska, K., Kabzinski, J., Sygut, A., Dziki, L., Dziki, A., & Majsterek, I. (2013). An association selected polymorphisms of XRCC1, OGG1 and MUTYH gene and the level of efficiency oxidative DNA damage repair with a risk of colorectal cancer. *Mutat Res, 745-746*, 6-15. doi:10.1016/j.mrfmmm.2013.04.002

Qian, B., Zhang, H., Zhang, L., Zhou, X., Yu, H., & Chen, K. (2011). Association of genetic polymorphisms in DNA repair pathway genes with non-small cell lung cancer risk. *Lung Cancer, 73*(2), 138-146. doi:10.1016/j.lungcan.2010.11.018

Qin, Q., Lu, J., Zhu, H., Xu, L., Cheng, H., Zhan, L., . . . Sun, X. (2014). PARP-1 Val762Ala polymorphism and risk of cancer: a meta-analysis based on 39 case-control studies. *PLoS One, 9*(5), e98022. doi:10.1371/journal.pone.0098022

Rampal, R., Ahn, J., Abdel-Wahab, O., Nahas, M., Wang, K., Lipson, D., . . . Levine, R. L. (2014). Genomic and functional analysis of leukemic transformation of myeloproliferative neoplasms. *Proc Natl Acad Sci U S A, 111*(50), E5401-5410. doi:10.1073/pnas.1407792111

Rueff, J., & Rodrigues, A. S. (2016). Cancer Drug Resistance: A Brief Overview from a Genetic Viewpoint. *Methods Mol Biol, 1395*, 1-18. doi:10.1007/978-1-4939-3347-1_1

Rumi, E., Pietra, D., Ferretti, V., Klampfl, T., Harutyunyan, A. S., Milosevic, J. D., . . . Investigators, A. I. p. l. R. s. C. G. I. M. M. (2014). JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. *Blood, 123*(10), 1544-1551. doi:10.1182/blood-2013-11-539098

Santos, L. S., Branco, S. C., Silva, S. N., Azevedo, A. P., Gil, O. M., Manita, I., . . . Gaspar, J. F. (2012). Polymorphisms in base excision repair genes and thyroid cancer risk. *Oncol Rep, 28*(5), 1859-1868. doi:10.3892/or.2012.1975

Scott, L. M., Tong, W., Levine, R. L., Scott, M. A., Beer, P. A., Stratton, M. R., . . . Green, A. R. (2007). JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. *N Engl J Med, 356*(5), 459-468. doi:10.1056/NEJMoa065202

Silva, S. N., Azevedo, A. P., Teixeira, V., Pina, J. E., Rueff, J., & Gaspar, J. F. (2009). The role of GSTA2 polymorphisms and haplotypes in breast cancer susceptibility: a case-control study in the Portuguese population. *Oncol Rep, 22*(3), 593-598.

Silva, S. N., Moita, R., Azevedo, A. P., Gouveia, R., Manita, I., Pina, J. E., . . . Gaspar, J. (2007). Menopausal age and XRCC1 gene polymorphisms: role in breast cancer risk. *Cancer Detect Prev, 31*(4), 303-309. doi:10.1016/j.cdp.2007.07.001

Solé, X., Guinó, E., Valls, J., Iniesta, R., & Moreno, V. (2006). SNPStats: a web tool for the analysis of association studies. *Bioinformatics, 22*(15), 1928-1929. doi:10.1093/bioinformatics/btl268

Srivastava, A., Srivastava, K., Pandey, S. N., Choudhuri, G., & Mittal, B. (2009). Single-nucleotide polymorphisms of DNA repair genes OGG1 and XRCC1: association with gallbladder cancer in North Indian population. *Ann Surg Oncol, 16*(6), 1695-1703. doi:10.1245/s10434-009-0354-3

Srivastava, K., Srivastava, A., & Mittal, B. (2010). Polymorphisms in ERCC2, MSH2, and OGG1 DNA repair genes and gallbladder cancer risk in a population of Northern India. *Cancer, 116*(13), 3160-3169. doi:10.1002/cncr.25063

Swerdlow, S. H., Campo, E., Harris, N. L., Jaffe, E. S., Pileri, S. A., Stein, H., . . . Vardiman, J. W. (2008). *WHO Classification of Tumours of Haematopioetic and Lymphoid Tissues*. Lyon: World Health Organization.

Talhaoui, I., Lebedeva, N. A., Zarkovic, G., Saint-Pierre, C., Kutuzov, M. M., Sukhanova, M. V., . . . Ishchenko, A. A. (2016). Poly(ADP-ribose) polymerases covalently modify strand break termini in DNA fragments in vitro. *Nucleic Acids Res, 44*(19), 9279-9295. doi:10.1093/nar/gkw675

Tao, H., Shinmura, K., Suzuki, M., Kono, S., Mibu, R., Tanaka, M., . . . Sugimura, H. (2008). Association between genetic polymorphisms of the base excision repair gene MUTYH and increased colorectal cancer risk in a Japanese population. *Cancer Sci, 99*(2), 355-360. doi:10.1111/j.1349-7006.2007.00694.x

Tefferi, A., & Pardanani, A. (2015). Myeloproliferative Neoplasms: A Contemporary Review. *JAMA Oncol, 1*(1), 97-105. doi:10.1001/jamaoncol.2015.89

Tefferi, A., & Vardiman, J. W. (2008). Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia, 22*(1), 14-22. doi:10.1038/sj.leu.2404955

Walter, D., Lier, A., Geiselhart, A., Thalheimer, F. B., Huntscha, S., Sobotta, M. C., . . . Milsom, M. D. (2015). Exit from dormancy provokes DNA-damage-induced attrition in haematopoietic stem cells. *Nature, 520*(7548), 549-552. doi:10.1038/nature14131

Wang, C. Q., Krishnan, V., Tay, L. S., Chin, D. W., Koh, C. P., Chooi, J. Y., . . . Osato, M. (2014). Disruption of Runx1 and Runx3 leads to bone marrow failure and leukemia predisposition due to transcriptional and DNA repair defects. *Cell Rep, 8*(3), 767-782. doi:10.1016/j.celrep.2014.06.046

Wang, W., Wang, M., Chen, Y., Zhang, Z., Wang, S., Xu, M., . . . Zhao, Q. (2012). The hOGG1 Ser326Cys polymorphism contributes to cancer susceptibility: evidence from 83 case-control studies. *Mutagenesis, 27*(3), 329-336. doi:10.1093/mutage/ger083

Wei, B., Zhou, Y., Xu, Z., Xi, B., Cheng, H., Ruan, J., . . . Lu, P. (2011). The effect of hOGG1 Ser326Cys polymorphism on cancer risk: evidence from a meta-analysis. *PLoS One, 6*(11), e27545. doi:10.1371/journal.pone.0027545

Wei, H., & Yu, X. (2016). Functions of PARylation in DNA Damage Repair Pathways. *Genomics Proteomics Bioinformatics, 14*(3), 131-139. doi:10.1016/j.gpb.2016.05.001

Wenjuan, C., Jianzhong, L., Chong, L., Yanjun, G., Keqing, L., Hanzhang, W., & Zhiping, W. (2016). The hOGG1 Ser326Cys gene polymorphism and susceptibility for bladder cancer: a meta-analysis. *Int Braz J Urol, 42*(5), 883-896.

Wilson, D. M., Kim, D., Berquist, B. R., & Sigurdson, A. J. (2011). Variation in base excision repair capacity. *Mutat Res, 711*(1-2), 100-112. doi:10.1016/j.mrfmmm.2010.12.004

Wolanskyj, A. P., Schwager, S. M., McClure, R. F., Larson, D. R., & Tefferi, A. (2006). Essential thrombocythemia beyond the first decade: life expectancy, long-term complication rates, and prognostic factors. *Mayo Clin Proc, 81*(2), 159-166. doi:10.4065/81.2.159

Wu, B., Liu, H. L., Zhang, S., Dong, X. R., & Wu, G. (2012). Lack of an association between two BER gene polymorphisms and breast cancer risk: a meta-analysis. *PLoS One, 7*(12), e50857. doi:10.1371/journal.pone.0050857

Yan, L., Li, Q., Li, X., Ji, H., & Zhang, L. (2016). Association Studies Between XRCC1, XRCC2, XRCC3 Polymorphisms and Differentiated Thyroid Carcinoma. *Cell Physiol Biochem, 38*(3), 1075-1084. doi:10.1159/000443058

Zhai, X., Liu, J., Hu, Z., Wang, S., Qing, J., Wang, X., . . . Shen, H. (2006). Polymorphisms of ADPRT Val762Ala and XRCC1 Arg399Glu and risk of breast cancer in Chinese women: a case control analysis. *Oncol Rep, 15*(1), 247-252.

Zhang, Y., Newcomb, P. A., Egan, K. M., Titus-Ernstoff, L., Chanock, S., Welch, R., . . . Garcia-Closas, M. (2006). Genetic polymorphisms in base-excision repair pathway genes and risk of breast cancer. *Cancer Epidemiol Biomarkers Prev, 15*(2), 353-358. doi:10.1158/1055-9965.EPI-05-0653

Zhao, Z., Liu, C., Zeng, Y., Gu, L., Ying, M., Wang, N., . . . Ma, Y. (2014). The association between the APE1 Asp148Glu polymorphism and breast cancer susceptibility: a meta-analysis based on case-control studies. *Tumour Biol, 35*(5), 4727-4734. doi:10.1007/s13277-014-1618-5

Zhou, X., Wei, L., Jiao, G., Gao, W., Ying, M., Wang, N., . . . Liu, C. (2015). The association between the APE1 Asp148Glu polymorphism and prostate cancer susceptibility: a meta-analysis based on case-control studies. *Mol Genet Genomics, 290*(1), 281-288. doi:10.1007/s00438-014-0916-3

Zou, H., Li, Q., Xia, W., Liu, Y., Wei, X., & Wang, D. (2016). Association between the OGG1 Ser326Cys Polymorphism and Cancer Risk: Evidence from 152 Case-Control Studies. *J Cancer, 7*(10), 1273-1280. doi:10.7150/jca.15035

CHAPTER 9:
EFFECTS OF POLYMORPHIC DNA GENES INVOLVED IN BER AND CASPASE PATHWAYS ON THE CLINICAL OUTCOME OF MYELOPROLIFERATIVE NEOPLASMS UNDER TREATMENT WITH HYDROXYUREA

Submitted for publication in Molecular Medicine Reports

Abstract - Caspases and base excision repair genes polymorphisms and therapeutic response to hydroxyurea in Philadelphia-negative myeloproliferative neoplasms: a study in a Portuguese population.

(2017) Accepted for publication in Annals of Medicine

ABSTRACT

Several single nucleotide polymorphisms (SNPs), influencing DNA repair capacity and apoptotic status, may confer genetic predisposition to Philadelphia-chromosome negative myeloproliferative neoplasms (PN-MPNs), and influence therapeutic response and clinical course. Moreover, despite the development of more efficient drugs in the last years, some patients with PN-MPNs still progress to conditions more aggressive and difficult to treat such as myelodysplasia, myelofibrosis and acute leukaemia. The aim of this work was to help in evaluating whether SNPs in genes involved in apoptosis and base excision repair (BER) pathway and of some known risk factors in PN-MPNs may have an effect on survival and therapeutic response to hydroxyurea, the standard drug used, in terms of disease progression and predisposition to new non-myeloid neoplasms and thrombotic events in patients according to the type of MPN.

This study comprise 133 Caucasian Portuguese patients diagnosed with PN-MPNs, during the period 1992-2016, and under treatment with hydroxyurea (isolated in 104 patients and in combination with other agents in 6 patients), with a total of 17 cases with progression to myelofibrosis/leukemia, 11 who developed new non-myeloid neoplasms and 22 who presented thrombotic events. All apoptosis and BER pathway SNPs included in this study were genotyped using real-time PCR (RT-PCR 7300 Applied Biosystem), through TaqMan® SNP genotyping assays (Life Technology), according to manufacturer instructions, and were evaluated using regression analysis by SPSS 22.0 (SPSS Inc.). The informed consent of the subjects and acceptance of the study protocol by a local ethics committee has been obtained.

Concerning progression to secondary myelofibrosis/leukemia, there is an association with the survival and the exposure to cytoreductive agents. Globally, the presence of at least one variant allele carriers for CASP8 3'UTR variant is associated with a decreased effect in disease progression (OR=0.24; 95% CI, 0.08-0.69) and the presence of XRCC1 Arg194Trp variant showed a border-line effect (OR=3.58; 95%CI, 0.98-13.01) for the same outcome, representing a worse prognosis. When stratified for ET patients, the presence of at least one variant allele for CASP9 Arg173His polymorphism is associated with a worse prognosis for these patients, increasing the frequency of development of secondary myelofibrosis/leukemia (OR=11.27; 95%CI, 1.13-112.28). On the other hand, BER polymorphism APEX1 Asp148Glu showed a border-line effect for the presence of at least one variant allele representing a better prognosis (OR=0.28; 95%CI, 0.74-1.03). Conversely, an increased effect was found for XRCC1 Arg194Trp variant (OR=6.60; 95%CI, 1.60-27.06) for the progression to secondary myelofibrosis/leukemia.

Concerning the development of new nonmyeloid malignancies, globally, the presence of CASP8 Asp270His variant is associated with a worse prognosis (OR=5.90; 95%CI, 1.42-24.62) and the presence of at least one variant allele carriers for XRCC1 Arg399Gln polymorphism revealed a better prognosis for these patients concerning the same factor (OR=0.27; 95%CI, 0.07-1.03).

With regard to the presence of thrombotic events, there is a decreased incidence to develop these episodes in patients who carrier at least one variant allele carriers for XRCC1 Gln399Arg (OR=0.35; 95%CI, 0.14-0.88).

No significant change in OR was observed for any of the other genotypes considered.

According to present reported results, progression to secondary myelofibrosis/leukemia is influenced by the exposure to cytoreductive agents and caspase and BER polymorphisms. Moreover, caspase and BER polymorphisms also influence the development of new nonmyeloid malignancies. On the other hand, only BER pathway has a role in the presence of thrombotic events. According to our results, the presence of JAK2 mutation has no influence for the development of any of these complications.

Larger studies are required to confirm these results and to provide conclusive evidence of association between these and other variants with PN-MPNs therapeutic response and clinical evolution.

Identification of the main molecules that are altered in MPNs and the concurrent presence of polymorphisms/variants in key genes involved in DNA repair and cell survival (e.g. caspases) may allow the development of drugs more directly targeted to the pathophysiology of the disease, with high efficacy, fewer adverse effects, contributing to compliance of the patients with treatments. Also, the clinical indication of the classical drugs (e.g. hydroxyurea) may be guided by the variant genes which may influence its beneficial effects.

CHAPTER 9: EFFECTS OF POLYMORPHIC DNA GENES INVOLVED IN BER AND CASPASE PATHWAYS ON THE CLINICAL OUTCOME OF MYELOPROLIFERATIVE NEOPLASMS UNDER TREATMENT WITH HYDROXYUREA

9.1 – INTRODUCTION

Polycythemia vera (PV), Essential thrombocythemia (ET) and Primary myelofibrosis (PMF) are included in Philadelphia-chromosome negative myeloproliferative neoplasms (PN-MPNs).

There is an intrinsic tendency of PN-MPNs to progress to acute myeloid leukemia (AML), in occasions preceded by a phase of secondary myelofibrosis or myelodysplastic syndromes (MDS) (Mesa et al., 2016), and to concurrently present thrombohaemorhagic complications, to an extent that is not fully known (Björkholm, Hultcrantz, & Derolf, 2014; Hernández-Boluda et al., 2012) and with a life expectancy reduced compared with that in the general population (Passamonti et al., 2004; Wolanskyj, Schwager, McClure, Larson, & Tefferi, 2006).

It is well known that high doses of alkylating agents and combined cytoreductive treatments are associated with enhancement of malignant transformation in PN-MPNs (Björkholm et al., 2014; Hernández-Boluda et al., 2012), reported in the literature to occur in 5-10% of patients 10 years following the initial diagnosis, correlated with a bad prognosis (Rampal et al., 2014; Swerdlow et al., 2008).

Moreover, there are other determinant factors not related to therapy that cannot be ruled out, affecting the clinical course of these disorders and the response to therapy, namely mutational burden, polymorphic variants of several genes, ambient/dietary exposure and immune system (Beer et al., 2010; Bolufer et al., 2006; Delhommeau, Jeziorowska, Marzac, & Casadevall, 2010; Hasselbalch et al., 2014; Kilpivaara & Levine, 2008).

Another important concern refers to the tendency of development of new nonhematological and nonmyeloid neoplasms in MPNs, with an incidence ratio of 1.2-1.4 and 3.4, respectively, when compared to the general population (Hernández-Boluda et al., 2012). There is evidence that this risk is higher when *JAK2* V617F mutation is present and other patient factors may be also involved, although the association with cytoreductive therapy is not so well established (Hernández-Boluda et al., 2012; Santoro et al., 2017).

Reported thromboembolic complications range from 7% to 57% at diagnosis and rise to 41%-91% during follow-up, and arterial thrombotic events being much more common than venous ones in both PV and ET (Andıç et al., 2016).

Thrombotic and haemorrhagic events are observed in up to 39% and 39.6% of PV patients, respectively (Griesshammer, Gisslinger, & Mesa, 2015). The cumulative rate of nonfatal thrombosis in PV is 3.8 events per 100 persons per year, and in ET the rate of fatal and nonfatal thrombotic events ranges from 2% to 4% per 100 persons per year (Andıç et al., 2016). PMF seems less susceptible for thrombotic events, with a cumulative percentage of 2.23% per 100 persons per year. Age, previous thrombosis, leukocytosis and the presence of *JAK2* mutation are known risk factors for thrombosis occurrence in MPNs (Andıç et al., 2016). Extreme thrombocytosis (count over 1000 or 1500x109/L) is found to be related to haemorrhagic complications but not thrombosis, due to induced reduction of high-molecular-weight von Willebrand factor levels (Andıç et al., 2016).

On the other hand, several single nucleotide polymorphisms (SNPs) at various loci, influencing DNA repair capacity and apoptotic status, and additional somatic genetic effects may confer genetic predisposition to PN-MPNs (A. P. Azevedo et al., 2017), influencing phenotype definition and determining therapeutic response (Bănescu et al., 2014; Campregher, Santos, Perini, & Hamerschlak, 2012; Delhommeau et al., 2010; Levine, 2009; Nangalia et al., 2013; Tefferi & Pardanani, 2015; Tognon, Nunes, & Castro, 2013; Wang et al., 2014). Moreover, despite the development of more efficient drugs in the last years, some patients with PN-MPNs still evidence disease progression to conditions more aggressive and difficult to treat (Björkholm et al., 2014; Mesa et al., 2016).

As a matter of fact, DNA damage induced to haematopoietic precursor cells would appear to be crucial for leukemic transformation, despite DNA repair systems act to repair the DNA lesions, thus maintaining genetic integrity (Batar, Güven, Bariş, Celkan, & Yildiz, 2009; Baute & Depicker, 2008; Bănescu et al., 2014). Several polymorphisms in DNA repair genes have been associated to protein dysfunction, compromising DNA damage repair (Batar et al., 2009; Bolufer et al., 2006; Bănescu et al., 2014; Hoeijmakers, 2001; Wang et al., 2014).

Previous data described in the literature reported the identification and study of various SNPs in genes involved in the BER pathway [*APEX1*, mutY DNA glycosylase, earlier mutY homolog (*E. coli*) (*MUTYH*), *OGG1*, *PARP1*, *PARP4* and *XRCC1*] for their association with progression to leukaemia and disease outcome (Annamaneni et al., 2013; Bolufer et al., 2006; Bănescu et al., 2014) in patients with ET and PV. Likewise, there is also evidence that a nucleotide excision repair gene polymorphism is strongly associated with leukaemic transformation and development of non-myeloid malignancies in these disorders (Hernández-Boluda et al., 2012).

On the other hand, apoptosis is considered as the most important pathway of cell death, through regulatory proteins, the caspases, organized in both intrinsic and extrinsic pathways, acting as a defense mechanism against damaged, stressed, or stimulated cells by any agents, preventing accumulation of non-functional cells in the tissues.

The haematopoietic system is subject to a high cellular turnover rate, which makes it particularly sensitive to disturbance in the apoptosis process (Zaman, Wang, & Gandhi, 2014). Altered control of pro- and anti-apoptotic genes and in the relation with *JAK2* or *STAT5* signaling routes, seem to lead to myeloaccumulation and myeloproliferation, participating in the pathogenesis of MPNs (Malherbe et al.,

2016; Nunes et al., 2013; Olsson & Zhivotovsky, 2011; Testa, 2004; Tognon et al., 2011; Tognon et al., 2012; Tognon et al., 2013; Tognon et al., 2016).

Since the end of 60's, several groups as the American Polycythemia Vera study Group (PVSG) and the Italian group for hematological diseases in adults (GIMEMA) devoted their efforts to the study of natural history of PV, ET, and PMF and the best treatment options (Tefferi, 2016).

Nowadays therapeutic goals in PV and ET are mostly directed to prevention of thrombohemorrhagic complications and relieve of symptoms, without curative potential nor capacity of prolonging life or preventing disease progression (Griesshammer et al., 2015). Drug therapy towards PMF intend especially to control symptoms and splenomegaly, with the possibility of remission in a limited number of patients undergoing allogeneic stem cell transplant (ASCT) (Rumi & Cazzola, 2017; Tefferi, 2016).

According to contemporary treatment algorithms, based on risk stratification (Tefferi, 2016), all the patients with PV require phlebotomies to a hematocrit target of <45%. In PV and ET patients it is very important to identify those whose risk of vascular and thrombotic complications is high enough to justify the use of risk-adapted treatment strategies, including aspirin, anticoagulants, hydroxyurea (HU), anagrelide, interferon alfa (IFN-alpha) or the recent JAK2 inhibitors (eg. Ruxolitinib) (Tefferi, 2016). Patients are stratified and treatment is prescribed according to evaluation of the three major risk factors for thrombosis, namely previous history of arterial or venous thrombosis, eventual presence of *JAK2* mutation and age >60 years (Tefferi, 2016; Treliński, Chojnowski, Cebula-Obrzut, & Smolewski, 2012). The risk of thrombosis in older *JAK2*-unmutated without thrombosis history the risk is low enough to occasionally consider, on an individual basis, skipping cytoreductive therapy (Tefferi, 2016).

HU is widely used as first-line cytoreductive therapy in "high risk" patients, as result of consensus of randomized clinical trials (Rumi & Cazzola, 2017; Treliński et al., 2012). This drug is an antimetabolite, capable of inhibition of ribonucleoside diphosphate reductase necessary for synthesis and repair of DNA, with a subsequent myelosuppressive activity (Treliński et al., 2012). Moreover, HU affects polymorphonuclear leukocytes function and interferes in their interactions with platelets, having an antithrombotic effect (Treliński et al., 2012). Several authors have shown that HU can also induce apoptosis in many types of cells (endothelial cells, human mesenchymal stem cells and mouse embryonic stem cells), promoting cell death by regulating the expression levels of Bcl-2 and the tumor suppressor p53 protein (Treliński et al., 2012).

However, patients treated with HU can develop intolerance or become resistant to therapy, leading to an increased risk of death and progression to secondary myelofibrosis (Griesshammer et al., 2015).

An alternative to first-line treatment is INF-alpha, which has antiproliferative effects on hematopoietic primordial cells, reduce *JAK2* V617F allele burden and induce cytogenetic remission, but it also associated with intolerable adverse effects, leading to noncompliance and therapy discontinuation (Griesshammer et al., 2015).

Second line cytoreductive therapy include busulfan or ^{32}P, but these agents have been associated with a very high propensity to leukemic transformation, being reserved to very precise situations (Griesshammer et al., 2015).

The discovery of *JAK2* mutation led to the study and development of more targeted agents with the propose of JAK2 inhibition (ex: ruxolitinib), revealing better tolerability, clinical and quality of life improvement and increased survival (Griesshammer et al., 2015).

In this study, we intend to characterize the way as SNPs in caspase and base excision repair (BER) genes might be relevant in PN-MPNs patients' survival and therapeutic response concerning their role in disease progression and risk predisposition to new non-myeloid neoplasms and thrombotic events.

9.2 – MATERIALS AND METHODS

9.2.1 – STUDY SUBJECTS

The present study involves 133 Caucasian Portuguese patients diagnosed with PN-MPNs (80 with ET, 39 with PV and 14 with PMF), during the period of 1992-2016, for evaluation of the predisposition to fibrotic/leukemic progression, development of new primary non-myeloid neoplasms and thrombotic events. Among these patients, 104 were medicated with isolated HU and 6 with HU in combination with other agents.

The study population included a total of 17 cases of ET/PV with fibrotic/leukemic (2^{ry} MF/AML) progression and 76 ET/PV patients who did not progress, 11 cases of ET/PV/PMF who developed new primary non-myeloid neoplasms and 30 patients who did not and 22 cases of ET/PV/PMF who presented thrombotic events and 56 patients who did not present this type of event.

For each case patient 1 to several control patients were selected from the group who did not presented the event in question. Cases and controls were matched for the type of PN-MPN and duration of follow-up, ensuring that controls were followed at least for the same amount of time than the matched case, having the same chance to develop the event.

The patients were selected within the Portuguese population, with Portuguese ascendants, recruited in the Departments of Clinical Hematology and of Clinical Pathology, Hospital of S. Francisco Xavier, West Lisbon Hospital Centre, a public general hospital that provides health care to the western population of Lisbon, where those patients were followed and treated. Diagnosis criteria for all patients were those updated by the World Health Organization (Arber et al., 2016; Tefferi & Vardiman, 2008) and all clinical, hematologic and treatment data were obtained from registries.

A written informed consent was obtained from all those involved, prior to blood withdrawal, in agreement with the Declaration of Helsinki. The blood samples were coded to guarantee anonymity.

This study was also conducted with approval by the institutional ethics' boards of the involved institutions.

General characteristics for PN-MPNs patients at time of diagnosis and related to disease outcome are summarized in Table 9.1.

9.2.2 – DNA EXTRACTION

The methodology used is described in Chapter 3, section 3.2.2.

9.2.3 – SNP SELECTION (CASPASES AND BASE EXCISION REPAIR PATHWAY)

The appropriate SNPs analized in the present study were selected according the described in Chapters 7 and 8, sections 7.2.3 and 8.2.3 (Table 9.2).

The selected apoptosis polymorphisms were rs2227309 and rs2227310 (*CASP7*), rs1045485 and rs1035142 (*CASP8*), rs2308950, rs1132312 and rs1052571 (*CASP9*) and rs13006529 (*CASP10*) and BER pathway polymorphisms (A. P. Azevedo et al., 2017) were rs1130409 (APEX1), rs3219489 (MUTYH), rs1052133 (OGG1), rs1136410 (PARP1), rs13428 and rs1050112 (PARP4), and rs1799782 and rs25487 (XRCC1) (Table 9.2).

9.2.4 – GENOTYPING

The apoptosis and BER polymorphisms were genotyped and *JAK2* V617F (rs77375493) mutational status was determined using the same methodology described in Chapter 3, section 3.2.3

The SNP genotyping assay information for apoptosis and BER polymorphisms (A. P. Azevedo et al., 2017), according to the event in question and aspect to analyze is summarized in Tables 9.3-9.7.

JAK2 V617F mutational status (A. P. Azevedo et al., 2017) is summarized in Table 9.1.

9.2.5 – STATISTICAL ANALYSIS

The analysis of Hardy-Weinberg frequencies for all alleles in the control and patient populations was carried out using exact probability tests available in SNPStat software. Differences in genotype frequency, gender, hematological values, therapeutic and pathology distributions, progression to secondary 2^{ry} MF/AML, development of a new primary nonmyeloid malignancy and thrombotic events

occurrence distributions between PN-MPNs patient cases and controls were evaluated by the Chi-Square (χ^2) test.

Hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated for each variable using the Cox univariate model. For the purpose of these calculations, the association between selected SNPs and their effect over 2^{ry} MF/AML progression, was evaluated using logistic regression conditional for the matched cases and controls. The covariates selected, including clinic-laboratorial data at the time of MPN diagnosis, *JAK2* mutational status and therapy related aspects (exposure to HU and/or other cytoreductive agents), were reported in the literature from previous studies as associated with disease progression (Griesshammer et al., 2015; Hernández-Boluda et al., 2012).

Because there were few patients who received other therapy than HU, exposure to cytoreductive therapy was considered as "no exposure" (including IFN or anagrelide isolated, as these drugs are not leukemogenic), "HU only" and "other agents alone or in combination".

All analyses were performed using the Statistical Package for the Social Sciences for Windows 22.0 version (SPSS, Inc.) (Tables 9.3-9.7). Since this is not a conclusive final study but an exploratory one on the role of apoptosis and BER pathway polymorphisms and some known risk factors in PN-MPNs clinical outcome, and the data to be obtained should be looked at as proof of concept, the Bonferroni adjustment was deemed as not necessary as it is too conservative.

9.3 – RESULTS

9.3.1 – GENERAL CHARACTERISTICS OF PATIENTS AND SURVIVAL

This study included 133 PN-MPNs patients, whose general characteristics are summarized in Table 9.1. According to diagnosis criteria patients' distribution was as follows: 80 (60.2%) with ET, 39 (29.3%) with PV and 14 (10.5%) with PMF.

Table 9.1 – Characteristics of patients according to the type of MPN.

Characteristic	ET	PV	PMF
No. of patients	80	39	14
Sex, male/female, n/%	32 (40.0) / 48 (60.0)	20 (51.3) / 19 (48.7)	9 (64.3) / 5 (35.7)
Age, y[a]	66 (33-100)	69 (46-96)	73 (55-84)
Hb, g/L[a]	131 (85-168)	165 (129-213)	114 (74-151)
HTC	0.4 (0.261-0.498)	0.5 (0.429-0.660)	0.4 (0.218-0.491)
WBC, x10^9/L[a]	10.0 (2.7-26.6)	12.7 (4.5-33.5)	10.8 (2.2- 17.7)
Platelets, x10^9/L[a]	777 (241-2485)	411 (164-1316)	232 (34-461)

***JAK2* V617F mutation, n(%)**			
Val/Val	21 (25.6)	5 (12.8)	7 (50.0)
Val/Phe	56 (70.9)	31 (79.5)	5 (35.7)
Phe/Phe	2 (2.5)	3 (7.7)	2 (14.3)
Exposure to cytoreductive agentes, n (%)			
No exposure	8 (10.1)	5 (12.8)	5 (35.7)
HU only	65 (82.3)	33 (84.6)	6 (42.9)
HU+other agents	6 (7.6)	0 (0.0)	0 (0.0)
Other agents	0 (0.0)	1 (2.6)	3 (21.4)
Follow-up, y[a]	8.6 (1-25)	7.4 (1-17)	6.0 (1-15)
Death[b]**, n (%)**	12 (54.5)	7 (31.8)	3 (13.6)
≤ 5 years	7 (58.3)	2 (28.6)	3 (100.0)
6–10 years	0 (0.0)	3 (42.9)	0 (0.0)
10–20 years	5 (41.7)	2 (28.6)	0 (0.0)

[a]Median (range)
[b]*P*=0.044; *P*=0.146; OR, 95% CI: 1.42 (0.89-2.28)

Globally, the study included 72 (54.1%) females and 61 (45.9%) male patients, with an overall mean age of 68 years, in agreement with the gender distribution usually observed in this type of pathology.

Mean values for laboratorial data at time of diagnosis for each disorder are listed in Table 9.1, reflecting the different clinical-hematological pattern characteristic of each one of them. Hemoglobin was significantly higher in PV than in other groups and significantly lower in PMF than in other groups. Platelet count was higher in ET than in other groups.

Concerning the presence of *JAK2* V617F mutation, 73.4% of ET, 87.2% of PV and 50.0% of PMF patients were positive (Table 9.1).

Hydroxyurea was the first choice drug in the majority of ET, PV, and PMF cases, used alone in 104 patients and in combination with other agents in 6 patients. Anagrelide and interferon-alpha were used in a minority of patients. Acetylsalicylic acid was used in approximately 35% of both PV and ET cases (Table 9.1).

Patients were followed up for a mean of 7.6 years. Myelofibrosis patients presented the shorter survival, with the majority of them dying less than five years after diagnosis, followed by PV (20.5%) and ET (13.8%) patients (Table 9.1).

Survival is influenced by progression to 2^{ry}MF/AML and the presence of *JAK2* V617F mutation (Table 9.3). None of the other evaluated factors evidenced to influence PN-MPNs survival.

The characteristics of each SNP under study are described in Table 9.2, while the genotype frequencies and therapeutic distribution determined according to survival are shown in Table 9.3.

Table 9.2 – Selected caspases and BER pathway SNPs and detailed information on the corresponding base and amino acid exchanges as well as MAF.

Gene	dsSNP	Codon	Nucleotide exchange	Minor allele frequency (%)[a]
Caspases SNPs				
CASP7	rs2227309	249	G→A (Arg/ Lys)	28.0
	rs2227310	255	C→G (Asp/Glu)	29.
CASP8	rs1045485	270	G→C (Asp/His)	13.0
	rs1035142	---	G→T 3'UTR	49.0
CASP9	rs2308950	173	G→A (Arg/His)	4.0
	rs1132312	136	C→T (Phe/Phe)	49.6
	rs1052571	28	C→T (Ala/Val)	49.6
CASP10	rs13006529	522	A → T (Ile/Leu)	44.0
BER SNPs				
APEX1	rs1130409	148	T→G (Asp/Glu)	44.0
MUTYH	rs3219489	335	G→C (Gln/His)	31.9
OGG1	rs1052133	326	C→G (Ser/Cys)	29.9
PARP1	rs1136410	762	T→C (Val/Ala)	24.4
PARP4	rs13428	1,280	G→C (Gly/Arg)	45.8
	rs1050112	1,328	C→A (Pro/Thr)	45.8
XRCC1	rs1799782	194	C→T (Arg/Trp)	13.1
	rs25487	399	G→A (Arg/Gln)	26.6

[a]According to http://www.ncbi.nlm.nih.gov/snp/.
MAF, minor allele frequency; APEX1, apurinic/apyrimidinic endonuclease 1; MUTYH, mutY DNA glycosylase, earlier mutY homolog (*E. coli*); OGG1, 8-oxoguanine DNA glycosylase 1; PARP, poly (ADP-ribose) polymerase; XRCC1, X-ray repair cross-complementing 1.

Table 9.3 – Factors influencing survival.

	Alive (n=109	Died: ≤ 5 years (n=12)	Died: 6– 10 years (n=6)	Died: 10-20 years (n=6)	*P*[a]
Cases (n=17) with progression to 2[ry] MF/AML, n (%)	10 (58.8)	2 (11.8)	0 (0.0)	5 (29.4)	**<0.001**
Cases with non-myeloid neoplasms (n=11), n (%)	7 (63.6)	2 (18.2)	1 (9.1)	1 (9.1)	0.720
Cases with thrombotic events (n=22), n (%)	16 (72.7)	2 (9.1)	2 (9.1)	2 (9.1)	0.088
Exposure to cytoreductive agentes, n (%)					0.205
No exposure	13 (12.0)	2 (16.7)	3 (50.0)	0 (0.0)	
HU only	88 (81.5)	8 (66.7)	3 (50.0)	5 (83.3)	
HU+other agents	4 (3.7)	1 (8.3)	0 (0.0)	1 (16.7)	
Other agents	3 (2.8)	1 (8.3)	0 (0.0)	0 (0.0)	
***JAK2* V617F mutation genotype, n (%)**					**0.009**
Val/Val	26 (24.1)	5 (41.7)	2 (33.3)	0 (0.0)	
Val/Phe	79 (73.1)	6 (50.0)	2 (33.3)	5 (83.3)	
Phe/Phe	3 (2.8)	1 (8.3)	2 (33.3)	1 (16.7)	
Caspases SNPs					
CASP7 (Arg249Lys; rs2227309)					0.902
Arg/Arg	63 (57.8)	6 (50.0)	4 (66.7)	4 (66.7)	
Arg/Lys	40 (36.7)	6 (50.0)	2 (33.3)	2 (33.3)	
Lys/Lys	6 (5.5)	0 (0.0)	0 (0.0)	0 (0.0)	
CASP7 (Asp255Glu; rs2227310)					0.883
Asp/Asp	58 (53.7)	6 (50.0)	4 (66.7)	4 (66.7)	
Asp/Glu	43 (39.8)	6 (50.0)	2 (33.3)	2 (33.3)	
Glu/Glu	7 (6.5)	0 (0.0)	0 (0.0)	0 (0.0)	
CASP8 (3'UTR G>T; rs1035142)					0.668
G/G	41 (37.6)	5 (41.7)	3 (50.0)	1 (16.7)	
G/T	51 (46.8)	4 (33.3)	3 (50.0)	3 (50.0)	
T/T	17 (15.6)	3 (25.0)	0 (0.0)	2 (33.3)	
CASP8 (Asp270His; rs1045485)					0.570
Asp/Asp	82 (75.9)	9 (75.0)	5 (83.3)	5 (83.3)	
Asp/His	22 (20.4)	3 (25.0)	0 (0.0)	1 (16.7)	
His/His	4 (3.7)	0 (0.0)	1 (16.7)	0 (0.0)	
CASP9 (Arg173His; rs2308950)					0.989
Arg/Arg	105 (96.3)	12 (100.0)	6 (100.0)	6 (100.0)	
Arg/His	3 (2.8)	0 (0.0)	0 (0.0)	0 (0.0)	
His/His	1 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	
CASP9 (Phe136Phe; rs1132312)					0.902
TTC/TTC	25 (22.9)	3 (25.0)	2 (33.3)	1 (16.7)	
TTC/TTT	60 (55.0)	6 (50.0)	4 (66.7)	4 (66.7)	
TTT/TTT	24 (22.0)	3 (25.0)	0 (0.0)	1 (16.7)	
CASP9 (Ala28Val; rs1052571)					0.913
Ala/Ala	21 (19.3)	3 (25.0)	0 (0.0)	1 (16.7)	
Ala/Val	60 (55.0)	6 (50.0)	4 (66.7)	4 (66.7)	
Val/Val	28 (25.7)	3 (25.0)	2 (33.3)	1 (16.7)	
CASP10 (Ile522Leu; rs13006529)					0.611
Ile/Ile	29 (26.6)	6 (50.0)	1 (16.7)	2 (33.3)	
Ile/Leu	55 (50.5)	3 (25.0)	4 (66.7)	3 (50.0)	
Leu/Leu	25 (22.9)	3 (25.0)	1 (16.7)	1 (16.7)	
BER SNPs					
APEX1 (Asp148Glu; rs1130409)					0.141
Asp/Asp	28 (25.7)	2 (16.7)	3 (50.0)	4 (66.7)	
Asp/Glu	55 (50.5)	5 (41.7)	3 (50.0)	1 (16.7)	
Glu/Glu	26 (23.9)	5 (41.7)	0 (0.0)	1 (16.7)	

MUTYH (Gln335His; rs3219489)					0.381
His/His	59 (54.1)	6 (50.0)	1 (16.7)	2 (33.3)	
His/Gln	41 (37.6)	5 (41.7)	3 (50.0)	3 (50.0)	
Gln/Gln	9 (8.3)	1 (8.3)	2 (33.3)	1 (16.7)	
OGG1 (Ser326Cys; rs1052133)					0.837
Ser/Ser	67 (61.5)	8 (66.7)	5 (83.3)	3 (50.0)	
Ser/Cys	34 (31.2)	3 (25.0)	1 (16.7)	3 (50.0)	
Cys/Cys	8 (7.3)	1 (8.3)	0 (0.0)	0 (0.0)	
PARP1 (Val762Ala; rs1136410)					0.174
Val/Val	89 (81.7)	8 (66.7)	4 (66.7)	3 (50.0)	
Val/Ala	20 (18.3)	4 (33.3)	2 (33.3)	3 (50.0)	
Ala/Ala	-	-	-	-	
PARP4 (Gly1280Arg; rs13428)					0.506
Gly/Gly	45 (41.3)	4 (33.3)	4 (66.7)	1 (16.7)	
Gly/Arg	51 (46.8)	6 (50.0)	1 (16.7)	3 (50.0)	
Arg/Arg	13 811.9)	2 (16.7)	1 (16.7)	2 (33.3)	
XRCC1 (Arg194Trp; rs1799782)					0.211
Arg/Arg	93 (85.3)	11 (91.7)	6 (100.0)	4 (66.7)	
Arg/Trp	7 (6.4)	1 (8.3)	0 (0.0)	2 (33.3)	
Trp/Trp	9 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	
XRCC1 (Gln399Arg; rs25487)					0.292
Arg/Arg	38 (34.9)	6 (50.0)	5 (83.3)	3 (50.0)	
Arg/Gln	48 (44.0)	5 (41.7)	1 (16.7)	2 (33.3)	
Gln/Gln	23 (21.1)	1 (8.3)	0 (0.0)	1 (16.7)	

*Median (range)
**Patients who received IFN or anagrelide as the only cytoreductive drugs were included in the "no exposure" group
[a] *P*value determined by $\chi 2$ test
APEX1, apurinic/apyrimidinic endonuclease 1; MUTYH, mutY DNA glycosylase, earlier mutY homolog (*E. coli*); OGG1, 8-oxoguanine DNA glycosylase 1; PARP, poly (ADP-ribose) polymerase; XRCC1, X-ray repair cross-complementing 1; OR, odds ratio; CI, confidence interval; ND, none determined.

9.3.2 – ANALYSIS OF PATIENTS WHO PROGRESSED TO SECONDARY MF/AML

The study included 17 patients with PV (8 patients) or ET (9 patients) who progressed to 2^{ry}MF/AML, corresponding to 20.5% and 11.2% of each disorder population respectively, and 76 controls who did not (Table 9.1 and 9.4).

The characteristics of these cases/controls patients are summarized in Table 9.4.

Median follow-up time for diagnosis of progression in cases patients was 7.6 years, ranging from 1 to 18 years. There was no significant difference in age among cases and controls patients (median of 67.7 years), but the first ones had higher WBC counts at presentation and displayed more frequently *JAK2* mutation. *JAK2* V617F mutation was also tested, but no association was found with the propensity of leukemic transformation (Table 9.4). Regarding the number of individuals with marked thrombocytosis (>1000x10^9/L), there is no significant difference among cases and controls patients (Table 9.4).

Globally, there is an association with progression to 2^{ry} MF/AML and the exposure to cytoreductive agents (Table 9.4). Twelve of the total patients who progressed to 2^{ry}MF/AML were medicated with HU only (Table 9.4). The result obtained in the group of patients medicated with "other agents" than HU is not statistically relevant because it represents typical statistical error type I.

Genotypes distribution was found to be in Hardy-Weinberg equilibrium.

The presence of at least one variant allele carriers for *CASP8* 3'UTR variant is associated with a lower effect in disease progression to 2ry MF/AML (OR=0.24; 95%CI, 0.08-0.69) and the presence of *XRCC*1 Arg194Trp variant showed a border-line effect (OR=3.58; 95%CI, 0.98-13.01) (Tables 9.4 and 9.7) suggesting a higher risk of developing 2ry MF/AML, representing a worse prognosis.

When stratified for ET patients, the presence of at least one variant allele carriers for *CASP9* Arg173His polymorphisms is associated with a worse effect in progression to 2ry MF/AML (OR=11.27; 95%CI, 1.13-112.28). While for BER polymorphisms the *APEX1* Asp148Glu showed a border-line effect related to a probable better prognosis concerning the progression to 2ry MF/AML for patients who present at least one variant allele (OR=0.28; 95%CI, 0.74-1.03), an increased effect in disease progression was found for *XRCC*1 Arg194Trp variant (OR=6.60; 95%CI, 1.60-27.06), which might be related to a worse prognosis (Table 9.7).

No significant change in OR was observed for any of the other genotypes considered (Tables 9.4 and 9.7).

Table 9.4 – Factors investigated for their association with fibrotic/leukemic progression in ET and PV. Distribution in case patients who progressed to 2ry MF/AML and control patients who did not.

Characteristic	Progression to 2ry MF/AML Cases (n=17)	Non progression Controls (n=76)	*P*[a]	OR[b] (95% CI)
ET/PV, n/%	9 (52.9)/8 (47.1)	54 (71.1)/22 (28.9)	0.149	--
Sex, male/female, n/%	8 (47.1)/9 (52.9)	31 (40.8)/45 (59.2)	0.636	1.25 (0.46-3.42)
Age, y*	67.3 (46-91)	68.0 (37-100)	0.231	1.01 (0.97-1.05)
Years to progression*	7.6 (1-18)	--	--	--
WBC*				
>10 x10^9/L*	9 (52.9)	33 (43.4)	--	--
>15 x10^9/L*	4 (23.5)	12 (15.8)	--	--
PLT >1000x10^9/L	4 (23.5)	16 (21.1)	--	--
***JAK2* V617F mutation, n/%**			0.716	
Val/Val	2 (11.8)	15 (19.7)		1 (Reference)
Val/Phe	14 (82.4)	58 (76.3)		1.59 (0.36-7.09)
Phe/Phe	1 (5.9)	3 (3.9)		0.69 (0.15-18.29)
Exposure to cytoreductive agentes**			**0.011**	
No exposure	1 (5.9)	9 (11.8)		1 (Reference)
HU only	12 (70.6)	65 (85.5)		1.12 (0.14-8.82)
HU+other agents	3 (17.6)	2 (2.6)		6.04 (0.61-60.15)
Other agents	1 (5.9)	0 (0.0)		29.41 (1.64-528.37)[c]
Caspases SNPs				
CASP7 (Arg249Lys; rs2227309)			0.201	
Arg/Arg	6 (35.3)	45 (59.2)		1 (Reference)
Arg/Lys	10 (58.8)	28 (36.8)		2.43 (0.83-7.13)
Lys/Lys	1 (5.9)	3 (3.9)		4.07 (0.47-35.31)
Arg/Lys+Lys/Lys	11 (64.7)	31 (40.7)		2.53 (0.88-7.28)
CASP7 (Asp255Glu; rs2227310)			0.353	
Asp/Asp	1 (5.9)	3 (4.0)		1 (Reference)
Asp/Glu	10 (58.8)	31 (41.3)		3.74 (0.43-32.53)
Glu/Glu	6 (35.3)	41 (54.7)		2.09 (0.71-6.14)
Asp/Glu+Glu/Glu	16 (94.1)	72 (96.0)		2.18 (0.76-6.30)

CASP8 (3'UTR; rs1035142)			0.084	
G/G	10 (58.8)	23 (30.3)		1 (Reference)
G/T	5 (29.4)	39 (51.3)		0.36 (0.08-1.64)
T/T	2 (11.8)	14 (18.4)		**0.19 (0.05-0.70)[d]**
G/T + T/T	7 (41.2)	53 (69.7)		**0.24 (0.08-0.69)[e]**
CASP8 (Asp270His; rs1045485)			0.468	
Asp/Asp	12 (70.6)	59 (78.7)		1 (Reference)
Asp/His	4 (23.5)	15 (20.0)		1.90 (0.58-6.21)
His/His	1 (5.9)	1 (1.3)		4.45 (0.55-35.73)
Asp/His+His/His	5 (29.4)	16 (21.3)		2.14 (0.71-6.44)
CASP9 (Arg173His; rs2308950)			0.710	
Arg/Arg	16 (94.1)	73 (96.1)		1 (Reference)
Arg/His	1 (5.9)	2 (2.6)		ND
His/His	0 (0.0)	1 (1.3)		4.24 (0.53-34.09)
Arg/His+His/His	1 (5.9)	3 (3.9)		3.31 (0.41-26.77)
CASP9 (Phe136Phe; rs1132312)			0.119	
TTC/TTC	7 (41.2)	16 (21.1)		1 (Reference)
TTC/TTT	9 (52.9)	43 (56.6)		0.51 (0.18-1.43)
TTT/TTT	1 (5.9)	17 (22.3)		0.16 (0.02-1.35)
TTC/TTT+TTT/TTT	10 (58.8)	60 (78.9)		0.42 (0.15-1.14)
CASP9 (Ala28Val; rs1052571)			0.261	
Val/Val	7 (41.2)	19 (25.0)		1 (Reference)
Val/Ala	9 (52.9)	43 (56.6)		0.61 (0.22-1.69)
Ala/Ala	1 (5.9)	14 (18.4)		0.25 (0.03-2.02)
Val/Ala +Ala/Ala	10 (58.8)	57 (75.0)		0.52 (0.19-1.42)
CASP10 (Ile522Leu; rs13006529)			0.461	
Ile/Ile	7 (41.2)	20 (26.3)		1 (Reference)
Ile/Leu	6 (35.3)	36 (47.4)		0.62 (0.20-1.92)
Leu/Leu	4 (23.5)	20 (26.3)		0.70 (0.19-2.52)
Ile/Leu+Leu/Leu	10 (61.8)	56 (73.7)		0.65 (0.23-1.80)
BER SNPs				
APEX1 (Asp148Glu; rs1130409)			0.128	
Asp/Asp	6 (35.3)	17 (22.4)		1 (Reference)
Asp/Glu	5 (29.4)	43 (56.6)		0.33 (0.09-1.16)
Glu/Glu	6 (35.3)	16 (21.0)		1.12 (0.35-3.59)
Asp/Glu+Glu/Glu	11 (64.7)	59 (77.6)		0.55 (0.20-1.55)
MUTYH (Gln335His; rs3219489)			0.315	
His/His	2 (11.8)	5 (6.6)		1 (Reference)
His/Gln	9 (52.9)	29 (38.2)		1.43 (0.48-4.27)
Gln/Gln	6 (35.3)	42 (55.3)		1.64 (0.33-8.20)
His/Gln+Gln/Gln	15 (88.2)	71 (93.5)		1.47 (0.52-4.15)
OGG1 (Ser326Cys; rs1052133)			0.861	
Ser/Ser	1 (5.9)	7 (9.2)		1 (Reference)
Ser/Cys	6 (35.3)	23 (30.3)		1.46 (0.51-4.15)
Cys/Cys	10 (58.8)	46 (60.5)		0.95 (0.12-7.60)
Ser/Cys+Cys/Cys	16 (94.1)	69 (90.8)		1.35 (0.50-3.68)
PARP1 (Val762Ala; rs1136410)			0.456	
Val/Val	12 (70.6)	60 (78.9)		1 (Reference)
Val/Ala	5 (29.4)	16 (21.1)		1.37 (0.47-3.99)
Ala/Ala	0 (0.0)	0 (0.0)		-
Val/Ala+Ala/Ala	-	-		-
PARP4 (Gly1280Arg; rs13428)			**0.019**	
Gly/Gly	5 (29.4)	35 (46.1)		1 (Reference)
Gly/Arg	6 (35.3)	34 (44.7)		1.12 (0.34-3.68)
Arg/Arg	6 (35.3)	7 (9.2)		2.78 (0.78-9.98)
Gly/Arg+Arg/Arg	12 (70.6)	41 (53.9)		1.50 (0.51-4.41)
XRCC1 (Arg194Trp; rs1799782)			0.099	
Arg/Arg	14 (82.4)	64 (84.2)		1 (Reference)
Arg/Trp	3 (17.6)	4 (5.3)		**3.58 (0.98-13.01)[f]**
Trp/Trp	0 (0.0)	8 (10.5)		ND
Arg/Trp+Trp/Trp	3 (17.6)	12 (15.8)		1.68 (0.47-6.06)

XRCC1 (Gln399Arg; rs25487)			0.911	
Arg/Arg	6 (35.3)	31 (40.8)		1 (Reference)
Arg/Gln	8 (47.1)	32 (42.1)		1.10 (0.37-3.30)
Gln/Gln	3 (17.6)	13 (17.1)		1.25 (0.30-5.29)
Arg/Gln+Gln/Gln	11 (64.7)	45 (59.2)		1.14 (0.40-3.20)

*Median (range)
**Patients who received IFN or anagrelide as the only cytoreductive drugs were included in the "no exposure" group
[a] *P* value determined by $\chi 2$ test
[b] *P* determined by conditional logistic regression
[c] *P*=0.022; [d] *P*=0.013; [e] P= 0.009; [f] *P*= 0.053 (borderline effect)
APEX1, apurinic/apyrimidinic endonuclease 1; MUTYH, mutY DNA glycosylase, earlier mutY homolog (*E. coli*); OGG1, 8-oxoguanine DNA glycosylase 1; PARP, poly (ADP-ribose) polymerase; XRCC1, X-ray repair cross-complementing 1; OR, odds ratio; CI, confidence interval; ND, none determined.

9.3.3 – ANALYSIS OF PATIENTS WHO DEVELOPED NEW NONMYELOID MALIGNANCIES

The study included 11 ET/PV/PMF patients who developed a new primary nonmyeloid malignancy during follow-up and 30 control patients who did not (Tables 9.1 and 9.5). One of these case patients developed a lymphoid malignancy and the others developed solid organ new malignancies, the most affected being lung, thyroid, adrenal gland and digestive tract.

The characteristics of these cases/controls patients are summarized in Table 9.5.

There is a predominance of male patients among cases, but there is no significant difference in age among cases and controls patients (median of 70.1 years) (Table 9.5).

PMF patients present the highest incidence of new primary nonmyeloid malignancies (21.4% against 10.2 and 5.0% of PV and ET patients, respectively) (Tables 9.1 and 9.5).

No significant association with exposure to cytoreductive agents or the presence of *JAK2* mutation was found (Table 9.5).

Globally, the presence of *CASP8* Asp270His variant allele is associated with an increased incidence to develop new non-myeloid malignancies (OR=5.90; 95%CI, 1.42-24.62), while the presence of at least one variant allele carriers for *XRCC*1 Arg399Gln (OR=0.27; 95%CI, 0.07-1.03) showed a border-line effect related to a decreased incidence to develop new non-myeloid malignancies (Tables 9.5 and 9.7).

Genotypes distribution was found to be in Hardy-Weinberg equilibrium.

No significant change in OR was observed for any of the other genotypes considered.

Table 9.5 – Factors investigated for their association with new nonmyeloid malignancy in ET, PV and PMF.
Distribution in case patients who developed a new nonmyeloid cancer and control patients who did not.

Characteristic	Development of new nonmyeloid malignancy Cases (n=11)	No development Controls (n=30)	*P*[a]	OR[b] (95% CI)
ET/PV/PMF, n/%	4 (36.4)/4 (36.4)/ 3 (27.3)	17 (56.7)/7 (23.3)/ 6 (20.0)	0.509	--
Sex, male/female, n/%	8 (72.7)/3 (27.3)	17 (56.7)/13 (43.3)	0.350	--
Age, y*	70.3 (60-84)	69.9 (45-100)	0.552	--
***JAK2* V617F mutation, n/%**			**0.027**	
Val/Val	5 (45.5)	3 (10.0)		1 (Reference)
Val/Phe	6 (54.5)	23 (76.7)		0.62 (0.18-2.07)
Phe/Phe	0 (0.0)	4 (13.3)		ND
Exposure to cytoreductive agentes**			0.649	
No exposure	1 (9.1)	5 (16.7)		
HU only	9 (81.8)	24 (80.0)		
Other agents alone or in combination	1 (9.1)	1 (3.3)		
Caspases SNPs				
CASP7 (Arg249Lys; rs2227309)			0.137	
Arg/Arg	5 (45.5)	21 (70.0)		1 (Reference)
Arg/Lys	6 (54.5)	7 (23.3)		1.42 (0.40-5.00)
Lys/Lys	0 (0.0)	2 (6.7)		ND
Arg/Lys+Lys/Lys	6 (54.5)	9 (30.0)		1.23 (0.35-4.32)
CASP7 (Asp255Glu; rs2227310)			0.124	
Asp/Asp	5 (45.5)	20 (66.7)		1 (Reference)
Asp/Glu	6 (54.5)	7 (23.3)		1.22 (0.34-4.36)
Glu/Glu	0 (0.0)	3 (10.0)		ND
Asp/Glu+Glu/Glu	6 (54.5)	10 (33.3)		0.92 (0.26-3.33)
CASP8 (3'UTR; rs1035142)			0.174	
G/G	6 (54.5)	10 (33.3)		1 (Reference)
G/T	5 (45.5)	13 (43.3)		0.72 (0.22-2.36)
T/T	0 (0.0)	7 (23.3)		ND
G/T + T/T	5 (45.5)	20 (66.6)		0.56 (0.17-1.84)
CASP8 (Asp270His; rs1045485)			**0.011**	
Asp/Asp	6 (54.5)	25 (83.3)		1 (Reference)
Asp/His	2 (18.2)	5 (16.7)		0.90 (0.17-4.69)
His/His	3 (27.3)	0 (0.0)		**5.90 (1.42-24.62)**[c]
Asp/His+His/His	5 (45.5)	5 (16.7)		1.96 (0.59-6.54)
CASP9 (Arg173His; rs2308950)			-	
Arg/Arg	11 (100.0)	30 (100.0)		1 (Reference)
Arg/His	0 (0.0)	0 (0.0)		-
His/His	0 (0.0)	0 (0.0)		-
CASP9 (Phe136Phe; rs1132312)			0.815	
TTC/TTC	3 (27.3)	11 (36.7)		1 (Reference)
TTC/TTT	5 (45.5)	13 (43.3)		1.53 (0.35-6.74)
TTT/TTT	3 (27.3)	6 (20.0)		1.20 (0.24-6.02)
TTC/TTT+TTT/TTT	8 (72.8)	19 (63.3)		1.38 (0.36-5.28)
CASP9 (Ala28Val; rs1052571)			0.977	
Val/Val	4 (36.4)	12 (40.0)		1 (Reference)
Val/Ala	5 (45.5)	13 (43.3)		1.26 (0.32-4.93)
Ala/Ala	2 (18.2)	5 (16.7)		0.73 (0.13-4.04)
Val/Ala+Ala/Ala	7 (63.6)	18 (60.0)		1.03 (0.30-3.56)
CASP10 (Ile522Leu; rs13006529)			0.476	
Ile/Ile	4 (36.4)	6 (20.0)		1 (Reference)
Ile/Leu	6 (54.5)	18 (60.0)		0.48 (0.13-1.75)
Leu/Leu	1 (9.1)	6 (20.0)		0.7 (0.08-6.59)
Ile/Leu+Leu/Leu	7 (63.6)	24 (80.0)		0.50 (0.14-1.77)

BER SNPs				
APEX1 (Asp148Glu; rs1130409)			0.341	
Asp/Asp	4 (36.4)	11 (36.7)		1 (Reference)
Asp/Glu	3 (27.3)	14 (46.7)		0.96 (0.20-4.63)
Glu/Glu	4 (36.4)	5 (16.7)		0.92 (0.22-3.87)
Asp/Glu+Glu/Glu	7 (63.6)	19 (62.3)		0.94 (0.26-3.38)
MUTYH (Gln335His; rs3219489)			0.647	
His/His	5 (45.4)	9 (30.0)		1 (Reference)
His/Gln	5 (45.5)	17 (56.7)		1.02 (0.27-3.84)
Gln/Gln	1 (9.1)	4 (13.3)		0.48 (0.05-4.34)
His/Gln+Gln/Gln	6 (54.6)	21 (70.0)		0.86 (0.24-3.06)
OGG1 (Ser326Cys; rs1052133)			0.673	
Ser/Ser	8 (72.7)	21 (70.0)		1 (Reference)
Ser/Cys	2 (18.2)	8 (26.7)		0.87 (0.18-4.26)
Cys/Cys	1 (9.1)	1 (3.3)		4.93 (0.49-49.59)
Ser/Cys+Cys/Cys	3 (27.3)	9 (30.0)		1.18 (0.30-4.65)
PARP1 (Val762Ala; rs1136410)			0.252	
Val/Val	6 (54.5)	22 (73.3)		1 (Reference)
Val/Ala	5 (45.5)	8 (26.7)		1.73 (0.52-5.72)
Ala/Ala	0 (0.0)	0 (0.0)		-
PARP4 (Gly1280Arg; rs13428)			0.795	
Gly/Gly	4 (36.4)	8 (26.6)		1 (Reference)
Gly/Arg	5 (45.5)	17 (56.7)		0.64 (0.17-2.40)
Arg/Arg	2 (18.2)	5 (16.7)		1.17 (0.21-6.66)
Gly/Arg+Arg/Arg	9 (81.8)	25 (83.3)		0.73 (0.21-2.53)
XRCC1 (Arg194Trp; rs1799782)			0.964	
Arg/Arg	9 (81.8)	25 (83.3)		1 (Reference)
Arg/Trp	1 (9.1)	2 (6.7)		1.14 (0.14-9.29)
Trp/Trp	1 (9.1)	3 (10.0)		1.49 (0.17-12.97)
Arg/Trp+Trp/Trp	2 (18.2)	5 (16.7)		1.29 (0.27-6.19)
XRCC1 (Gln399Arg; rs25487)			**0.047**	
Arg/Arg	8 (72.7)	10 (33.3)		1 (Reference)
Arg/Gln	3 (27.3)	12 (40.0)		0.51 (0.13-1.95)
Gln/Gln	0 (0.0)	8 (26.7)		ND
Arg/Gln+Gln/Gln	3 (27.3)	20 (66.7))		**0.27 (0.07-1.03)[d]**

*Median (range)
**Patients who received IFN or anagrelide as the only cytoreductive drugs were included in the "no exposure" group
[a] *P*value determined by $\chi 2$ test
[b] *P* determined by conditional logistic regression
[c] *P*=0.015; [d] *P*=0.056
APEX1, apurinic/apyrimidinic endonuclease 1; MUTYH, mutY DNA glycosylase, earlier mutY homolog (*E. coli*); OGG1, 8-oxoguanine DNA glycosylase 1; PARP, poly (ADP-ribose) polymerase; XRCC1, X-ray repair cross-complementing 1; OR, odds ratio; CI, confidence interval; ND, none determined.

9.3.4 – ANALYSIS OF PATIENTS WHO PRESENTED WITH THROMBOTIC EVENTS

The study included 22 ET/PV/PMF patients who developed a thrombotic event and 56 patients who did not (Table 9.1 and 9.6). Thrombotic events are slightly more common in PV patients (17.9%), followed by ET and PMF patients (Tables 9.1 and 9.6). In our studied population contrariwise, more than a half are ET patients (Table 9.6).

In the population here studied, the most frequent types of major thrombosis observed in our patients included stroke, myocardial infarction, peripheral arterial thrombosis and deep vein thrombosis.

There is no significant difference in age (median of 67.9 years) and platelets count (difference in median values of $100x10^9$/L, higher in control patients) between cases and controls patients (Table 9.6).

No significant association with the presence of *JAK2* V617F mutation, nor the exposure to cytoreductive agents were found (Table 9.6).

Globally, the thrombotic events where less frequent, revealing a decreased incidence, in patients with at least one variant allele carriers for *XRCC1* Gln399Arg (OR=0.35; 95%CI, 0.14-0.88) (Tables 9.6 and 9.7).

Genotypes distribution was found to be in Hardy-Weinberg equilibrium.

No significant change in OR was observed for any of the other genotypes considered.

Table 9.6 – Factors investigated for their association with thrombotic events in ET, PV and PMF.
Distribution in case patients who developed a thrombotic event and control patients who did not.

Characteristic	Development of thrombotic event Cases (n=22)	No development Controls (n=56)	*P*[a]	OR[b] (95% CI)
ET/PV/PMF, n/%	13 (59.1) / 7 (31.8) / 2 (9.1)	34 (60.7)/ 19 (33.9)/ 3 (5.4)	0.830	
Sex, male/female, n/%	13 (59.1)/9 (40.9)	24 (42.9)/32 (57.1)	0.196	
Age, y*	68.5 (45-88)	67.3 (54-96)	0.810	
Platelets, $x10^9$/L*	576.6 (134-1518)	676.9 (63-1473)	--	--
***JAK2* V617 mutation, n/%**			0.945	
Val/Val	5 (22.7)	12 (21.4)		1 (Reference)
Val/Phe	15 (68.2)	40 (71.4)		1.03 (0.37-2.90)
Phe/Phe	2 (9.1)	4 (7.1)		0.70 (0.13-3.69)
Exposure to cytoreductive agentes**			0.624	
No exposure	2 (9,1)	8 (14.3)		
HU only	17 (77.3)	45 (80,4)		
HU+other agents	2 (9.1)	2 (3.6)		
Other agents	1 (4.5)	1 (1.8)		
Caspases SNPs				
CASP7 (Arg249Lys; rs2227309)			0.743	
Arg/Arg	14 (63.6)	32 (57.1)		1 (Reference)
Arg/Lys	8 (36.4)	23 (41.1)		1.28 (0.52-3.15)
Lys/Lys	0 (0.0)	1 (1.8)		ND
Arg/Lys+Lys/Lys	8 (36.4)	24 (42.9)		1.26 (0.51-3.12)
CASP7 (Asp255Glu; rs2227310)			0.654	
Asp/Asp	12 (54.5)	30 (54.5)		1 (Reference)
Asp/Glu	10 (45.5)	23 (41.8)		1.53 (0.63-3.70)
Glu/Glu	0 (0.0)	2 (3.7)		ND
Asp/Glu+Glu/Glu	10 (45.5)	25 (45.5)		1.42 (0.59-3.45)
CASP8 (3'UTR; rs1035142)			0.765	
G/G	9 (40.9)	19 (33.9)		1 (Reference)
G/T	10 (45.5)	26 (46.4)		0.52 (0.20-1.38)
T/T	3 (13.6)	11 (19.6)		0.63 (0.17-2.36)
G/T + T/T	13 (59.1)	37 (66.1)		0.55 (0.22-1.35)
CASP8 (Asp270His; rs1045485)			0.497	
Asp/Asp	16 (72.7)	40 (72.7)		1 (Reference)
Asp/His	6 (27.3)	12 (21.8)		1.54 (0.59-4.06)
His/His	0 (0.0)	3 (5.5)		ND
Asp/His+His/His	6 (27.3)	15 (27.3)		1.48 (0.56-3.89)

CASP9 (Arg173His; rs2308950)			0.268	
Arg/Arg	20 (90.9)	54 (96.4)		1 (Reference)
Arg/His	1 (4.5)	2 (3.6)		1.75 (0.23-13.42)
His/His	1 (4.5)	0 (0.0)		6.19 (0.78-49.23)
Arg/His+His/His	2 (9.1)	2 (3.6)		2.74 (0.63-12.04)
CASP9 (Phe136Phe; rs1132312)			0.601	
TTC/TTC	6 (27.3)	10 (17.9)		1 (Reference)
TTC/TTT	12 (54.5)	32 (57.1)		0.92 (0.34-2.49)
TTT/TTT	4 (18.2)	14 (25.0)		0.55 (0.13-2.27)
TTC/TTT+TTT/TTT	16 (72.7)	46 (82.1)		0.82 (0.31-2.15)
CASP9 (Ala28Val; rs1052571)			0.306	
Val/Val	7 (31.8)	10 (17.9)		1 (Reference)
Ala/Val	12 (54.5)	32 (57.1)		1.10 (0.41-2.95)
Ala/Ala	3 (13.6)	14 (25.0)		0.83 (0.20-3.35)
Val/Ala+Ala/Ala	15 (68.2)	46 (82.1)		1.03 (0.40-2.69)
CASP10 (Ile522Leu; rs13006529)			0.232	
Ile/Ile	3 (13.6)	18 (32.1)		1 (Reference)
Ile/Leu	13 (59.1)	24 (42.9)		1.88 (0.53-6.65)
Leu/Leu	6 (27.3)	14 (25.0)		1.60 (0.38-6.80)
Ile/Leu+Leu/Leu	19 (86.4)	38 (67.9)		1.80 (0.53-6.15)
BER SNPs				
APEX1 (Asp148Glu; rs1130409)			0.575	
Asp/Asp	5 (22.7)	19 (33.9)		1 (Reference)
Asp/Glu	11 (50.0)	26 (46.5)		1.25 (0.42-3.68)
Glu/Glu	6 (27.3)	11 (19.6)		1.15 (0.34-3.82)
Asp/Glu+Glu/Glu	17 (77.3)	37 (66.1)		1.21 (0.44-3.34)
MUTYH (Gln335His; rs3219489)			0.629	
His/His	9 (40.9)	27 (48.2)		1 (Reference)
His/Gln	10 (45.5)	25 (44.6)		1.06 (0.42-2.67)
Gln/Gln	3 (13.6)	4 (7.2)		2.00 (0.54-7.50)
His/Gln+Gln/Gln	13 (59.1)	29 (51.8)		1.20 (0.50-2.85)
OGG1 (Ser326Cys; rs1052133)			0.308	
Ser/Ser	16 (72.7)	36 (64.3)		1 (Reference)
Ser/Cys	3 (13.6)	16 (28.6)		0.54 (0.15-1.88)
Cys/Cys	3 (13.6)	4 (7.1)		1.54 (0.44-5.42)
Ser/Cys+Cys/Cys	6 (27.3)	20 (35.7)		0.80 (0.30-2.09)
PARP1 (Val762Ala; rs1136410)			0.215	
Val/Val	19 (86.4)	41 (73.2)		1 (Reference)
Val/Ala	3 (13.6)	15 (26.8)		0.70 (0.20-2.39)
Ala/Ala	0 (0.0)	0 (0.0)		-
PARP4 (Gly1280Arg; rs13428)			0.067	
Gly/Gly	12 (54.5)	15 (26.8)		1 (Reference)
Gly/Arg	8 (36.4)	32 (57.1)		0.45 (0.18-1.14)
Arg/Arg	2 (9.1)	9 (16.1)		0.60 (0.13-2.74)
Gly/Arg+Arg/Arg	10 (45.5)	41 (73.2)		0.47 (0.20-1.13)
XRCC1 (Arg194Trp; rs1799782)			0.382	
Arg/Arg	19 (86.4)	47 (83.9)		1 (Reference)
Arg/Trp	3 (13.6)	5 (8.9)		2.24 (0.64-7.84)
Trp/Trp	0 (0.0)	4 (7.1)		ND
Arg/Trp+Trp/Trp				1.51 (0.43-5.28)
XRCC1 (Gln399Arg; rs25487)			0.386	
Arg/Arg	10 (45.5)	19 (33.9)		1 (Reference)
Arg/Gln	10 (45.5)	25 (44.6)		0.40 (0.15-1.07)
Gln/Gln	2 (9.1)	12 (21.4)		**0.22 (0.05-1.05)[c]**
Arg/Gln+Gln/Gln				**0.35 (0.14-0.88)[d]**

*Median (range)

**Patients who received IFN or anagrelide as the only cytoreductive drugs were included in the "no exposure" group

[a] *P* value determined by $\chi 2$ test

[b] *P* determined by conditional logistic regression

[c] P=0.058; [d] P=0.025;

APEX1, apurinic/apyrimidinic endonuclease 1; MUTYH, mutY DNA glycosylase, earlier mutY homolog (*E. coli*); OGG1, 8-oxoguanine DNA glycosylase 1; PARP, poly (ADP-ribose) polymerase; XRCC1, X-ray repair cross-complementing 1; OR, odds ratio; CI, confidence interval; ND, none determined.

Table 9.7 – ORs (95% CI) for polymorphisms and PN-MPNs association.

Pathology stratification	n	SNP	OR (95% CI)	*P* value
PROGRESSION TO 2RY MF/AML (n=17)				
Globally	**17**	***CASP8* (3'UTR; rs1035142)**		
		G/G[a]	1 (Reference)	
		G/T	0.19 (0.05-0.70)	0.013
		G/T+T/T	0.24 (0.08-0.69)	0.009
		***XRCC1_194* (Arg194Trp; rs1799782)**		
		Arg/Arg[a]	1 (Reference)	
		Arg/Trp	3.58 (0.98-13.01)	0.053
TE	**9**	**CASP9 (Arg173His; rs2308950)**		
		Arg/Arg[a]	1 (Reference)	
		Arg/His	12.73 (1.30-124,41)	0.029
		Arg/His+His/His	11.27 (1.13-112.28)	0.039
		***APEX1* (Asp148Glu; rs1130409)**		
		Asp/Asp[a]	1 (Reference)	
		Asp/Glu	0.19 (0.04-1.0)	0.049
		Asp/Glu+Glu/Glu	0.28 (0.74-1.03)	0.055
		***XRCC1_194* (Arg194Trp; rs1799782)**		
		Arg/Arg[a]	1 (Reference)	
		Arg/Trp	6.60 (1.60-27.06)	0.009
DEVELOPMENT OF NEW NONMYELOID NEOPLASMS (n=11)				
Globally	**11**	**CASP8 (Asp270His; rs1045485)**		
		Asp/Asp[a]	1 (Reference)	
		His/His	5.90 (1.42-24.62)	0.015
		***XRCC1_399* (Gln399Arg; rs25487)**		
		Arg/Arg[a]	1 (Reference)	
		Arg/Gln+Gln/Gln	0.27 (0.07-1.03)	0.056
PRESENCE OF THROMBOTIC EVENTS (n=22)				
Globally	**22**	***XRCC1_399* (Gln399Arg; rs25487)**		
		Arg/Arg[a]	1 (Reference)	
		Gln/Gln	0.22 (0.05-1.05)	0.058
		Arg/Gln+Gln/Gln	0.35 (0.14-0.88)	0.025

[a] The genotype considered as reference class
APEX1, apurinic/apyrimidinic endonuclease 1; XRCC1, X-ray repair cross-complementing 1; OR, odds ratio; CI, confidence interval; ND, none determined.

9.4 – DISCUSSION AND CONCLUSIONS

This study was aimed to evaluate the general characteristics and clinical outcome of PN-MPNs in a Portuguese population, the majority of patients under therapeutic scheme with HU .

Median age of 68 years old at time of diagnosis and gender distribution are similar to what is usually described in the literature (Andıç et al., 2016; Srour et al., 2016). Moreover, there is knowledge

in the literature that ET is more common in women and PV more common in men (Bai et al., 2008; Passamonti et al., 2008), sustained in our study especially in the case of ET patients.

General laboratorial characteristics of the patients included in this study were in agreement with diagnostic WHO classification criteria (Arber et al., 2016; Swerdlow et al., 2008). Hemoglobin, hematocrit and leukocytes' levels were higher in PV and platelet levels were higher in ET, and they were lower in PMF, as expected.

We found *JAK2* mutation in 87.2% of the PV group, 72.5% of the ET group, and 50.0% of PMF group. Although, the frequency of *JAK2* mutation in the PV and ET groups is similar to that reported by another author, from a work developed in Turkey (Andıç et al., 2016), these results differ from what is described in the general literature (Ana Paula Azevedo et al., 2017). Regarding PMF group, the current results are in accordance with previous ones we had obtained (Ana Paula Azevedo et al., 2017).

Survival was highest in ET and lowest in PMF cases. However, the majority of deaths occurred through five years after diagnosis and involved ET and PMF patients, followed by PV patients six to ten years after diagnosis. According to literature, PV has a life expectancy of 10 to 20 years (Passamonti et al., 2004), ET patients ranges from 13 to 22.3 years (Gangat et al., 2007; Passamonti et al., 2008), and PMF patients have a mean overall survival of 5.5 years (Tefferi, 2000).

Progression to 2^{ry} MF/AML and the presence of *JAK2* V617F mutation shortened the survival significantly, consistent with the literature (Andıç et al., 2016). However, according to our results development of new primary nonmyeloid malignancies and occurrence of thrombotic events did not influenced survival, not corroborating what is described in the literature (Andıç et al., 2016), probably because of the dimension of the studied populations. Likewise, none of the caspase and BER pathway studied polymorphisms influenced survival.

It is known that prognosis of MPNs is determined by progression to secondary MF and AML, development on new primary nonmyeloid malignancies and thromboembolic and hemorrhagic complications, reflecting the impact that therapeutics and other inherited genetic factors may play in disease outcome (Andıç et al., 2016; Hernández-Boluda et al., 2012).

Therapy should be directed towards preventing leukemic transformation in PN-MPNs. Progression to AML is a relatively rare complication, usually appearing late in the clinical course of ET and PV, requiring studies involving large cohorts of patients and extended follow-up periods (Hernández-Boluda et al., 2012). We here describe the development of a case control-study, comparing a population composed of ET and PV patients demonstrating fibrotic/leukemic progression with a control population including patients who did not progress, monitored for at least the same period of time, to investigate the role of clinical, genetic and therapy related factors potentially involved in fibrotic/leukemic transformation in PN-MPNs.

According to our results, 12.8% of all PN-MPNs patients progressed to 2^{ry} MF/AML, with a mean time of 7.6 years. ET and PV revealed the same incidence of progression, but none of the PMF cases

transformed to leukemia, similarly with other authors (Andıç et al., 2016). Patients who developed leukemia were not significantly different from others by means of sex and age.

Leukemic transformation was influenced by the exposure to cytoreductive agents (Hernández-Boluda et al., 2012), but the mutagenic and carcinogenic potential of HU, through reduction of DNA repair, in PN-MPNs is controversial, with significant discrepancies among the several studies reported in the literature (Hernández-Boluda et al., 2012).

Our results revealed that previous treatment with HU was not found to be an influencing factor for leukemic transformation. Although not statistically significant in our results, it appears that leukocytosis and marked thrombocytosis could be involved in leukemic transformation, in agreement with the literature (Griesshammer et al., 2015; Hernández-Boluda et al., 2012; Passamonti et al., 2008; Passamonti et al., 2004).

There is published evidence of the involvement of genetic polymorphisms and the susceptibility to leukemic progression (Hernández-Boluda et al., 2012), consistent with our results, in which an association between caspase and BER gene polymorphisms was found. Altogether, the presence of at least one variant allele in carriers for *CASP8* 3'UTR variant is associated with a lower effect in disease progression, and the presence of *XRCC*1 Arg194Trp variant showed a border-line effect, suggesting a higher effect associated with a worse prognosis for 2^{ry} MF/AML development.

When stratified for ET patients, the presence of at least one variant allele carriers for *CASP9* Arg173His polymorphisms is associated with an increased effect and a worse prognosis. Regarding BER polymorphisms, *APEX1* Asp148Glu showed a border-line effect related to an ensuing better prognosis for the presence of at least one variant allele carriers. A worse prognosis in disease progression was found for *XRCC*1 Arg194Trp variant.

Development of new nonmyeloid malignancies was observed in 8.3% of all PN-MPNs patients, approximately equally distributed among ET, PV and PMF, with a male predominance. Regarding age, it appears that there is no significant difference among case and control patients.

The majority of cases corresponded to solid organ new malignancies, the most affected being lung, thyroid, adrenal gland and digestive tract, and on case developed a lymphoid malignancy.

The association of long-term HU use with development of nonmyeloid malignancies is controversial, with some authors reporting no association and others a weak relation (Hernández-Boluda et al., 2012; Santoro et al., 2017). In fact, in the present study, no association was observed. According to previous studies (Hernández-Boluda et al., 2012), *JAK2* V617F mutation and caspase and BER polymorphisms constitute significant influencing factors for the occurrence of new primary nonmyeloid malignancies in PN-MPNs, conditioning these disorders clinical evolution. Our results revealed no association with the presence of *JAK2* mutation. However, globally, an increased incidence to develop new non-myeloid malignancies was found for the presence of *CASP8* Asp270His variant, while a border-line effect related to a decreased incidence to develop new non-myeloid malignancies was observed for the presence of at least one variant allele carriers for *XRCC*1 Arg399Gln.

Thrombotic complications were seen in 16.5% of all PN-MPNs patients in our study, the majority of them occurring during follow-up, with a similar incidence between PV, ET and PMF patients, slightly lower in the last group. In the literature, it is reported that about 41% of all deaths in PN-MPNs (1.5 deaths per 100 persons per year) were due to cardiovascular complications (Barbui, Finazzi, & Falanga, 2013), with an increase of thromboembolic events during follow-up (ranging from 41% to 91%), in comparison with a variation from 7% to 57% at PN-MPN diagnosis, with fewer PMF patients being affected compared to other PN-MPNs (Andıç et al., 2016). In our patients, arterial events were more frequent than venous, in agreement to what is described by other authors (Andıç et al., 2016), including stroke, myocardial infarction, peripheral arterial thrombosis and deep vein thrombosis. Apparently, according to our results, the occurrence of this type of events is not related to age, sex, platelet counts, the presence of *JAK2* mutation, nor the exposure to cytoreductive agents, differing from the results previously published by some authors (Andıç et al., 2016).

Leukocytosis and *JAK2* V617F allele burden have been identified as thrombotic risk factors and in the case of PV patients, leukocytosis at diagnosis has been considered to be associated with an increased risk to develop arterial thrombosis and progress to acute leukemia, resulting in shorter survival (Griesshammer et al., 2015).

In the present study, only BER pathway showed a role in the presence of thrombotic events, revealing a decreased incidence when at least one variant allele carriers for *XRCC1* Gln399Arg is present, making thrombotic episodes less frequent in these patients.

The caspases, a specific group of cysteine aspartate proteases, are a family of intracellular proteins involved in the initiation and execution of apoptosis processes, responsible for the dismantling and destruction of the cell (Green & Llambi, 2015; Riedl & Salvesen, 2007). There are 14 different caspases and they can be classified as initiator, effector and cytokine activators (Kiraz, Adan, Kartal Yandim, & Baran, 2016). The initiators caspases (caspase-2, 8, 9, 10) activate the effectors caspases (caspase 3, 6, 7 and 14), which are capable of degrading direct multiple substrates leading to deregulation of vital cellular processes and cellular death (Kiraz et al., 2016; Ng, Porter, & Jänicke, 1999; Oliver & Vallette, 2005; Philchenkov, Zavelevich, Kroczak, & Los, 2004; Riedl & Salvesen, 2007), and also the cytokine activators caspases (caspase 1, 4, 5, 11, 12 and 13). Several proteins have been described that promote pro- or anti- apoptotic activity in the cell. The ratio of these pro-and anti-apoptotic proteins plays an important role in the regulation of cell death, and disruption in the balance between these proteins has been established to contribute to carcinogenesis by reducing apoptosis in malignant cells (Ding et al., 2000; Goldar, Khaniani, Derakhshan, & Baradaran, 2015; Olsson & Zhivotovsky, 2011).

Several studies described modifications in the expression of molecules that participate in the regulation of intrinsic and extrinsic routes of apoptosis, as well as functional studies that showed resistance to apoptosis, indicating that the deregulation of apoptosis in MPNs is a mechanism involved in the pathophysiology and clinic-hematological outcome of these diseases (Malherbe et al., 2016;

Testa, 2004; Tognon et al., 2013).

Uphold of genetic integrity, through DNA repair mechanisms, is essential for preventing cellular damage and the development of leukaemia (A. P. Azevedo et al., 2017). Protein function and thus DNA damage repair may be affected by several polymorphisms in DNA repair genes, leading to susceptibility to malignancy (A. P. Azevedo et al., 2017). Previous reports have identified BER pathway polymorphisms associated solid tumors development (A. P. Azevedo et al., 2017), and a nucleotide excision repair gene polymorphism displayed strong association with leukaemic transformation and development of non-myeloid malignancies in ET and PV patients (Hernández-Boluda et al., 2012).

The BER pathway typically repairs a small region (1-13 nucleotides) around the damaged base, and several polymorphisms have been identified and studied for their association with progression to leukemia and disease outcome (A. P. Azevedo et al., 2017).

Previous studies performed by our working group in which we tested the contribution of apoptosis and BER related genes to individual susceptibility to MPNs, also revealed involvement of the same family of polymorphisms (A. P. Azevedo et al., 2017) that were found to be associated with disease outcome as described in this present work.

HU, an antimetabolite, and anagrelide are the most commonly used drugs in the treatment of all PN-MPNs groups. The former was shown to reduce the incidence of thrombotic events in several studies, but there is some evidence that it may increase the risk of leukemic transformation (Andıç et al., 2016; Hernández-Boluda et al., 2012). Anagrelide is effective in reducing platelet counts in ET and PV patients who are resistant or intolerant to HU. Increment of leukemia progression has not been shown for this drug (Andıç et al., 2016).

HU is the initial choice of treatment because of its proven efficiency, especially in reducing thrombotic complications. However, HU is recommended to be used with caution in young patients regarding the data showing an increment of leukemia incidence in long-term usage of HU (Andıç et al., 2016). In the ANAHYDRET study, it was shown that anagrelide is as effective as hydroxyurea. Secondary leukemia has not been reported with anagrelide treatment yet. Interferon alpha was the least commonly used agent in all MPN groups, probably because of its parenteral usage and poor tolerability (Andıç et al., 2016).

In our study, we did not find any relation between the complications of MPN and the treatment options.

In summary, in the present study there is evidence of influence of fibrotic/leukemic transformation and the presence of *JAK2* mutation in PN-MPNs survival. Caspase (*CASP8* and *CASP9*) and BER pathway (*XRCC1* and *APEX1*) polymorphisms are associated with leukemic transformation and occurrence of new primary nonmyeloid malignancies, whereas only BER pathway (*XRCC1*) polymorphisms are associated with the presence of thrombotic events. Apparently, therapeutics only influences the tendency to 2^{ry}MF/AML transformation.

Despite all the advances in the last few years, leading to the development of more targeted treatments, there are still lacking long-term effective and well-tolerated therapies for both low and high

risk patients (Griesshammer et al., 2015).

Larger studies are required to confirm these results and to provide conclusive evidence of association between these and other variants and PN-MPNs and therapeutic response.

Identification of the main molecules that are altered in MPNs will allow the development of drugs more directly targeted to the pathophysiology of the disease, with high efficacy, fewer adverse effects, contributing to compliance of the patients with treatments.

In the future, these new data may contribute to a best knowledge of these diseases course and to a more rational and efficient choice of therapeutic strategies to be adopted in the treatment of PN-NMPs.

9.5 – REFERENCES

Andıç, N., Ünübol, M., Yağcı, E., Akay, O. M., Yavaşoğlu, İ., Kadıköylü, V. G., & Bolaman, A. Z. (2016). Clinical Features of 294 Turkish Patients with Chronic Myeloproliferative Neoplasms. *Turk J Haematol, 33*(3), 187-195. doi:10.4274/tjh.2015.0041

Annamaneni, S., Gorre, M., Kagita, S., Addepalli, K., Digumarti, R. R., Satti, V., & Battini, M. R. (2013). Association of XRCC1 gene polymorphisms with chronic myeloid leukemia in the population of Andhra Pradesh, India. *Hematology, 18*(3), 163-168. doi:10.1179/1607845412Y.0000000040

Arber, D. A., Orazi, A., Hasserjian, R., Thiele, J., Borowitz, M. J., Le Beau, M. M., . . . Vardiman, J. W. (2016). The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood, 127*(20), 2391-2405. doi:10.1182/blood-2016-03-643544

Azevedo, A. P., Silva, S., Reichert, A., Lima, F., Júnior, E., & Rueff, J. (2017). Prevalence of the Janus kinase 2 V617F mutation in Philadelphia-negative myeloproliferative neoplasms in a Portuguese population. *Biomedical Reports*(7), 370-376. doi:10.3892/br.2017.977

Azevedo, A. P., Silva, S. N., De Lima, J. P., Reichert, A., Lima, F., Júnior, E., & Rueff, J. (2017). DNA repair genes polymorphisms and genetic susceptibility to Philadelphia-negative myeloproliferative neoplasms in a Portuguese population: The role of base excision repair genes polymorphisms. *Oncol Lett, 13*(6), 4641-4650. doi:10.3892/ol.2017.6065

Bai, J., Xue, Y., Ye, L., Yao, J., Zhou, C., Shao, Z., . . . Zheng, Y. (2008). Risk factors of long-term incidences of thrombosis, myelofibrosis and evolution into malignance in polycythemia vera: a single center experience from China. *Int J Hematol, 88*(5), 530-535. doi:10.1007/s12185-008-0188-y

Barbui, T., Finazzi, G., & Falanga, A. (2013). Myeloproliferative neoplasms and thrombosis. *Blood, 122*(13), 2176-2184. doi:10.1182/blood-2013-03-460154

Batar, B., Güven, M., Bariş, S., Celkan, T., & Yildiz, I. (2009). DNA repair gene XPD and XRCC1 polymorphisms and the risk of childhood acute lymphoblastic leukemia. *Leuk Res, 33*(6), 759-763. doi:10.1016/j.leukres.2008.11.005

Baute, J., & Depicker, A. (2008). Base excision repair and its role in maintaining genome stability. *Crit Rev Biochem Mol Biol, 43*(4), 239-276. doi:10.1080/10409230802309905

Beer, P. A., Delhommeau, F., LeCouédic, J. P., Dawson, M. A., Chen, E., Bareford, D., . . . Green, A. R. (2010). Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. *Blood, 115*(14), 2891-2900. doi:10.1182/blood-2009-08-236596

Björkholm, M., Hultcrantz, M., & Derolf, Å. (2014). Leukemic transformation in myeloproliferative neoplasms: therapy-related or unrelated? *Best Pract Res Clin Haematol, 27*(2), 141-153. doi:10.1016/j.beha.2014.07.003

Bolufer, P., Barragan, E., Collado, M., Cervera, J., López, J. A., & Sanz, M. A. (2006). Influence of genetic polymorphisms on the risk of developing leukemia and on disease progression. *Leuk Res, 30*(12), 1471-1491. doi:10.1016/j.leukres.2006.01.016

Bănescu, C., Trifa, A. P., Demian, S., Benedek Lazar, E., Dima, D., Duicu, C., & Dobreanu, M. (2014). Polymorphism of XRCC1, XRCC3, and XPD genes and risk of chronic myeloid leukemia. *Biomed Res Int, 2014*, 213790. doi:10.1155/2014/213790

Campregher, P. V., Santos, F. P., Perini, G. F., & Hamerschlak, N. (2012). Molecular biology of Philadelphia-negative myeloproliferative neoplasms. *Rev Bras Hematol Hemoter, 34*(2), 150-155. doi:10.5581/1516-8484.20120035

Delhommeau, F., Jeziorowska, D., Marzac, C., & Casadevall, N. (2010). Molecular aspects of myeloproliferative neoplasms. *Int J Hematol, 91*(2), 165-173. doi:10.1007/s12185-010-0530-z

Ding, H. F., Lin, Y. L., McGill, G., Juo, P., Zhu, H., Blenis, J., . . . Fisher, D. E. (2000). Essential role for caspase-8 in transcription-independent apoptosis triggered by p53. *J Biol Chem, 275*(49), 38905-38911. doi:10.1074/jbc.M004714200

Gangat, N., Wolanskyj, A. P., McClure, R. F., Li, C. Y., Schwager, S., Wu, W., & Tefferi, A. (2007). Risk stratification for survival and leukemic transformation in essential thrombocythemia: a single institutional study of 605 patients. *Leukemia, 21*(2), 270-276. doi:10.1038/sj.leu.2404500

Goldar, S., Khaniani, M. S., Derakhshan, S. M., & Baradaran, B. (2015). Molecular mechanisms of apoptosis and roles in cancer development and treatment. *Asian Pac J Cancer Prev, 16*(6), 2129-2144.

Green, D. R., & Llambi, F. (2015). Cell Death Signaling. *Cold Spring Harb Perspect Biol, 7*(12). doi:10.1101/cshperspect.a006080

Griesshammer, M., Gisslinger, H., & Mesa, R. (2015). Current and future treatment options for polycythemia vera. *Ann Hematol, 94*(6), 901-910. doi:10.1007/s00277-015-2357-4

Hasselbalch, H. C., Thomassen, M., Riley, C. H., Kjær, L., Larsen, T. S., Jensen, M. K., . . . Skov, V. (2014). Whole blood transcriptional profiling reveals deregulation of oxidative and antioxidative defence genes in myelofibrosis and related neoplasms. Potential implications of downregulation of Nrf2 for genomic instability and disease progression. *PLoS One, 9*(11), e112786. doi:10.1371/journal.pone.0112786

Hernández-Boluda, J. C., Pereira, A., Cervantes, F., Alvarez-Larrán, A., Collado, M., Such, E., . . . Guillem, V. (2012). A polymorphism in the XPD gene predisposes to leukemic transformation and new nonmyeloid malignancies in essential thrombocythemia and polycythemia vera. *Blood, 119*(22), 5221-5228. doi:10.1182/blood-2012-02-411215

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature, 411*(6835), 366-374. doi:10.1038/35077232

Kilpivaara, O., & Levine, R. L. (2008). JAK2 and MPL mutations in myeloproliferative neoplasms: discovery and science. *Leukemia, 22*(10), 1813-1817. doi:10.1038/leu.2008.229

Kiraz, Y., Adan, A., Kartal Yandim, M., & Baran, Y. (2016). Major apoptotic mechanisms and genes involved in apoptosis. *Tumour Biol, 37*(7), 8471-8486. doi:10.1007/s13277-016-5035-9

Levine, R. L. (2009). Mechanisms of mutations in myeloproliferative neoplasms. *Best Pract Res Clin Haematol, 22*(4), 489-494. doi:10.1016/j.beha.2009.08.006

Malherbe, J. A., Fuller, K. A., Mirzai, B., Kavanagh, S., So, C. C., Ip, H. W., . . . Erber, W. N. (2016). Dysregulation of the intrinsic apoptotic pathway mediates megakaryocytic hyperplasia in myeloproliferative neoplasms. *J Clin Pathol*. doi:10.1136/jclinpath-2016-203625

Mesa, R., Miller, C. B., Thyne, M., Mangan, J., Goldberger, S., Fazal, S., . . . Mascarenhas, J. O. (2016). Myeloproliferative neoplasms (MPNs) have a significant impact on patients' overall health and productivity: the MPN Landmark survey. *BMC Cancer, 16*(1), 167. doi:10.1186/s12885-016-2208-2

Nangalia, J., Massie, C. E., Baxter, E. J., Nice, F. L., Gundem, G., Wedge, D. C., . . . Green, A. R. (2013). Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med, 369*(25), 2391-2405. doi:10.1056/NEJMoa1312542

Ng, P. W., Porter, A. G., & Jänicke, R. U. (1999). Molecular cloning and characterization of two novel pro-apoptotic isoforms of caspase-10. *J Biol Chem, 274*(15), 10301-10308.

Nunes, N. S., Tognon, R., Moura, L. G., Kashima, S., Covas, D. T., Santana, M., . . . Castro, F. A. (2013). Differential expression of apoptomiRs in myeloproliferative neoplasms. *Leuk Lymphoma, 54*(9), 2047-2051. doi:10.3109/10428194.2013.767453

Oliver, L., & Vallette, F. M. (2005). The role of caspases in cell death and differentiation. *Drug Resist Updat, 8*(3), 163-170. doi:10.1016/j.drup.2005.05.001

Olsson, M., & Zhivotovsky, B. (2011). Caspases and cancer. *Cell Death Differ, 18*(9), 1441-1449. doi:10.1038/cdd.2011.30

Passamonti, F., Rumi, E., Arcaini, L., Boveri, E., Elena, C., Pietra, D., . . . Lazzarino, M. (2008). Prognostic factors for thrombosis, myelofibrosis, and leukemia in essential thrombocythemia: a study of 605 patients. *Haematologica, 93*(11), 1645-1651. doi:10.3324/haematol.13346

Passamonti, F., Rumi, E., Pungolino, E., Malabarba, L., Bertazzoni, P., Valentini, M., . . . Lazzarino, M. (2004). Life expectancy and prognostic factors for survival in patients with polycythemia vera and essential thrombocythemia. *Am J Med, 117*(10), 755-761. doi:10.1016/j.amjmed.2004.06.032

Philchenkov, A., Zavelevich, M., Kroczak, T. J., & Los, M. (2004). Caspases and cancer: mechanisms of inactivation and new treatment modalities. *Exp Oncol, 26*(2), 82-97.

Rampal, R., Ahn, J., Abdel-Wahab, O., Nahas, M., Wang, K., Lipson, D., . . . Levine, R. L. (2014). Genomic and functional analysis of leukemic transformation of myeloproliferative neoplasms. *Proc Natl Acad Sci U S A, 111*(50), E5401-5410. doi:10.1073/pnas.1407792111

Riedl, S. J., & Salvesen, G. S. (2007). The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol, 8*(5), 405-413. doi:10.1038/nrm2153

Rumi, E., & Cazzola, M. (2017). Diagnosis, risk stratification, and response evaluation in classical myeloproliferative neoplasms. *Blood, 129*(6), 680-692. doi:10.1182/blood-2016-10-695957

Santoro, C., Sperduti, I., Latagliata, R., Baldacci, E., Anaclerico, B., Avvisati, G., . . . Mazzucconi, M. G. (2017). Role of treatment on the development of secondary malignancies in patients with essential thrombocythemia. *Cancer Med, 6*(6), 1233-1239. doi:10.1002/cam4.1081

Srour, S. A., Devesa, S. S., Morton, L. M., Check, D. P., Curtis, R. E., Linet, M. S., & Dores, G. M. (2016). Incidence and patient survival of myeloproliferative neoplasms and myelodysplastic/myeloproliferative neoplasms in the United States, 2001-12. *Br J Haematol, 174*(3), 382-396. doi:10.1111/bjh.14061

Swerdlow, S. H., Campo, E., Harris, N. L., Jaffe, E. S., Pileri, S. A., Stein, H., . . . Vardiman, J. W. (2008). *WHO Classification of Tumours of Haematopioetic and Lymphoid Tissues*. Lyon: World Health Organization.

Tefferi, A. (2000). Myelofibrosis with myeloid metaplasia. *N Engl J Med, 342*(17), 1255-1265. doi:10.1056/NEJM200004273421706

Tefferi, A. (2016). Myeloproliferative neoplasms: A decade of discoveries and treatment advances. *Am J Hematol, 91*(1), 50-58. doi:10.1002/ajh.24221

Tefferi, A., & Pardanani, A. (2015). Myeloproliferative Neoplasms: A Contemporary Review. *JAMA Oncol, 1*(1), 97-105. doi:10.1001/jamaoncol.2015.89

Tefferi, A., & Vardiman, J. W. (2008). Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia, 22*(1), 14-22. doi:10.1038/sj.leu.2404955

Testa, U. (2004). Apoptotic mechanisms in the control of erythropoiesis. *Leukemia, 18*(7), 1176-1199. doi:10.1038/sj.leu.2403383

Tognon, R., Gasparotto, E. P., Leroy, J. M., Oliveira, G. L., Neves, R. P., Carrara, R. e. C., . . . de Castro, F. A. (2011). Differential expression of apoptosis-related genes from death receptor pathway in chronic myeloproliferative diseases. *J Clin Pathol, 64*(1), 75-82. doi:10.1136/jcp.2010.080895

Tognon, R., Gasparotto, E. P., Neves, R. P., Nunes, N. S., Ferreira, A. F., Palma, P. V., . . . Castro, F. A. (2012). Deregulation of apoptosis-related genes is associated with PRV1 overexpression and JAK2 V617F allele burden in Essential Thrombocythemia and Myelofibrosis. *J Hematol Oncol, 5*, 2. doi:10.1186/1756-8722-5-2

Tognon, R., Nunes, N. e. S., & Castro, F. A. (2013). Apoptosis deregulation in myeloproliferative neoplasms. *Einstein (Sao Paulo), 11*(4), 540-544.

Tognon, R., Nunes, N. S., Ambrosio, L., Souto, E. X., Perobelli, L., Simões, B. P., . . . Attié de Castro, F. (2016). Apoptosis- and cell cycle-related genes methylation profile in myeloproliferative neoplasms. *Leuk Lymphoma, 57*(5), 1201-1204. doi:10.3109/10428194.2015.1071491

Treliński, J., Chojnowski, K., Cebula-Obrzut, B., & Smolewski, P. (2012). Impaired apoptosis of megakaryocytes and bone marrow mononuclear cells in essential thrombocythemia: correlation with JAK2V617F mutational status and cytoreductive therapy. *Med Oncol, 29*(4), 2388-2395. doi:10.1007/s12032-012-0202-3

Wang, C. Q., Krishnan, V., Tay, L. S., Chin, D. W., Koh, C. P., Chooi, J. Y., . . . Osato, M. (2014). Disruption of Runx1 and Runx3 leads to bone marrow failure and leukemia predisposition due to transcriptional and DNA repair defects. *Cell Rep, 8*(3), 767-782. doi:10.1016/j.celrep.2014.06.046

Wolanskyj, A. P., Schwager, S. M., McClure, R. F., Larson, D. R., & Tefferi, A. (2006). Essential thrombocythemia beyond the first decade: life expectancy, long-term complication rates, and prognostic factors. *Mayo Clin Proc, 81*(2), 159-166. doi:10.4065/81.2.159

Zaman, S., Wang, R., & Gandhi, V. (2014). Targeting the apoptosis pathway in hematologic malignancies. *Leuk Lymphoma, 55*(9), 1980-1992. doi:10.3109/10428194.2013.855307.

CHAPTER 10:
CONCLUSIONS AND FINAL REMARKS

Myeloproliferative neoplasms are classically divided into *BCR-ABL1* (Philadelphia chromosome) positive chronic myeloid leukemia and PN-MPNS, including PV, ET and PMF, according to WHO classification (Arber et al., 2016; Swerdlow et al., 2008). It has been on these last three entities that this dissertation has bent over.

PN-MPNs are rare diseases worldwide, predominantly diagnosed in the age range of 50–60 years (Bellanné-Chantelot et al., 2006). Among the various registries worldwide, this group of disorders has a combined annual incidence rate of 0.4-2.8 for PV, 0.38-1.7 for ET and 0.1-1.0 for PMF, per 100 000 persons (Moulard et al., 2014; Titmarsh et al., 2014).

From the various published reports, PN-MPNs patients overall have a reduced life expectancy compared with general population, with PMF having the lowest overall survival, followed by PV and ET (Rumi & Cazzola, 2017; Tefferi, 2008). It was also reported that mortality in PN-MPNs patients is decreasing in the last few years and is generally attributed to disease progression to other hematologic malignancies, such as AML and MDS, bacterial infections and cardio- and cerebrovascular diseases, especially in younger patients (Hultcrantz et al., 2015; Rumi & Cazzola, 2017).

The discovery of *JAK2* V617F in 2005 and the other mutations that followed, such as *JAK2* exon12, *MPL* and *CALR*, which took place as driver mutations, was a very important milestone in the knowledge and understanding of the biological and molecular mechanisms that were behind the pathogenesis of these disorders, contributing to and influencing the definition of the phenotype and having a prognostic impact. Although they are different mutations located in distinct genes in several chromosomes, all of them share the same concept of having been the result of a clonal malignant transformation of hematopoietic stem-cells, characterized by evidencing cytokine hypersensitivity and deregulation of downstream signaling pathways, leading to abnormal amplification and proliferation of myeloid lineages and a tendency to transform to AML (Cross, 2011; Duletić et al., 2012; Tefferi & Pardanani, 2015).

JAK2 V617F is found in approximately 95% of PV cases, and 50 to 60% of ET and PMF patients. Mutations in *MPL* W515 and *CALR* genes have practically only been detected in ET (3-4% and 20-25%, respectively) and PMF (6-7% and 20-25%, respectively) patients. These three mutations are mutually exclusive in the majority of cases.

It is known that the different driver mutations involved in the pathogenesis of PN-MPNs can lead to different clinical effects and that a single mutation may be associated with distinct phenotypes and clinical outcomes (Rumi & Cazzola, 2017). For example, homozygosity for *JAK2* V617F is more common in PV compared with ET, leading to the suggestion that increased *JAK2* V617F signaling is associated with a more erythrocytic phenotype; approximately one third of *JAK2* V617F positive PV

cases are homozygous with mutation burdens greater than 50%, associated with a more aggressive phenotype; one third of the remaining *JAK2* V617F negative PV cases carry *JAK2* exon 12 mutations, often accompanied by an apparently isolated erythrocytosis; *MPL* and *CALR* mutations are usually associated to a thrombocytosis phenotype, with *CALR* revealing better prognosis (Jones & Cross, 2013).

According to WHO, classical PN-MPNs classification is based on molecular evaluation but also in specific standardized clinical-histopathological criteria, in order to an accurate diagnosis and capacity of predicting prognosis. Phenotypic differences depend on which myeloid cell lineage is predominantly expanded in the peripheral blood, namely elevated red cell mass in PV, and elevated platelet numbers in essential ET, while PMF patients display bone marrow fibrosis, a variable myeloid cell number, extramedullary hematopoiesis, and hepatosplenomegaly. Patients with PV and ET are at an increased risk of developing thrombotic or hemorrhagic events and may progress to an accelerated myelofibrosis phase, but all three subtypes are associated with a long-term risk of transformation to AML or MDS (Jones & Cross, 2013).

Considering the lack of Portuguese epidemiological data, the present dissertation had as the initial purpose to show data related to the characterization of the studied population, according to the type of PN-MPN, its prevalence and the presence of *JAK2* mutation. To achieve this aim, case-control studies, which are powerfull tools for the study of candidate polymorphic genes and their potential association to a given disease in a population, were carried out in a Caucasian Portuguese sample of 133 patients and 281 age- and sex-matched control subjects, recruited in a hospital setting, from 2009 until 2016.

All the patients included in the study were diagnosed accordingly WHO Classification criteria, on the basis of molecular, clinical, pathologic and morphologic/histologic features.

The results from this study revealed that in the studied population, ET was the more prevalent, corresponding to 60.2% of patients, followed by PV (29.3%) and finally PMF (10.5%), with a discrete predominance of females (54.1%). No significant differences between the case and control groups were found, concerning smoking habits or age distribution (median of 68 years old at time of diagnosis), in agreement with the literature (Andıç et al., 2016; Bai et al., 2008; Passamonti et al., 2008; Srour et al., 2016). A total of 75.0% of patients were positive for the presence of the *JAK2* V617F mutation (Azevedo *et al.*, 2017, for detailed information see Chapter 3), with an increased incidence in ET (73.4%) and a decreased incidence in PV (87.2%) patients than expected, when compared to the published literature, indicating that the population of the present study has a different pattern regarding the presence of this mutation, in comparison with other already studied populations from other countries. On the other hand, the fact that patients with the wild type *JAK2* V617F or exon 12 mutation are extremely rare as it is reinforced in very recent reports (Rumi & Cazzola, 2017), wereas our results reveal a prevalence of 12.8% of these patients, supports the idea that the *JAK2* mutation acting alone may not be sufficient to develop the PV phenotype.

The clinical-hematological features of the patients were also accessed and described throughout the work, detailed in Chapter 9 (Azevedo *et al.,* submitted for publication), and were accordingly to what is defined for each entity. As expected, hemoglobin levels were significantly higher in PV and lower in PMF, whereas platelet count was higher in ET than in other groups.

Among the patients that were *JAK2* V617F mutation negative, it was highlighted a very interesting and unique patient with a new *JAK2* exon 12 mutation - c.1605G>T (p.Met535Ile), associated with another mutation in the same exon already identified (Pita & Azevedo *et al.*, 2017, for detailed information see Chapter 4), among the great diversity of exon 12 mutations (Vorechovsky, Jones, & Cross, 2013). This patient revealed the peculiarity of presenting a marked thrombocytosis, unlike the usual phenotype for exon 12 mutation patients, mostly characterized by erythrocytosis and normal leukocyte and platelet counts (Arber et al., 2016; Godfrey et al., 2016; Koopmans, Schouten, & van Marion, 2015). This could be an example of how the result of the association of two abnormalities may give rise to a different pattern than one would expected to achieve with each individual change. The higher platelet count may be due to the fact that this patient harbors two lesions, both of which on their own have been associated with erythrocytosis phenotypes, resulting in an excess of JAK2 signaling or enhanced STAT activation and therefore more thrombocytosis than the usual (Williams, Kim, Rogers, Spivak, & Moliterno, 2007; Kim et al., 2016; Pardanani, Lasho, Finke, Hanson, & Tefferi, 2007; Park et al., 2016; Scott et al., 2007; Siemiatkowska, Bieniaszewska, Hellmann, & Limon, 2010; Wu et al., 2014; Scott, 2011).

Another peculiar clinical case that was presented and discussed in this dissertation refers to two patients who raised the suspition of the rare coexistence of *JAK2* V617F mutation and *BCR-ABL1* translocation (Mousinho & Azevedo *et al.*, for detailed information see Chapter 5). One of the major diagnostic criteria for ET is the absence of the Philadelphia chromosome (Ph), meaning that when present it would be almost indicative of CML. However, exceptionally there are rare situations in which ET patients present positive *BCR-ABL* translocation without features of CML (Pagnano et al., 2016; Qin et al., 2014). From the literature review, the frequency of coexistence of *JAK2* V617F mutation and *BCR-ABL* translocation in MPNs is low, and besides V617F there are no other types of *JAK2* mutation involved among the several cases reported. Several hypothesis have been advanced, but doubt still persists and it was not yet clarified which is the first anomaly to occur and to dominate, the amount of affected clones and if the presence of two molecular abnormalities may correspond to a new clinical entity, with the two mutations acting together and influencing one another, sharing phenotypes and varying levels of expression (Bee, Gan, Nadarajan, Latiff, & Menaka, 2010; Kwong, Chiu, Liang, Chan, & Chan, 1996; Qin et al., 2014; Zhou et al., 2015). Moreover, WHO classification does not address these atypical cases. Both patients reported revealed diagnostic clinical features matching ET, and in the first case also CML. Search for *JAK2* V6217F mutation was positive in both cases, and t(9,22) was positive in FISH analysis with an atypical pattern with only one fusion signal observed, being negative in molecular tests. One of the possible explanations is that this unique fusion signal detected by FISH can correspond only to der(9), and not to Ph chromosome with associated tyrosine kinase activity (on

chromosome 22). From literature review, there are no reports of a "true" positive *BCR-ABL* with an aberrant pattern similar to our cases, but instead there are descriptions of karyotipic aberrations associated with *JAK2* V617F mutation (Larsen, Hasselbalch, Pallisgaard, & Kerndrup, 2007). On the other hand, it has been published a der(9) chromosome coexistence with acute lymphoblastic leukemia (Specchia et al., 2003). So, these two patients are a good example of the probable importance and influence of the presence of der(9) chromosome, contributing to the phenotypic variations observed in some ET cases. Moreover, the coexistence of these two genetic abnormalities may also have therapeutic strategy implications.

Initially, it was thought to apply this study to MPNs both *BCR-ABL1* positive and negative, but it turned out to be limited to the study of PN-MPNs only. However, another particular patient was found and was decided to be presented in this work (Azevedo *et al*., 2017, for detailed information see Chapter 6). This was the case of a *BCR-ABL1* positive CML patient who developed resistance to imatinib. Quantitative real time polymerase chain reaction technique (qRT-PCR) testing was performed and revealed a new mutation - c.839T>G (p.Val280Gly). This is the first report describing this new *BCR-ABL* kinase domain mutation, which might be associated with resistance to imatinib. The identification of *BCR-ABL* expression as the defining leukemogenic event in CML and the introduction of *BCR-ABL* tyrosine kinase inhibitors in 2001 have revolutionized disease management, leading to a reduction in mortality rates and accordingly to an increase of the estimated prevalence of CML. However, approximately 15% to 30% of patients treated with imatinib discontinue treatment due to resistance or intolerance (Radich et al., 2014; Soverini et al., 2015; Soverini et al., 2011). More than 90 *BCR-ABL* mutations were detected so far, conferring variable degrees of drug resistance, with consequent clinical, therapeutic and prognostic impact (Nicolini et al., 2006; Soverini et al., 2006).

Although it is possible to identify a molecular marker in the majority of PN-MPNs patients, driver mutations cannot fully explain the phenotypic heterogeneity of PN-MPNs, nor the susceptibility of progression to myelofibrosis, acute myeloid leukemia (AML) or myelodysplastic syndromes (MDS) (Hinds et al., 2016). On the other hand, it is possible to identify one of the driver mutations in the majority of the patients, but genetic defects still await identification in about 10-15% of cases (triple-negative), mostly of ET and PMF patients, who meet the WHO criteria for MPN and are associated with a poor clinical outcome (Delhommeau et al., 2010; Nangalia et al., 2013; Rumi & Cazzola, 2017; Tefferi & Pardanani, 2015) . This finding may probably be due to the association with other commutated non-MPNs-driver genes (ex: *ASXL1, EZH2, TET2, IDH1/2, SF3B1, SRSF2*), karyotypic abnormalities, and the occurrence of genetic variation (SNPs) that contribute to disease onset, phenotypic variability and severity, phenotypic shifts and progression to more aggressive neoplasms (Rumi & Cazzola, 2017).

With the recent sequencing of the human genome, the study and discovery of genetic polymorphisms emerges as one of the research areas of modern genetics (Singh et al., 2008), in that it drives the studies of genetic association (Gaspar et al., 2006). In recent years, the study of polymorphic genes has been the main and most frequent approach in describing inter-individual

differences and showing consistent evidence of their involvement in different types of cancer. In the case of PN-MPNs, there are some studies developed in this field, mainly related to DNA repair (Adel Fahmideh, Schwartzbaum, Frumento, & Feychting, 2014; Batar, Güven, Bariş, Celkan, & Yildiz, 2009; Bănescu et al., 2014; Canbay et al., 2011; Chiang et al., 2014; Costa et al., 2007; Das et al., 2015; Du et al., 2014; Hernández-Boluda et al., 2012; Sorour, Ayad, & Kassem, 2013). However, to the best of our knowledge, no clinical association studies have been performed thus far to evaluate the role of caspases genes polymorphisms on PN-MPNs susceptibility.

Following this line of reasoning, and studying the same patient and control populations, different polymorphisms of caspases genes involved in apoptosis (nine polymorphisms of four genes) and base excision repair pathway (BER) (eight polymorphisms of six genes) were selected and genotyped using qRT-PCR, and statistical analysis performed using SPSS version 22.0 (Caspases: Azevedo et al., 2017, for detailed information see Chapter 7; and BER: Azevedo et al., 2017, for detailed information see Chapter 8).

The caspases are a specific group of cysteine aspartate proteases, a family of intracellular proteins involved in the initiation and execution of apoptosis processes, responsible for the dismantling and destruction of the cell (Green & Llambi, 2015; Riedl & Salvesen, 2007). The disruption in the balance between pro- or anti- apoptotic proteins has been established to contribute to carcinogenesis by reducing apoptosis in malignant cells (Ding et al., 2000; Goldar, Khaniani, Derakhshan, & Baradaran, 2015; Olsson & Zhivotovsky, 2011), and to be involved in the pathophysiology and clinical-hematological outcome of PN-MPNs (Malherbe et al., 2016; Testa, 2004; Tognon et al., 2013).

On the other hand, DNA repair mechanisms are essential for preventing cellular damage and the development of leukemia (Azevedo et al., 2017). Previous reports have identified BER pathway polymorphisms associated with overall solid tumors development (Azevedo et al., 2017), and leukemic transformation and development of non-myeloid malignancies in ET and PV patients (Hernández-Boluda et al., 2012).

We concluded that polymorphisms in apoptosis and BER related genes may contribute to individual susceptibility to MPNs and, hence, modify disease risk. Considering the polymorphisms investigated as a whole, our results revealed that none of the studied polymorphisms was involved in individual susceptibility to these disorders. However, potential associations were found after disease stratification. In fact, our results suggest the potential involvement of a *CASP9* (Phe136Phe) gene and a XRCC1_399 (Gln399Arg), and a *MUTYH* (Gln335His) gene polymorphisms after stratification by pathology diagnosis for ET female patients, and when they are *JAK2* positive an association with a *CASP9* (Phe136Phe) gene and a *XRCC1_399* (Gln399Arg) gene polymorphisms. In BER genes, combination of alleles also demonstrated an association with the disease for one specific haplogroup.

Although the cellular and molecular mechanisms involved in the pathophysiology of MPNs have not yet been fully clarified (Beer et al., 2010; Björkholm, Hultcrantz, & Derolf, 2014; Bolufer et al., 2006; Campregher, Santos, Perini, & Hamerschlak, 2012; Delhommeau et al., 2010; Hinds et al., 2016; Kilpivaara & Levine, 2008; Rice et al., 2011; Rueff & Rodrigues, 2016), there is evidence that in PN-

MPNs patients, hyperactive JAK/STAT signaling pathway appears to be a constant, even when the driver gene mutation is still unknown (Reuther, 2016; Skoda et al., 2015). Furthermore, JAK/STAT pathway regulates several biological processes including cell proliferation, differentiation, cell migration and also apoptosis (Becerra-Díaz *et al.*, 2011). Despite its capacity to induce a proliferative activity, JAK/STAT pathway has also an anti-apoptotic effect, through the upregulation of anti-apoptotic proteins, thereby allowing myeloid differentiation to proceed (Kieslinger et al., 2000). In turn, these anti-apoptotic proteins regulate the caspases that integrate apoptosis pathway. In particular in PN-MPNs, the deregulation of apoptosis is involved in the pathophysiology of these diseases (Malherbe et al., 2016; Testa, 2004; Tognon et al., 2013). Moreover, there is also evidence from previous studies that *CASP9* gene polymorphisms are involved in the pathogenesis of various types of cancer and that abnormalities affecting the intrinsic apoptotic cascade apparently promote megakaryocyte accumulation and thrombocytosis in PN-MPNs (Malherbe et al., 2016).

DNA repair is a ubiquitous process in all living systems. Its universality reflects the constant pressure that leads to altered genome integrity resulting from the intrinsic instability of the genetic material and limitations on the fidelity of DNA replication. Several agents induce different types of lesions in the DNA and according to the lesion they will create and activate a set of proteins responsible for the efficient repair of the lesion. Endogenously produced metabolites, namely reactive oxygen species, and also exogenous exposure, for example, to genotoxic compounds, have the ability to injure the cells and thereby increase genomic instability favoring the development of cancer. These specific DNA lesions caused by these metabolites can be repaired by mechanisms related to the BER pathway. Our findings may provide evidence for BER gene variants in susceptibility to PN-MPNs. Indeed, common variants in DNA repair genes may hamper the capacity to repair DNA, thus increasing cancer susceptibility.

The study of haplogroups is of more value, as it allows to test the genetic effect of combinations, not individualy, of relevant functional SNPs, which may contribute to the increased risk of cancer (Ponder, 2001).

It is well known that PN-MPNs have an intrinsic tendency to evidence progression to AML, sometimes preceded by a phase of secondary MF or MDS (Mesa et al., 2016), and to develop thrombohemorhagic complications, mainly arterial thrombotic events (Andıç et al., 2016), to an extent that is not fully known, but less frequent in PMF (Björkholm, Hultcrantz, & Derolf, 2014; Hernández-Boluda et al., 2012). These aspects have a considerable impact in PN-MPNs life expectancy, that is reduced when compared to general population (Passamonti et al., 2004; Wolanskyj, Schwager, McClure, Larson, & Tefferi, 2006). Moreover, therapy with cytoreductive and alkylating agents is also associated with an increased risk for malignant transformation, and consequently worse prognosis (Rampal et al., 2014; Swerdlow et al., 2008). On the other hand, regarding the development of new nonhematological and nonmyeloid neoplasms in PN-MPNs, there is evidence that the risk is higher when *JAK2* V617F mutation is present and that other individual factors may be also involved. However

the association with cytoreductive therapy is not so well established (Hernández-Boluda et al., 2012; Santoro et al., 2017).

Besides their role in genetic susceptibility, documented by some authors and evidenced by this work (Azevedo et al., 2017), it appears that SNPs involved in DNA repair capacity and apoptotic status, as well as additional somatic genetic effects, may also influnce phenotype definition, determine therapeutic response and modulate clinical outcome in PN-MPNs (Bănescu et al., 2014; Campregher, Santos, Perini, & Hamerschlak, 2012; Delhommeau et al., 2010; Levine, 2009; Nangalia et al., 2013; Tefferi & Pardanani, 2015; Tognon, Nunes, & Castro, 2013; Wang et al., 2014).

In this way, one of the last chapters of this dissertation (Azevedo *et al.*, for detailed information see Chapter 9), was dedicated to the presentation of the obtained results regarding the impact that SNPs in caspase and BER pathway genes might have in PN-MPNs patients' survival and therapeutic response, their role in disease progression and risk predisposition to new non-myeloid neoplasms and thrombotic events, in patients under treatment. This is probably one of the most complex sections in this dissertation, not only for the large amount of data obtained, but also for the multiplicity of factors/variants tested, requiring special approach strategies.

Patients were followed up for a mean of 7.6 years and hydroxyurea (HU) was the first option drug in the majority of ET, PV, and PMF cases included in the study. According to international guidelines, HU is the initial option for treatment because of its proven efficiency, especially in reducing thrombotic complications (Andıç et al., 2016).

According to our results, survival was influenced by progression to 2^{ry}MF/AML and the presence of *JAK2* V617F mutation, in agreement with the literature (Andıç et al., 2016), being shorter in PMF patients, with the majority of deaths occurring through five years after diagnosis and involving ET and PMF patients. However, according to our results survival was not influenced by the development of new primary nonmyeloid malignancies, nor the occurrence of thrombotic events, not corroborating what is described in the literature (Andıç et al., 2016). None of the caspase and BER pathway studied polymorphisms influenced survival.

Regarding the study of the role of clinical, genetic and therapy related factors potentially influencing PN-MPNs prognosis, namely progression to 2^{ry} MF/AML, the study included 12.8% of all PN-MPNs case patients (8 with PV and 9 with ET) that progressed and were matched with 76 control patients who did not, monitored for at least the same period of time. There was evidence of no significant difference in age (median of 67.7 years), nor in the number of patients with marked thrombocytosis, but with PV patients having higher white blood cells count at presentation and more frequently *JAK2* mutation, both not associated with the propensity for leukemic transformation. On the other hand, globally, there is an association with progression to 2^{ry} MF/AML and the exposure to cytoreductive agents, consistent with the literature (Hernández-Boluda et al., 2012), however our results showed that previous treatment with HU was not found to be an influencing factor for leukemic transformation. On the other hand, although not statistically significant in our results, leukocytosis and marked thrombocytosis could be involved in leukemic transformation, in agreement with the literature

(Griesshammer et al., 2015; Hernández-Boluda et al., 2012; Passamonti et al., 2008; Passamonti et al., 2004).

Concerning SNPs, it appears to exist a correlation in the studied population, consistent with other authors reports (Hernández-Boluda et al., 2012). Globally, our results revealed a lower effect of a *CASP8* (3'UTR) gene polymorphism in disease progression to 2ry MF/AML, and a higher risk associated with the presence of a *XRCC*1 (Arg194Trp) gene polymorphism, representing a worse prognosis. Stratification for ET patients revealed a *CASP9* (Arg173His) and a *XRCC*1 (Arg194Trp) genes polymorphisms associated with a worse effect in progression to 2ry MF/AML, whereas an *APEX1* (Asp148Glu) gene polymorphism showed a probable better prognosis.

The development of new primary nonmyeloid malignancies during follow-up occurred in 8.3% (11 patients) PN-MPNs cases patients, which were matched with 30 control patients who did not, with solid organ malignancies being more frequent than hematological ones, affecting more frequently PMFpatients. There was a predominance among males, but no significant difference in age, and no significant association was found for the presence of *JAK2* mutation, nor for the exposure to cytoreductive agents. The association of long-term HU use with development of nonmyeloid malignancies is controversial among the several authors (Hernández-Boluda et al., 2012; Santoro et al., 2017).

Globally, a *CASP8* (Asp270His) gene polymorphism evidenced association with an increased incidence, while a *XRCC*1 (Arg399Gln) gene polymorphism appeared to be related to a decreased incidence for new non-myeloid malignancies development.

Finally, thrombotic events were reported in 16.5% (22 patients) PN-MPNs cases patients, more frequently with PV and ET, which were matched with 56 control patients. In our population, the most frequent types of major thrombosis occur in arterial territories, along with deep vein thrombosis, in agreement with the literature (Andıç et al., 2016). There was no significant difference in age, nor platelets levels between cases and controls patients. In the same way, no significant association was found for exposure to cytoreductive agents, nor the presence of *JAK2* mutation, differing from some authors (Andıç et al., 2016).

In general, only BER pathway evidenced an association with the presence of thrombotic events, patients bearing a *XRCC1 (*Gln399Arg) gene polymorphism have a decreased incidence for thrombotic events.

In conclusion, the results obtained in the present dissertation helped to characterize a population of PN-MPNs patients, which can reflect Portuguese reality, and have suggested that some of the polymorphic variations studied, which are part of different mechanistic pathways, may constitute an important risk factor for the development and clinical progression of PN-MPNs.

Additional studies involving larger and diversified populations are required to further clarify the potential value of different genotypes as predictive biomarkers of susceptibility to PN-MPNs, so that the obtained results can be validated.

The use of association studies has been criticized for several reasons, namely that retrospective determination of an association between being an allele or genotype and a given pathology does not necessarily mean that there is an etiological link between them. Only prospective studies (cohort studies) in large, primarily healthy populations can provide direct and reliable evidence. (Tempfer et al., 2006).

It is also possible to continue this work through the evaluation of other polymorphisms present in both the pathways described and in other pathways that are thought to be relevant, such as those involved in DNA repair (ex. homologous, non-homologous and nucleotide excision repair). Another possibility is the study of specific regulatory proteins involve in apoptosis, such as SOCs. The relevance of the association studies can also be consolidated with the use of functional studies.

Some works for the development of new techniques of genetic classification of these tumors are in course, involving important reference centres and relevant resources. We hope these data may be a contribute to the clarification of the pathophysiology of these disorders, to the identification of subgroups of patients and to the elaboration of tighter therapeutic strategies, which may allow to identify in advance what will be the potential response of a patient to a given therapeutic protocol, with foreseeable implications in increasing the survival.

APPENDIX

APPENDIX 1 – INFORMED CONSENT AND MEDICAL QUESTIONNAIRE

APPENDIX 2 – CENTRO HOSPITALAR LISBOA OCIDENTAL ETHICAL COMISSION APPROVAL

APPENDIX 3 – NOVA MEDICAL SCHOOL ETHICAL COMISSION APPROVAL

APPENDIX 1: INFORMED CONSENT AND MEDICAL QUESTIONNAIRE

CENTRO HOSPITALAR DE LISBOA OCIDENTAL, E.P.E.

HOSPITAL SÃO FRANCSICO XAVIER

Ex^{mo} Senhor/a

O Serviço de Patologia Clínica do Hospital de São Francisco Xavier e o Departamento de Genética da Faculdade de Ciências Médicas da Universidade Nova de Lisboa encontram-se envolvidos num projecto de investigação com vista a identificar factores de pré-disposição para tumores hematológicos.

A identificação destes factores de risco genético poderá vir a ser de extrema importância uma vez que poderá permitir no futuro a identificação de indivíduos de risco para este tipo de patologia. Esta abordagem, a ser conseguida, poderá permitir um conjunto de dados visando, num futuro próximo, o rasteio precoce de indivíduos com risco acrescido para este tipo de patologia e consequentemente o diagnóstico precoce.

Em função destes objectivos solicitamos a sua colaboração, a qual se traduzirá apenas pela resposta a um pequeno questionário confidencial e anónimo e a colheita de um pequeno volume de sangue, que ficará guardado no Departamento de Genética da FCM para estudo.

Agradecendo antecipadamente a sua colaboração,

Dra. Ana Paula Azevedo

Serviço de Patologia Clínica do HSFX – CHLO

DECLARAÇÃO

Eu, _____, declaro ter sido devidamente esclarecido sobre a natureza e objectivos do estudo “Polimorfismos genéticos: potenciais factores de risco para doença oncológica” tendo decidido colaborar voluntariamente neste estudo.

Lisboa, _____ de _____ de 2_____

_____

(Assinatura)

INQUÉRITO

Código: _____ (a preencher pelo Médico)

Data de Nascimento: _____/_____/_____ Sexo: _____ Etnia: _____

Diagnóstico: _____ Data de Diagnóstico: _____/_____/_____

Data de colheita: _____/_____/_____ Terapêutica: _____

Peso: _____ Altura: _____ Naturalidade: _____

Local de residência: _____

Reside numa rua com muito trânsito? _____

Reside perto de alguma fábrica, refinaria, bomba de gasolina? Especifique: ._____

_____

Profissão: _____

Trabalha com produtos químicos? _____ Especifique tanto quanto possível: _____

_____

No seu ambiente de trabalho está exposta a produtos químicos? _____ Especifique tanto quanto possível: _____

_____

Trabalha numa rua com muito trânsito? _____

Trabalha perto de alguma fábrica, refinaria, bomba de gasolina? Especifique: _____

_____

Esteve exposto a radiação ionizante por razões ocupacionais?_____Especifique tanto quanto possível:

_____

Esteve exposto (durante algum período de tempo) a radiação ionizante por razões médicas (ex: RX, TAC)?-_____ Especifique tanto quanto possível: _____-

_____

Realizou transfusões de sangue nos últimos 12 meses? (Se sim qual a razão?)

_____

Hábitos alcoólicos:

Vinho tinto (Quantidade/Anos de Consumo): _____

Vinho branco (Quantidade/Anos de Consumo): _____

Cerveja (Quantidade/Anos de Consumo): _____

Outras bebidas (Quantidade/Anos de Consumo): _____

Hábitos tabágicos: n.º de cigarros por dia _____ anos de consumo _____

Toma regularmente algum tipo de medicação? _____(Qual, Duração):

_____

Está a tomar, ou tomou, suplementos vitamínicos? _____ (Qual, Duração):

_____

Qual o seu tipo de alimentação?

	Todas as refeições	Frequentemente (4-6 vezes/semana)	Raramente (< 3 vezes/semana)	Ocasionalmente
Sopa:	☐	☐	☐	☐
Vegetais:	☐	☐	☐	☐
Fruta:	☐	☐	☐	☐
Grelhados/ churrascos:	☐	☐	☐	☐
Fumados:	☐	☐	☐	☐
Enlatados:	☐	☐	☐	☐

1.1.1 Chá: sim/não _____ Quantas vezes por dia?

_____

1.1.2 Café: sim/não _____ Quantas vezes por dia?

_____

1.1.3 Água: só às refeições _____ também fora das refeições _____ Quantos litros por dia (aproximadamente)? _____

Existem antecedentes familiares de cancro, nomeadamente hematológicos?_____-

_____

Especifique o(s) tipo(s) de tumor(es) e o parentesco do familiar. _____

_____

_____

Foi-lhe diagnosticado anteriormente qualquer outro tipo de tumor? _____ Qual? Em que data?

_____

_____

Outra doença associada: _____

_____

_____

Exame anatomo-patológico: _____

_____

_____

_____

_____

FIM

APPENDIX 2: CENTRO HOSPITALAR LISBOA OCIDENTAL ETHICAL COMISSION APPROVAL

Ministério da Saúde

CENTRO HOSPITALAR DE LISBOA OCIDENTAL, E.P.E.
HOSPITAL DE SANTA CRUZ

PARECER DA COMISSÃO DE ÉTICA

Estudo Título	N.º
"Susceptibilidade genética para Síndromes Mieloproliferativos Crónicos e eficácia terapêutica"	----------

Descrição:

Investigador: Dr.ª Ana Paula Azevedo

Local: Serviço de Patologia Clínica HSFX

A Comissão de Ética deliberou em reunião de 6 de Abril de 2009 emitir parecer favorável á realização do estudo **"Susceptibilidade genética para Síndromes Mieloproliferativos Crónicos e eficácia terapêutica"**, com a recomendação de quer os inquéritos sejam feitos sempre pelo mesmo Investigador.

Ouvido o Relator, o processo foi votado pelos Membros da Comissão de Ética do Centro Hospitalar de Lisboa Ocidental presentes em reunião de 6 de Abril de 2009:

Presidente: Prof.ª Doutora Teresa Marques
Vice-Presidente: Pe. João Vaiente

Dr.ª Paula Peixe, Enf.ª Clara Carvalho, Dr.ª Helena Farinha e Dr. Santana Carlos

Pelo exposto emitiu-se em reunião de 6 de Abril de 2009, **Parecer favorável**.

Presidente da Comissão de Ética

Profª Doutora Maria Teresa Marques

Avenida Prof. Doutor Reinaldo dos Santos 2799-523 CARNAXIDE Telf: +351 214 163 400 Fax +351 214 188 095
e-mail: comissao.etica@hsc.min-saude.pt

APPENDIX 3: NOVA MEDICAL SCHOOL ETHICAL COMISSION APPROVAL

Decisão final sobre o projeto "Genetic susceptibility for Myeloproliferative Neoplasms and therapeutic efficacy"

A Comissão de Ética da NMS|FCM-UNL (CEFCM) decidiu, por unanimidade, aprovar o projeto de investigação intitulado "Genetic susceptibility for Myeloproliferative Neoplasms and therapeutic efficacy" (nº34/2015/CEFCM), submetido pela Dra. Ana Azevedo.

Lisboa, 27 de Novembro de 2015

O Presidente da Comissão de Ética,

(Prof. Doutor Diogo Pais)

TO WHOM IT MAY CONCERN

The Ethics Research Committee NMS|FCM-UNL (CEFCM) has unanimously approved the Project entitled "Genetic susceptibility for Myeloproliferative Neoplasms and therapeutic efficacy" (nr.34/2015/CEFCM), submitted by Dr. Ana Azevedo.

Lisbon, November 27th, 2015

The Chairman of the Ethics Research Committee,

(Diogo Pais, MD, PhD)

Campo dos Mártires da Pátria, 130 | 1169-056 Lisboa | Portugal | T. +351 218 803 000 – Ext. 20447 E-mail cefcm@fcm.unl.pt