

## CA18233 - European Network for Innovative Diagnosis and Treatment of Chronic Neutropenias

May 2, 2020

Dear colleagues,

Please, find below the protocol for the investigation of patients with unexplained neutropenia for the SNP associated with ethnic neutropenia (ENP).

By sharing the protocol,

1. We share the genotyping/phenotyping techniques that you may want to **apply for your routine** practice.
2. We encourage partners from different countries within the consortium to **estimate the frequency of the SNP among patients with unexplained neutropenia** using the techniques described below. The genotype/phenotype techniques can be easily applied in any Laboratory using the local informed consent forms and approval of the study. However, in case you would like to send the samples for genotyping to Petter Höglund's or Helen Papadaki's Lab, this can be easily arranged; in such a case we have to send you the informed consent document for the patients.
3. We would also like to share a number of research questions that you may want to explore further based on your expertise:

Specifically, it has been shown that (i) ENP confers a change of the compartmentalization of neutrophils, from the blood stream to the tissues, particularly into the spleen (in rodents), (ii) absence of ACKR1 expression on early erythroid cells in the bone marrow (but normal on endothelial cells) confers an immune activation, expressed as increased expression of CD16 on neutrophils, probably also other immune-related molecules, e.g. MMP9 etc. (*Duchene J et al. Nature Immunol 2017;18:753*).

It would be then interesting to know:

(a) Is the proposed change of compartmentalization of neutrophils also true in humans? Is the total body neutrophil count (TBNC) normal in ENP individuals and increased number of neutrophils dwell in the tissues? This can be assessed by means of increased emigration of neutrophils into skin windows or gingival pockets. **Novel techniques are needed to assess the TBNC.**

(b) Are more neutrophils accumulating in the spleen without causing splenomegaly?

(c) Will more neutrophils accumulate in the lungs of pneumonic ENP subjects (e.g. in severe Covid19 pneumonitis. Or in the joints of rheumatoid arthritis patients? In the guts or abdomen of colitis patients, in the hearts of myocardial infarction patients ?)

(d) Is production and release of neutrophils lower in the BM than in controls? This has been approached in the past (with epinephrine, steroid, LPS administration and vigorous physical activity) **but novel techniques are needed for better understanding of these phenomena.**

(e) Is it true that newborn neutrophils leave the BM with increased CD16 expression and what are the consequences of this? Will this lead to an immune response to CD16, manifested as a positive MAIGA test for autoantibodies to CD16 epitopes on the neutrophil surface (or to other epitopes)? Will an increased CD16 expression translate into enhanced binding of CD16 ligands (opsonizing antibodies, therapeutic FcR-binding antibodies such as the Fc-part of rituximab) with risk of more side effects or enhanced efficacy of the treatment?

On behalf of EuNet-INNOCHRON WG2

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## **Investigation of the single nucleotide polymorphism of the DARC/ACKR1 gene associated with ethnic neutropenia in Chronic Neutropenia Patients within EuNet-INNOCHRON**

### **1. Introduction**

Ethnic neutropenia (ENP) is a situation when individuals mostly of African and Near and Middle East ancestry present lower ANCs than individuals of European ancestry and is commonly associated with absence of Duffy antigens from red blood cells [1, 2]. Duffy antigens are encoded by the Duffy antigen receptor for chemokines (DARC), also known as Atypical Chemokine Receptor-1 (ACKR1), gene [3]. The Duffy blood group has two major codominant alleles, FY\*A and FY\*B, differentiated by a single nucleotide polymorphism (SNP) (G125A, rs12075) resulting in the main blood group variants Fya and Fyb, respectively [3, 4]. The most common genetic determinant associated with ENP is a second SNP (rs2814778) at the GATA promoter region of the DARC/ACKR1 gene associated with a T to C substitution (-46T>C, rs2814778); the homozygous C/C polymorphism prevents the Duffy antigens' expression on erythroid cells resulting in the Duffy-null Fy(a-b-) (also known as erythroid silent, ES) phenotype [3, 5]. This polymorphism is usually haplotypically associated with the FY\*B allele (FY\*BES). The C/C rs2814778 (FY\*BES/FY\*BES) genotype is rare or absent among white European individuals [6]. Therefore, the investigation of unexplained neutropenia in European patients does not usually include Duffy polymorphisms genotyping and thus, data on Duffy phenotype/genotype/allele frequencies in neutropenic compared to normal individuals have been missing. A recent study however [7] showed for the first-time an increased frequency of the genotype C/C (rs2814778) and the associated Duffy-null phenotype in European patients with Chronic Idiopathic Neutropenia - a term used to define patients with Chronic Neutropenia (CNP) who do not have an apparent underlying cause despite thorough testing [1]- but not in healthy individuals.

Therefore, it is suggested to study the Duffy genotype of patients with unexplained neutropenia of European origin, after informed consent is obtained. The results could identify the frequency of the genotype C/C (rs2814778) and substantiate the notion of inclusion of this test in the diagnostic algorithm of CNP patients.

Notably, in cases that Duffy genotyping is not readily available, Duffy phenotyping on Red Blood Cells can be performed as a complimentary method in collaboration with the nearest blood bank. However, on the occasion that a patient is serological Fy null, DUFFY genotyping should still be performed to validate the result, as rare mutations of another kind might also exhibit a Fy null phenotype.

## 2. Method

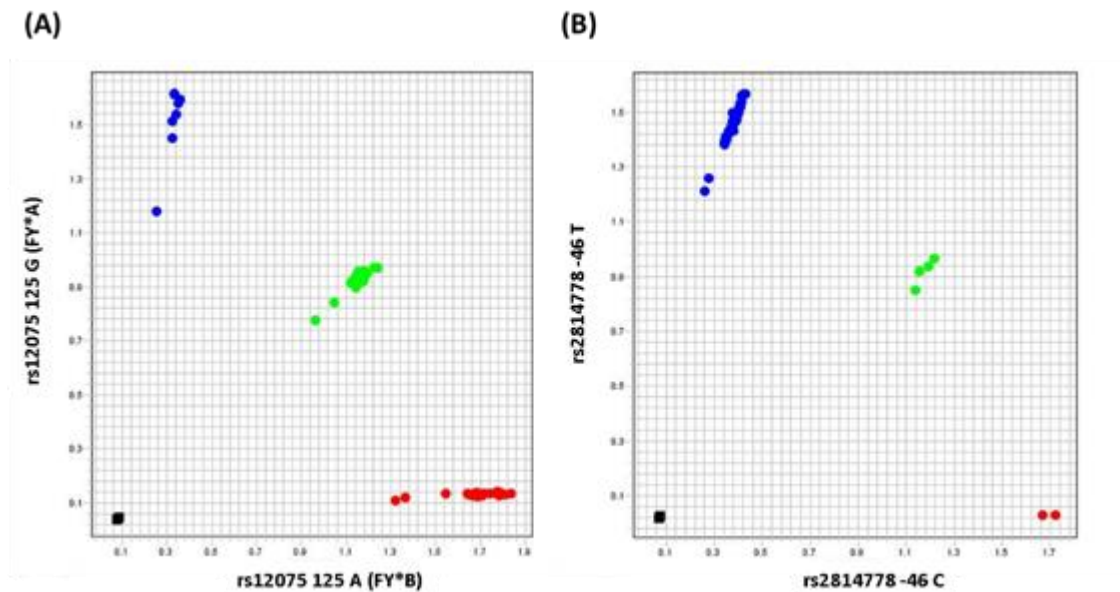
### 2.1 Genotyping of *FY\*A*, *FY\*B* and *FY\*BES* alleles

Genotyping is performed using high purity DNA isolated from ethylenediaminetetraacetic acid (EDTA)-preserved peripheral blood (PB). DNA can be extracted efficiently from both fresh or frozen blood samples (stored at -80°C), using PureLink® Genomic DNA Mini Kit (Invitrogen, ThermoFisher Scientific, Waltham, Massachusetts, USA.), or any commercial kit of choice, according to the manufacturer's instructions. It is of great importance for the precision and reproducibility of the method, to use high quality DNA. For this reason, both quantity and purity of each DNA sample are determined spectrophotometrically at 260nm, using NanoDrop Spectrophotometer. In order to exclude contamination of the DNA sample with protein or organic compounds during extraction, the ratio of the absorbance at 260 and 280nm ( $A_{260}/280$ ) is used, that typically ranges between 1.8 and 2.0. Extracted DNA can be stored at -20 °C until analyzed.

Allelic discrimination of *DARC/ACKR1* rs12075 and rs2814778 SNPs is carried out using pre-designed TaqMan SNP Genotyping real-time PCR assays from Applied Biosystems (Foster City, CA, USA), according to the manufacturer's protocol (cat. nos. C\_\_2493442\_10 and C\_\_15769614\_10, respectively). A 96-well plate is prepared with a mixture on an Applied Biosystems ViiA™ 7 Real Time PCR system of 1X TaqMan SNP Genotyping assay, 1X TaqMan Universal Master mix (both from Applied Biosystems) and 20 ng DNA per well for each SNP. Accurate quantification of DNA is of great importance, since loading different amounts of DNA, could produce misleading results.

The PCR conditions are: denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C denaturation for 15 sec and a 60 °C annealing for 1 min. After PCR, the plates are read, and data is analysed using Quant Studio™ Real-Time PCR Software (Applied Biosystems). For efficient clustering, a sufficient number of DNA samples is assayed per run. Finally, negative controls and samples of known genotypes (if available) should be used. Allelic discrimination plots must be all reviewed individually for quality.

Representative allelic discrimination plots generated by real-time PCR genotyping of the *DARC/ACKR1* SNPs rs12075 and rs2814778 are shown in Figure 1 below.



**Fig.1 Representative allele discrimination plots generated by real-time PCR genotyping of the DARC/ACKR1 polymorphisms rs12075 and rs2814778.**

(A) The plot depicts the allele discrimination genotyping of the SNP rs12075 (G125A). The ancestral allele A at rs12075 (FY\*B) is plotted on x-axis versus the derived allele G (FY\*A) on y-axis. (B) The plot shows the allelic discrimination genotyping of the SNP rs2814778 (-46T > C). The ancestral allele T (FY\*B) is plotted on y-axis versus the derived allele C (FY\*BES) on x-axis. Samples that are homozygous for the derived alleles are shown on the lower right corner of the plots (red clusters), whereas samples that are homozygous for the ancestral alleles are shown on the upper left corner (blue clusters). Heterozygous genotypes are shown in the center of the plots (green clusters). No-template control is depicted in the left bottom corner.

### Strengths and Weaknesses of the Method

SNP genotyping analysis Using TaqMan assays is considered as the “gold-standard” technology by many researchers in genotyping and genetic variation analysis. This is mainly due to the simplicity and reproducibility of the method. Reactions are easily set up, requiring only three reaction components for PCR: purified genomic DNA (20 ng), the assay solution, and TaqMan Genotyping Master Mix. Furthermore, the use of predesigned TaqMan SNP Genotyping Assays allows the analysis of a high number of test samples, that can be assayed simultaneously. SNP genotyping results are obtained in a fast and simple way.

However, due to the high sensitivity of the method technical handling issues may arise from one user to another. For example, accurate determination of DNA concentration and pipetting are essential for efficient clustering. Therefore, precise handling and quantitative measurements are critical to the success of the assay. Additionally, evaporation can occur if the plate is not properly sealed, leading to failure of the PCR reaction and subsequent wasting of expensive reagents and valuable samples. Finally, efficient clustering is observed during analysis of a large number of test samples, thus preventing efficient assaying of separate samples or a very low number of test samples.

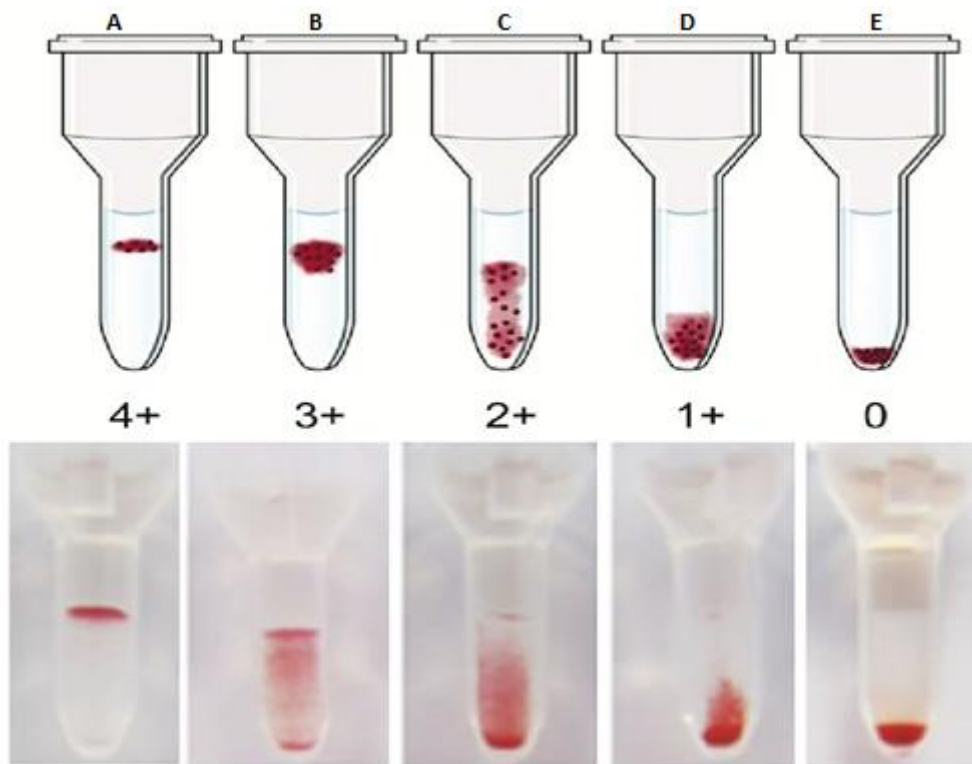
## 2.2 DUFFY phenotyping

Duffy phenotyping of red blood cells can be determined in peripheral blood samples by means of a direct antiglobulin test using the commercially available anti-Fya, anti-Fyb reagents and the LISS/Coombs ID-cards (Bio-Rad; DiaMed GmbH, Cressier FR, Switzerland) according to the manufacturer's instructions. For optimal results, the determination should be performed using a freshly drawn sample. Blood samples drawn less than 24 hours prior to testing and refrigerated are also acceptable. Preferably, blood samples should be drawn into citrate, EDTA or CPD-A anticoagulant.

In brief, a 0.8% red cell suspension is prepared using 1 mL of Diluent-2 (Bio-Rad) and 10 µL of the red blood sediment or 20 µL of whole blood and used immediately. Fifty microliters of the red cell suspension are then mixed with 50 µL of the corresponding antiserum (anti-Fy<sup>a</sup> or anti-Fy<sup>b</sup>, polyclonal antibodies from human serum) within the ID-Cards containing polyspecific anti-human globulin serum supplied by the manufacturer. The mixture containing ID-Cards is then incubated for 15 minutes at 37°C and then centrifuged at 3000 rpm for 10 minutes at room temperature. The results are evaluated based on the manufacturer's instructions as follows (Fig.2):

1. **Positive:** Agglutinated cells forming a red line on the surface of the gel or agglutinates dispersed in the gel. Positive reactions of + to +++ indicate the presence of the corresponding antigen. Strong ++++ positive reactions are rare.
2. **Negative:** Compact button of cells on the bottom of the microtube. Negative reactions indicate the absence of the corresponding antigen.

Cells that are direct antiglobulin test (DAT) positive due to in vivo coating with IgG will be agglutinated by the AHG within the gel matrix. A DAT should be performed on the sample prior to antigen typing to eliminate this possibility.



**Fig.2 Representative red cells Fya or Fyb phenotype results. (A-D) Positive results, (E) Negative result**

### **Strengths and Weaknesses of the Method**

Duffy Phenotyping is a quick method lasting for less than 45min of preparation and analysis for approximately 10 samples, with a lot of off-hands time. It is a simple method, that can be done in almost all blood banks, and requires only small volume of blood to be tested (10-50 $\mu$ L). Reagents are available in the form of kits with user-friendly instructions and it is a flexible method, since in a rare case that no Fya/Fyb ID-cards are available, the ID-cards for Coombs method can be used with the appropriate Fya/Fya antiserum.

However, weak positive signals might be misinterpreted as low number of cell aggregates might be formed on a negative sample, therefore, in the absence of control specimen the procedure must be repeated. In rare occasions bacteremia or other circulating antibodies might affect the result.

### 3. References

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